U.S. Environmental Protection Agency, National Center for Computational Toxicology (U.S. EPA NCCT)

TOXICITY FORECASTER (TOXCAST) *IN VITRO* ASSAYS

Assay Documentation for Non-Guideline *In Vitro* Test Methods: Endocrine Assays

25 April 2019

Correspondence: contact U.S. EPA NCCT
109 T.W. Alexander Drive (MD-B-205-1)
Research Triangle Park, NC 27711
919-541-4219

Assay Endpoint ID: 2

ACEA_T47D_80hr_Positive

Assay Title: ACEA 80-hr T47-D Human Breast Cell Proliferation Assay

Overview

Assay Summary:

One possible effect of endocrine disrupting chemicals is increased cell growth through perturbation of endocrine pathways linked to cell cycle regulation. Activation of the estrogen receptor (ER) signaling pathway, for example, is one possible mechanism that underlies cell proliferation in hormonally sensitive tissues such as mammary and endometrial tissue. The role of steroid hormones in the regulation of some mammary tumors has been well established (Russo and Russo 2006, Yager and Davidson 2006) and has motivated the development of estrogen pathway-based chemotherapeutics. This assay was designed to identify those chemicals in the ToxCast chemical library with the potential to affect cell growth by activating the estrogen receptor-mediated cell proliferation pathway. These impacts were observed by monitoring changes in electrical impedance on the surface of an electronic cell culture growth plate (E-plates) following 80-hour incubation with test chemicals.

Assay Definition

Assay Throughput:

The assay is conducted on 96-well plates with each plate containing positive controls for proliferation (17β-estradiol) and cytotoxicity (MG132), negative controls (assay media, RPMI 1640), and two concentrations (0.5% and 0.125%) of DMSO solvent controls. Following a 24-hour incubation period, the cells are exposed to test chemicals for 80 hours and response is monitored no less than once per hour.

Experimental System:

T-47D human breast carcinoma ductal cell line, originally derived in 1974 from pleural effusion of a 57-year-old patient, which exhibits epithelial-like morphology (Horwitz et al. 1978, Keydar et al. 1979).

Xenobiotic Biotransformation Potential:

T-47D cells contain specific high affinity receptors for estradiol, progesterone, glucocorticoid and androgen (Horwitz et al. 1978). Some potential for P450 mediated metabolism is present, e.g. CYP1A1, CYP1A2, CYP1B1 (Angus et al. 1999, Hevir et al. 2011, MacPherson and Matthews 2010, Spink et al. 2002, Spink et al. 1998), CYP2B6 (Lo et al. 2010), CYP3A4 (Nagaoka et al. 2006) and CYP2C8 (Mitra et al. 2011), as well as some experimental evidence for the capacity to retain expression of some phase II metabolizing enzymes, e.g., UGTs (Harrington et al. 2006, Hevir et al. 2011), GSTs (Hevir et al. 2011) and sulphotransferases (e.g., SULT1A3(Miki et al. 2006), SULT1E1, SULT2B1 (Hevir et al. 2011)).

Basic Procedure:

Materials

	Product	Source	Cat. No.
Cells	T-47D	ATCC	HTB-133
Growth media	RPMI1640	Hyclone	SH30027FS
Growth media serum	10% FBS	Hyclone	SH3007103
Test media	RPMI 1640	Gibco.	11835030
Test media serum	10% charcoal stripped FBS	Hyclone	SH3006803HI
Positive control	17β-estradiol	Tocris	2824
Reference compound	dexamethasone	Sigma-Aldrich	D1756
Reference compound	hydrocortisone	Sigma-Aldrich	H4001
Reference compound	progesterone	Sigma-Aldrich	P8783
Reference compound	aldosterone	Sigma-Aldrich	A9477
Reference compound	T3 (3,3',5-Triiodo-L-thyronine sodium salt)	Sigma-Aldrich	T6397
Reference compound	T4 (thyroxine)	Sigma-Aldrich	T2376

Protocols

T-47D cells purchased from American Tissue Culture Collection (ATCC) were maintained in RPMI1640 media supplemented with 10% characterized fetal bovine serum (FBS) until testing. Before screening, cells were preconditioned in assay medium: phenol red-free RPMI1640 supplemented with 10% charcoal-stripped FBS. Cells were then detached and seeded in 96-well E-Plates in assay medium. After overnight monitoring of growth once every hour, chemicals were added to T-47D cells and remained in the medium until the end of the experiment. Each chemical in the ToxCast library was tested in an 8-point, 1:4 serial dilution series starting at a maximum final concentration of 100 µM and was tested in duplicate using two separate E-plates for each dilution series. A maximum starting concentration of 0.5% DMSO was present in the 100 µM chemical samples and subsequent dilutions used a final concentration of 0.125% DMSO. Positive controls (MG132 for cytotoxicity and 17β-estradiol for proliferation) and a negative control (assay media) were tested in quadruplicate on each testing plate along with 0.5% and 0.125% DMSO tested in duplicates on each plate to serve as solvent controls for the highest concentration of testing chemicals and all lower dilutions, respectively. Reference chemicals were tested with 8 concentrations with 1:5 serial dilutions. The xCELLigence system Multi-E-Plate stations employing real-time cell analysis (RTCA) were used to measure cellular responses recorded once every 5 min for the first 5 h, and once every hour for an additional 100h.

Proprietary Elements:

Assay is non-proprietary; xCELLigence RTCA software and biosensor technology are available from ACEA Biosciences, Inc. and T-47D cells are commercially available from American Type Culture Collection (ATCC HTB-133) with signed Material Transfer Agreement (MTA).

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to activate endogenous estrogenic signaling pathways, and is intended to provide information

on a large number of diverse chemicals. Cell proliferation may result from both estrogenic pathways and non-estrogenic pathways so results from this assay in isolation do not ensure estrogenic activity for a test chemical. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

Status:

The assay is fully developed, and data are publicly available.

Assay References

Assay Source Contact Information:

ACEA Biosciences

6779 Mesa Ridge Road #100, San Diego, CA 92121 USA

Tel: +1 858-724-0928 | 1 866-308-2232

Fax: +1 858-724-0927

info@aceabio.com

Assay Publication Year:

2006

Assay Publication:

Xing, J. Z., Zhu, L., Gabos, S., & Xie, L. (2006). "Microelectronic cell sensor assay for detection of cytotoxicity and prediction of acute toxicity". Toxicol In Vitro 20(6), 995-1004. (PMID: 16481145)

Method Updates / Confirmatory Studies:

None Reported

Assay Component Descriptions

Assay Objectives:

The ACEA T47D 80-hour Positive assay exposed human breast carcinoma cell (T-47D) cultures to the ToxCast library of diverse environmental chemicals using an eight-point, 1:4 dilution series concentration-response format (starting at a maximum final concentration of 100µM), using MG132 (cytotoxicity) and Estradiol (E2) (proliferation) as positive controls and assay media and DMSO as a negative control and solvent control, respectively. All control chemicals were tested in quadruplicate on each plate.

The ACEA_T47D ER assay analyzed changes in cell adhesion and morphology at the electrode: solution interface (located on the bottom of 96-well E-plate culture wells) using electronic microsensors. Changes

in electrical impedance were monitored in real-time at the plate surface to investigate the potential activation of the estrogen signaling pathway and subsequent increases in growth or changes in cell structure following 80-hour incubation with the test chemicals. The electrical signal produced by the experimental system can be used to detect changes in cell number, morphology and adhesion which occur in response to xenoestrogenic activation of ER-mediated pathways, and concentration-response curves were modeled for each chemical to determine half-maximal activity levels.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogenic signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The activity of estrogenic chemicals is generally probed in vitro by monitoring ligand-binding in experimental systems, however estrogenic potency is also a function of interaction with transcriptional machinery and other signaling pathways. This assay was designed to identify chemical perturbagens which can affect a cell proliferation response in human breast carcinoma cells by acting as xenoestrogenic compounds which impact estrogen signaling pathways. While cell proliferation rates can be altered via multiple pathways, growth responses in T47D cells are considered to be particularly reliable indicators of estrogenic activation.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds which potentially affect endocrine systems in exposed populations by interacting with estrogen receptor mediated signaling pathways. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE) in a developing Adverse Outcome Pathways (AOP) leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the leading to reproductive dysfunction in oviparous vertebrates (AOPs under development), and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (under development). ER antagonism has strong evidence as the MIE for an AOP describing reduction of vitellogenin synthesis in liver (under review), which can lead to reduced cumulative fecundity in repeat-spawning fish species. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER interference in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Xiao, C., Lachance, B., Sunahara, G., & Luong, J. H. (2002). "Assessment of cytotoxicity using electric cell-substrate impedance sensing: concentration and time response function approach". Analytical Chemistry 74(22), 5748-5753. (PMID: 12463358)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.66

Neutral control median absolute deviation, by plate:	0.11
Positive control well median response value, by plate:	3.00
Positive control well median absolute deviation, by plate:	0.19
Negative control well median, by plate:	0.03
Negative control well median absolute deviation value, by plate:	0.06
Z' (median across all plates, using positive control wells):	0.31
Z' (median across all plates, using negative control wells):	0.69
SSMD (median across all plates, using positive control wells):	6.00
SSMD (median across all plates, using negative control wells):	-13.00
Signal-to-noise (median across all plates, using positive control wells):	12.94
Signal-to-noise (median across all plates, using negative control wells):	-15.00
Signal-to-background (median across all plates, using positive control wells):	1.82
Signal-to-background (median across all plates, using negative control wells):	0.02
CV (median across all plates):	0.07

Assay Endpoint Descriptions

Data Interpretation

Biological Response:

Increase cell proliferation in response to xenoestrogenic interference with ER-mediated pathways as measured by monitoring electrical impedance at the cell-plate interface.

Analytical Elements:

Data were collected from the xCELLigence system which converts raw impedance values into the Cell Index (CI) value; this is a measure of adhesion where CI = (impedance at time point n – impedance in the absence of cells)/nominal impedance value. These data were then converted to a Normalized Cell Index according to the equation NCI(Ti) = [CI(Ti)]/[CI(Tk)], {i = 1,2,3,….N where CI(Tk) is the last time point before chemical addition, CI(Ti) is the cell index at the i-th measured time point, and N is the total number of time points. Data were grouped by chemical and smoothed to combine replicates using a simple moving average (as the replicates were assessed in duplicate on separate plates so the time points were not identical). DMSO controls were considered as baseline for activity, and 17β-Estradiol was used as a positive control and 100% activity for all the test chemicals on that plate. If a chemical sample was run on two different plates, then the maximum NCI values for the positive and negative controls were averaged. Concentration response curves were generated using smoothed NCI values and all statistical analyses were conducted using R programming language, employing tcpl package to generate model

parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC50 (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive

ATG_ERE_CIS_up

ATG_ERa_TRANS_up

NVS_NR_bER

NVS_NR_hER

NVS_NR_mERa

OT_ER_ERaERa_0480

OT_ER_ERaERa_1440

OT_ER_ERaERb_0480

OT_ER_ERaERb_1440

OT_ERa_ERELUC_AG_1440

OT_ERa_ERELUC_ANT_1440

OT_ERa_EREGFP_0120

OT_ERa_EREGFP_0480

Tox21_ERa_BLA_Agonist_ratio

Tox21_ERa_BLA_Antagonist_ratio

Tox21_ERa_LUC_BG1_Agonist

Tox21_ERa_LUC_BG1_Antagonist

Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8

Target (nominal) number of replicates: 2

Standard minimum concentration tested: 0.39 µM

Standard maximum concentration tested: 200 µM

Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.77

The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 23.86

Reference Chemicals / Predictive Capacity:

ER reference chemicals (list adapted from OECD Test Guideline No. 457, as according to methods described by Judson et al. 2015 (Judson et al. 2015)):

CASRN	Chemical Name	Agonist Potency	Antagonist Potency	Activity in Assay
57-63-6	17alpha-Ethinyl estradiol	Strong	Inactive	Yes
50-28-2	17beta-Estradiol	Strong	NA	Yes
56-53-1	Diethylstilbestrol (DES)	Strong	Inactive	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
57-91-0	17alpha-Estradiol	Moderate	NA	Yes
140-66-9	4-tert-Octylphenol	Moderate	NA	Yes
53-16-7	Estrone	Moderate	NA	Yes
599-64-4	4-Cumylphenol	Weak	NA	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Inactive	Yes
80-05-7	Bisphenol A	Weak	Inactive	Yes
77-40-7	Bisphenol B	Weak	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
446-72-0	Genistein	Weak	Inactive	Yes
143-50-0	Kepone	Weak	Inactive	No
789-02-6	o,p'-DDT	Weak	NA	Yes
58-18-4	17alpha-Methyltestosterone	Very Weak	NA	Yes
520-36-5	Apigenin	Very Weak	Inactive	Yes
85-68-7	Butylbenzyl phthalate	Very Weak	Inactive	Yes
480-40-0	Chrysin	Very Weak	Inactive	No

115-32-2	Dicofol	Very Weak	Inactive	No
117-81-7	Diethylhexyl phthalate	Very Weak	Inactive	No
84-74-2	Di-n-butyl phthalate	Very Weak	Inactive	No
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
520-18-3	Kaempferol	Very Weak	NA	Yes
72-43-5	Methoxychlor	Very Weak	NA	Yes
72-55-9	p,p'-DDE	Very Weak	Inactive	No
104-40-5	p-n-Nonylphenol	Very Weak	NA	Yes
1912-24-9	Atrazine	Inactive	NA	No
50-22-6	Corticosterone	Inactive	NA	Yes
66-81-9	Cycloheximide	Inactive	NA	No
13311-84-7	Flutamide	Inactive	NA	Yes
52-86-8	Haloperidol	Inactive	NA	No
52806-53-8	Hydroxyflutamide	Inactive	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
57-30-7	Phenobarbital Sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	Yes
68392-35-8	4-Hydroxytamoxifen (E/Z)	NA	Active	Yes
82640-04-8	Raloxifene	NA	Active	Yes
10540-29-1	Tamoxifen	NA	Active	Yes
54965-24-1	Tamoxifen citrate	NA	Active	Yes
57-83-0	Progesterone	NA	Inactive	Yes

Agonist Activity	ToxCast Active	ToxCast Inactive
Active	21	7
Inactive	6	6

Antagonist Activity	ToxCast Active	ToxCast Inactive
Active	4	0
Inactive	8	6

Agonist Sensitivity = 75%

Agonist Specificity = 50%

Balanced Accuracy = 62.5%

Antagonist Sensitivity = 100%

Antagonist Specificity = 42.9%

Balanced Accuracy = 71.4%

Overall balanced accuracy for assay = 62.1%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [16].

Assay Documentation

References

[1] Russo, J. and I. H. Russo (2006). J Ster Biochem Mol Biol 102(1): 89-96. (PMID: 17113977)

[2] Yager, J. D. and N. E. Davidson (2006). New Engl J Med 354(3): 270-282.

[3] Keydar, I., et al. (1979). Eur J Cancer 15(5): 659-670. (PMID: 228940)

[4] Horwitz, K. B., et al. (1978). Cancer Res 38(8): 2434-2437. (PMID: 667841)

[5] Spink, D. C., et al. (1998). Carcinogenesis 19(2): 291-298. (PMID: 9498279)

[6] Spink, D. C., et al. (2002). Drug Metab Disposition 30(3): 262-269. (PMID: 11854143)

[7] Angus, W. G., et al. (1999). Carcinogenesis 20(6): 947-955. (PMID: 10357772)

[8] MacPherson, L. and J. Matthews (2010). Cancer Lett 299(2): 119-129. (PMID: 20846786)

[9] Hevir, N., et al. (2011). Chem-Biol Interact 191(1): 206-216. (PMID: 21182832)

[10] Lo, R., et al. (2010). Biochimica et Biophysica Acta 1799(5): 469-479. (PMID: 20079471)

[11] Nagaoka, R., et al. (2006). Endocrine 30(3): 261-268. (PMID: 17526937)

[12] Mitra, R., et al. (2011). J Biol Chem 286(20): 17543-17559. (PMID: 21402692)

[13] Harrington, W. R., et al. (2006). Endocrinology 147(8): 3843-3850. (PMID: 16690804)

[14] Miki, Y., et al. (2006). Cancer Res 66(1): 535-542. (PMID: 16397270)

[15] Judson, R. S., et al. (2015). Toxicol Sci 148(1): 137-154. (PMID)

[16] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

ATCC, American Tissue Culture Collection

CV, Coefficient of Variation

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

EDC, Endocrine disrupting chemicals

ER, Estrogen Receptor

FBS, Fetal Bovine Serum

GST, Glutathione S-Transferase

MIE, Molecular Initiating Event

NCI, Normalized Cell Index

NR, Nuclear Receptor

RTCA, Real-Time Cell Analysis

SSMD, Strictly Standardized Mean Difference

SULT, Sulfotransferases

UGT, UDP-glucuronosyltransferase

Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

15 November 2016

Author of Revisions:

EPA NCCT

Potential Regulatory Applications:

Context of use:

Examples of end use scenarios could include, but are not limited to:

-Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

-Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

-Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

-Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA

ATG_AR_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Androgen Receptor Activation Assay

Assay Descriptions

Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL^{TM}) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 (Aden et al. 1979), which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 (Westerink and Schoonen 2007a) with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions (Hewitt and Hewitt 2004); some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes (Guo et al. 2010). Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 (Hart et al. 2010, Walle et al. 2000, Westerink and Schoonen 2007b) and UGTs (1A1, 1A6 and 2B7) (Hart et al. 2010). In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein (Boehme et al. 2010) and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g.,

ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) (Adachi et al. 2007).

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

Assay Component Descriptions

Assay Objectives:

The Attagene TRANS Androgen Receptor assay measures changes in human androgen-receptor (AR) activation using the mammalian one-hybrid assay format, which monitored transcriptional activity of

the AR ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection. The presence of AR agonists alters the transactivation function of Gal4-AR RTU and modulates reporter transcription. Following 24-hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures used). Following MTC determination, concentration-response assays where conducted by exposing a single replicate to a 6-8 point concentration series, starting at the MTC and followed by 3-fold serial dilutions. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls.

ATG_AR_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_AR_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-AR proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an AR-specific reporter sequence. Chemical-AR activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting androgen signaling pathways. The androgen receptor mediates gene expression in response to androgenic exposures, and modulates the activity for a wide variety of physiological processes. The ATG_AR_TRANS assay used a hepatoma cell-based platform to monitor androgen receptor transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for endocrine disrupting activity via interactions with androgen receptor.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.1575
Neutral control median absolute deviation, by plate:	0.2646
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.23

Assay Endpoint Descriptions

Data Interpretation

Biological Response:

Androgen receptor agonism - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of human androgen receptor as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_AR_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.176
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	0.881

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

Assay Documentation

References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)
[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). Journal of Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EAGMST, Extended Advisory Group on Molecular Screening and Toxicogenomics
EDC, Endocrine disrupting chemicals
GST, Glutathione S-Transferase
MRTU, Multiple Reporter Transcription Unit
MIE, Molecular Initiating Event
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

Supporting Information (existing annotations):

ATG_ERa_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Estrogen Receptor Alpha Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by chimeric GAL4-NR transcription factors utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by the yeast GAL4 DNA-binding domain. This family of Attagene assays employ a recently developed profiling technology (Factorial™) which consists of multiple RTU construct sequences that are identical with the exception of processing tag sequences assigned to each TF which create a unique restriction enzyme cleavage site for individual RTUs, and allow for specific determination of NR activity. A specific MRTU paired with a specific chimeric GAL4-NR protein are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19) and, 24 hr after transfection, transfected cells for all 25 nuclear receptors and MRTU's are mixed and plated. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by quantitating transcribed reporter RNA through cDNA synthesis, fluorescent labeling, and restriction enzyme digestion to yield specific reporter products. This trans-format Factorial™ assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by a single reaction creating highly homogeneous detection conditions.

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates

genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The estrogen receptor used in this assay is a partial receptor consisting of the ligand-binding domain and hinge region and may not represent the physiological form of the receptor. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.2. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS Estrogen Receptor alpha assay measures changes in human estrogen-receptor alpha (hERα) activation using the mammalian one-hybrid assay format, which monitored transcriptional activation using hERα ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection, and one replicate of each compound was screened at eight concentrations of each test compound. The presence of agonists/antagonists of ERα alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_ERa_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_ERa_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-ERα proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an ERα-specific reporter sequence. Chemical-ERα activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERa_TRANS assay used a hepatoma cell-based platform to monitor estrogen receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity. This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates (AOP under development), and there is some evidence that estrogen

receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.77
Neutral control median absolute deviation, by plate:	0.50
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
CV (median across all plates):	0.28

(no positive control used for this assay)

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen receptor agonism - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of human estrogen receptor α as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERa_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a signal baseline. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_LUC_BG1_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.09 µM
Standard maximum concentration tested: 200 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.23
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 1.13

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	No
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	Yes

104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No

57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	24	13
Inactive	17	5

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	34	8
Inactive	7	10

In Vitro Sensitivity = 64.9%
In Vitro Specificity = 22.7%
Balanced Accuracy = 43.8%

In Vivo Sensitivity = 81.0%
In Vivo Specificity = 58.8%
Balanced Accuracy = 69.9%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)
[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
ERE, Estrogen Response Element
E2, Estradiol
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

15 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

ATG_ERb_TRANS2_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Estrogen Receptor Beta Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The second series of trans-acting Attagene assays (TRANS2) track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (Factorial™) which consists of multiple RTU construct sequences that are identical with the exception of processing tag sequences assigned to each TF which create a unique restriction enzyme cleavage site for individual RTUs, and allow for specific determination of NR activity. A specific MRTU paired with a specific chimeric GAL4-NR protein are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19) and, 24 hr after transfection, transfected cells for all 25 nuclear receptors and MRTU's are mixed and plated. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by quantitating transcribed reporter RNA through cDNA synthesis, fluorescent labeling, and restriction enzyme digestion to yield specific reporter products. This trans-format Factorial™ assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 23 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by a single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells

also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The estrogen receptor used in this assay is a partial receptor consisting of the ligand-binding domain and hinge region and may not represent the physiological form of the receptor. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None Reported

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS2 Estrogen Receptor beta assay measures changes in estrogen-receptor beta (ERβ) activation using the mammalian one-hybrid assay format, which monitored transcriptional activation using ERβ ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection, and one replicate of each compound was screened at eight concentrations of each test compound. the presence of agonists/antagonists of ERβ alters the transactivation function of Gal4-NR and modulates the reporter signal. Following 24-hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_ERb_TRANS2 series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_ERb_TRANS2 is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-ERβ proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an ERβ-specific reporter sequence. Chemical-ERβ activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERa_TRANS assay used a hepatoma cell-based platform to monitor estrogen receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity. This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates (AOP under development), and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (AOPs under development). Chemical-activity profiles derived from this assay can inform

prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.6625
Neutral control median absolute deviation, by plate:	0.09118
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.14

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen receptor agonism - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of human estrogen receptor β as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERb_TRANS2_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a signal baseline. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor beta activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test

chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_LUC_BG1_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	2
Standard minimum concentration tested:	0.04 µM
Standard maximum concentration tested:	10 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.2125
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.063

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and

ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
ERE, Estrogen Response Element
E2, Estradiol
GST, Glutathione S-Transferase
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

ATG_ERE_CIS_up

Assay Title: Attagene CIS-FACTORIAL HepG2 Estrogen Response Element Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene CIS assays track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual transcription factor response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL™) which consists of cis-regulating element (promoter) binding by RTUs. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and the CIS- format assay measures changes in RTU expression resulting from TF binding to response element DNA-binding sites. Response to 24-hour incubation of test chemicals with cells in a 24-well plate is monitored by examining fluorescent activity produced by transcribed mRNA. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions in a single-replicate 8-point chemical concentration series.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These

transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None Reported

2. Assay Component Descriptions

Assay Objectives:

The Attagene CIS estrogen response element assay measures changes in the mRNA production controlled by a cis-acting element (promoter). Multiple RTU constructs are transfected into the human liver hepatoma cell line HepG2, and the cis- format assay measures changes in RTU expression resulting from endogenous TF binding, i.e. estrogen receptor α or β, to estrogen response element (ERE) DNA-binding sites following 24 hour exposures to the ToxCast chemical library. TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by

quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_CIS series assays consist of multiplexed (FACTORIAL™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP) cDNA and each tagged with *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurable with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. The ATG_ERE_CIS describes MRTU FACTORIAL™ assays reporting activity in endogenous cis-regulatory estrogen response element (ERE) constructs (which are responsive to endogenous human estrogen receptor α and β).

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERE_CIS assay used a hepatoma cell-based platform to monitor estrogen receptor transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds which potentially affect endocrine systems in exposed populations by interacting with estrogen receptor mediated signaling pathways. There is evidence that estrogen receptor activation in early life is a molecular initiating event (MIE) in a developing Adverse Outcome Pathways (AOP) leading to endometrial carcinoma in the mouse (currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER interference in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.88
Neutral control median absolute deviation, by plate:	0.19
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using positive control wells):	NA

Signal-to-noise (median across all plates, using positive control wells): NA
Signal-to-background (median across all plates, using positive control wells): NA
CV (median across all plates): 0.21

(no positive control used for this assay)

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity; increased production of mRNA transcripts production in response to active transcription following TF interaction with ERE promoter sequences as measured by RT_PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERE_CIS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor transactivation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_LUC_BG1_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.09 µM
Standard maximum concentration tested: 200 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.10
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 0.50

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes

480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	28	9
Inactive	17	5

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	36	6
Inactive	9	8

In Vitro Sensitivity = 75.7%

In Vitro Specificity = 22.7%
Balanced Accuracy = 49.2%

In Vivo Sensitivity = 85.7%
In Vivo Specificity = 47.1%
Balanced Accuracy = 66.4%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)
[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)
[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
ERE, Estrogen Response Element

E2, Estradiol
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

ATG_ERRa_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Estrogen-Related Receptor Alpha Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (Factorial^{TM}) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format Factorial^{TM} assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells

also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene Estrogen-Related Receptor alpha TRANS assay measures changes in an ERRα activation using the mammalian one-hybrid assay format. A chimeric yeast GAL4 DNA binding domain-human ERRα ligand binding domain protein regulates expression of a specific RTU through the UAS promoter that binds the GAL4-DNA binding domain. The presence of agonists/antagonists of ERRα alters the transactivation function of Gal4-NR and modulates reporter transcription. ERRs are orphaned nuclear receptors with significant homology to the estrogen receptor family; they share target genes, co-regulators and promoters, and have an almost identical DNA binding domain and bind similarly to EREs in the nucleus, but are responsive to different ligands. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC50) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_ERRa_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_ERRa_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-NR proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an ERRα-specific reporter sequence. Chemical-ERRα activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. Estrogenic DNA binding mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERRa_TRANS assay used a hepatoma cell-based platform to monitor estrogen-related receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.45
Neutral control median absolute deviation, by plate:	0.40
Positive control well median response value, by plate:	NA

Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.27

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity; increased production of mRNA transcripts in response to ligand binding to estrogen-related receptor α as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERRa_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a signal baseline. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. ERR activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_ERRa_TRANS_up
ATG_ERRb_TRANS2_up
ATG_ERRg_TRANS_up

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM

Standard maximum concentration tested:	200.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.24
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

EDC, Endocrine disrupting chemicals

ER, Estrogen Receptor

ERE, Estrogen Response Element
E2, Estradiol
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

ATG_ERRb_TRANS2_up

Assay Title: Attagene TRANS2-FACTORIAL HepG2 Human Estrogen-Related Receptor Beta Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The second series of trans-acting Attagene assays (TRANS2) track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against all 48 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells

also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene Estrogen-Related Receptor beta TRANS2 assay measures changes in an ERRβ activation using the mammalian one-hybrid assay format. A chimeric yeast GAL4 DNA binding domain-human ERRβ ligand binding domain protein regulates expression of a specific RTU through the UAS promoter that binds the GAL4-DNA binding domain. The presence of agonists/antagonists of ERRβ alters the transactivation function of Gal4-NR and modulates reporter transcription. ERRs are orphaned nuclear receptors with significant homology to the estrogen receptor family; they share target genes, co-regulators and promoters, and have an almost identical DNA binding domain and bind similarly to EREs in the nucleus, but are responsive to different ligands. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_ERRβ_TRANS2 series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_ERRβ_TRANS2 is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-NR proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an ERRβ-specific reporter sequence. Chemical-ERRβ activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. Estrogenic DNA-binding mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERRb_TRANS2 assay used a hepatoma cell-based platform to monitor estrogen-related receptor beta transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.72
Neutral control median absolute deviation, by plate:	0.478
Positive control well median response value, by plate:	NA

Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.28

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity; increased production of mRNA transcripts in response to ligand binding of estrogen-related receptor β as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERRβ_TRANS2_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a signal baseline. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. ERR beta activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_ERRa_TRANS_up
ATG_ERRb_TRANS2_up
ATG_ERRg_TRANS_up

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	2
Standard minimum concentration tested:	0.04 µM

Standard maximum concentration tested:	10 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.457
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	2.284

Reference Chemicals / Predictive Capacity:

Rationale for selection of chemical library:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). Journal of Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

EDC, Endocrine disrupting chemicals

ER, Estrogen Receptor

ERE, Estrogen Response Element
E2, Estradiol
GST, Glutathione S-Transferase
MIE, Molecular Initiating Event
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

ATG_ERRg_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Estrogen-Related Receptor Gamma Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (Factorial^{TM}) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format Factorial^{TM} assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells

also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene Estrogen-Related Receptor gamma TRANS assay measures changes in an ERRγ activation using the mammalian one-hybrid assay format. A chimeric yeast GAL4 DNA binding domain-human ERRγ ligand binding domain protein regulates expression of a specific RTU through the UAS promoter that binds the GAL4-DNA binding domain. The presence of agonists/antagonists of ERRγ alters the transactivation function of Gal4-NR and modulates reporter transcription. ERRs are orphaned nuclear receptors with significant homology to the estrogen receptor family; they share target genes, co-regulators and promoters, and have an almost identical DNA binding domain and bind similarly to EREs in the nucleus, but are responsive to different ligands. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_ERRg_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_ERRg_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-NR proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an ERRγ-specific reporter sequence. Chemical-ERRγ activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. Estrogenic DNA-binding mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The ATG_ERRg_TRANS assay used a hepatoma cell-based platform to monitor estrogen-related receptor gamma transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenoestrogenic activity.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	3.31
Neutral control median absolute deviation, by plate:	1.56
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA

Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.47

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity; increased production of mRNA transcripts in response to ligand binding of estrogen-related receptor gamma as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_ERRβ_TRANS2_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a signal baseline. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. ERR gamma activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_ERRa_TRANS_up
ATG_ERRb_TRANS2_up
ATG_ERRg_TRANS_up

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.2777
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.386

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). Journal of Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

EDC, Endocrine disrupting chemicals

ER, Estrogen Receptor

ERE, Estrogen Response Element

E2, Estradiol

GST, Glutathione S-Transferase
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 120

ATG_FXR_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Farnesoid X Receptor Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL™) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export

pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo (dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote FXR mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene's second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS Farnesoid X Receptor alpha assay measures changes in FXR activation using the mammalian one-hybrid assay format, which monitored transcriptional activity of the FXR

ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection. The presence of agonists/antagonists of FXR alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_FXR_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_FXR_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-FXR proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of a FXR-specific reporter sequence. Chemical-FXR activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Farnesoid-X-receptor (FXR) is a ligand-activated nuclear whose primary function is to act as a bile acid sensor, protecting the liver from bile acid toxicity by regulating the transcription of genes involved in bile acid homeostasis [10]. Genes associated with bile acid biosynthesis and recycling (CYP7A1 and IBABP, respectively) are known targets of FXR [11]. FXR has a role in the regulation of glucose and lipid metabolic pathways. FXR is primarily expressed in the liver, kidney, intestine and adrenal cortex, and regulates the expression of target genes by binding as a heterodimer with the retinoid X receptor (RXR). The FXR/RXR heterodimer is "permissive" in that the pair becomes transcriptionally active in the presence of either an RXR-selective ligand or a FXR ligand [8]. The FXR-RXR heterodimer, when bound to DNA, can act as transcriptional activators or inhibitors. FXR is activated by bile acids and the main endogenous ligand for FXR is chenodeoxycholic acid (CDCA). FXR reduces bile acid concentration in the liver by repressing genes involved in bile acid synthesis and regulates lipid metabolism. Numerous studies have reported that FXR exerts protective function during cholestasis, diabetes, liver regeneration, and cancer. The ATG_FXR_TRANS assay used a hepatoma cell-based platform to monitor farnesoid X receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for endocrine disrupting activity via interactions with farnesoid X receptor.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system

enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.24
Neutral control median absolute deviation, by plate:	0.286
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.23

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of farnesoid X receptor as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_FXR_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up

NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.169
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	0.846

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)
[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Kim, I., et al. (2007). J Lipid Res 48(12): 2664-2672. (PMID: 17720959)
[11]Laudet, V. and H. Gronemeyer (2001). The Nuclear Receptor Factsbook, Gulf Professional Publishing.
[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
FXR, Farnesoid-X-Receptor
GST, Glutathione-S-Transferase
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

20 October 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 85

ATG_IR1_CIS_up

Assay Title: Attagene CIS-FACTORIAL HepG2 Farnesoid X Response Element IR1 Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene CIS assays track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual transcription factor response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL™) which consists of cis-regulating element (promoter) binding by RTUs. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and the CIS- format assay measures changes in RTU expression resulting from TF binding to response element DNA-binding sites. Response to 24-hour incubation of test chemicals with cells in a 24-well plate is monitored by examining fluorescent activity produced by transcribed mRNA. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions in a single-replicate 8-point chemical concentration series.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2 to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These

transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote FXR-IR1 mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene CIS IR1 FXR response element assay measures changes in the mRNA production controlled by a cis-acting element (promoter). Multiple RTU constructs are transfected into the human liver hepatoma cell line HepG2, and the cis- format assay measures changes in RTU expression resulting from endogenous TF binding, i.e. FXR ligand binging to inverted repeat sequences separated by 1 base pair (IR1) which are responsive to FXR-RXR heterodimer formation, following 24 hour exposures to the ToxCast chemical library. TF activity was reported via cDNA

synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Half-maximal activity (IC50) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_CIS series assays consist of multiplexed (FACTORIAL) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP) cDNA and each tagged with HpaI restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurable with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. The ATG_IR1_CIS describes MRTU FACTORIAL assays reporting activity in endogenous cis-regulatory FXR response element (IR1) constructs.

Scientific Principles:

Farnesoid-X-receptor (FXR) is a ligand-activated nuclear whose primary function is to act as a bile acid sensor, protecting the liver from bile acid toxicity by regulating the transcription of genes involved in bile acid homeostasis [10]. Genes associated with bile acid biosynthesis and recycling (CYP7A1 and IBABP, respectively) are known targets of FXR [11]. FXR has a role in the regulation of glucose and lipid metabolic pathways. FXR is primarily expressed in the liver, kidney, intestine and adrenal cortex, and regulates the expression of target genes by binding as a heterodimer with the retinoid X receptor (RXR). The FXR/RXR heterodimer is "permissive" in that the pair becomes transcriptionally active in the presence of either an RXR-selective ligand or a FXR ligand [8]. The FXR-RXR heterodimer, when bound to DNA, can act as transcriptional activators or inhibitors. FXR is activated by bile acids and the main endogenous ligand for FXR is chenodeoxycholic acid (CDCA). FXR reduces bile acid concentration in the liver by repressing genes involved in bile acid synthesis and regulates lipid metabolism. Numerous studies have reported that FXR exerts protective function during cholestasis, diabetes, liver regeneration, and cancer. The ATG_FXR_TRANS assay used a hepatoma cell-based platform to monitor farnesoid X receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for endocrine disrupting activity via interactions with farnesoid X receptor.

Method Development Reference:

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.633
Neutral control median absolute deviation, by plate:	0.156
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z’ (median across all plates, using positive control wells):	NA
Z’ (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA

Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.25

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to DNA binding of farnesoid X receptor response element IR1 as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_IR1_CIS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR transactivation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_FXR_TRANS_up
NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.0976
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	0.488

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). Journal of Experimental Therapeutics and Oncology 6(4): 335-348. (PMID: 18038766)

[10] Kim, I., et al. (2007). J Lipid Res 48(12): 2664-2672. (PMID: 17720959)

Laudet, V. and H. Gronemeyer (2001). The Nuclear Receptor Factsbook, Gulf Professional Publishing.

[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

EDC, Endocrine disrupting chemicals
FXR, Farnesoid-X-Receptor
GST, Glutathione-S-Transferase
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

6 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

ATG_PPARa_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Peroxisome Proliferator-activated Receptor Alpha (PPARα) Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL^{TM}) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export

pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95 °C, 20 s at 68 °C and 10 min at 72 °C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37 °C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote PPAR mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS Peroxisome Proliferator-Receptor (PPAR) α assay measures changes in human PPAR alpha activation using the mammalian one-hybrid assay format, which monitored transcriptional activity of the AR ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection. The presence of AR agonists alters the transactivation function of Gal4-AR RTU and modulates reporter transcription. Following 24-hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures used). Following MTC determination, concentration-response assays where conducted by exposing a single replicate to a 6-8 point concentration series, starting at the MTC and followed by 3-fold serial dilutions. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls.

ATG_AR_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_AR_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-AR proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of an AR-specific reporter sequence. Chemical-AR activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated nuclear receptor which regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of energy homeostasis. PPARα plays a crucial role in the regulation of proteins involved in fatty acid transport and hepatic uptake, is expressed predominantly in metabolically active tissues, including liver, kidney, skeletal muscle, and brown fat and is a target for hyperlipidemia drugs, fatty acids (and their derivative eicosanoids) and xenobiotics. The ATG_PPARa_TRANS assay used a hepatoma cell-based platform to monitor PPARα transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenobiotic PPARα ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARa) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that PPARa activation in utero is the molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to impaired fertility in males (AOP Under EAGMST Review), and there is some evidence that PPARa activation is the MIE for a putative pathways leading to hepatocellular adenomas and carcinomas and may

be involved in increased pancreatic acinar tumors (in mouse and rat models) (AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPARa activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	3.455
Neutral control median absolute deviation, by plate:	1.334
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.39

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPARα agonism; nuclear steroid hormone receptor initiated production of mRNA transcripts in response to ligand binding of peroxisome proliferator-activated receptor alpha as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_PPARa_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR alpha activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss

functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publically available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
NVS_NR_hPPARa

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.24
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.17

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)
[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)
[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
GST, Glutathione S-Transferase
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
PPAR, Peroxisome Proliferator-Activated Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 September 2016

Date of Revisions:

21 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

ATG_PPARd_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Peroxisome Proliferator-activated Receptor Delta (PPARd) Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL^{TM}) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24 hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export

pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

Factorial™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS peroxisome proliferator-activated receptor delta (PPARδ) assay measures changes in human PPARδ activation using the mammalian one-hybrid assay format, which monitored transcriptional activation using PPARδ ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection, and one replicate of each compound was screened at eight concentrations of each test compound. The presence of agonists/antagonists of PPARδ alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_PPARd_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_PPARd_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-ERα proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of a PPARδ-specific reporter sequence. Chemical-PPARδ activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Peroxisome proliferator-activated receptor delta is a ligand-activated nuclear receptor which is expressed ubiquitously and may have a role in regulating the differentiation of adipocytes, in keratinocyte differentiation and in the regulation of cholesterol and lipid metabolism [10, 11]. The ATG_PPARd_TRANS assay used a hepatoma cell-based platform to monitor PPARδ transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenobiotic PPARδ ligand-binding activity.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.781
Neutral control median absolute deviation, by plate:	0.337
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA

Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.43

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPARd agonism; nuclear steroid hormone receptor initiated production of mRNA transcripts in response to ligand binding of peroxisome proliferator-activated receptor delta as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_PPARd_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR delta receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
Tox21_PPARd_BLA_agonist_ratio
Tox21_PPARd_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.225
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.123

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). Journal of Experimental Therapeutics and Oncology 6(4): 335-348. (PMID: 18038766)

[10] Schmuth, M., et al. (2004). Journal of Investigative Dermatology 122(4): 971-983.

[11] Seimandi, M., et al. (2005). Anal Biochem 344(1): 8-15. (PMID: 16038868)

[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

GST, Glutathione S-Transferase

MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
PPAR, Peroxisome Proliferator-Activated Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 September 2016

Date of Revisions:

21 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

ATG_PPARg_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Peroxisome Proliferator-activated Receptor Gamma (PPARg) Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL^{TM}) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export

pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

Factorial™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

Attagene second generation of TRANS-FACTORIAL assays were conducted with an expanded NR platform, which covers all 48 human NRs.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS peroxisome proliferator-activated receptor gamma (PPARγ) assay measures changes in human PPARγ activation using the mammalian one-hybrid assay format, which monitored transcriptional activation using PPARγ ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection, and one replicate of each compound was screened at eight concentrations of each test compound. The presence of agonists/antagonists of PPARγ alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24-hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_PPARg_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_PPARg_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-ERα proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of a PPARγ-specific reporter sequence. Chemical-PPARγ activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor which regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of energy homeostasis. PPARγ is primarily expressed in adipose tissue, macrophages and in the colon where it controls adipocyte differentiation, lipid storage and inflammatory responses. PPARγ agonists, the thiazolidinediones (TZDs), improve insulin sensitivity, lower glucose levels, and lower plasma triglycerides and free fatty acid (FFA) levels by enhancing their uptake into adipocytes. The ATG_PPARg_TRANS assay used a hepatoma cell-based platform to monitor PPARγ transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for xenobiotic PPARγ ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARg) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is some evidence to support a putative AOP linking PPAR gamma receptor activation with increased occurrence of sarcomas in rats, mice, and hamsters (AOP currently under development). Chemical-activity profiles derived from this assay

can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPAR activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	2.66
Neutral control median absolute deviation, by plate:	1.08
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.41

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPARg agonism; nuclear steroid hormone receptor initiated production of mRNA transcripts in response to ligand binding of peroxisome proliferator-activated receptor gamma as measured by RT-PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_PPARg_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR gamma activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical.

Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
NVS_NR_hPPARg
OT_PPARg_PPARgSRC1_0480
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_Agonist_ratio
Tox21_PPARg_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.235
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.17

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)
[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)
[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)
[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)
[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)
[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)
[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)
[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
GST, Glutathione S-Transferase
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
PPAR, Peroxisome Proliferator-Activated Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
TF, Transcription Factor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 September 2016

Date of Revisions:

21 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 102

ATG_PPRE_CIS_up

Assay Title: Attagene CIS-FACTORIAL HepG2 Peroxisome Proliferator-activated Response Element Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene CIS assays track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual transcription factor response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL^{TM}) which consists of cis-regulating element (promoter) binding by RTUs. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and the CIS- format assay measures changes in RTU expression resulting from TF binding to response element DNA-binding sites. Response to 24-hour incubation of test chemicals with cells in a 24-well plate is monitored by examining fluorescent activity produced by transcribed mRNA. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions in a single-replicate 8-point chemical concentration series.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo(dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

FACTORIAL™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

Due to low expression levels of estrogen receptor in HepG2 cells, a full-length, human estrogen receptor α cDNA was co-transfected in to the cells together with the MRTUs. The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene CIS peroxisome proliferator-activated response element assay measures changes in the mRNA production controlled by a cis-acting element (promoter) (PPRE). Multiple RTU constructs are transfected into the human liver hepatoma cell line HepG2, and the cis- format assay

measures changes in RTU expression resulting from endogenous TF binding, i.e. PPAR activation, to response element (PPRE) DNA-binding sites following 24 hour exposures to the ToxCast chemical library. TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Modulation of PPRE activity was monitored using a positive control of Rosiglitazone. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_CIS series assays consist of multiplexed (FACTORIAL™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP) cDNA and each tagged with *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurable with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. The ATG_PPRE_CIS describes MRTU FACTORIAL™ assays reporting activity in endogenous cis-regulatory peroxisome proliferator-activated response element (PPRE) constructs.

Scientific Principles:

Peroxisome proliferator-activated receptors are transcription factors belonging to the family of ligand-inducible nuclear receptors; they regulate the expression of genes involved in many cellular and metabolic processes, and are involved in fatty acid-oxidation, energy homeostasis and function in epidermal differentiation and repair. They regulate glucose, lipid, and cholesterol metabolism in response to fatty acids and their derivatives, eicosanoids, and are targets for drugs used in the treatment of hyperlipidemia and diabetes. There are three distinct subtypes; PPAR alpha, PPAR delta (also called PPAR beta) and PPAR gamma, each of which show distinct tissue distribution and ligand preference. All these subtypes heterodimerize with Retinoid X receptor (RXR) and these heterodimers regulate transcription of various genes. PPARα is expressed predominantly in metabolically active tissues, including liver, kidney, skeletal muscle, and brown fat, and its ligands include fatty acids, hypolipidemic drugs, and xenobiotics, where PPARβ/δ is the target for antidiabetic agents of the thiazolidinedione class, and is expressed ubiquitously, and may be involved in the regulation of cholesterol and lipid metabolism. PPARγ is highly expressed in adipocytes, is involved in control of lipid storage and is thought to be important in the induction of adipogenesis. The ATG_PPRE_CIS assay used a hepatoma cell-based platform to monitor gene expression resulting from PPAR response element activation which is regulated by PPAR receptor ligand-binding activity and this assay is designed to help identify environmental compounds with a capacity for xenobiotic PPAR ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARa) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that PPARa activation in utero is the molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to impaired fertility in males (AOP Under EAGMST Review), and there is some evidence that PPARa activation

is the MIE for a putative pathways leading to hepatocellular adenomas and carcinomas and may be involved in increased pancreatic acinar tumors (both AOPs based on evidence in mouse and rat models) (AOPs currently under development). There is also a putative AOP linking PPAR gamma receptor activation with increased occurrence of sarcomas in rats, mice, and hamsters (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPAR activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.26
Neutral control median absolute deviation, by plate:	0.627
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.5

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity – mRNA transcript production in response to active transcription following TF interaction with PPRE promoter sequences as measured by RT_PCR and capillary electrophoretic detection of fluorescently labeled mRNA.

Analytical Elements:

ATG_PPRE_CIS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Negative and zero values are removed before analysis and raw values are log transformed. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR receptor transactivation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise

band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPARa_TRANS_up
ATG_PPARd_TRANS_up
ATG_PPARg_TRANS_up
NVS_NR_hPPARa
NVS_NR_hPPARg
OT_PPARg_PPARgSRC1_0480
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_Agonist_ratio
Tox21_PPARd_BLA_agonist_ratio
Tox21_PPARd_BLA_antagonist_ratio
Tox21_PPARg_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.180
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	0.900

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical

inventory used in this assay includes the “e1k” chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

GST, Glutathione S-Transferase

MRTU, Multiple Reporter Transcription Unit

NR, Nuclear Receptors

PPAR, Peroxisome Proliferator-Activated Receptors

RTU, Reporter Transcription Unit

SEAP, Secreted Embryonic Alkaline Phosphatase

SULT, Sulfotransferases

TF, Transcription Factor

UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

12 September 2016

Date of Revisions:

22 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 143

ATG_THRa1_TRANS_up

Assay Title: Attagene TRANS-FACTORIAL HepG2 Human Thyroid Receptor Alpha Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Attagene TRANS assay tracks changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL™) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (variant HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against 25 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST μ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo (dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

Factorial™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote thyroid receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS Thyroid Receptor alpha assay measures changes in thyroid-receptor alpha (TRα) activation using the mammalian one-hybrid assay format, which monitored transcriptional activity of the TRα ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection. The presence of

agonists/antagonists of TRα alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the RTU sequences followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_TRa_TRANS series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_THRa1_TRANS is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-TRα proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of a TRα-specific reporter sequence. Chemical-TRα activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting thyroid hormone signaling pathways. The thyroid receptor mediates gene expression in response to thyroid hormones T3 and T4, and modulates the activity for a wide variety of physiological processes. The ATG_THRa1_TRANS assay used a hepatoma cell-based platform to monitor thyroid receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for endocrine disrupting activity via interactions with thyroid receptor.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.9215
Neutral control median absolute deviation, by plate:	0.2454
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA

Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.27

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of thyroid receptor α.

Analytical Elements:

ATG_THRa1_TRANS_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Each chemical is run as a single replicate in a 6-point concentration series, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Thyroid receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_dn
LTEA_HepaRG_THRSP_up
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Agonist
Tox21_TR_LUC_GH3_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	7
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.09 µM
Standard maximum concentration tested:	200.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.225
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.127

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
GST, Glutathione S-Transferase
MRTU, Multiple Reporter Transcription Unit
NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
T3, Triiodothyronine
T4, Thyroxine
TF, Transcription Factor

TH, Thyroid Hormone
TR, Thyroid Receptor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

20 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

ATG_THRb_TRANS2_up

Assay Title: Attagene TRANS2-FACTORIAL HepG2 Human Thyroid Receptor Beta Activation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The second series of trans-acting Attagene assays (TRANS2) track changes in transcription factor (TF) activity in response to chemical perturbations by utilizing a library of multiple reporter transcription unit (MRTU) constructs regulated by individual TF response elements. This family of Attagene assays employ a recently developed profiling technology (FACTORIAL) which consists of trans-acting TF DNA binding sites. The multiple RTU construct sequences are identical with the exception of processing tag sequences assigned to each TF which create a unique cleavage site for individual RTUs, and allow for precise determination of NR activity. The MRTUs are transfected into an in-house clone of human liver hepatoma cell line HepG2 (HG19), and each RTU expresses a chimeric GAL4-NR protein that regulates transcription of a reporter sequence. Nuclear receptor binding by exogenous compounds alters the transactivation function of Gal4-NR and modulates reporter transcription. The chemical-NR activity is monitored by examining fluorescent activity produced by transcribed mRNA. This trans-format FACTORIAL assay was used to evaluate agonistic/antagonistic properties of the ToxCast chemical library against all 48 human nuclear receptors following 24-hour incubation with cells in a 24-well plate in a single-replicate 8-point concentration series. All reporters are detected simultaneously in the same assay well and by single reaction creating highly homogeneous detection conditions.

1.2. Assay Definition

Assay Throughput:

Transfected HepG2 cells are aliquoted into 24-well microtiter plates and incubated with test compounds for 24 hours prior to PCR detection of total RNA transcription using capillary electrophoresis.

Experimental System:

The HepG2 cell line is a permanent cell culture isolated from the liver tumor lobectomy of a 15-yr-old Caucasian male from Argentina in 1975 [1], which has been cloned and transfected with a library of multiple reporter transcription units (– see section 2, Assay Component Descriptions, for detailed definition of MRTUs).

Xenobiotic Biotransformation Potential:

The HepG2 cells used in this assay are variant HG19, a cell line selected for enhanced xenobiotic metabolism. These cells express 2- to 13 times more cytochrome P450 activity than parental HepG2 (Attagene, personal communication). The parental HepG2 cell line has been shown by others to retain the potential for Phase I and Phase II metabolic responses to xenobiotics, e.g., expression of CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6/3A, 2E1, and 3A4/5 [2] with CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A activities reported at levels similar to human hepatocytes although variable depending on source and culture conditions [3]; some enzymes (e.g., CYP2W1) have even been observed at higher rates than in primary hepatocytes [4]. Phase II enzyme activities identified in HepG2 cells include SULTS (1A1, 1A2, 1E1 and 2A1), GSTs (mGST-1, GST µ1), NAT1, EPHX1 [5-7] and UGTs (1A1, 1A6 and 2B7) [7]. In addition, HepG2 cells can potentially express xenobiotic regulation activities via functionally active p53 protein [8] and Nrf2, a transcription factor which regulates genes containing antioxidant response element (ARE) sequences in their promoters; HepG2 cells also possess the capacity to express a number of ATP-binding cassette (ABC) xenobiotic export

pumps (e.g., ABCC1, C2, C3 and G2 – membrane-bound proteins also regulated in part by Nrf2 TF DNA-binding) [9].

Basic Procedure:

Human liver HepG2 cells are transiently transfected with multiple reporter transcription units (MRTUs) in 6-well plates using FuGene 6 reagent (Roche; 3 ml FuGene/1 mg DNA). These transfected cells are aliquoted into wells of a 24-well plate, exposed to the chemical library of environmental compounds for 24 hours, and total RNA was isolated using TriZol reagent (Invitrogen). RNA was reverse-transcribed using oligo (dT) primer and Mo-MLV reverse transcriptase (Invitrogen) and treated with DNAse I (Ambion) for 30 min. Then one-tenth of the produced cDNA was amplified by PCR using Taq DNA polymerase (Invitrogen) and two reporter sequence–specific primers. The PCR products were fluorescently labeled by primer extension with 6-arboxyfluorescein (6-FAM) 5'-labeled reporter sequence–specific primer (2 min at 95°C, 20 s at 68°C and 10 min at 72°C) and these products were digested with 5U of *Hpa*I (New England Biolabs) for 2h at 37°C. The fragments were purified using Qiaquick PCR columns (Qiagen), analyzed on an ABI 3130xL genetic analyzer (Applied Biosystems) with peak positions identified by using a set of X-rhodamine (ROX)-labeled MapMarker1000 molecular weight standards (BioVentures). The raw capillary electrophoresis data was processed using Attagraph software (Attagene).

Proprietary Elements:

Factorial™ is a novel pathway profiling technology trademarked and patented by Attagene, Inc.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote thyroid receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Attagene, Inc.
7020 Kit Creek Road Suite 260
Morrisville, NC 27560
Tel: (888)721-2121

Assay Publication Year:

2010

Assay Publication:

Martin, M. T., Dix, D. J., Judson, R. S., Kavlock, R. J., Reif, D. M., Richard, A. M., Rotroff, D. M., Romanov, S., Medvedev, A., Poltoratskaya, N., Gambarian, M., Moeser, M., Makarov, S. S., & Houck, K. A. (2010). Impact of environmental chemicals on key transcription regulators and correlation to toxicity end points within EPA's ToxCast program. Chem Res Toxicol, 23(3), 578-590. (PMID: 20143881)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Attagene TRANS2 Thyroid Receptor beta assay measures changes in thyroid-receptor β (TRβ) activation using the mammalian one-hybrid assay format, which monitored transcriptional

activation using TRβ ligand-binding domain (LBD) fused with the yeast GAL4 DNA-binding domain (DBD) transiently transfected into HepG2 cells with a 5X upstream activator sequence (UAS) reporter. Each individual trans-RTU system (receptor and reporter gene) was separately transfected into the cell, followed by pooling and plating of the transfected cells prior to detection. The presence of agonists/antagonists of TRβ alters the transactivation function of Gal4-NR and modulates reporter transcription. Following 24 hour incubation with compounds from the ToxCast chemical library, TF activity was reported via cDNA synthesis and RT-PCR of the fluorescently-labeled MRTU mRNA followed by quantitation by capillary electrophoresis. Half-maximal activity (IC_{50}) was determined for each chemical using changes in transcription factor activity expressed as log2 fold induction over DMSO controls in a single replicate, 8-point concentration response series. Cytotoxicity assessments were performed concurrently using a MTT tetrazolium assay to establish maximum tolerated concentrations (MTC) (see ATG_XTT_Cytotoxicity assay description for MTT procedures).

ATG_THRb_TRANS2 series assays consist of multiplexed (Factorial™) assay endpoints which assessed transcription factor activity using reporter transcription unit (RTU) quantitation in the HepG2 human liver hepatoma cell line. The multiple RTUs were constructed from essentially identical reporter gene sequences (representing a functionally inactivated fragment of human SEAP cDNA) and each tagged with unique *Hpa*I restriction cleavage sites at variable positions to allow discrimination of individual TF activities. When co-introduced into HepG2 cells, the RTUs produce reporter RNAs in amounts commensurate with the activities of the corresponding TFs present in a cell. This homogeneous construct creates a uniform environment for detecting the activity of many transcription factors simultaneously, and allowing for a more detailed assessment of xenobiotic impacts on the cellular regulatory network. ATG_THRb_TRANS2 is a MRTU Factorial™ assay reporting activity in exogenous, chimeric Gal4-TRβ proteins that bind to a GAL4-specific upstream activation sequence promoter (5X-UAS-TATA) and regulate the transcription of a TRβ-specific reporter sequence. Chemical-THRβ activity can be monitored by measuring changes in fluorescence relative to DMSO controls.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting thyroid hormone signaling pathways. The thyroid receptor mediates gene expression in response to T3 / T4, and modulates the activity for a wide variety of physiological processes. The ATG_THRb_TRANS2 assay used a hepatoma cell-based platform to monitor thyroid receptor alpha transcriptional activity and this assay is designed to help identify environmental compounds with a capacity for endocrine disrupting activity.

Method Development Reference:

Romanov, S., Medvedev, A., Gambarian, M., Poltoratskaya, N., Moeser, M., Medvedeva, L., Gambarian, M., Diatchenko, L., & Makarov, S. (2008). Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. Nat Methods, 5(3), 253-260. (PMID: 18297081)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.303
Neutral control median absolute deviation, by plate:	0.0385
Positive control well median response value, by plate:	NA
Positive control well median absolute deviation, by plate:	NA
Negative control well median, by plate:	NA

Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	NA
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	NA
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	NA
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	NA
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.13

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Transcription factor activity - nuclear steroid hormone receptor initiated pathway production of mRNA transcripts in response to ligand binding of thyroid receptor β.

Analytical Elements:

ATG_THRb_TRANS2_up readout data was analyzed in the positive (gain of signal) fitting direction using log2 fold-induction over DMSO controls which provide a baseline signal. Each chemical is run as a single replicate in a 6-point concentration series, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Thyroid receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up
LTEA_HepaRG_THRSP_dn
LTEA_HepaRG_THRSP_up
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Agonist
Tox21_TR_LUC_GH3_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	2
Standard minimum concentration tested:	0.04 µM

Standard maximum concentration tested:	10.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.278
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	1.390

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Aden, D. P., et al. (1979). Nature 282: 615-616. (PMID: 233137)

[2] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1581-1591. (PMID: 17637504)

[3] Hewitt, N. and P. Hewitt (2004). Xenobiotica 34(3): 243-256. (PMID: 15204697)

[4] Guo, L., et al. (2010). Drug Metab Disposition 39(3): 528-538. (PMID: 21149542)

[5] Westerink, W. M. and W. G. Schoonen (2007). Toxicol In Vitro 21(8): 1592-1602. (PMID: 17716855)

[6] Walle, T., et al. (2000). Drug Metab Disposition 28(9): 1077-1082. (PMID: 10950852)

[7] Hart, S. N., et al. (2010). Drug Metab Disposition 38(6): 988-994. (PMID: 20228232)

[8] Boehme, K., et al. (2010). Toxicol Lett 198(2): 272-281. (PMID: 20655369)

[9] Adachi, T., et al. (2007). J Exper Therap Oncol 6(4): 335-348. (PMID: 18038766)

[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

ATCC, American Tissue Culture Collection

CYP, Cytochrome P450s

DMSO, Dimethyl Sulfoxide

EDC, Endocrine disrupting chemicals

GST, Glutathione S-Transferase

MRTU, Multiple Reporter Transcription Unit

NR, Nuclear Receptors
RTU, Reporter Transcription Unit
SEAP, Secreted Embryonic Alkaline Phosphatase
SULT, Sulfotransferases
T3, Triiodothyronine
T4, Thyroxine
TF, Transcription Factor
TH, Thyroid Hormone
TR, Thyroid Receptor
UGT, UDP-Glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

20 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

CEETOX_H295R_11DCORT_dn

Assay Name: CeeTox H295R High-throughput Steroidogenesis Assay – 11-Deoxycortisol Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

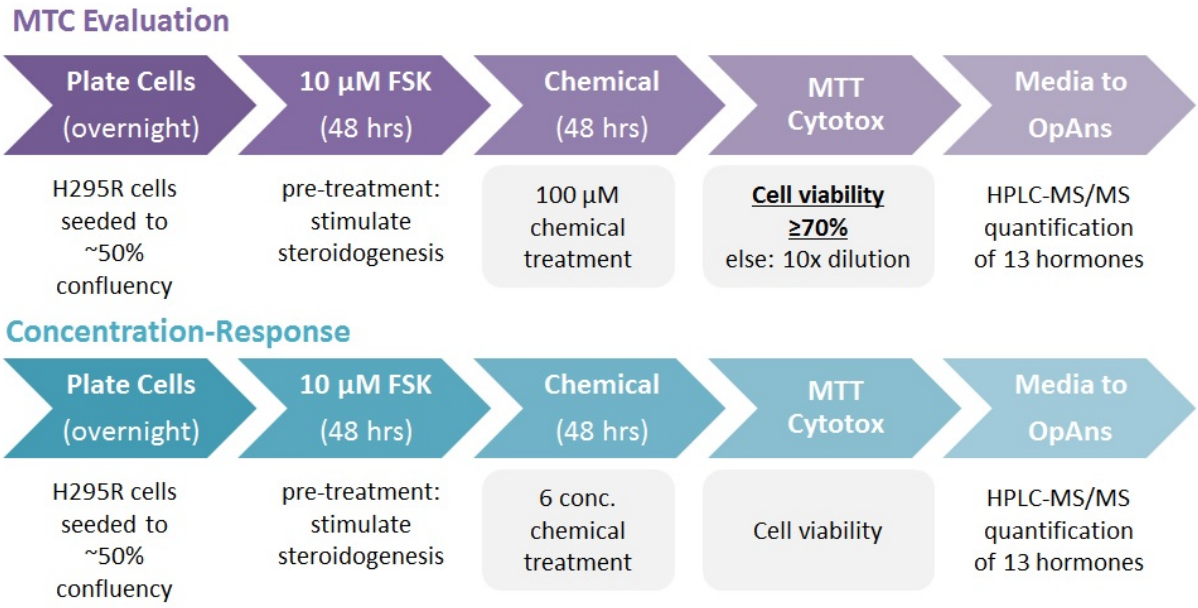


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 11-deoxycortisol assay was used to screen a large chemical library for changes in 11-deoxycortisol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for 11-deoxycortisol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of 11-deoxycortisol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 11-deoxycortisol were reported as 5 ng/mL and 1000 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 5.0% and accuracy of 101.7% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

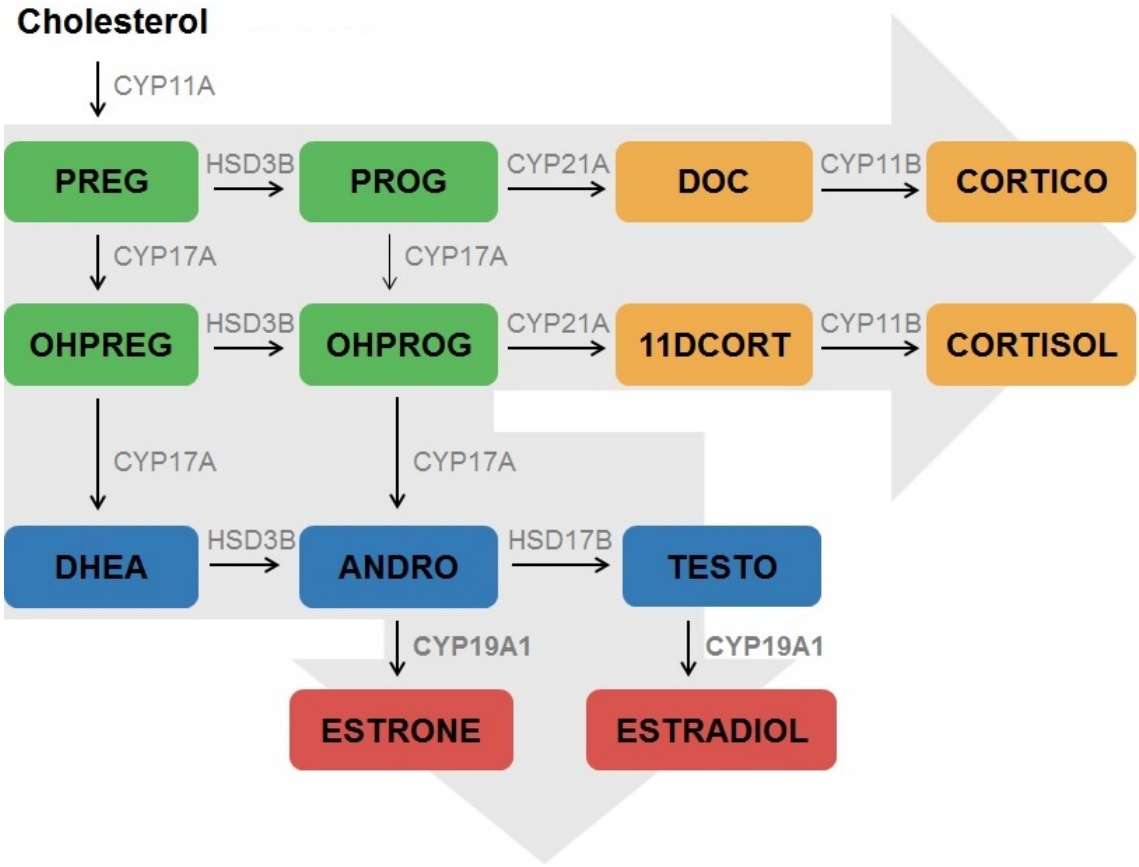


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 15 for 11DCORT. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -27 for 11DCORT. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of 11-deoxycortisol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_11DCORT_dn readout data was analyzed in the negative (loss of signal) fitting direction, using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in 11-deoxycortisol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. 11-deoxycortisol inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 1.50 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_11-DEOXYCORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn

CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For 11DCORT, the $r^2 = 0.82$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)

[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information (existing annotations):

CEETOX_H295R_11DCORT_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – 11-deoxycortisol Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

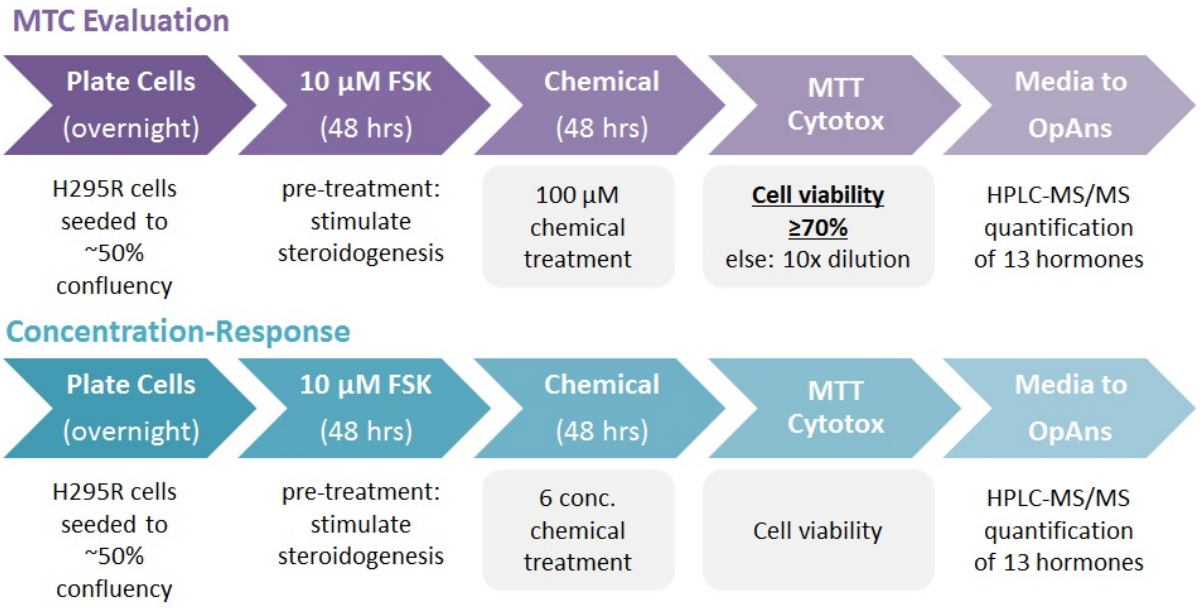


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 11-deoxycortisol assay was used to screen a large chemical library for changes in 11-deoxycortisol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application a three-tiered screening approach was employed The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for 11-deoxycortisol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of 11-deoxycortisol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 11-deoxycortisol were reported as 5 ng/mL and 1000 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 5.0% and accuracy of 101.7% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis) resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

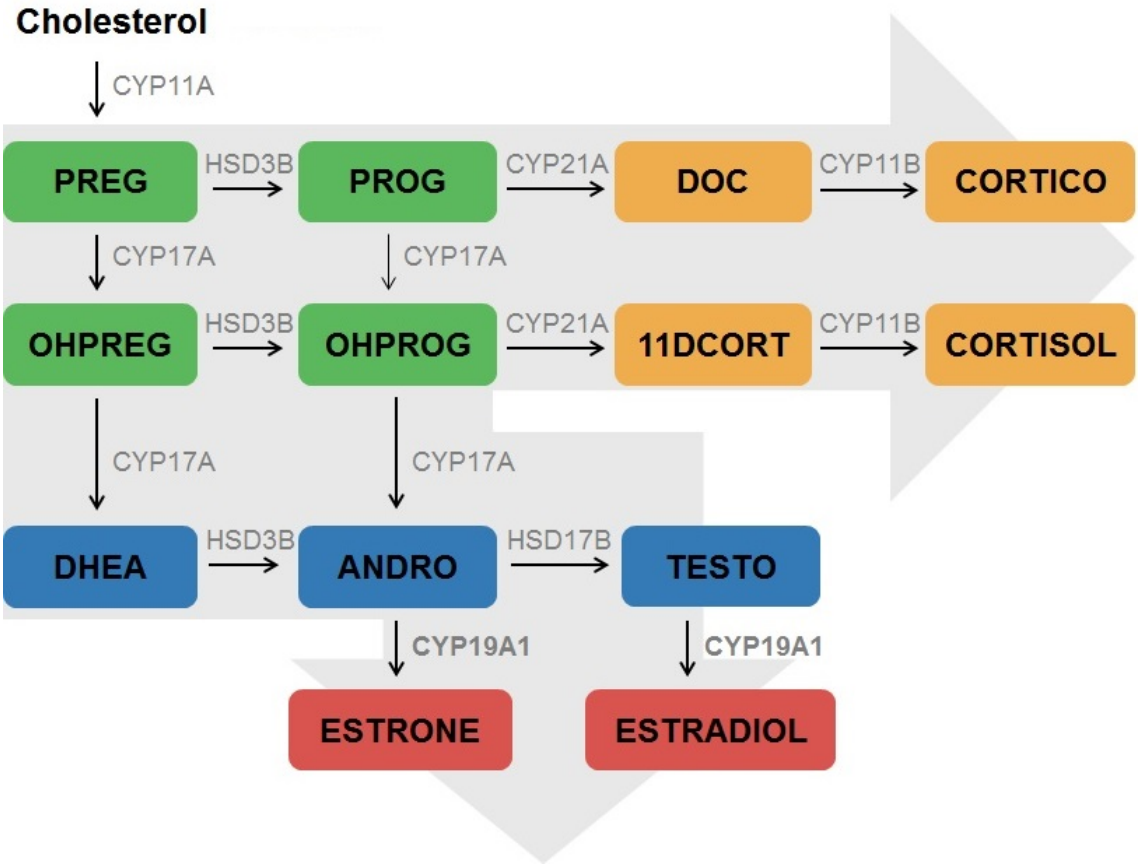


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 15 for 11DCORT. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -27 for 11DCORT. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of 11-deoxycortisol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_11DCORT_up readout data was analyzed in the positive (gain of signal) fitting direction, using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in 11-deoxycortisol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were $\log_2$ transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. 11-deoxycortisol stimulation was determined based on a chemical fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in $\log_2$ fold-change which was approximately 1.50 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_11-DEOXYCORTISOL_dn
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up

CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For 11DCORT, the $r^2 = 0.82$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)

[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

Assay Endpoint ID: 896

CEETOX_H295R_ANDR_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Androstenedione Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

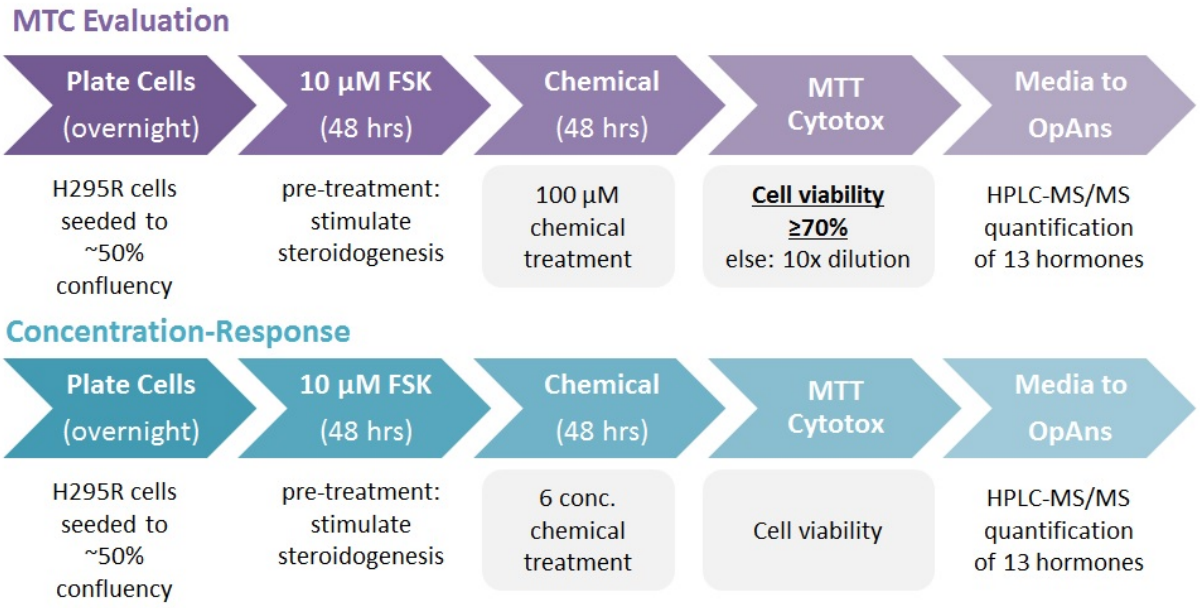


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R androstenedione assay was used to screen a large chemical library for changes in androstenedione levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for androstenedione quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of androstenedione from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for androstenedione were reported as 1 ng/mL and 200 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 4.7% and accuracy of 99.9% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

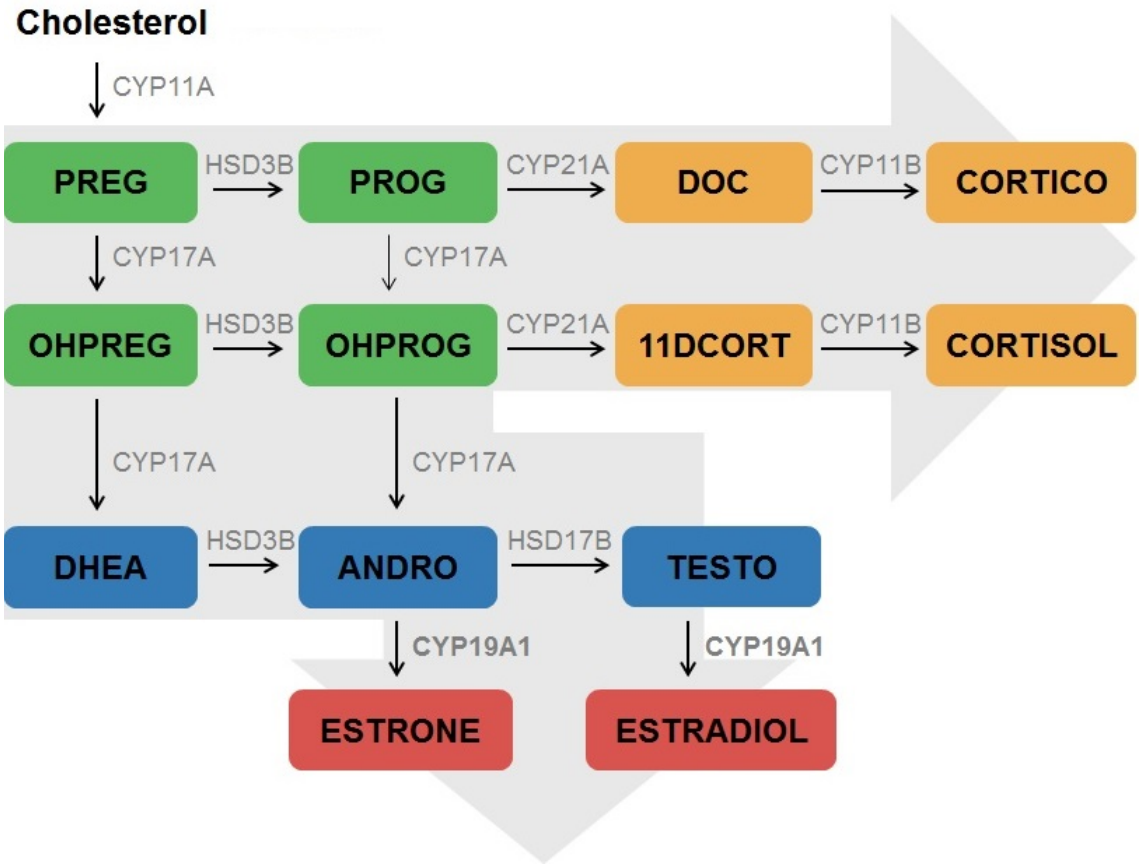


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.68 for androstenedione. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.84 for androstenedione. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 11 for androstenedione. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -19 for androstenedione. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of androstenedione following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ANDROSTENEDIONE_dn readout data was analyzed in the negative (loss of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in androstenedione activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Androstenedione inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; which was approximately a 1.70 fold-change cut-off); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up

CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For androstenedione, the $r^2 = 0.81$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

13 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

Assay Endpoint ID: 897

CEETOX_H295R_ANDR_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Androstenedione Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

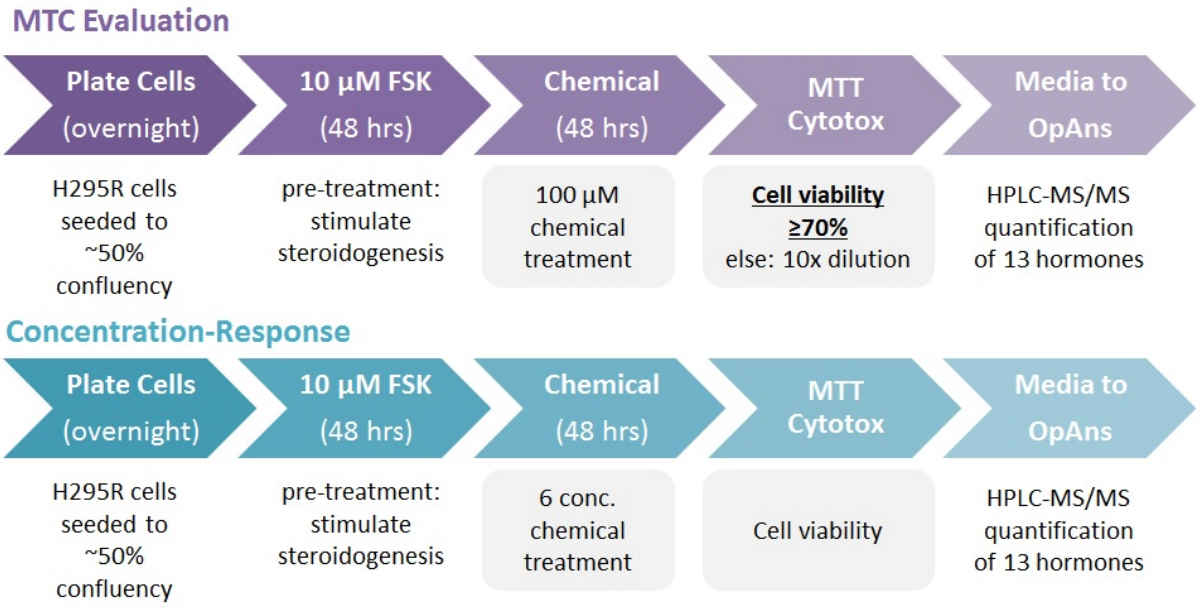


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R androstenedione assay was used to screen a large chemical library for changes in androstenedione levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for androstenedione quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of androstenedione from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for androstenedione were reported as 1 ng/mL and 200 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 4.7% and accuracy of 99.9% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

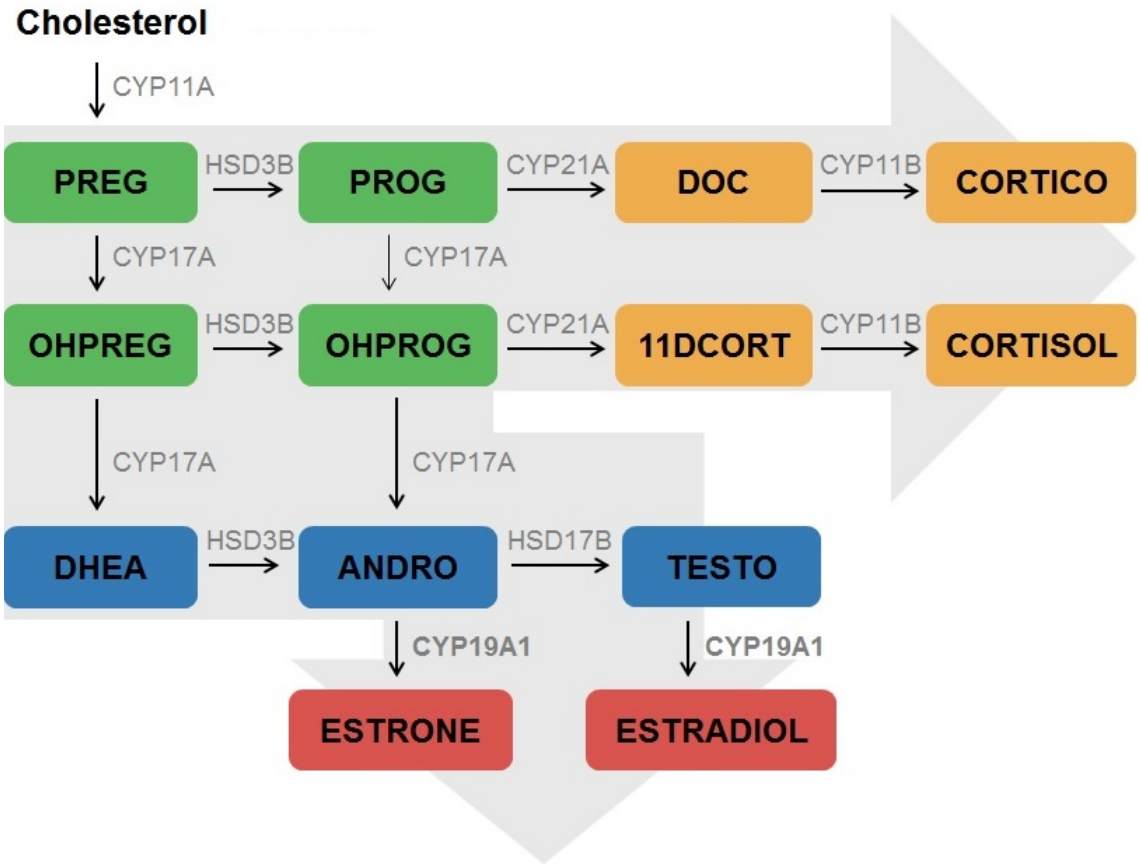


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.68 for androstenedione. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.84 for androstenedione. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 11 for androstenedione. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -19 for androstenedione. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of androstenedione following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ANDROSTENEDIONE_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls (baseline signal), and was reported as log2 fold-change increase in androstenedione activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androstenedione stimulation was determined based on a chemical fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the androstenedione assay this created a 1.70 fold-change cut-off); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up

CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median $\log_2$-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For androstenedione, the $r^2 = 0.81$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

CEETOX_H295R_CORTIC_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Corticosterone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

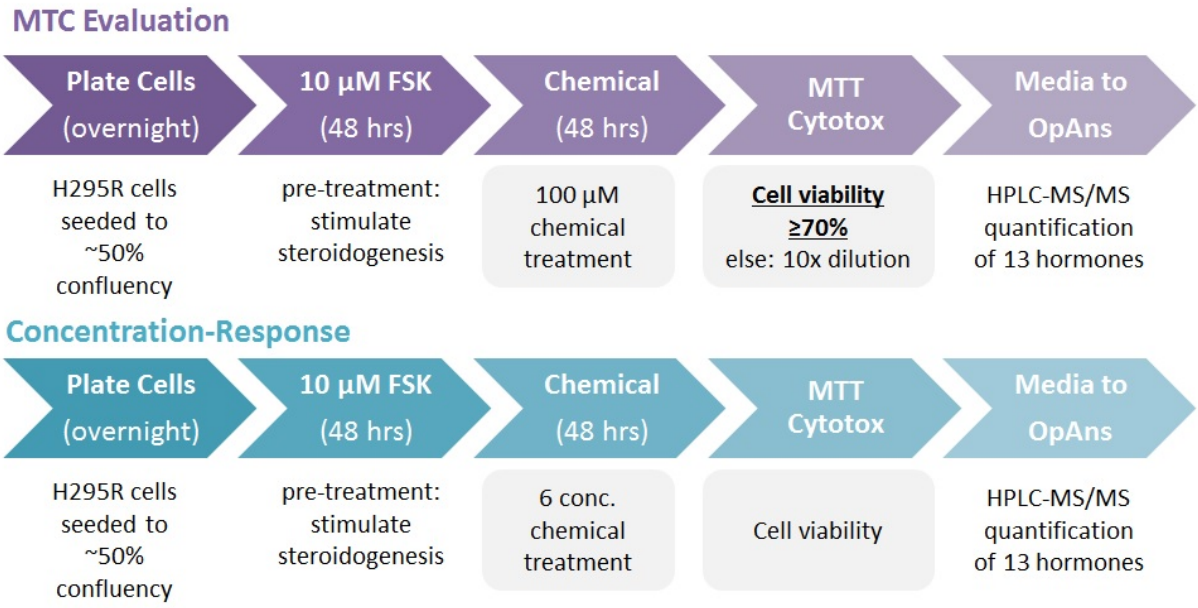


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Corticosterone assay was used to screen a large chemical library for changes in Corticosterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for corticosterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of corticosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10 µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for corticosterone were reported as 0.5 ng/mL and 100 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 4.7% and accuracy of 100.5% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

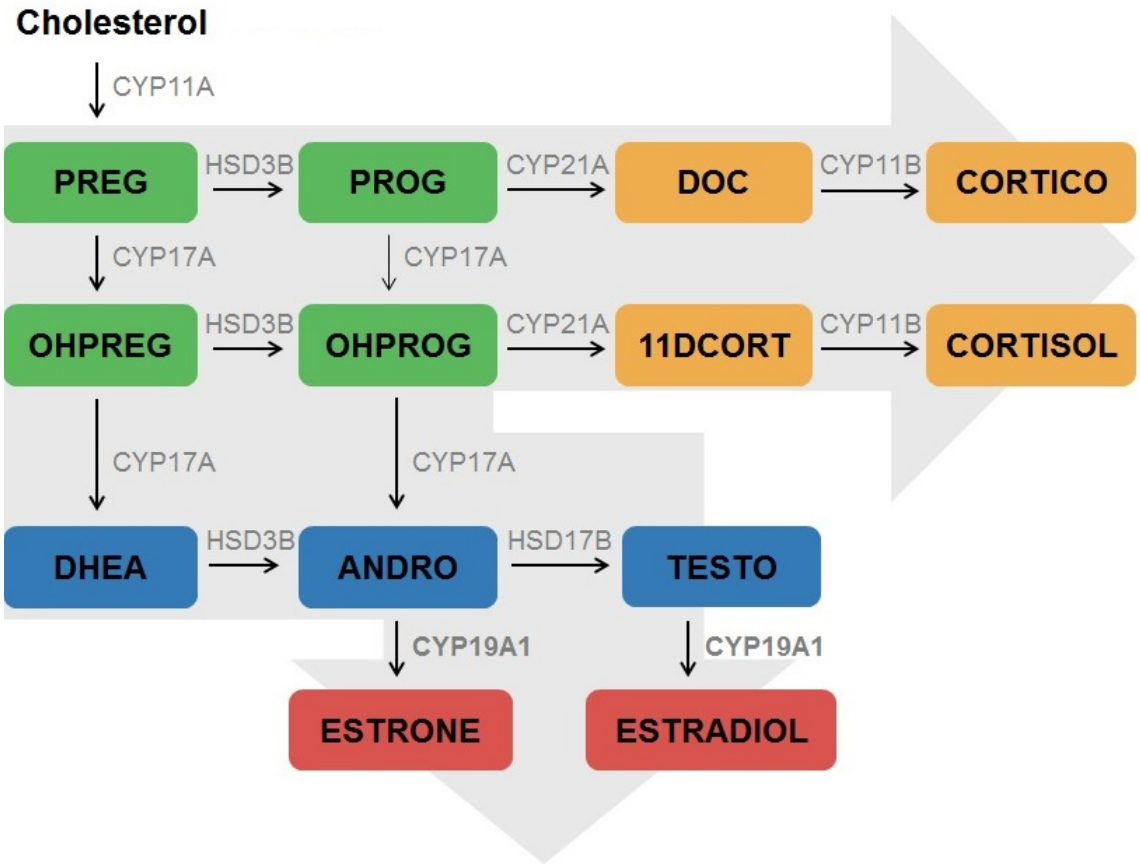


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18. SSMD-prochloraz ranged from -27-7. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of corticosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_CORTIC_dn readout data was analyzed in the negative (loss of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in corticosterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Corticosterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn

CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.

[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

Assay Endpoint ID: 899

CEETOX_H295R_CORTIC_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Corticosterone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified/adapted version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, seeking ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC. If a test chemical altered the levels of at least 4 different hormones, the chemical was selected for follow-up concentration-response evaluation to establish whether changes in hormone levels were concentration-dependent. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones such as androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

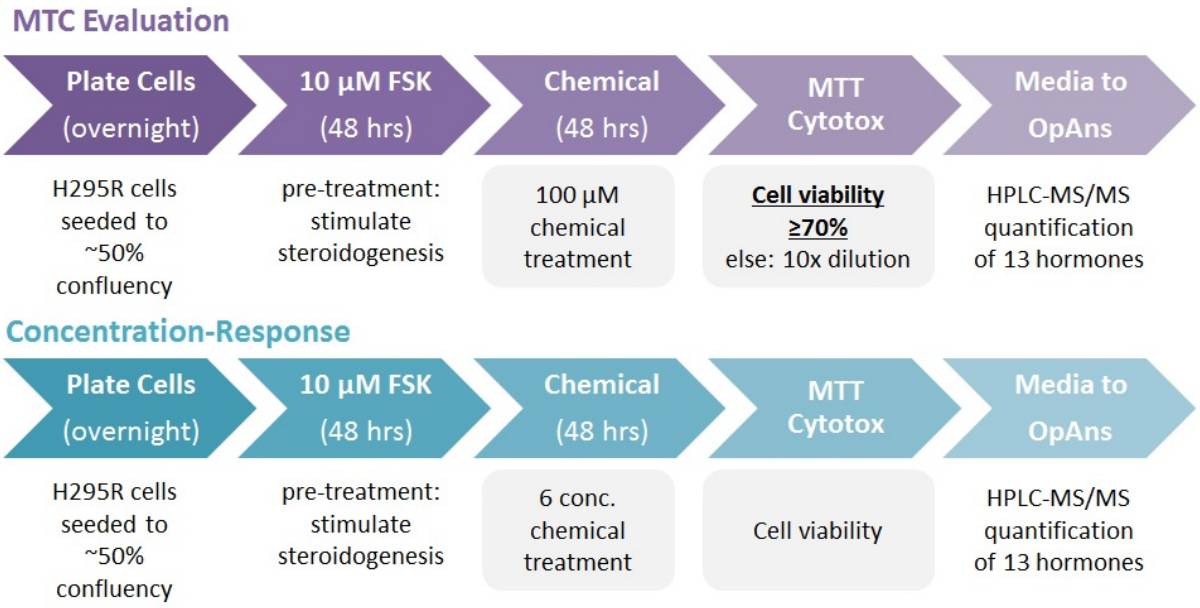


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Corticosterone assay was used to screen a large chemical library for changes in corticosterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for corticosterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of corticosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for corticosterone were reported as 0.5 ng/mL and 100 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 4.7% and accuracy of 100.5% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

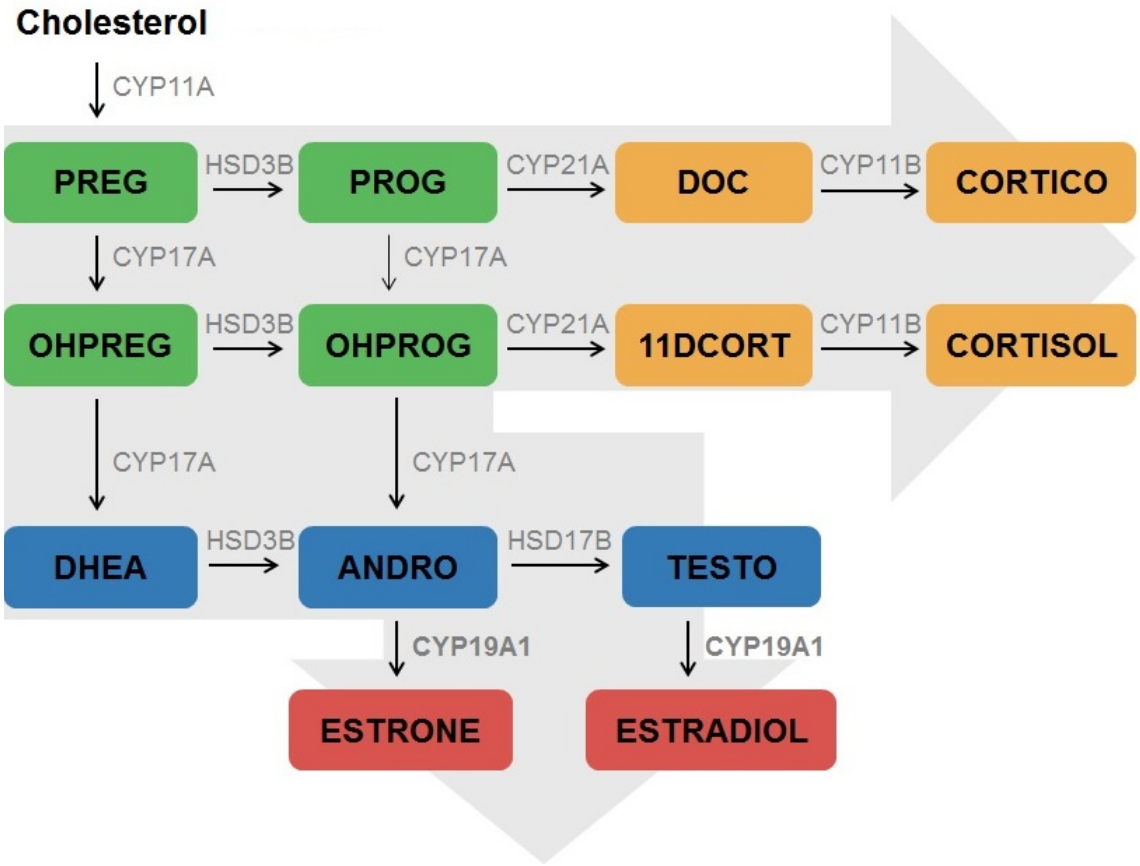


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18. SSMD-prochloraz ranged from -27-7. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of corticosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_CORTIC_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls as a baseline signal, and was reported as log2 fold-change increase in corticosterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Corticosterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn

CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median $\log_2$-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)

[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

CEETOX_H295R_CORTISOL_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Cortisol Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

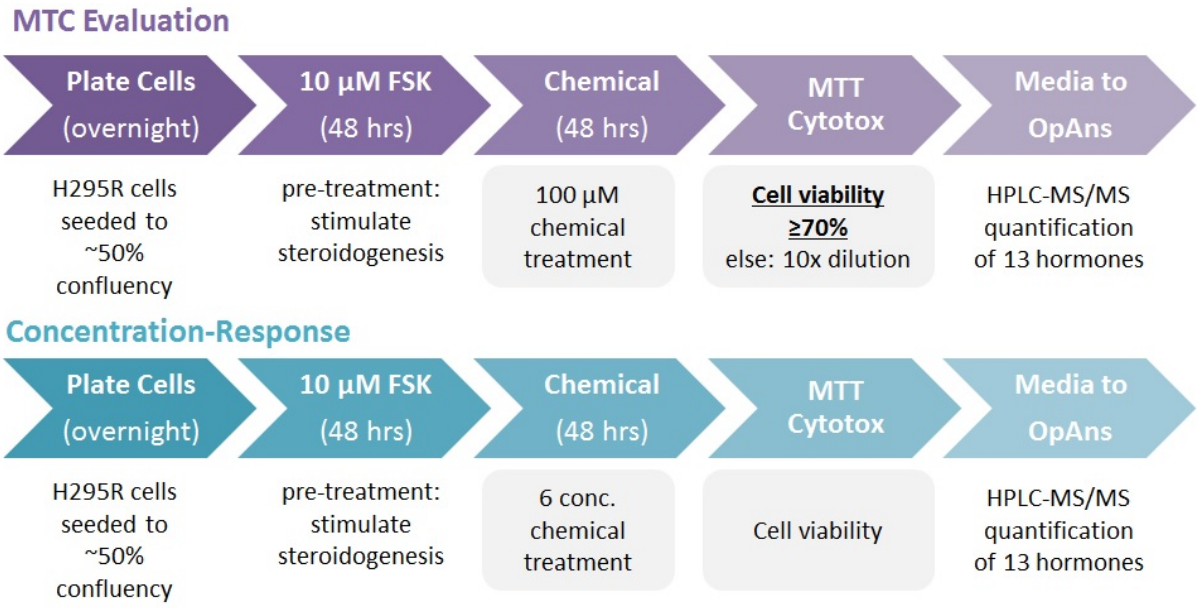


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Cortisol assay was used to screen a large chemical library for changes in cortisol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for cortisol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of cortisol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for cortisol were reported as 0.5 ng/mL and 100 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 3.3% and accuracy of 99.7% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

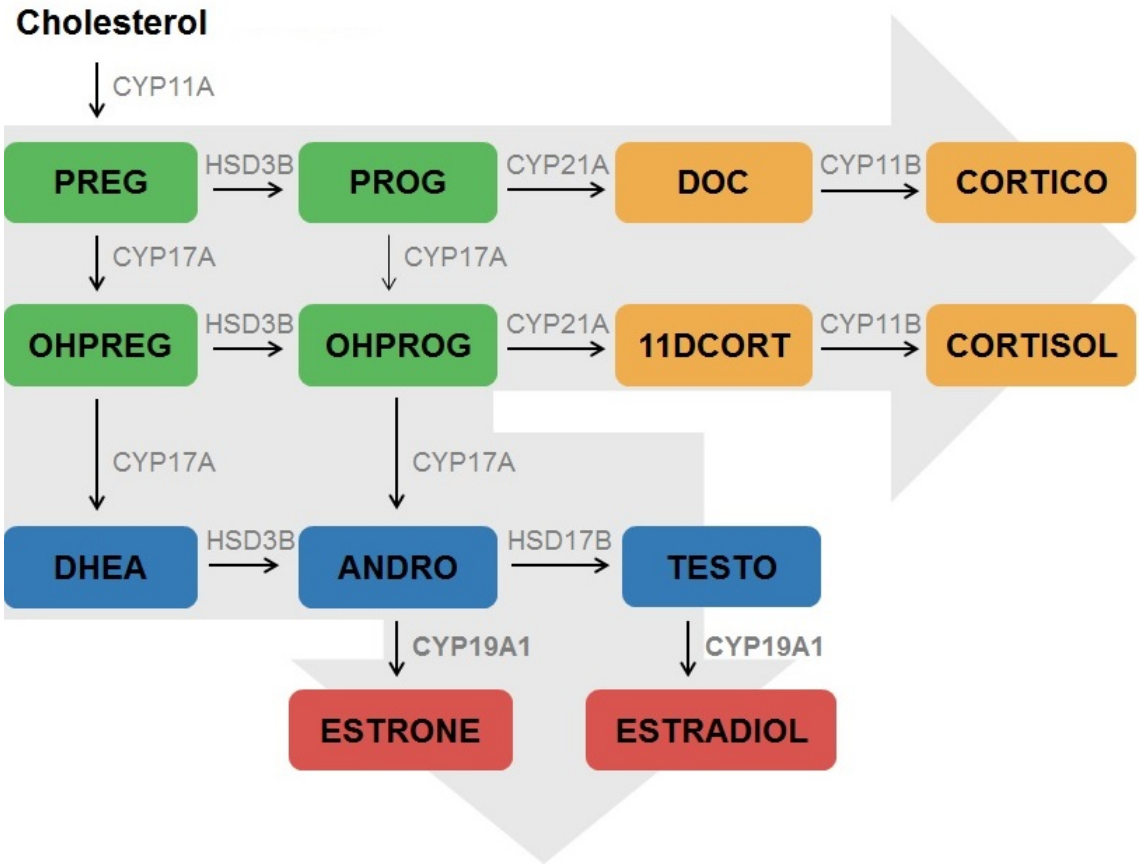


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.67 for cortisol. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.83 for cortisol. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 11 for cortisol. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -18 for cortisol. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of cortisol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_CORTISOL_dn readout data was analyzed in the negative (loss of signal) fitting direction using the DMSO controls as a baseline signal, and was reported as log2 fold-change increase in cortisol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Cortisol inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 1.98 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up

CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For cortisol, the $r^2 = 0.78$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

CEETOX_H295R_CORTISOL_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Cortisol Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

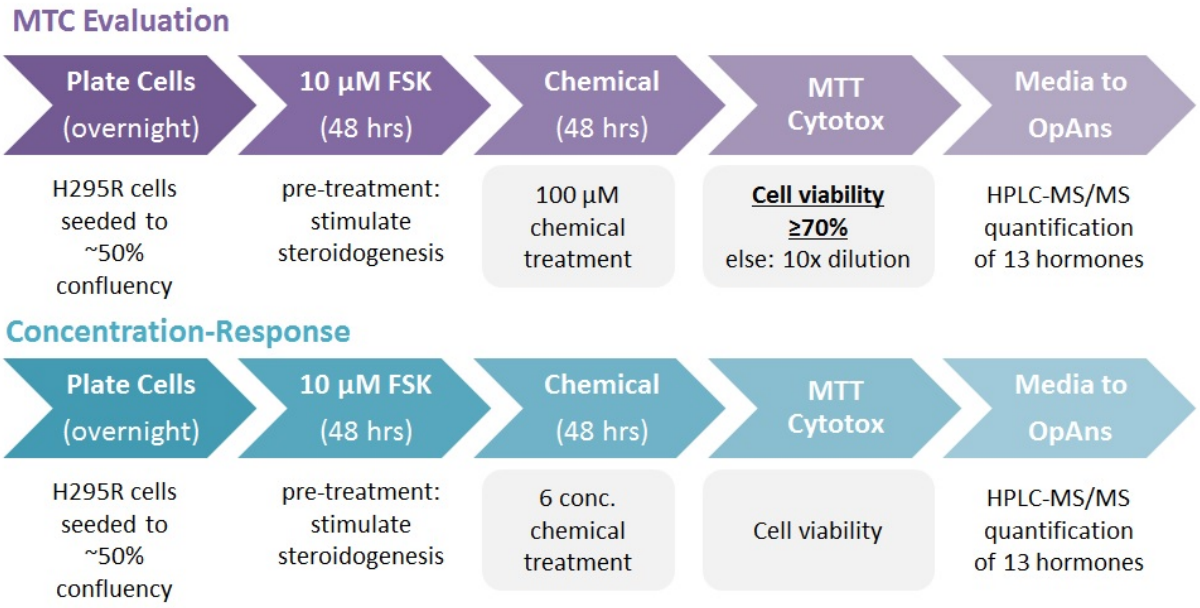


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Cortisol assay was used to screen a large chemical library for changes in cortisol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for cortisol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of cortisol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for cortisol were reported as 0.5 ng/mL and 100 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 3.3% and accuracy of 99.7% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

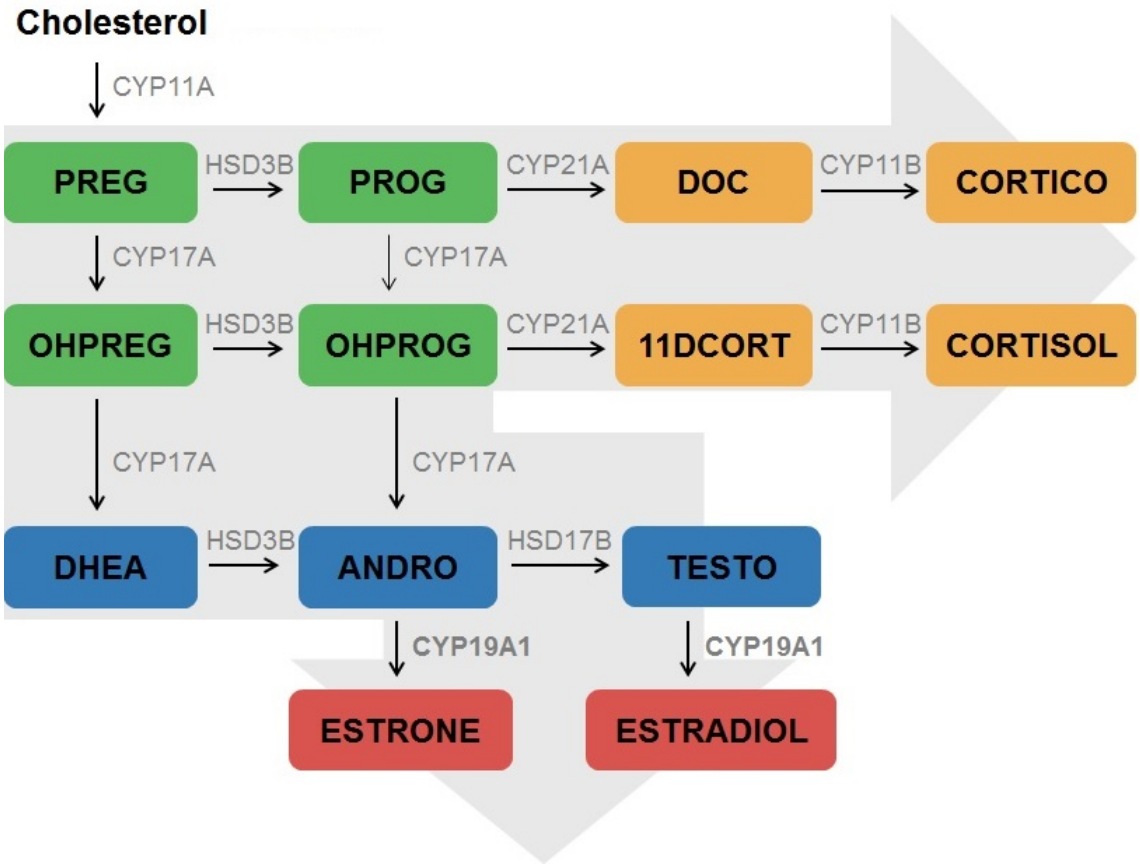


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.67 for cortisol. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.83 for cortisol. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 11 for cortisol. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -18 for cortisol. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of cortisol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_CORTISOL_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls as a baseline signal, and was reported as log2 fold-change increase in cortisol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Cortisol inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 1.98 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_DHEA_dn

CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For cortisol, the $r^2 = 0.78$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

Assay Endpoint ID: 902

CEETOX_H295R_DHEA_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – DHEA Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

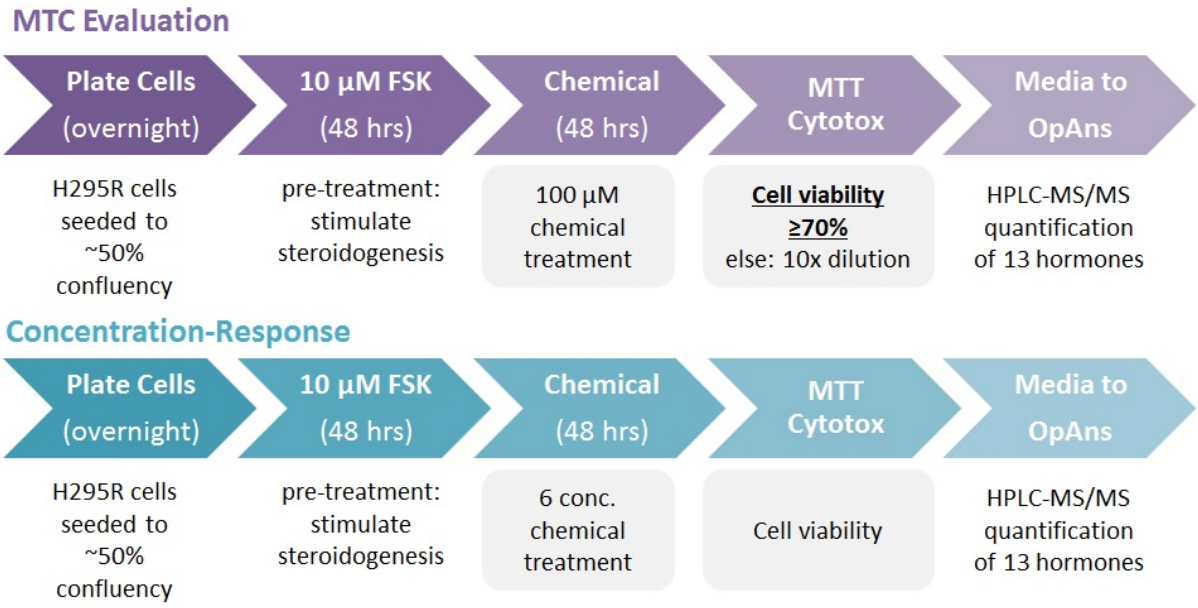


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Dehydroepiandrosterone (DHEA) assay was used to screen a large chemical library for changes in DHEA levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for DHEA quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of DHEA from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10 µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for DHEA were reported as 3 ng/mL and 600 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 4.0% and accuracy of 100.1% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

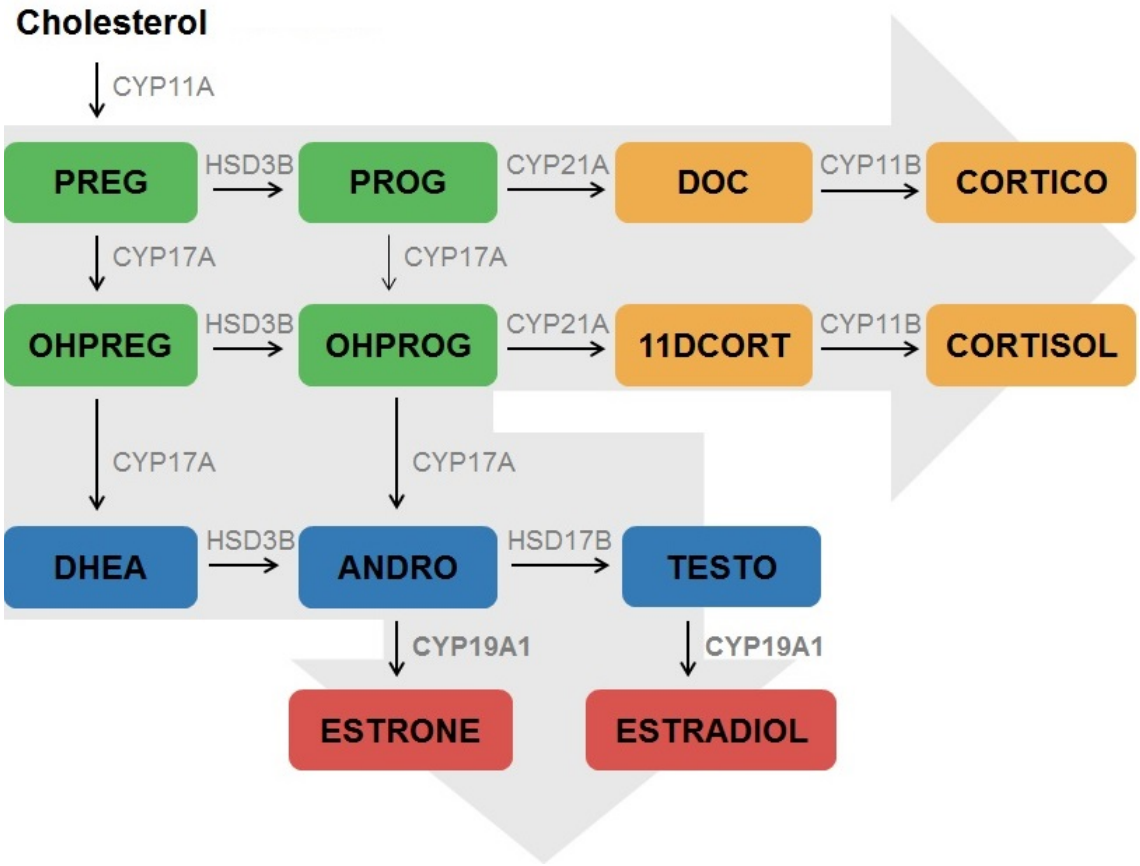


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18. SSMD-prochloraz ranged from -27-7. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of DHEA following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_DHEA_dn readout data was analyzed in the negative (loss of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in DHEA activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. DHEA inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn

CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DHEA, Dehydroepiandrosterone
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

13 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

CEETOX_H295R_DHEA_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – DHEA Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

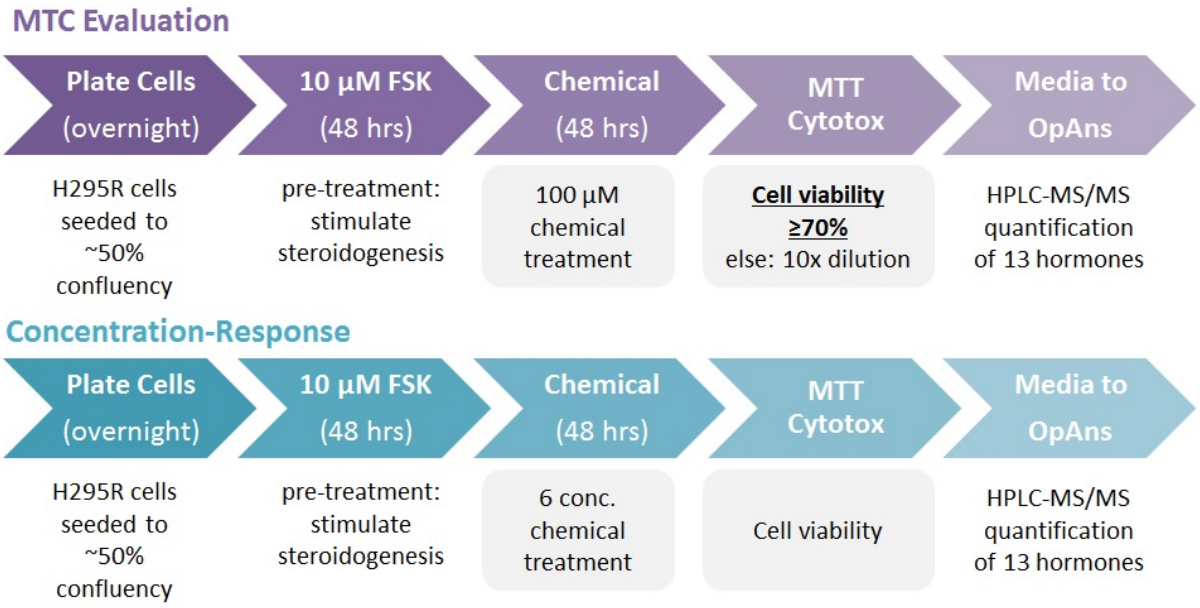


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18. SSMD-prochloraz ranged from -27-7. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Dehydroepiandrosterone (DHEA) assay was used to screen a large chemical library for changes in DHEA levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for DHEA quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of dehydroepiandrosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for DHEA were reported as 3 ng/mL and 600 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 4.0% and accuracy of 100.1% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by

CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

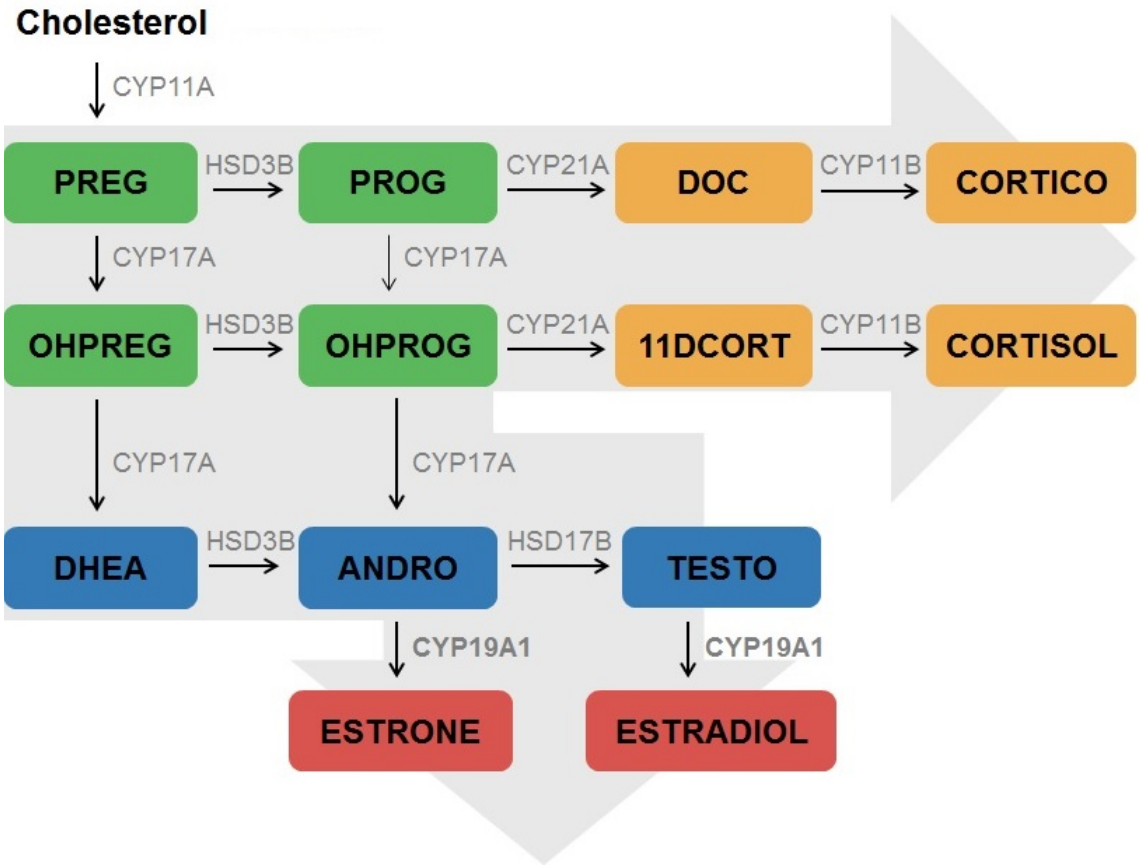


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of dehydroepiandrosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_DHEA_up readout data was analyzed in the positive (gain of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in DHEA activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. DHEA stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn

CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DHEA, Dehydroepiandrosterone
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry

MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Supporting Information:

CEETOX_H295R_DOC_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Deoxycorticosterone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

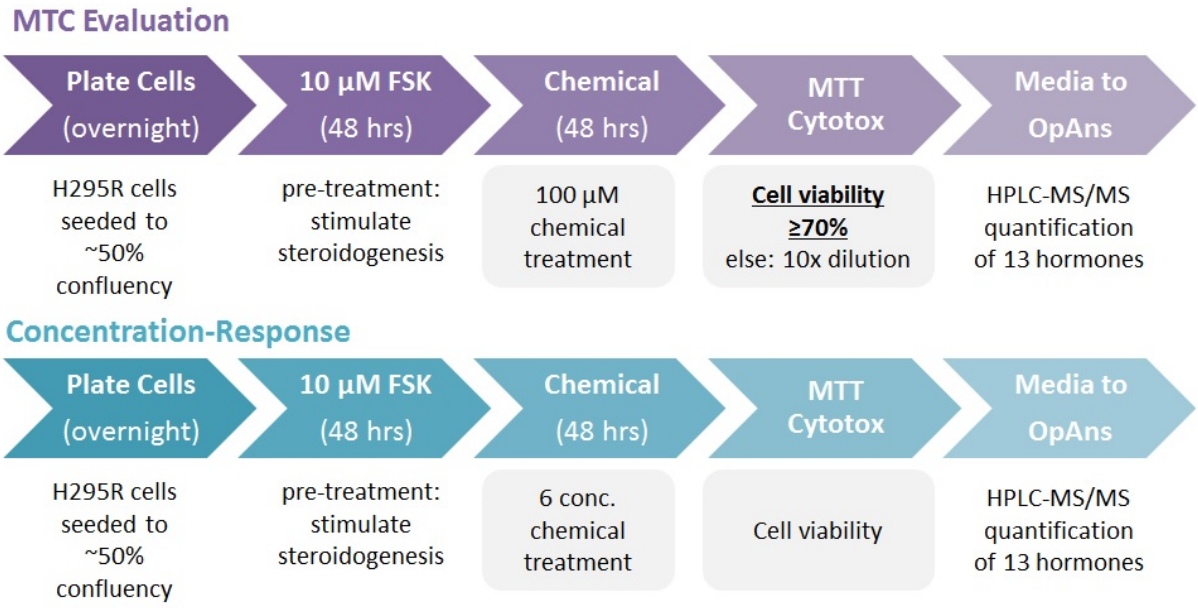


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 11-deoxycorticosterone assay was used to screen a large chemical library for changes in deoxycorticosterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for deoxycorticosterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of deoxycorticosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 11-deoxycorticosterone were reported as 0.5 ng/mL and 100 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 3.7% and accuracy of 99.9% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

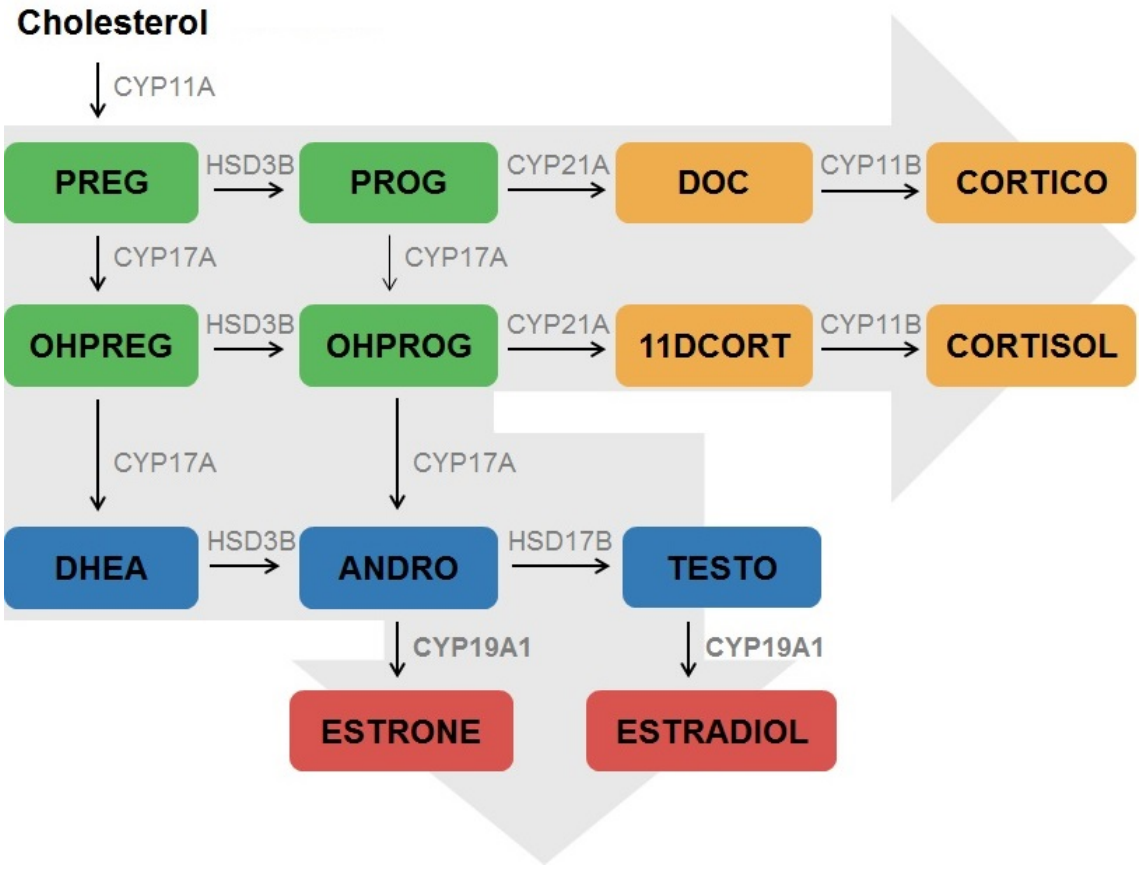


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.65 for deoxycorticosterone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.51 for deoxycorticosterone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 10 for deoxycorticosterone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of 7 for deoxycorticosterone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of 11-deoxycorticosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_DOC_dn readout data was analyzed in the negative (loss of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in deoxycorticosterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Deoxycorticosterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 1.90 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_up

CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For deoxycorticosterone, the $r^2 = 0.45$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_DOC_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Deoxycorticosterone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

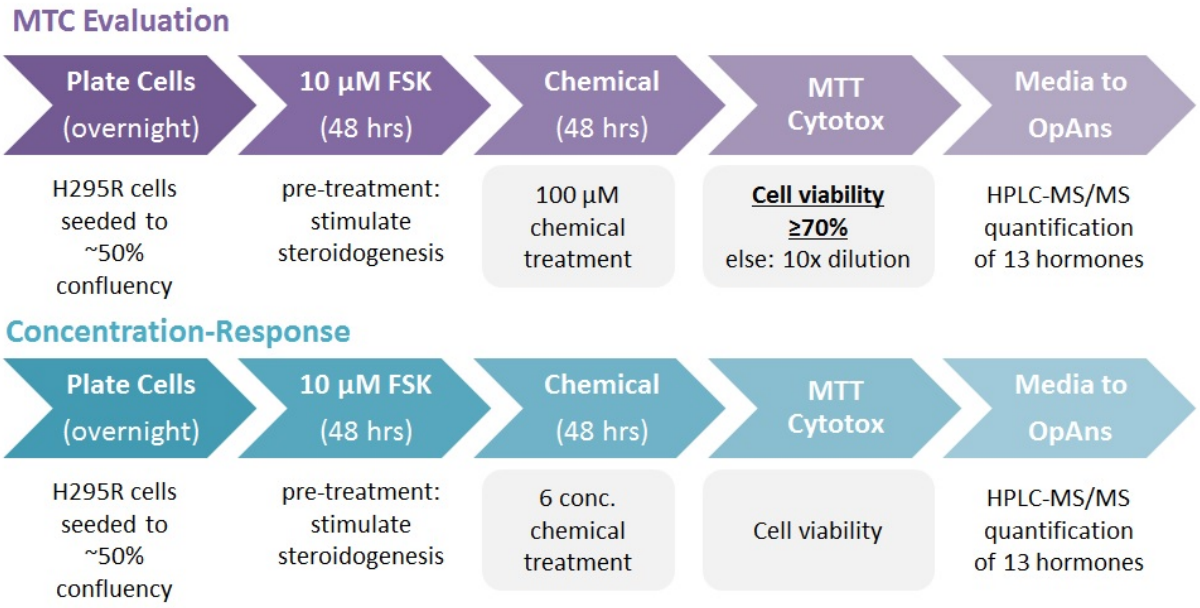


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 11-deoxycorticosterone assay was used to screen a large chemical library for changes in deoxycorticosterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for deoxycorticosterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of deoxycorticosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 11-deoxycorticosterone were reported as 0.5 ng/mL and 100 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 3.7% and accuracy of 99.9% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

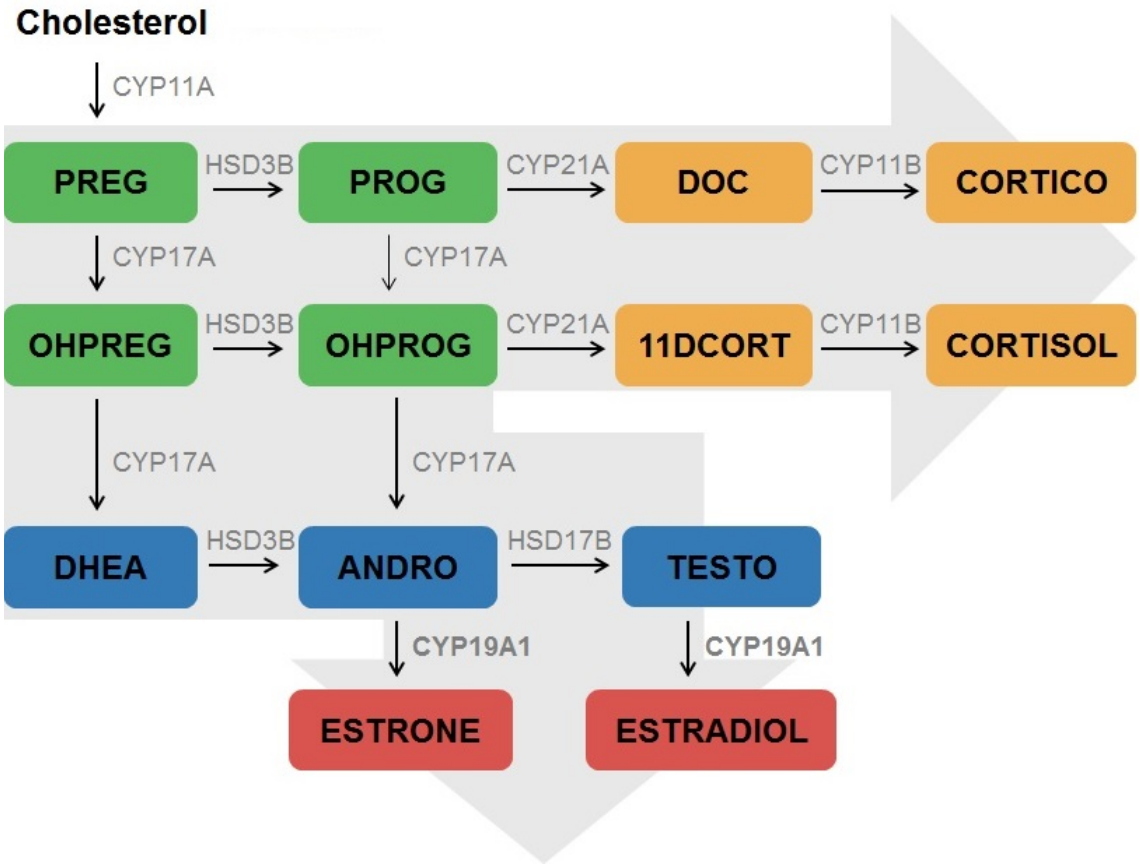


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.65 for deoxycorticosterone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.51 for deoxycorticosterone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 10 for deoxycorticosterone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of 7 for deoxycorticosterone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of 11-deoxycorticosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_DOC_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls as a baseline signal, and was reported as log2 fold-change increase in deoxycorticosterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Deoxycorticosterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 1.90 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up

CEETOX_H295R_DOC_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For deoxycorticosterone, the $r^2 = 0.45$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_ESTRADIOL_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Estradiol Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

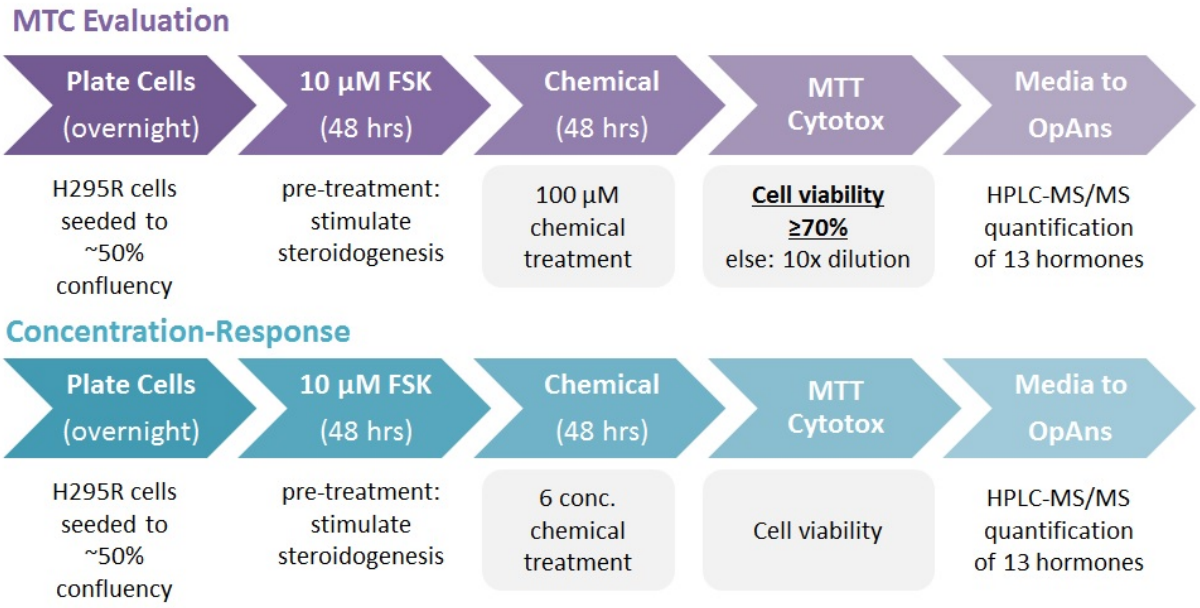


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R estradiol assay was used to screen a large chemical library for changes in estradiol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for estradiol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of estradiol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for estradiol were reported as 0.03 ng/mL and 6 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 6.3% and accuracy of 101.4% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

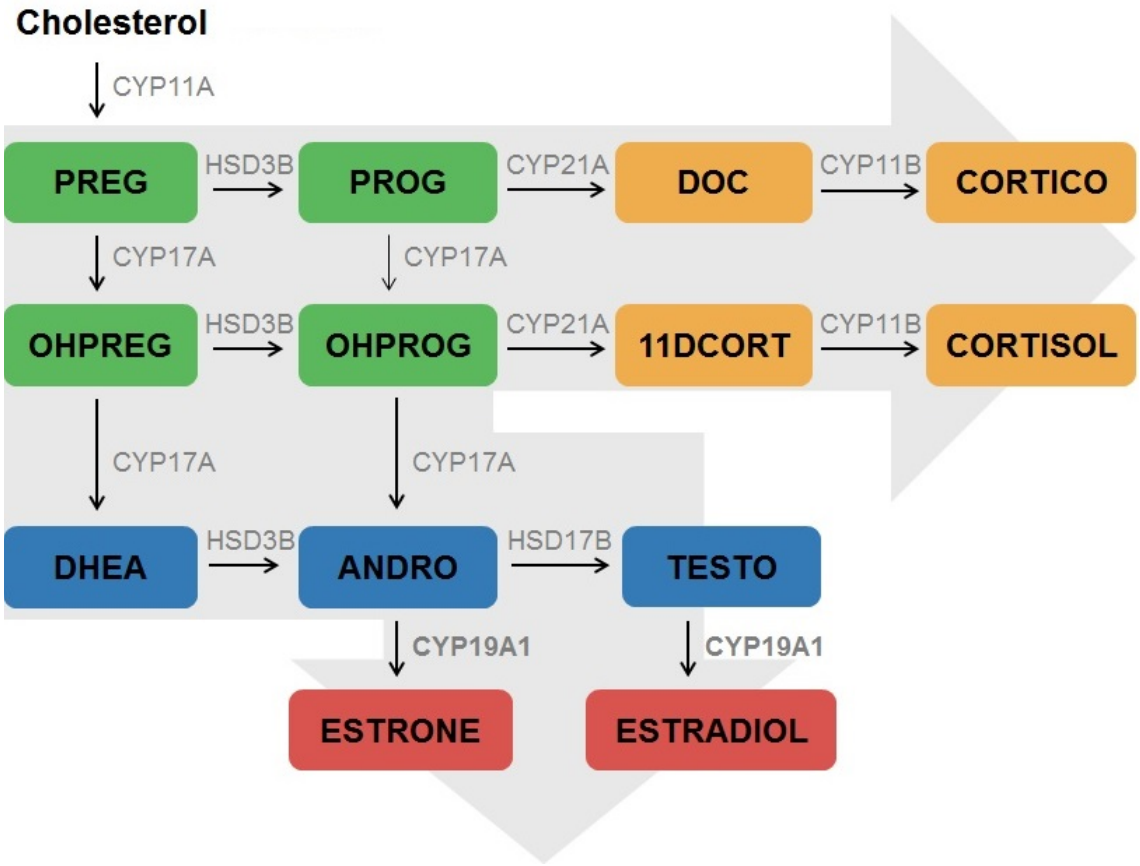


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.73 for estradiol. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.72 for estradiol. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 13 for estradiol. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -11 for estradiol. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of estradiol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ESTRADIOL_dn readout data was analyzed in the negative (loss of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in estradiol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Estradiol inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in $\log_2$ fold-change, which was approximately a 2.05 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up

CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For estradiol, the $r^2 = 0.64$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)

[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

13 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_ESTRADIOL_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Estradiol Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

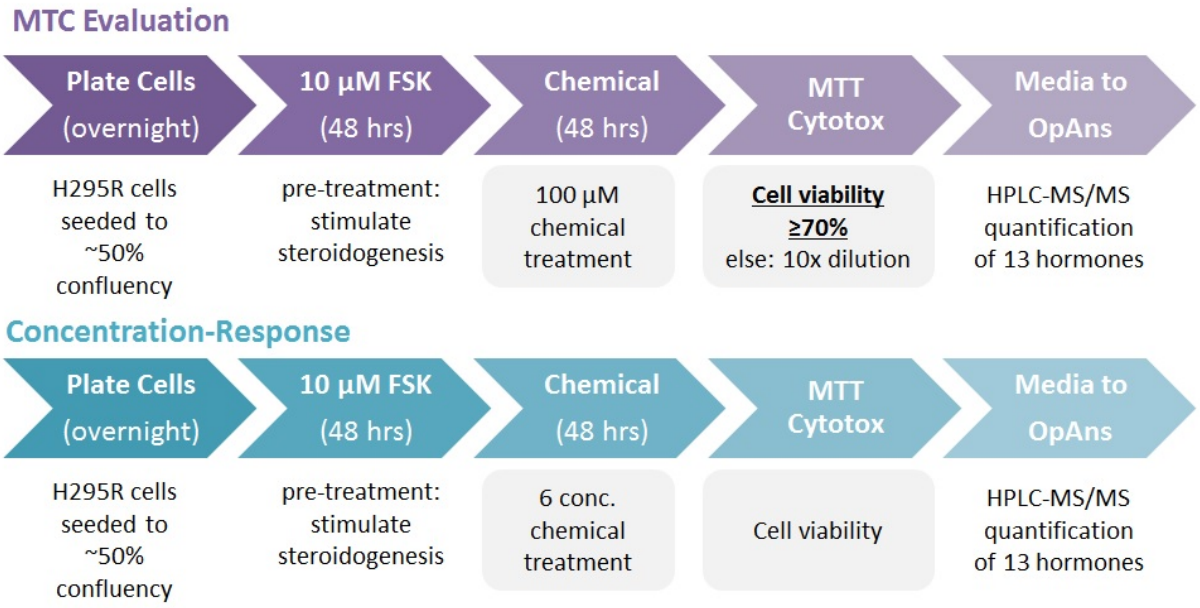


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R estradiol assay was used to screen a large chemical library for changes in estradiol levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for estradiol quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of estradiol from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for estradiol were reported as 0.03 ng/mL and 6 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 6.3% and accuracy of 101.4% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

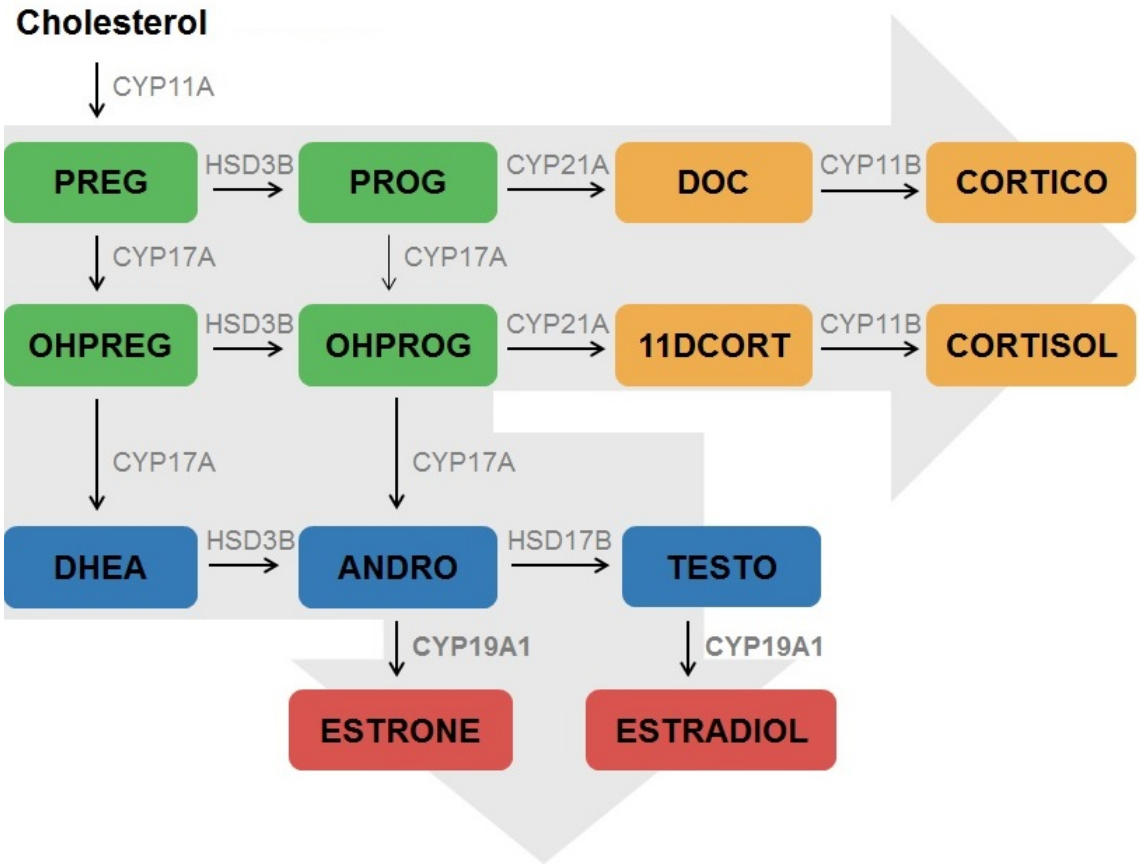


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.73 for estradiol. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.72 for estradiol. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 13 for estradiol. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -11 for estradiol. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of estradiol following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ESTRADIOL_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in estradiol activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Estradiol stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 2.05 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up

CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For estradiol, the $r^2 = 0.64$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_ESTRONE_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Estrone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

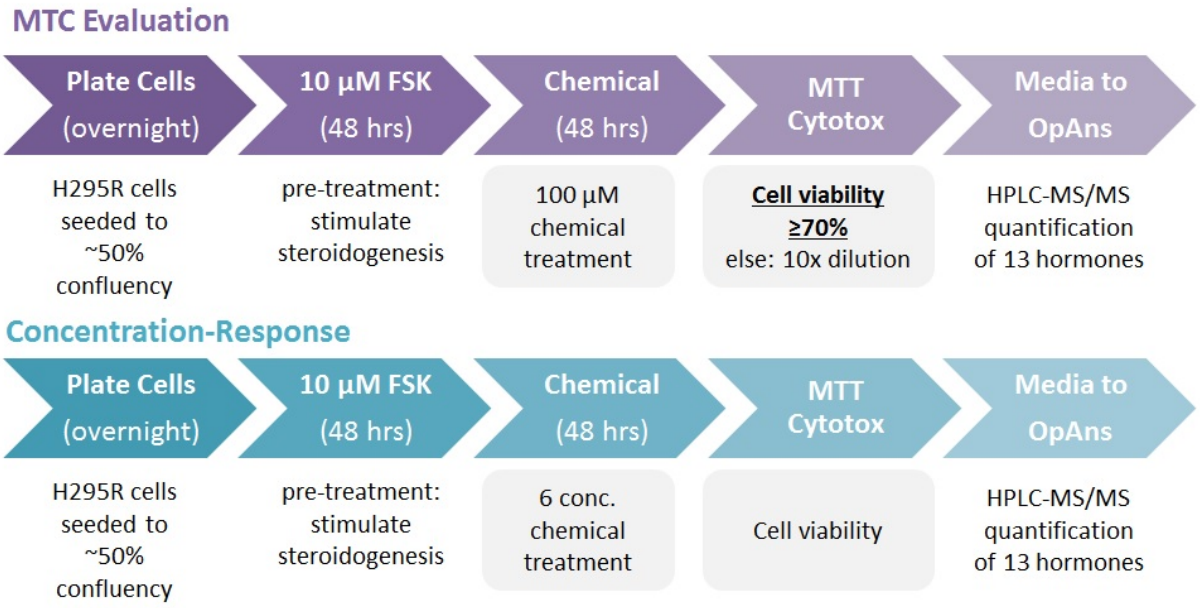


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R estrone assay was used to screen a large chemical library for changes in estrone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for estrone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of estrone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for estrone were reported as 0.03 ng/mL and 6 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 5.0% and accuracy of 100.4% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

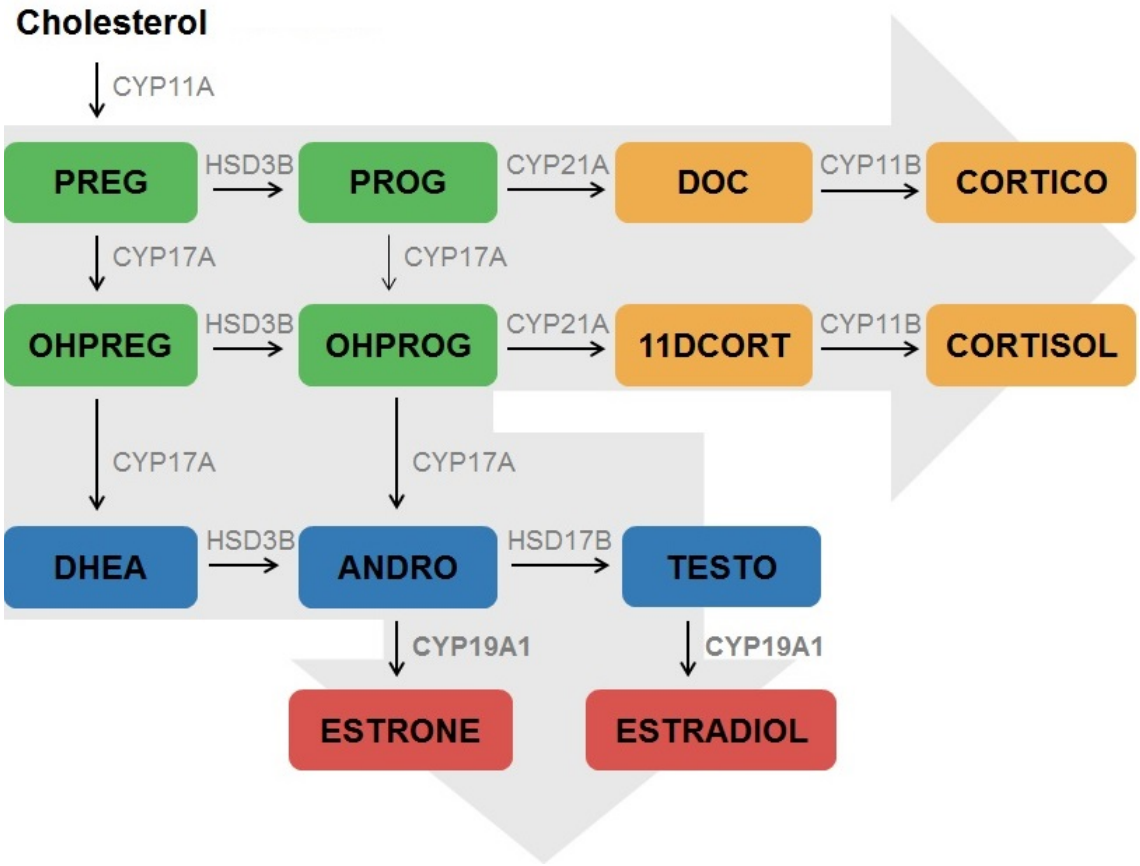


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.79 for estrone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.76 for estrone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 18 for estrone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -14 for estrone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of estrone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ESTRONE_dn readout data was analyzed in the negative (loss of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in estrone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Estrone inhibition was determined based on a chemical fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the estrone assay this created a 1.75 fold-change cut-off); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up

CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For estrone, the $r^2 = 0.65$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

13 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_ESTRONE_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Estrone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

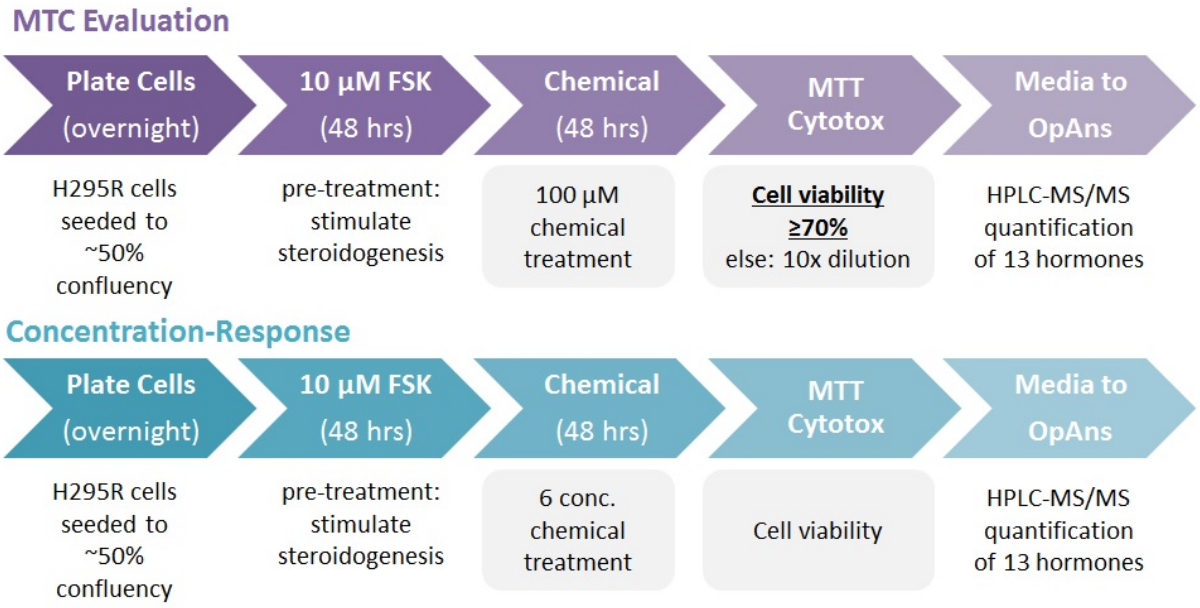


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R estrone assay was used to screen a large chemical library for changes in estrone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for estrone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of estrone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for estrone were reported as 0.03 ng/mL and 6 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 5.0% and accuracy of 100.4% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

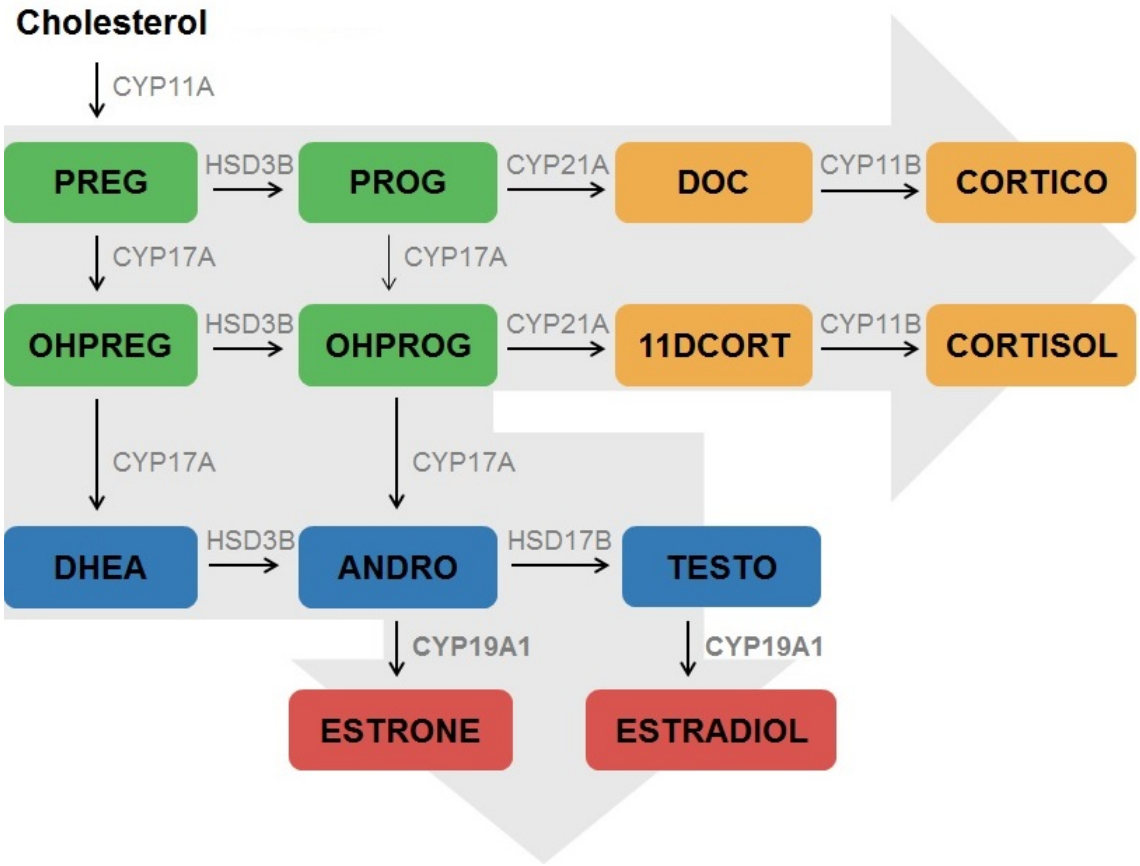


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.79 for estrone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.76 for estrone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 18 for estrone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -14 for estrone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of estrone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_ESTRONE_up readout data was analyzed in the positive (gain of signal) fitting direction using the DMSO controls as the baseline signal, and was reported as log2 fold-change increase in estrone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Estrone stimulation was determined based on a chemical fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the estrone assay this created a 1.75 fold-change cut-off); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up

CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For estrone, the $r^2 = 0.65$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

13 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Assay Endpoint ID: 892

CEETOX_H295R_OHPREG_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – 17alpha-hydroxypregnenolone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for

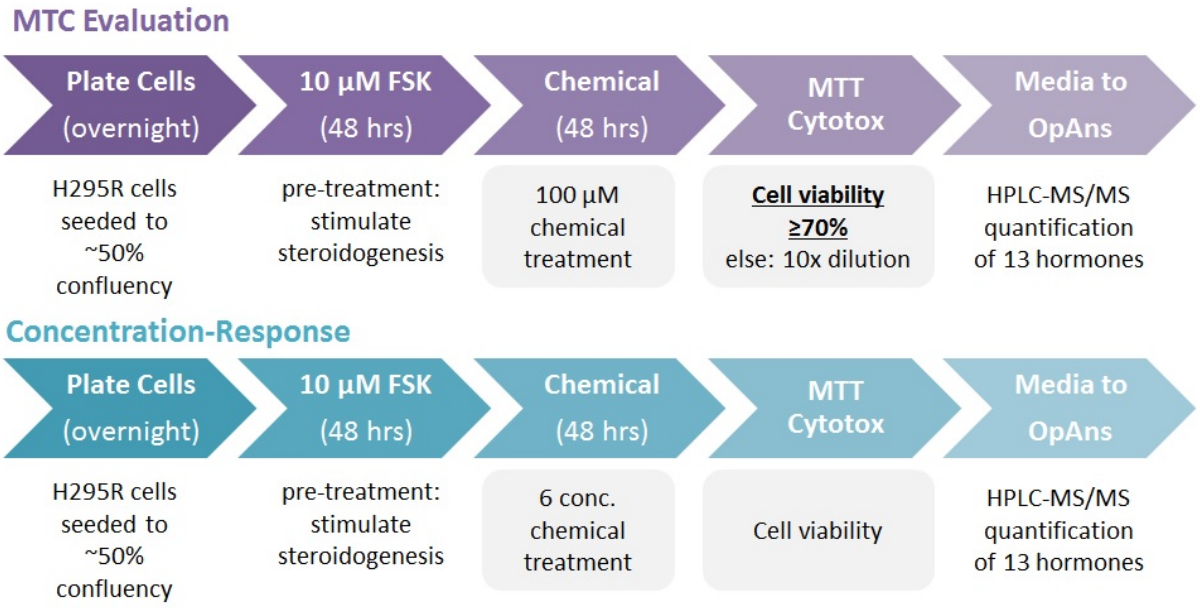
analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical



dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 17α-hydroxypregnenolone assay was used to screen a large chemical library for changes in 17α-hydroxypregnenolone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for 17α-hydroxypregnenolone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of 17α-hydroxypregnenolone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10 µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 17α-hydroxypregnenolone were reported as 5 ng/mL and 1000 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 6.7% and accuracy of 100% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by

CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

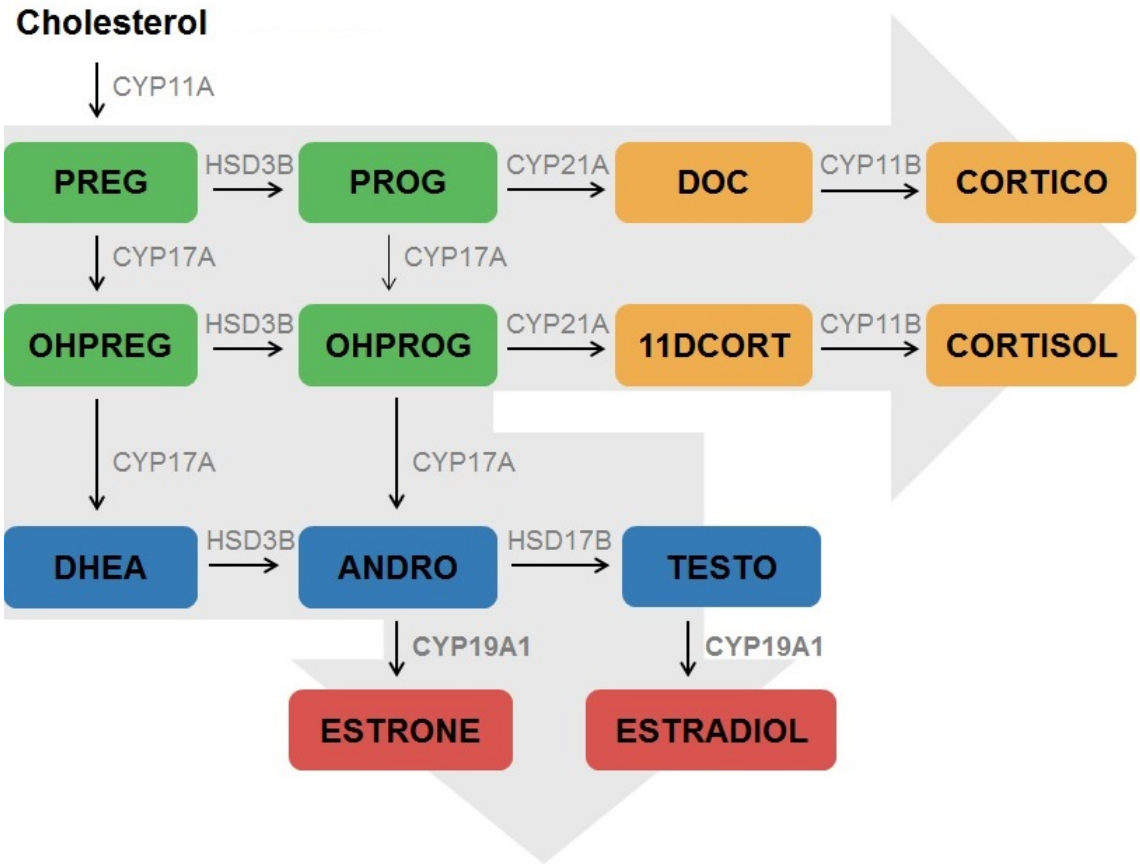


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.81 for OHPREG. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.77 for OHPREG. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 20 for OHPREG. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of 3 for OHPREG. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of 17α-hydroxypregnenolone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_OHPREG_dn readout data was analyzed in the negative (loss of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in 17α-hydroxypregnenolone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. 17α-hydroxypregnenolone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change, which was approximately a 1.64 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn

CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For OHPREG, the $r^2 = 0.64$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_OHPREG_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – 17alpha-hydroxypregnenolone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for

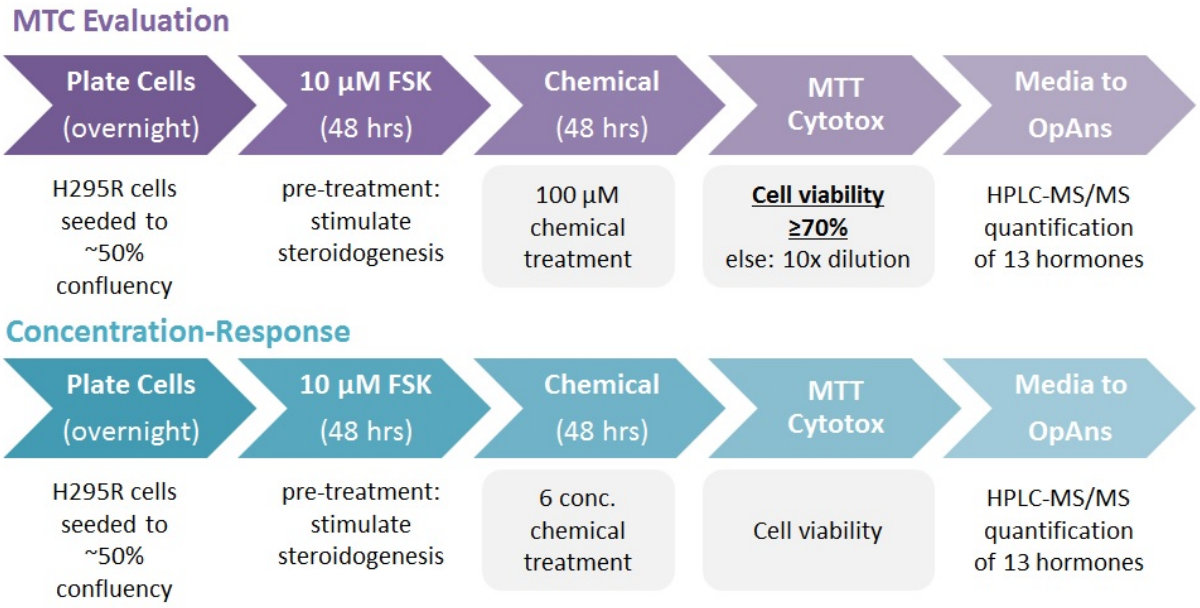
analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical



dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 17α-hydroxypregnenolone assay was used to screen a large chemical library for changes in 17α-hydroxypregnenolone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for 17α-hydroxypregnenolone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of 17α-hydroxypregnenolone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10 µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 17α-hydroxypregnenolone were reported as 5 ng/mL and 1000 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 6.7% and accuracy of 100% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

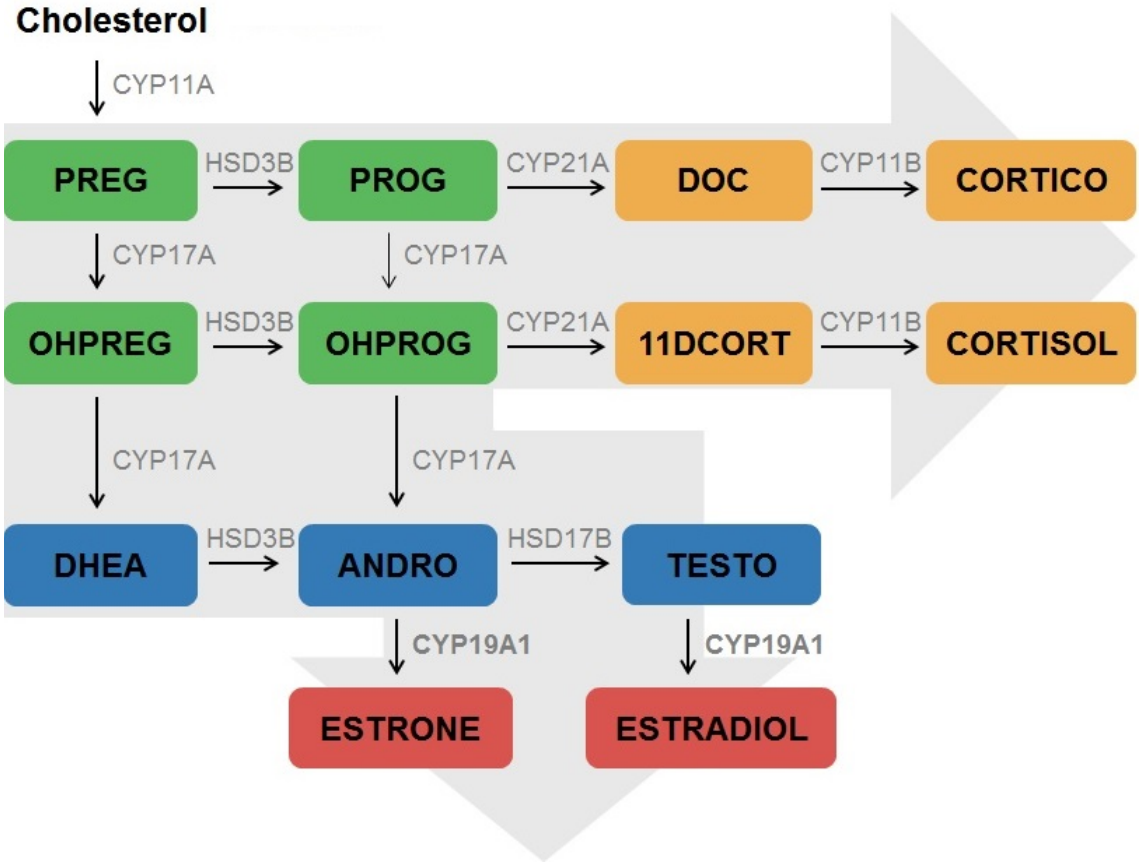


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.81 for OHPREG. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.77 for OHPREG. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 20 for OHPREG. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of 3 for OHPREG. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of 17α-hydroxypregnenolone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_OHPREG_up readout data was analyzed in the positive (gain of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in 17α-hydroxypregnenolone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were $\log_2$-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. 17α-hydroxypregnenolone stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in $\log_2$ fold-change, which was approximately a 1.64 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than

the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For OHPREG, the $r^2 = 0.64$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.

[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)

[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)

[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.

[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

ATCC, American Type Culture Collection

DMSO, Dimethyl Sulfoxide

HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry

MTC, Maximum Tolerated Concentration

MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide

R^2, correlation coefficient, R-squared

SSMD, strictly standardized mean difference

Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

CEETOX_H295R_OHPROG_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – 17alpha-hydroxyprogesterone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for

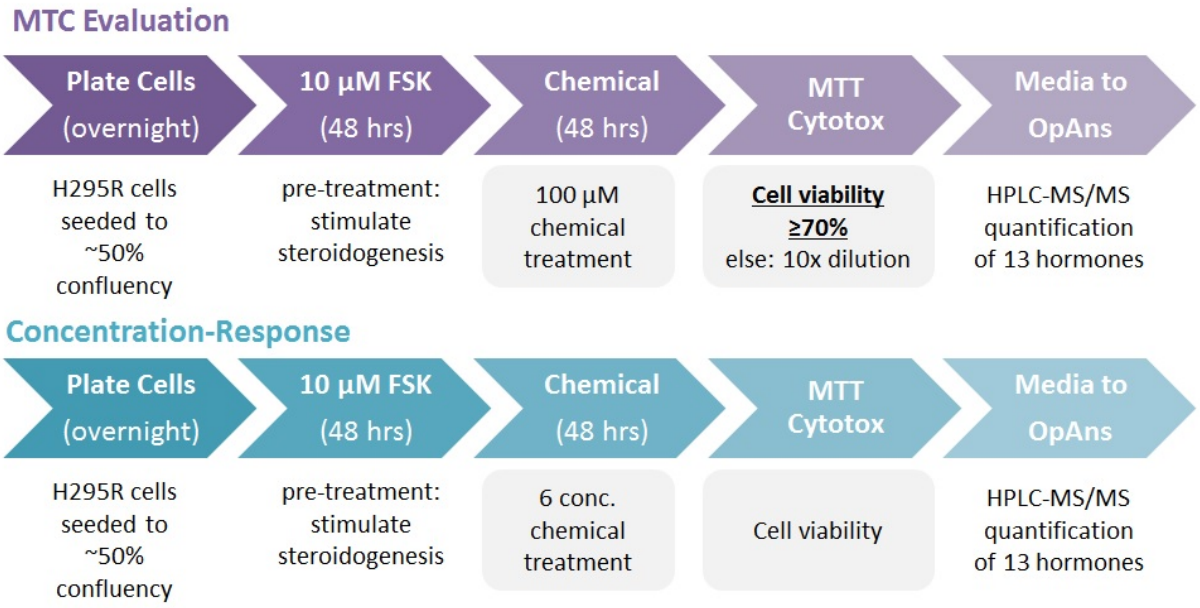
analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical



dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 17α-hydroxyprogesterone assay was used to screen a large chemical library for changes in 17α-hydroxyprogesterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for 17α-hydroxyprogesterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of 17α-hydroxyprogesterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10 µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 17α-hydroxyprogesterone were reported as 0.2 ng/mL and 40 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 4.0% and accuracy of 99.6% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by

CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

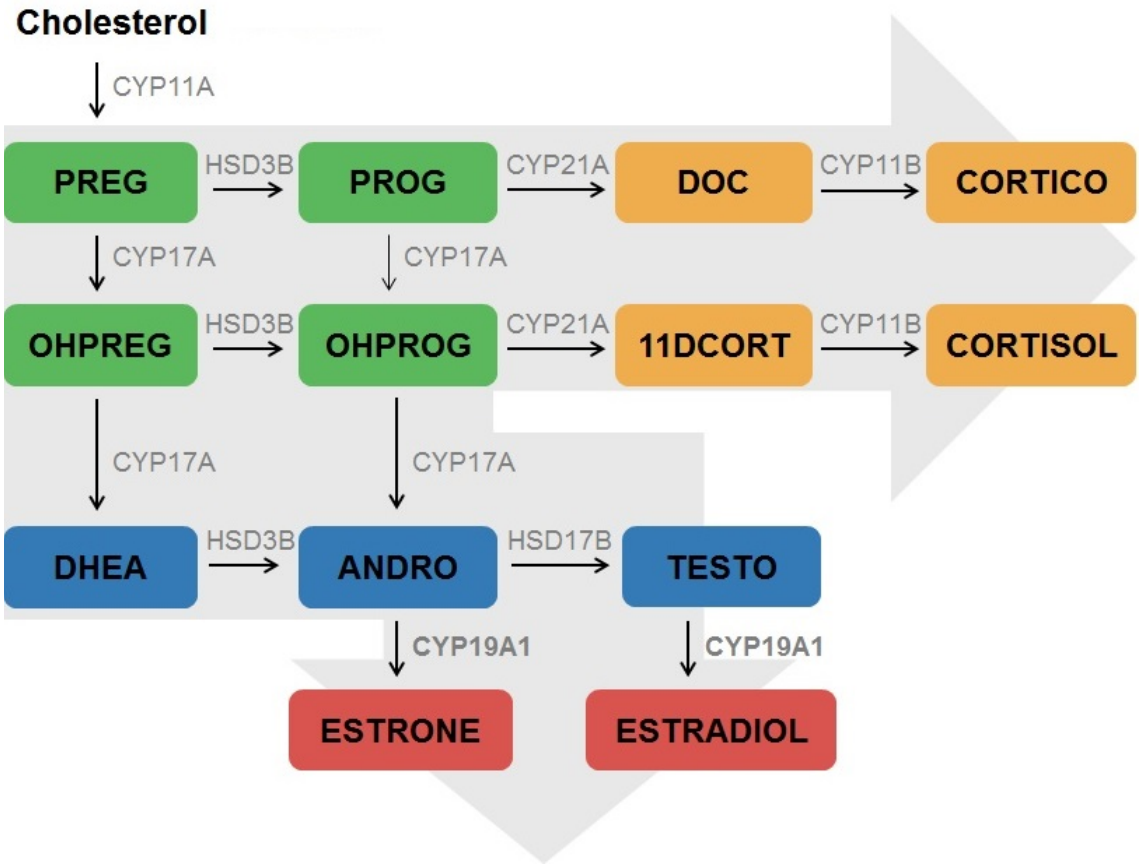


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.62 for OHPROG. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0 for OHPROG. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 10 for OHPROG. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -2 for OHPROG. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of 17α-hydroxyprogesterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_OHPROG_dn readout data was analyzed in the negative (loss of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in 17α-hydroxyprogesterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. 17α-hydroxyprogesterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the 17α-hydroxyprogesterone assay this was approximately a 1.87 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn

CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For OHPROG, the $r^2 = 0.71$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.

[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_OHPROG_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – 17alpha-hydroxyprogesterone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for

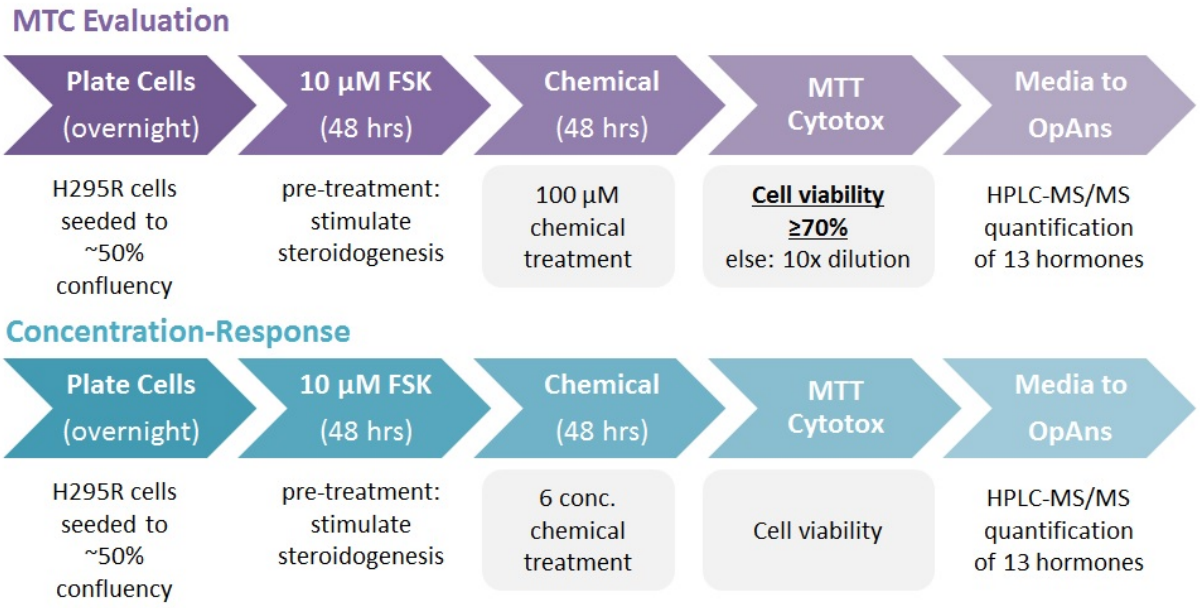
analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical



dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R 17α-hydroxyprogesterone assay was used to screen a large chemical library for changes in 17α-hydroxyprogesterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for 17α-hydroxyprogesterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of 17α-hydroxyprogesterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10 µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for 17α-hydroxyprogesterone were reported as 0.2 ng/mL and 40 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 4.0% and accuracy of 99.6% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

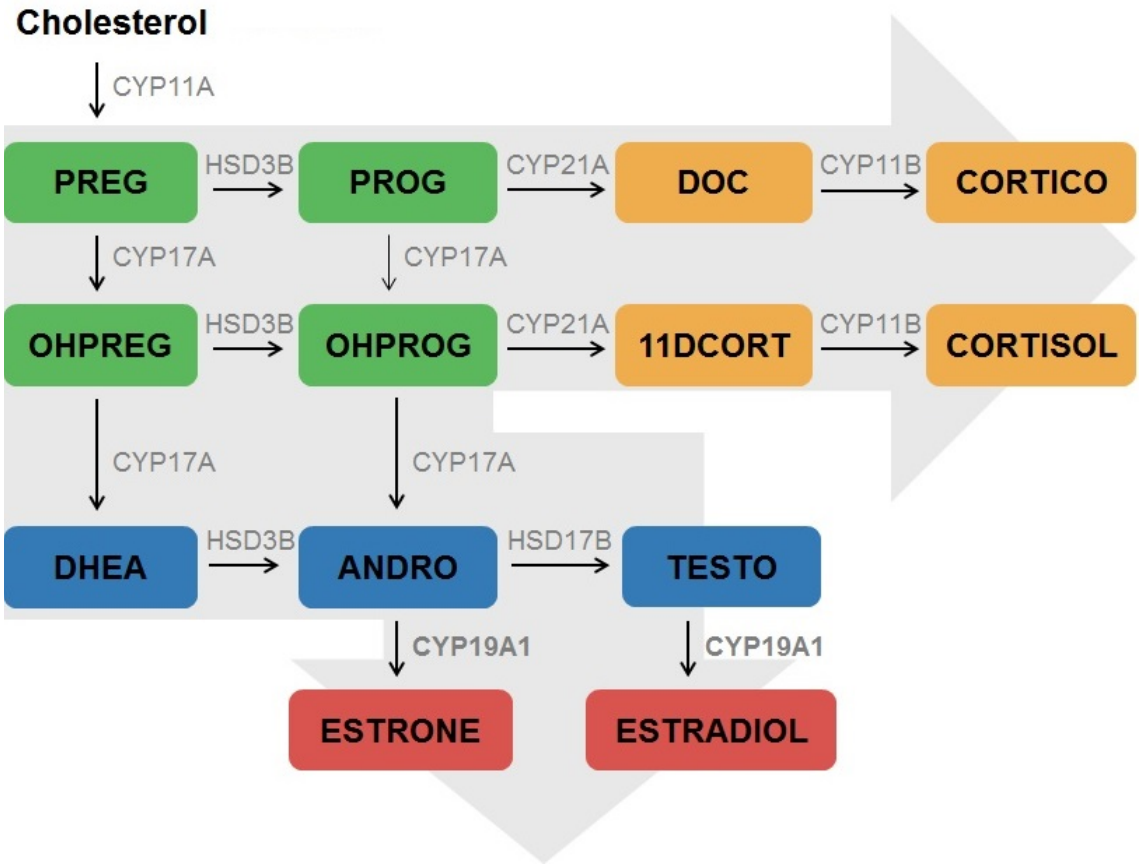


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.62 for OHPROG. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0 for OHPROG. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 10 for OHPROG. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -2 for OHPROG. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of 17α-hydroxyprogesterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_OHPROG_up readout data was analyzed in the positive (gain of signal) fitting direction uisng DMSO controls as the baseline signal, and was reported as log2 fold-change increase in 17α-hydroxyprogesterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. 17α-hydroxyprogesterone stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the 17α-hydroxyprogesterone assay this was approximately a 1.87 fold-change cut-off); the modeled top (modl_tp) of the curve was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn

CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For OHPROG, the $r^2 = 0.71$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.

[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Assay Endpoint ID: 910

CEETOX_H295R_PREG_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Pregnenolone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

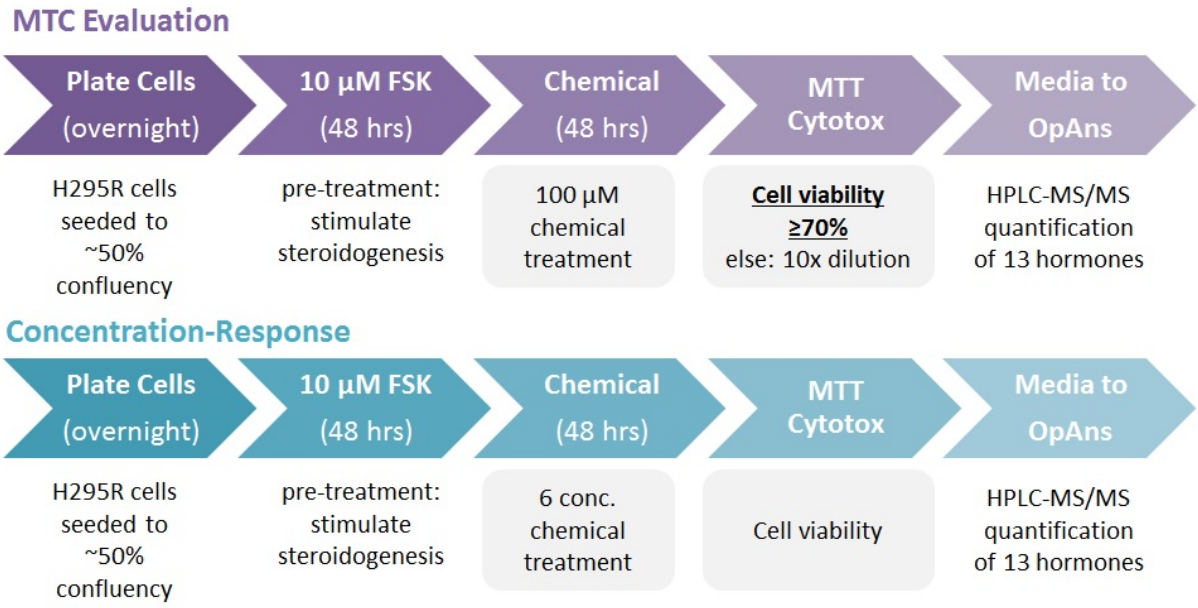


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Pregnenolone assay was used to screen a large chemical library for changes in pregnenolone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, media was subject to MBTE and dansyl chloride derivatization for pregnenolone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of pregnenolone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for pregnenolone were reported as 2 ng/mL and 400 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 10.0% and accuracy of 100.9% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

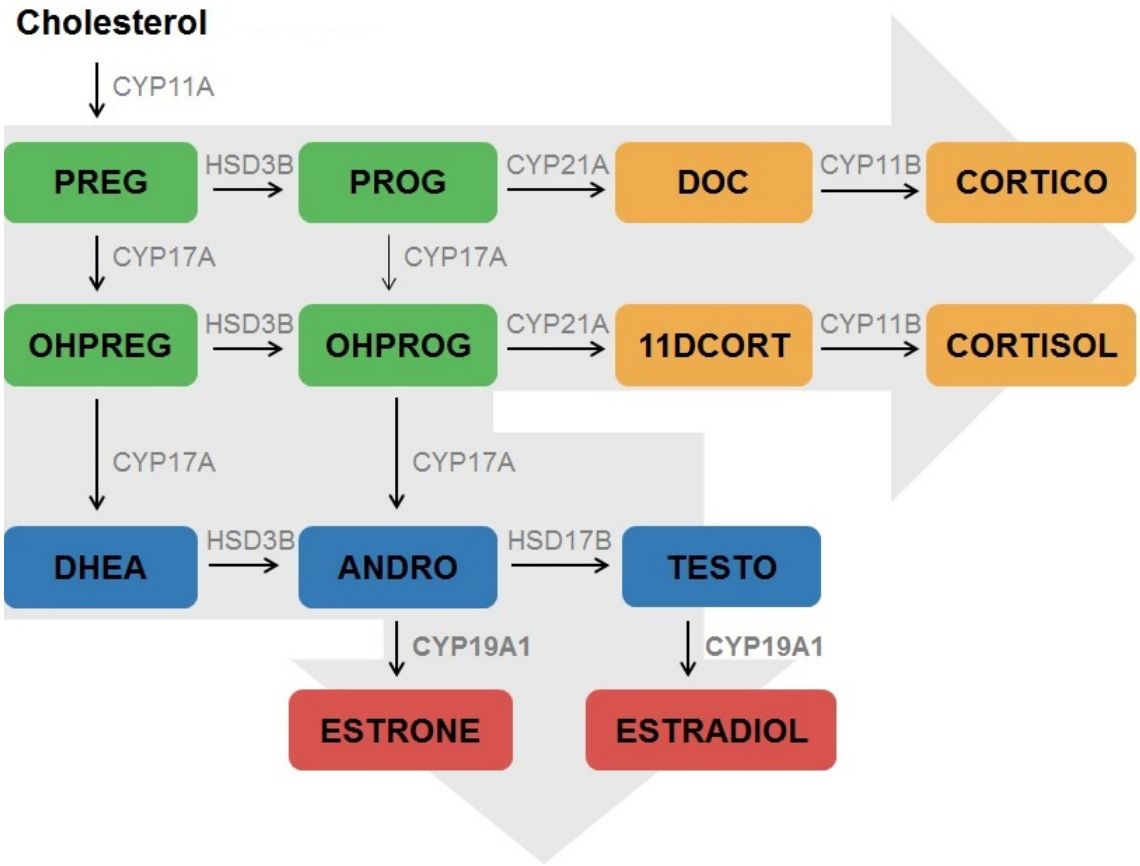


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18. SSMD-prochloraz ranged from -27-7. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of pregnenolone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_PREG_dn readout data was analyzed in the negative (loss of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in pregnenolone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Pregnenolone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up

CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_PREG_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Pregnenolone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

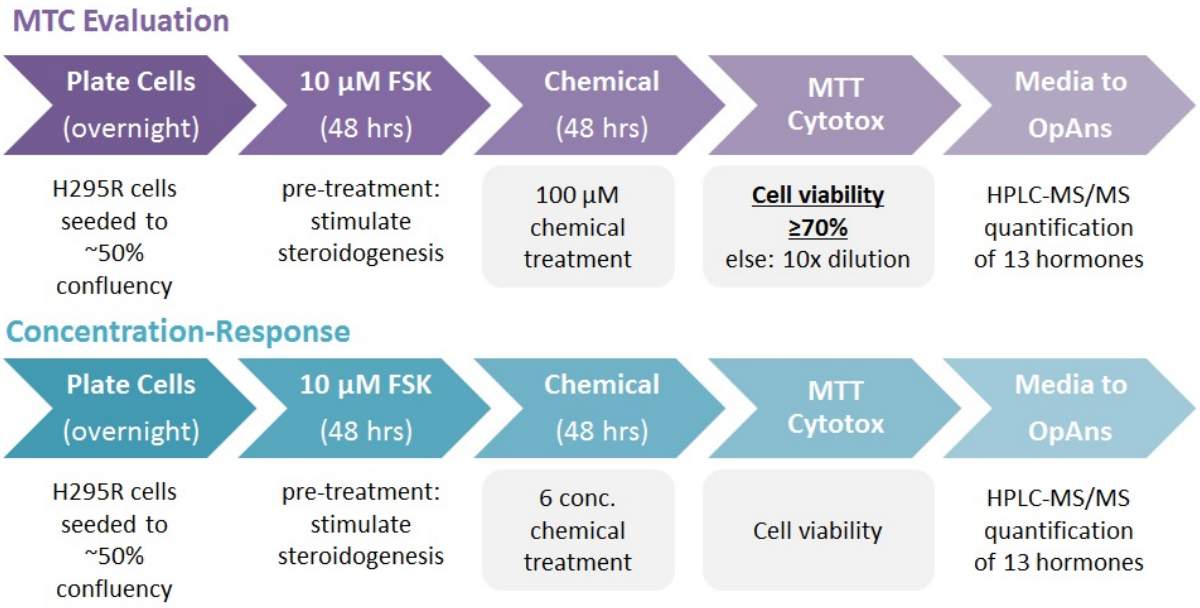


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Pregnenolone assay was used to screen a large chemical library for changes in pregnenolone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for pregnenolone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of pregnenolone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for pregnenolone were reported as 2 ng/mL and 400 ng/mL, respectively, using 20, 50, and 800 ng/mL standards, with precision of 10.0% and accuracy of 100.9% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

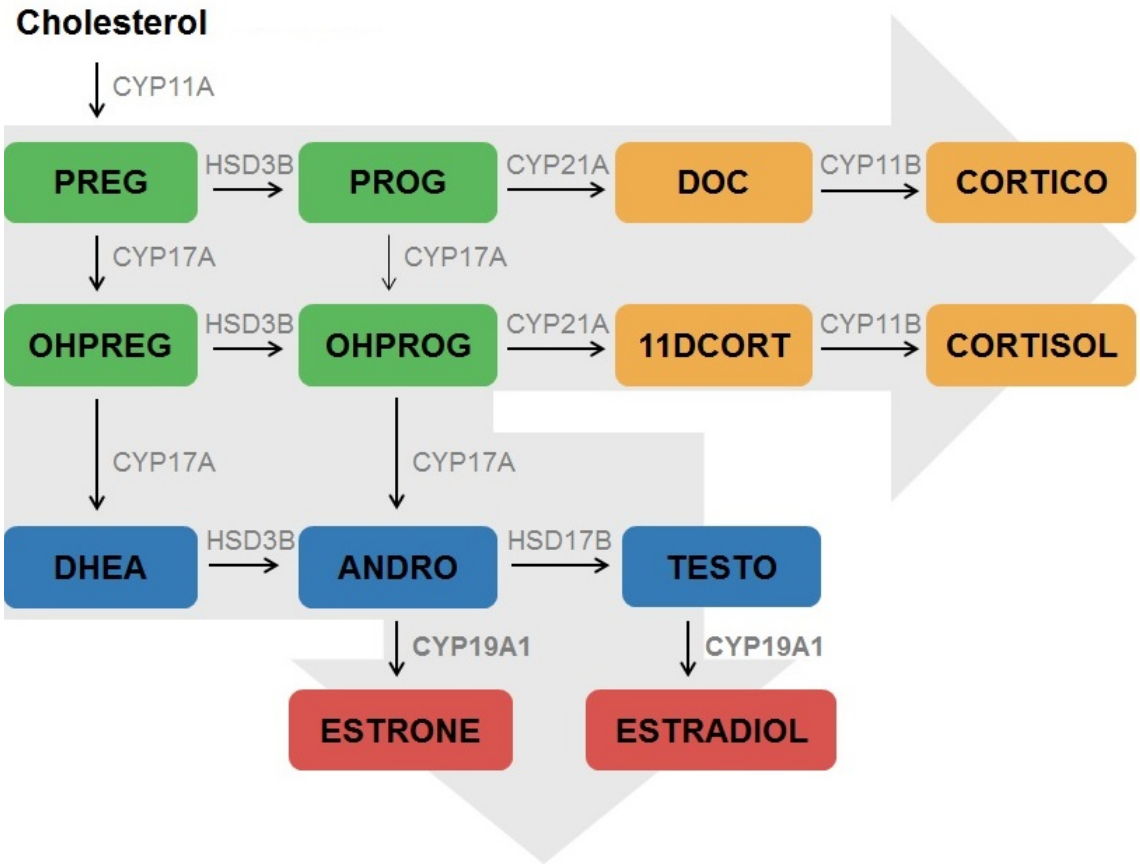


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.76 for 11DCORT. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.88 for 11DCORT. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18. SSMD-prochloraz ranged from -27-7. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of pregnenolone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_PROG_up readout data was analyzed in the positive (gain of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in pregnenolone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Pregnenolone stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change); the modeled top of the curve (modl_tp) was above the established response cutoff; and the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn

CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Assay Endpoint ID: 912

CEETOX_H295R_PROG_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Progesterone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

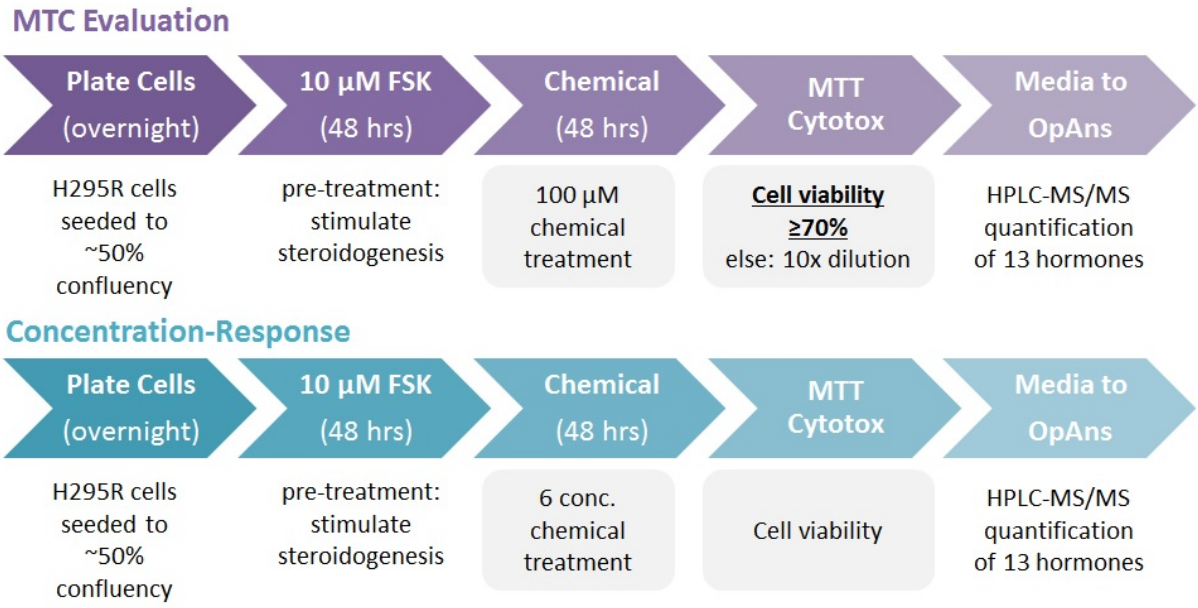


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R progesterone assay was used to screen a large chemical library for changes in progesterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for progesterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of progesterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for progesterone were reported as 0.2 ng/mL and 40 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 3.3% and accuracy of 98.1% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

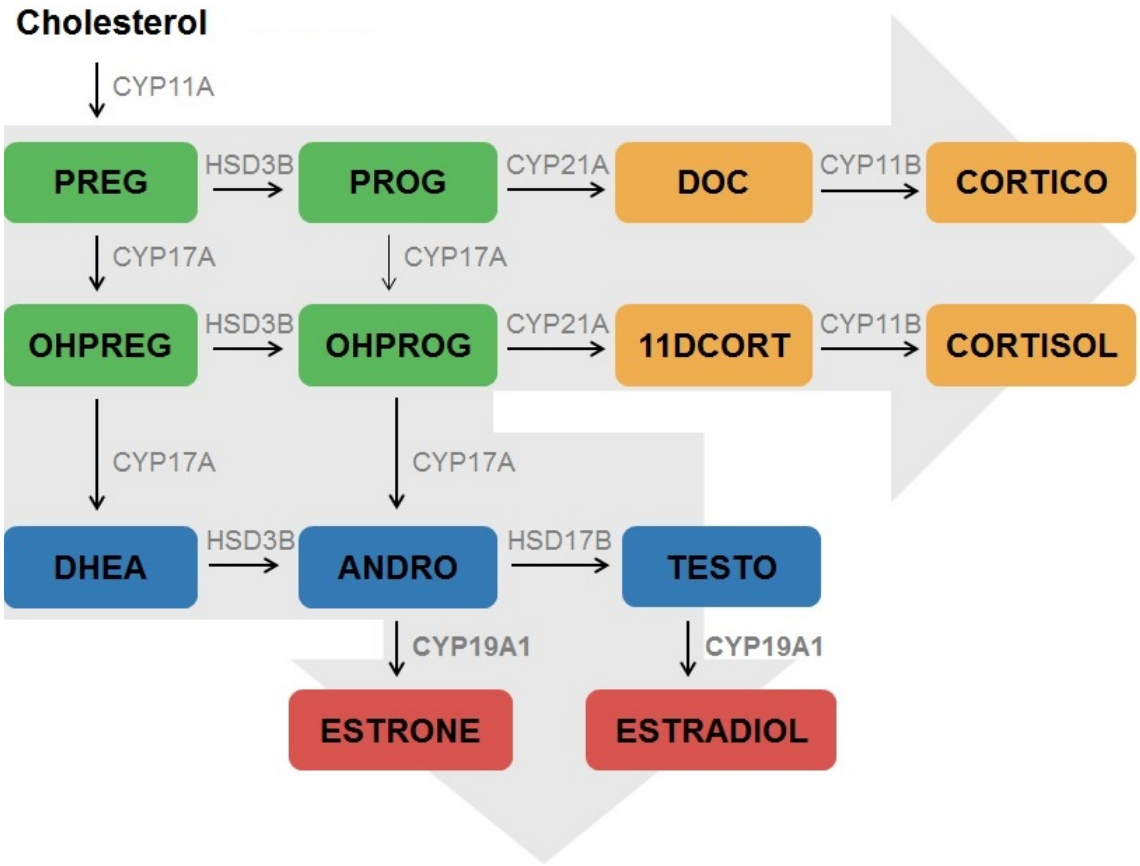


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.0 for progesterone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.85 for progesterone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 1 for progesterone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of 21 for progesterone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of progesterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_PROG_dn readout data was analyzed in the negative (loss of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in progesterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Progesterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the progesterone assay this was approximately a 1.97 fold-change cut-off); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn

CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For progesterone, the $r^2 = 0.79$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_PROG_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Progesterone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

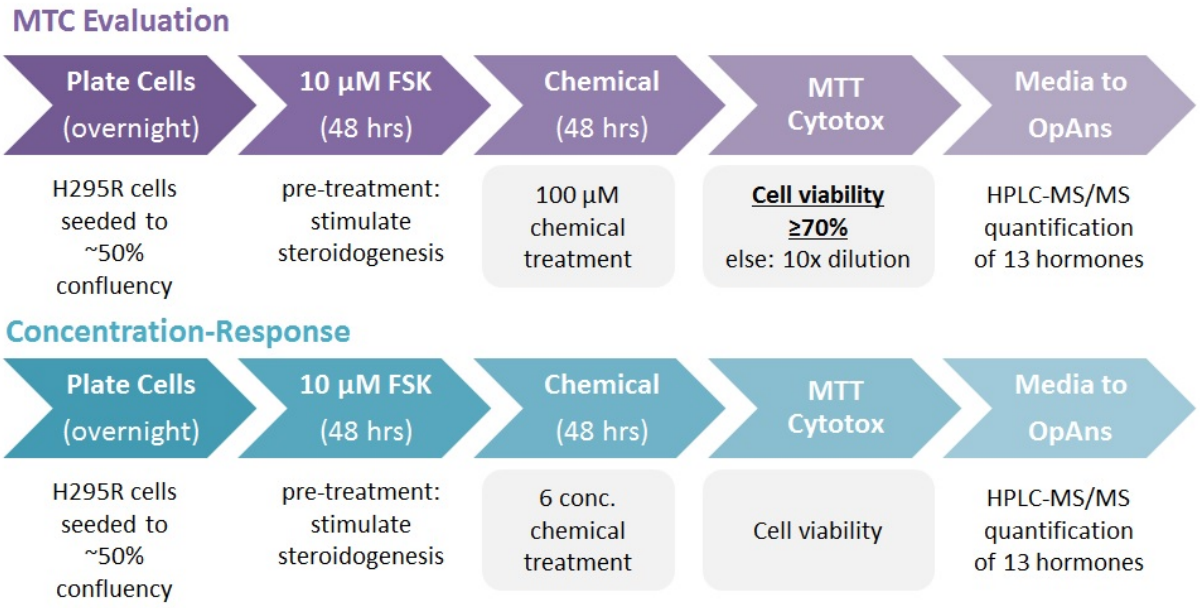


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R Progesterone assay was used to screen a large chemical library for changes in progesterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for progesterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of progesterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for progesterone were reported as 0.2 ng/mL and 40 ng/mL, respectively, using 4, 10, and 160 ng/mL standards, with precision of 3.3% and accuracy of 98.1% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

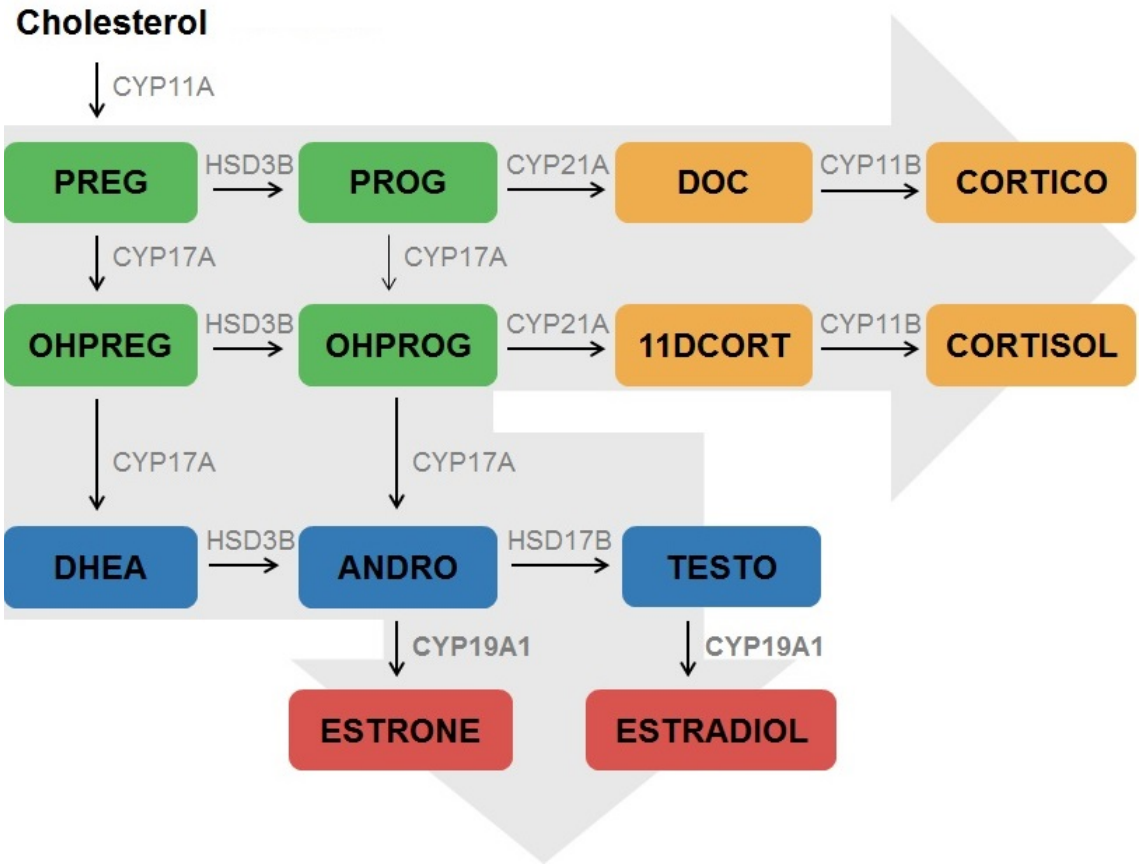


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.0 for progesterone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.85 for progesterone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 1 for progesterone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of 21 for progesterone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of progesterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_PROG_up readout data was analyzed in the positive (gain of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in progesterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Progesterone stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the progesterone assay this was approximately a 1.97 fold-change cut-off); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn

CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For progesterone, the $r^2 = 0.79$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.

[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Assay Endpoint ID: 914

CEETOX_H295R_TESTO_dn

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Testosterone Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

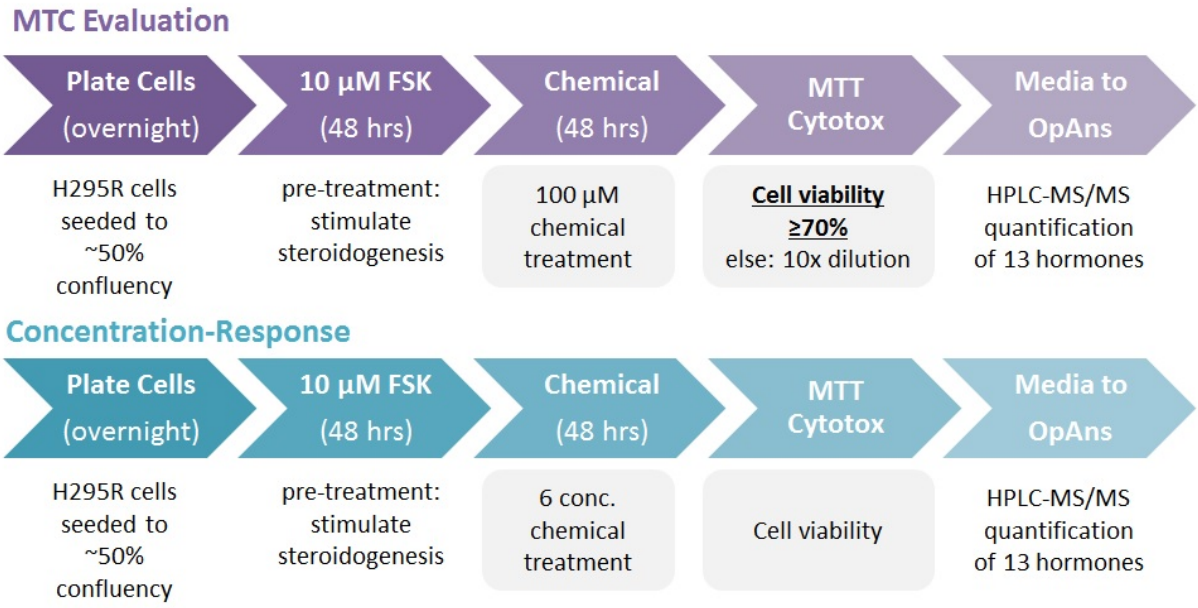


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R testosterone assay was used to screen a large chemical library for changes in testosterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for testosterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of testosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for testosterone were reported as 0.1 ng/mL and 20 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 5.7% and accuracy of 100.7% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

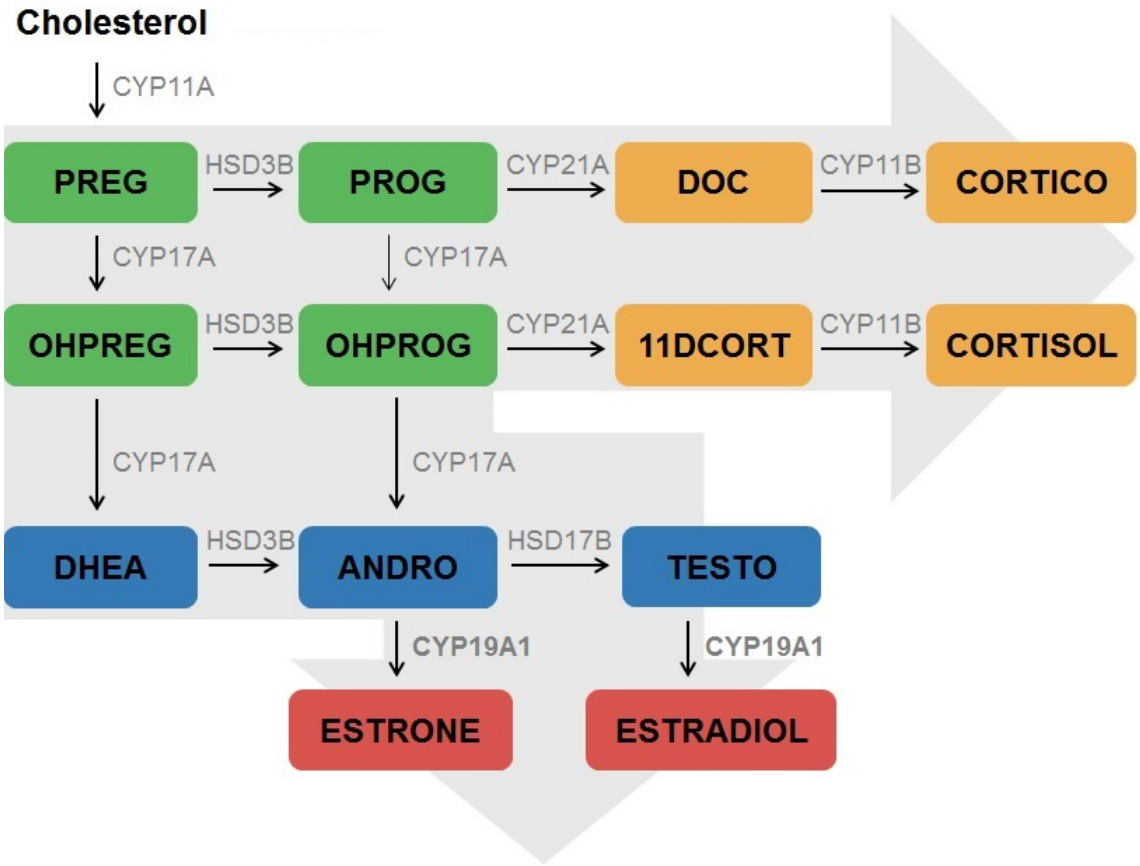


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.57 for testosterone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.75 for testosterone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 8 for testosterone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -13 for testosterone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7| demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Decreased production of testosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_TESTO_dn readout data was analyzed in the negative (loss of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in testosterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Testosterone inhibition was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the testosterone assay this created a 2.05 fold-change cut-off); if the modeled top of the curve (modl_tp) was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publically available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up

CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For testosterone, the $r^2 = 0.57$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.
[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)

[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

13 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

CEETOX_H295R_TESTO_up

Assay Title: CeeTox H295R High-throughput Steroidogenesis Assay – Testosterone Induction

1. Assay Descriptions

1.1. Overview

Assay Summary:

Endocrine disrupting chemicals have largely been studied by examining effects on nuclear receptor (primarily estrogen and androgen receptor) ligand binding or signaling pathways, but environmental chemicals can also disrupt endocrine systems by interfering with steroid hormone biosynthesis and metabolism (steroidogenesis). The CeeTox H295R steroidogenesis assays evaluate a human-derived, steroidogenically-competent adenocarcinoma cell line (H295R) in high-throughput (96-well) format to detect substances with the potential to disrupt steroid hormone production. To detect interference with steroidogenesis, this assay quantified 13 different steroid hormones using high-throughput HPLC-MS/MS. The assay is a modified version of the OECD H295R test guideline (OECD TG 456). Briefly, cells were seeded into 96-well plates and after overnight incubation were pre-treated with 10 µM forskolin for 48 hours to stimulate steroidogenesis. Following the pre-treatment, medium was replaced with test chemical for 48 hours. Following chemical exposure, medium was removed for steroid hormone analysis, and cells were subjected to the MTT assay as an indicator of cell viability. This assay involved a three-tiered screening strategy. The initial stage was designed to identify a maximum tolerated concentration (MTC) of test chemical in H295R cells. Briefly, chemicals were evaluated at up to 100 µM (solubility permitting) using the MTT assay, in order to determine the maximum concentration that maintained ≥70% cell viability. Following MTC determination, each chemical was screened for effects on steroidogenesis by quantifying changes in hormone levels at the MTC (single test concentration). Chemicals selected for follow-up were evaluated in dose-response to establish whether changes in hormone levels were concentration-dependent. In an initial screening exercise, chemicals were selected for concentration-response follow-up if at least 4 different hormones were significantly affected when screened at the MTC. Concentration-response experiments were conducted using six concentrations, serially diluted down from the MTC at half-log increments.

1.2. Assay Definition

Assay Throughput:

Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times (maximum passage of 10). Cells were seeded into 96-well plates at 50-60% confluency and allowed to adhere overnight. Prior to test chemical exposure, cells were pre-stimulated with forskolin for 48 hours, and chemical exposures were subsequently conducted for 48 hours in forskolin-free media. Following concentration-response and MTC assays, MTT cytotoxicity assays were conducted. The media samples were stored at -80 °C prior to HPLC-MS/MS quantification of steroid hormones.

Experimental System:

H295R is an immortalized cell line derived from an adrenocortical carcinoma isolated in 1980 from a 48-year-old African-American female patient. H295R cells express genes that encode for all the key enzymes involved in steroidogenesis [1, 2]. H295R cells have the physiological characteristics of zonally undifferentiated human fetal adrenal cells. H295R cells represent a unique in vitro system, as the cells retain the ability to produce many of the steroid hormones found in the adult adrenal cortex and the gonads (with the exception of dihydrotestosterone, DHT), which allows for analysis of xenobiotic effects on both corticosteroid synthesis and the production of sex steroid hormones, including androgens and estrogens.

Xenobiotic Biotransformation Potential:

H295R cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by endocrine-disrupting chemicals [3, 4].

Basic Procedure:

Cell culture and media preparation procedures were conducted in accordance with OECD Test No. 456 guidelines [1], with minor modifications. H295R cells (ATCC CRL-2128) were expanded for 5 passages and frozen in batches in liquid nitrogen. Prior to the steroidogenesis assay, H295R cells were thawed and passed at least 4 times. The maximum passage was 10. Cells were maintained in a 1:1 mixture of DMEM/F12 supplemented with 5ml/L ITS+ Premix (BD Bioscience) and 12.5 ml/L Nu-Serum (BD Bioscience). After seeding cells into 96-well plates at 50-60% confluency, cells were allowed to adhere overnight. Prior to chemical testing, culture medium was replaced with 175 µL medium containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Following pre-stimulation, forskolin medium was replaced with medium containing test chemical solubilized in DMSO (ensuring a final concentration of 0.1% DMSO). Test chemicals were incubated for 48 hours, then the medium was removed and split into 2 vials containing approximately 75µL each, and stored at -80 °C prior to hormone analysis. For steroid quantification, media samples were shipped to OpAns, LLC (Durham, NC) on dry ice, and samples were thawed to room temperature prior to liquid-liquid extraction. Steroid hormones were extracted from media samples using methyl tert-butyl ether (MTBE). An extra derivatization with dansyl chloride was included for estrogen (estrone and estradiol) detection only. Steroid hormones were separated by HPLC, eluted using a reverse phase C18 gradient with electrospray positive ionization, followed by quantification by tandem mass spectrometry (MS/MS). Data were acquired on a MassHunter Workstation Acquisition version B03.01 (Agilent Technologies, Inc.) and processed using MassHunter Quantitative Analysis for QQQ. Accuracy was determined for each hormone analyte from 3 standards to determine upper and lower limits of quantification (ULOQ and LLOQ, respectively) using a 7-point standard curve. Precision and accuracy of the extraction and quantification methods were calculated as the percent relative standard deviation (%RSD) of the spiked standards and percent spiked standard recovered, respectively. The goal was to achieve 100% accuracy (i.e., recover all spiked-in standard at quantification with minimal loss during run time) and good precision (i.e., have %RSD <15% to ensure reproducibility). Test medium was removed following chemical exposure, and cell viability was evaluated in the same wells by MTT [5]. MTT procedures were as follows: after removal of test medium, 500 µl of 0.5 mg/ml of 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide solution was added to the cells. Following a 4-hour incubation at 37 °C and 5% CO_2 to allow formazan-crystal formation, the MTT solution was removed and blue formazan salt crystals were solubilized using 500µl anhydrous isopropanol with shaking for 20 minutes. Absorbance was read at 570 and 650nm using a Packard Fusion microplate reader. Background correction of absorbance units was used to determine percent change relative to controls. All assay plates contained multiple control wells including four 10µM forskolin replicates to control for hormone stimulation, four 3µM procholaz replicates to control for hormone inhibition and 4 digitonin replicates to control for cell death. Cell viability, as indicated by the MTT assay, was used to establish maximum tolerated concentration (MTC) initially using a nominal concentration of 100µM (where feasible within solubility limits of the specific test chemical) and targeting cell viability ≥70%. When a test chemical reduced cell viability to 20% - 70%, the test chemical was diluted 10-fold and re-evaluated, but if cell viability was <20%, then the test chemical was diluted 100-fold and re-evaluated. Test chemical dilutions were made until cell viability was ≥70%. MTT assays were also conducted for duplicates of all concentrations of chemicals tested in concentration-response studies.

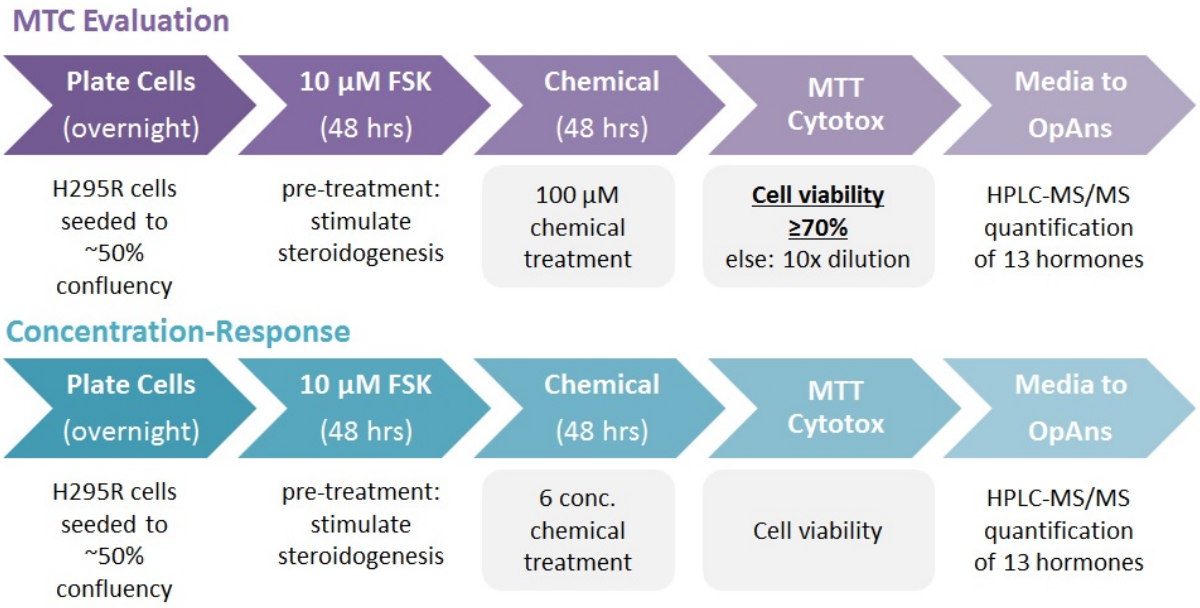


Figure 1. Schematic representation of general testing protocol for CeeTox Steroidogenesis assays. (Adapted from Karmaus et al. 2016.)

Proprietary Elements:

This assay is not proprietary; it is a modification of the existing OECD H295R *in vitro* steroidogenesis assay validated in 2011 (Test Guideline No. 456, [1]). NCI-H295R cells (ATCC CRL-2128) are commercially available from American Type Culture Collection with signed Material Transfer Agreement.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to interfere with endogenous steroidogenesis, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Cyprotex (Formerly CeeTox, Inc. in Kalamazoo, MI)
313 Pleasant St.
Watertown, MA 02472
1-888-297-7683
Fax: 1-617-812-0712
enquiries@cyprotex.com

Assay Publication Year:

2016

Assay Publication:

Karmaus AL, Toole CM, Filer DL, Lewis KC, Martin MT. 2016. High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells. Toxicological Sciences 150:323-332.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The CeeTox H295R testosterone assay was used to screen a large chemical library for changes in testosterone levels resulting from interference with steroidogenesis in H295R human adrenocortical carcinoma cells. For an initial screening application, a three-tiered screening approach was employed. The first stage involved establishing the maximum tolerated concentration (MTC) for each test chemical, respectively, which was identified using the MTT assay as an indicator of cell viability. Once MTC was determined, cells were treated with that concentration to evaluate steroid hormone changes at the MTC. In this single concentration "MTC screening" assay, any response ≥ |1.5-fold| relative to DMSO controls was considered to represent significant effects on hormone production. Following the MTC single concentration screen, those chemicals with significant effects on at least 4 hormones were considered active and subsequently assessed in concentration-response format using a 6-concentration series. After chemical treatment, which consists of 48hr forskolin pre-stimulation followed by 48hr chemical treatment, medium was subjected to MBTE and dansyl chloride derivatization for testosterone quantification. HPLC-MS/MS spectra were resolved and the absolute quantity of testosterone from the medium sample was reported in ng/mL. Chemicals were tested in duplicate, and each plate included duplicated controls for stimulation (10µM forskolin) and inhibition (3 µM procholaz) of steroidogenesis. 4 replicates per plate of DMSO-only solvent controls served as neutral controls. The LLOQ and LLOD for testosterone were reported as 0.1 ng/mL and 20 ng/mL, respectively, using 0.4, 1, and 16 ng/mL standards, with precision of 5.7% and accuracy of 100.7% (Karmaus *et al.*, 2016, Table 1).

Scientific Principles:

The steroidogenic pathway is a series of hydroxylation and dehydrogenation steps carried out by CYP450 and hydroxysteroid dehydrogenase enzymes. Environmentally-relevant chemicals can elicit endocrine disruption by altering steroid hormone biosynthesis and metabolism (steroidogenesis), resulting in adverse reproductive and developmental effects. The ToxCast HTS program adapted the OECD-validated H295R steroidogenesis assay using human adrenocortical carcinoma cells (TG No. 456; which established performance-based testing guidelines for the quantification of two steroid hormones, testosterone and estradiol) to quantitatively assess the concentration-dependent effects of chemicals on 13 steroid hormones in 4 hormone classes including progestagens, androgens, estrogens and glucocorticoids.

To identify xenobiotics with the capacity to disrupt the steroid hormone biosynthetic pathway, this assay quantitatively assesses changes in steroid hormone levels. The CeeTox Steroidogenesis assay also demonstrates a novel implementation of H295R cells as a high-throughput and multiplexed screening platform for screening a diverse chemical library for xenobiotic interference with steroidogenesis.

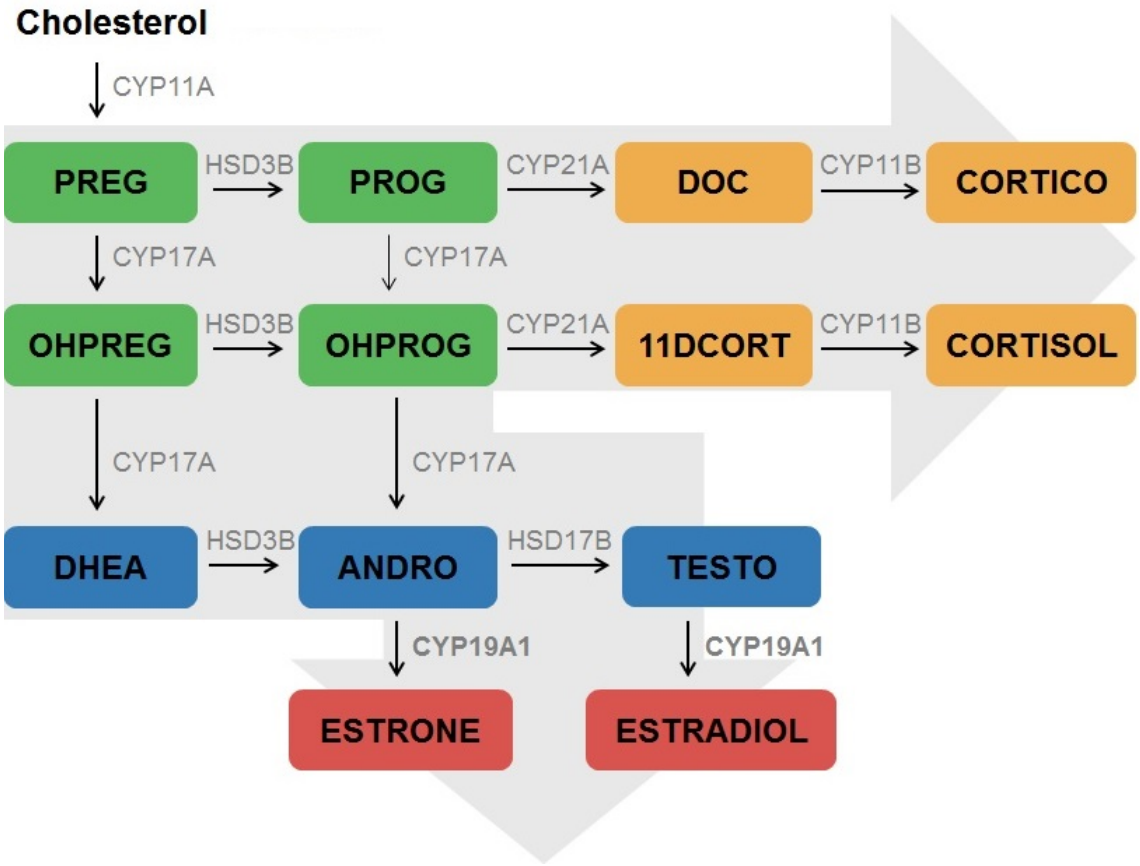


Figure 2. Subset of the steroidogenesis pathway targeted by the CeeTox H295R Steroidogenesis assays. (Modified from Karmaus *et al.* 2016.)

Method Development Reference:

Nielsen FK, Hansen CH, Fey JA, Hansen M, Jacobsen NW, Halling-Sørensen B, Björklund E, Styrishave B. (2012). "H295R cells as a model for steroidogenic disruption: a broader perspective using simultaneous chemical analysis of 7 key steroid hormones". Toxicology In Vitro 26:343-350.

OECD (2011). *Test No. 456 H295R Steroidogenesis Assay.* OECD Publishing, Paris

USEPA (2011). *Steroidogenesis (Human Cell Line – H295R)* OCSPP Guideline 890.1550. Standard Evaluation Procedure (SEP) Endocrine Disruptor Screening Program.

Assay Quality Statistics:

Robust Z-prime (Z') was calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and positive control (forskolin and prochloraz) wells. The Z' for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. Z'-forskolin ranged from 0.57-0.81, with a Z'-forskolin of 0.57 for testosterone. Z'-prochloraz ranged from 0.51-0.88, with a Z'-prochloraz of 0.75 for testosterone. Z' values ranging between 0.5 and 1 demonstrate that an assay signal can be reliably identified from variability around the baseline control.

The strictly standardized mean difference (SSMD) values were calculated per hormone analyte using the median of the raw response values across plates for the baseline control (DMSO) and

positive control (forskolin and prochloraz) wells. The SSMD for forskolin and prochloraz are presented in Table 2 of Karmaus et al. 2016, with separate values presented for each hormone to reflect the different positive control wells present on the plate included in the analysis of specific hormones. SSMD-forskolin ranged from 1-18, with a SSMD-forskolin of 8 for testosterone. SSMD-prochloraz ranged from -27-7, with a SSMD-prochloraz of -13 for testosterone. SSMD is a measure of effect size and a means of demonstrating directionality, i.e. differences between positive and negative control response; values ≥ |7|demonstrate excellent dynamic range and robust responses with the positive and negative controls.

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Increased production of testosterone following interference with steroidogenesis as quantified by HPLC-MS/MS.

Analytical Elements:

CEETOX_H295R_TESTOSTERONE_up readout data was analyzed in the positive (gain of signal) fitting direction using DMSO controls as the baseline signal, and was reported as log2 fold-change increase in testosterone activity (compared between duplicate chemical treatment and plate-matched quadruplet DMSO controls). Data were log_2-transformed, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest AIC value is considered the winning model and used in further analysis as the most appropriate predictor of assay effects. Testosterone stimulation was determined based on chemical-response data fulfilling the following criteria: the median of normalized response values at a single concentration exceeded the signal noise band (in this assay, any response over 6 times the baseline median absolute deviation in log_2 fold-change; in the testosterone assay this created a 2.05 fold-change cut-off); if the modeled top of the curve (modl_tp) was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up

CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_MTT_Cytotoxicity_dn
CEETOX_H295R_MTT_Cytotoxicity_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up

3.2. Assay Performance

Assay Performance Measures:

One measure of assay performance is reproducibility. For the initial screening exercise that employed single-concentration and concentration-response phases, a Pearson's linear correlation test, using the median log_2-fold change results from the MTC, was performed to demonstrate the concordance between the single-concentration and multi-concentration phases of screening. The correlation of maximum fold-change (r^2) is presented in Karmaus et al. 2016, Table 2. The correlation coefficients for each hormone between the two phases of that study, conducted independently, are presented by an average $r^2 = 0.70$. For testosterone, the $r^2 = 0.57$. These values demonstrate that the assay is highly reproducible.

Reference Chemicals / Predictive Capacity:

3.3. Assay Scope and Limitations

Chemical Library:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking [6].

4. Assay Documentation

4.1. References

[1] OECD (2011). Test No. 456 H295r Steroidogenesis Assay. Paris, OECD Publishing.

[2] Gracia, T., et al. (2006). Ecotoxicol Environ Saf 65(3): 293-305.
[3] Sanderson, J. T., et al. (2001). Toxicol Sci 61(1): 40-48. (PMID: 11294972)
[4] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[5] Mosmann, T. (1983). J Immunol Methods 65(1-2): 55-63.
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Type Culture Collection
DMSO, Dimethyl Sulfoxide
HPLC-MS/MS, High-Performance Liquid Chromatography, with tandem Mass Spectrometry
MTC, Maximum Tolerated Concentration
MTT, 3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazoliumbromide
R^2, correlation coefficient, R-squared
SSMD, strictly standardized mean difference
Z', Robust Z-prime

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

17 January 2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

ACEA_T47D_80hr_Positive
ATG_AR_TRANS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
ATG_ERE_CIS_up
ATG_ERRa_TRANS_up
ATG_ERRb_TRANS2_up
ATG_ERRg_TRANS_up
ATG_FXR_TRANS_up
ATG_IR1_CIS_up
ATG_PPARa_TRANS_up
ATG_PPARd_TRANS_up
ATG_PPARg_TRANS_up
ATG_PPRE_CIS_up
ATG_THRa1_TRANS_up
ATG_THRb_TRANS2_up
CEETOX_H295R_11DCORT_dn
CEETOX_H295R_11DCORT_up
CEETOX_H295R_ANDR_dn
CEETOX_H295R_ANDR_up
CEETOX_H295R_CORTIC_dn
CEETOX_H295R_CORTIC_up
CEETOX_H295R_CORTISOL_dn
CEETOX_H295R_CORTISOL_up
CEETOX_H295R_DHEA_dn
CEETOX_H295R_DHEA_up
CEETOX_H295R_DOC_dn
CEETOX_H295R_DOC_up
CEETOX_H295R_ESTRADIOL_dn
CEETOX_H295R_ESTRADIOL_up
CEETOX_H295R_ESTRONE_dn
CEETOX_H295R_ESTRONE_up
CEETOX_H295R_OHPREG_dn
CEETOX_H295R_OHPREG_up
CEETOX_H295R_OHPROG_dn
CEETOX_H295R_OHPROG_up
CEETOX_H295R_PREG_dn
CEETOX_H295R_PREG_up
CEETOX_H295R_PROG_dn
CEETOX_H295R_PROG_up
CEETOX_H295R_TESTO_dn
CEETOX_H295R_TESTO_up
LTEA_HepaRG_THRSP_dn
LTEA_HepaRG_THRSP_up
NVS_NR_bER
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_hER
NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist

NVS_NR_hPPARa
NVS_NR_hPPARg
NVS_NR_hTRa_Antagonist
NVS_NR_mERa
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERb_ERELUC_ANT_1440
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
OT_PPARg_PPARgSRC1_0480
OT_PPARg_PPARgSRC1_1440
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_BLA_Antagonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist
Tox21_AR_LUC_MDAKB2_Agonist_3uM_Nilutamide
Tox21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881
Tox21_AR_LUC_MDAKB2_Antagonist_10nM_R1881
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_VM7_Agonist
Tox21_ERa_LUC_VM7_Agonist_10nM_ICI182780
Tox21_ERa_LUC_VM7_Antagonist_0.1nM_E2
Tox21_ERa_LUC_VM7_Antagonist_0.5nM_E2
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio
Tox21_PPARd_BLA_Agonist_ratio
Tox21_PPARd_BLA_antagonist_ratio
Tox21_PPARg_BLA_Agonist_ratio
Tox21_PPARg_BLA_antagonist_ratio
Tox21_TR_LUC_GH3_Agonist
Tox21_TR_LUC_GH3_Antagonist

LTEA_HepaRG_THRSP_dn

Assay Title: Life Technologies / Expression Analysis HepaRG Cell-based High-Throughput Toxicogenomics Assay to Measure Thyroid Responsive Gene Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

A frequent concern with the extrapolation of in vitro assay data to in vivo toxicity is the potential lack of metabolic activity in the experimental platform used to assess chemical perturbations. Many xenobiotics impact biological systems following biotransformation in the liver, but most human hepatocyte cell lines express variable or negligible levels of liver-specific functions and P450 enzyme-related activities, making them unrepresentative models for in vivo toxicity. This assay was developed to further explore the extent to which liver specific functions and enzymes can impact toxicant responses in human hepatocytes. This assay assesses toxicogenomics in a metabolically competent human liver cell line (HepaRG) by screening the EPA ToxCast chemical library for gene-specific perturbations. Life Technologies exposed differentiated HepaRG cells in duplicate 96-well plates to 8-point half-log dilutions of test and control chemicals, and each assay includes positive controls for nuclear receptor activation (phenobarbital). Each plate assessed cell lysis (as measured by LDH release) following 48-hour incubation with a metabolically-activated cytotoxic agent (Aflatoxin B1). Following 48-hour concentration-response screening, cells were lysed and frozen and each plate was immediately shipped to Expression Analysis (Quintiles) labs for qRT-PCR analysis of changes in transcription levels for 93 genes related to biotransformation enzymes, nuclear receptors and NR mediated transporters, cell cycle regulation, and stress responses.

1.2. Assay Definition

Assay Throughput:

HepaRG cells are dispensed into 96-well microtiter plates and maintained in 770 media for 48 hours before media is removed and replaced with serum-free 750 induction media prior to test compound additions. Following 48-hour test exposures, 75 µL of spent media from each assay plate is transferred to black-walled microtiter plates to assess cytotoxicity (LDH release). Cells were then lysed with RLT buffer, sealed, frozen at -80 °C and sent for gene expression analysis (with universal human reference RNA added to each plate) using Fluidigm™ qRT-PCR.

Experimental System:

HepaRG is an immortalized cell line with a homogenous karyotype which was derived in 1999 from a hepatocarcinoma of a 66-year-old female Caucasian cholangiocarcinoma patient (Gripon et al. 2002). After proliferation, the cultures contain cells which retain the potential for inducible differentiation (with 2% DMSO addition) into hepatocytes and primitive biliary cells (Cerec et al. 2007, Gripon et al. 2002).

Xenobiotic Biotransformation Potential:

HepaRG cells have been shown to stably express various liver-specific functions after having reached confluency (Aninat et al. 2006). Under certain circumstances, HepaRG cells are capable of expressing cytochrome P450 enzymes (CYP1A2, 2B6, 2C9, 2D6, 2E1, and 3A4), metabolism-regulating nuclear receptors (AhR, PXR, CAR, and PPARα), phase 2 enzymes (UGT1A1, GSTA1, GSTA4, and GSTM1), and glutathione-related enzymes as well as liver-specific proteins (albumin, haptoglobin, and aldolase B), and functions (AFP and thioredoxin) have been detected (Aninat et al. 2006, Guillouzo et al. 2007). HepaRG cells seeded at high density show higher mRNA levels for these activities than in those seeded at low density and the highest values are observed in

differentiated cells exposed to 2% DMSO (Aninat et al. 2006, Guillouzo et al. 2007). With the exception of CYP2D6, the measured activities can be close or higher (CYP2B6 and CYP3A4) than those measured in primary human hepatocytes under similar culture conditions, and this capacity can be maintained in cultures over time (3-4 weeks) (Aninat et al. 2006, Anthérieu et al. 2010, Guillouzo et al. 2007, Josse et al. 2008, Lübberstedt et al. 2011). Therefore, HepaRG cells are thought to potentially possess both the metabolic performances of primary hepatocytes and growth capacity of hepatic cell lines (Andersson 2010, Guillouzo et al. 2007).

Basic Procedure:

Protocols

Cryopreserved HepaRG™ (LT catalog no. HPRGC10) in collagen (type I) coated 96-well plates were prepared according to standard methods and maintained in HepaRG™ 770 media for 48 hours post-plating. Prior to chemical additions, culture media was exchanged for serum-free 750 induction media and DMSO was back-added to stock chemicals to a final concentration of 0.5% for all dosing media. 8-point concentration-response curves were generated in half-log dose spacing ($10^{0.5}$-fold increments, maximum concentration 100 µM) for all chemicals. 48 hours after plating test cultures were dosed in duplicates and maintained in CO_2 incubator at 37 °C. Treated cells were imaged with an Essen IncuCyte™ FLR automated phase-contrast microscope with image analysis software at 24 and 48 hours post-exposure to assess morphological changes. The microscope was located inside the incubator to reduce cell disruption due to changes in environmental conditions. Each plate contained two total lysis control wells and four vehicle control wells (0.5% DMSO) as well as 1mM phenobarbital (induction positive control) and 100µM aflatoxin B1 (cytotoxicity positive control) in triplicate concentration response series. Total lysis controls were generated by removing spent culture media and replacing with 0.1% Triton X. Following 48-hour test exposures, 75µL of spent media from each assay plate was transferred to black-walled microtiter plates for cytotoxicity assessment using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) to assess LDH release. LDH activities were determined from each sample supernatant and were compared with mean responses of each total lysis control by calculation of total cell lysis activities for each replicate. Fluorescent emission was detected using a fluorometer set to optimal gain for each plate and raw data was converted from relative fluorescence units (RFU) to percent cytotoxicity, normalized to the vehicle and total lysis controls on each plate, as below:

% cytotoxicity = 100* [(Experimental Value - Culture Medium Background)/(Maximum LDH Release - Culture Medium Background)]

Cells were lysed with 75µL of RLT buffer, sealed, frozen at -80 °C and sent to EA for gene expression analysis using qRT-PCR using Fluidigm™ 96.96 microfluidic technology to determine ΔΔCt relative to DMSO and housekeeping genes. Each plate analyzed for gene expression included universal human reference RNA, a no template control and an enzyme-free control.

Proprietary Elements:

This assay is not proprietary. Cryopreserved HepaRG™ cells and media used are commercially available from ThermoFisher Scientific. Fluidigm 96.96 microfluidic technology is available from Fluidigm Corporation.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to activate endogenous thyroid signaling pathways, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to

provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Thermo Fisher Scientific (Formerly Life Technologies)
Global Headquarters
168 Third Avenue
Waltham, MA USA 02451

Phone:
781-622-1000
800-678-5599

Expression Analysis/Quintiles
US Headquarters
5827 South Miami Blvd
Morrisville, NC USA 27560

Phone:
919-998-7000

Assay Publication Year:

Assay Publication:

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The LTEA HepaRG thyroid responsive assay screened the ToxCast library of diverse environmental chemicals to probe for xenobiotic activity and potential to modulate expression of thyroid-hormone responsive gene (THRSP) in metabolically active human hepatocarcinoma cell line HepaRG. Bioactivity was monitored through analysis of cytotoxicity and morphological responses to 8-point half-log concentration series (100 to 0.1 µM) of duplicated chemical samples. Morphological changes were examined through phase-contrast microscopy at 24 and 48 hours post-exposure, and cytotoxicity was determined at 48 hours by LDH quantification. Metabolic activity of HepaRG cells was monitored by exposing the system to a 3-replicate 8-point concentration series of the mycotoxicant aflatoxin B1 (AFL), a cytotoxic agent requiring metabolic activation by cytochrome p450 dependent mixed-function oxidase to be converted to the reactive metabolite aflatoxin B1-8,9-epoxide (AFBO). A triplicate 8-point concentration series of the CAR-inducer phenobarbital was also included as a positive control for gene expression. Following the 48-hour concentration response screening, the cells were lysed and shipped to Expression Analysis/Quintiles for toxicogenomics analyses using real-time PCR conducted on Fluidigm 96.96 Dynamic Array IFCs (integrated fluidic circuits). This assay also included analysis of a chemical reference plate for gene expression inducers, including phenobarbital, omeprazole, fenofibric acid and chenodeoxycholic acid.

Scientific Principles:

Thyroid hormone signaling is essential for normal brain development both before and after birth and has profound effects on cellular metabolism in almost all organs. Thyroid hormone is important in lipid and glucose metabolism, lipolysis, and body weight, and variations in thyroid status in humans has been linked to significant changes in metabolic activity. Thyroidal regulation of metabolic pathways is a function of crosstalk with nuclear receptor signaling and thyroid hormone availability. One signaling pathway implicated in the link between thyroid status and body weight is regulated by THRSP, a primarily nuclear protein induced by TH and carbohydrate intake, which is closely related to lipid metabolism and breast cancer. An important component of an endocrine disruptor screening program should be the inclusion of assays designed to screen TH disrupting chemicals. The HepaRG cell line is a metabolically active hepatic system which was used to evaluate the potential for xenobiotic chemicals to alter THRSP activity. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous thyroid signaling by monitoring the expression of THRSP (fold-change relative to DMSO and housekeeping genes) following 48 hour incubation with test chemicals.

Method Development Reference:

Rotroff DM, Beam AL, Dix DJ, Farmer A, Freeman KM, Houck KA, Judson RS, LeCluyse EL, Martin MT, Reif DM. 2010. Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by ToxCast chemicals. Journal of Toxicology and Environmental Health, Part B 13:329-346. (PMID: 20574906)

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Inhibition of thyroid-hormone responsive gene (THRSP) monitored through qRT-PCR analysis of mRNA expression.

Analytical Elements:

Raw data were converted from raw relative fluorescence units (RFU) to percent cytotoxicity, normalized to the vehicle and total lysis controls on each plate, as in the following formula: % cytotoxicity = 100* [(Experimental - Culture Medium Background)/(Maximum LDH Release - Culture Medium Background)]. Gene expression was measured as ΔΔCt (log2 fold-change) relative to DMSO and housekeeping genes. Decreased (loss-of-signal) levels of mRNA indicate down regulation of THRSP by test chemical. Concentration-response relationships were determined based on a range of 8 chemical concentrations, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for

each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up
ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_up
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Agonist
Tox21_TR_LUC_GH3_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [9].

4. Assay Documentation

4.1. References

[1] Gripon, P., et al. (2002). PNAS 99(24): 15655-15660. (PMID: 12432097)
[2] Cerec, V., et al. (2007). Hepatology 45(4): 957-967. (PMID: 17393521)
[3] Aninat, C., et al. (2006). Drug Metab Disposition 34(1): 75-83. (PMID: 16204462)
[4] Guillouzo, A., et al. (2007). Chem-Biol Interact 168(1): 66-73. (No PMID)
[5] Anthérieu, S., et al. (2010). Drug Metab Disposition 38(3): 516-525. (PMID: 20019244)
[6] Lübberstedt, M., et al. (2011). J Pharmacol Toxicol Methods 63(1): 59-68. (PMID: 19328226)
[7] Josse, R., et al. (2008). Drug Metab Disposition 36(6): 1111-1118. (PMID: 18347083)
[8] Andersson, T. B. (2010). Hepatocytes: Methods Protocols: 375-387. (PMID: 20645063)
[9] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EA, Expression Analysis
EDC, Endocrine disrupting chemicals
FBS, Fetal Bovine Serum

GST, Glutathione S-Transferase
LDH, Lactate Dehydrogenase
LT, Life Technologies
NR, Nuclear Receptor
SULT, Sulfotransferases
THRSP, Thyroid-Hormone Responsive
UGT, UDP-glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

23 August 2016

Date of Revisions:

20 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

LTEA_HepaRG_THRSP_up

Assay Title: Life Technologies / Expression Analysis HepaRG Cell-based High-Throughput Toxicogenomics Assay to Measure Thyroid Responsive Gene Activation

1. Assay Descriptions

1.1. Overview

Assay Summary:

A frequent concern with the extrapolation of in vitro assay data to in vivo toxicity is the potential lack of metabolic activity in the experimental platform used to assess chemical perturbations. Many xenobiotics impact biological systems following biotransformation in the liver, but most human hepatocyte cell lines express variable or negligible levels of liver-specific functions and P450 enzyme-related activities, making them unrepresentative models for in vivo toxicity. This assay was developed to further explore the extent to which liver specific functions and enzymes can impact toxicant responses in human hepatocytes. This assay assesses toxicogenomics in a metabolically competent human liver cell line (HepaRG) by screening the EPA ToxCast chemical library for gene-specific perturbations. Life Technologies exposed differentiated HepaRG cells in duplicate 96-well plates to 8-point half-log dilutions of test and control chemicals, and each assay includes positive controls for nuclear receptor activation (phenobarbital). Each plate assessed cell lysis (as measured by LDH release) following 48-hour incubation with a metabolically-activated cytotoxic agent (Aflatoxin B1). Following 48-hour concentration-response screening, cells were lysed and frozen and each plate was immediately shipped to Expression Analysis (Quintiles) labs for qRT-PCR analysis of changes in transcription levels for 93 genes related to biotransformation enzymes, nuclear receptors and NR mediated transporters, cell cycle regulation, and stress responses.

1.2. Assay Definition

Assay Throughput:

HepaRG cells are dispensed into 96-well microtiter plates and maintained in 770 media for 48 hours before media is removed and replaced with serum-free 750 induction media prior to test compound additions. Following 48-hour test exposures, 75 µL of spent media from each assay plate is transferred to black-walled microtiter plates to assess cytotoxicity (LDH release). Cells were then lysed with RLT buffer, sealed, frozen at -80 °C and sent for gene expression analysis (with universal human reference RNA added to each plate) using Fluidigm™ qRT-PCR.

Experimental System:

HepaRG is an immortalized cell line with a homogenous karyotype which was derived in 1999 from a hepatocarcinoma of a 66-year-old female Caucasian cholangiocarcinoma patient [1]. After proliferation, the cultures contain cells which retain the potential for inducible differentiation (with 2% DMSO addition) into hepatocytes and primitive biliary cells [1, 2].

Xenobiotic Biotransformation Potential:

HepaRG cells have been shown to stably express various liver-specific functions after having reached confluency [3]. Under certain circumstances, HepaRG cells are capable of expressing cytochrome P450 enzymes (CYP1A2, 2B6, 2C9, 2D6, 2E1, and 3A4), metabolism-regulating nuclear receptors (AhR, PXR, CAR, and PPARα), phase 2 enzymes (UGT1A1, GSTA1, GSTA4, and GSTM1), and glutathione-related enzymes as well as liver-specific proteins (albumin, haptoglobin, and aldolase B), and functions (AFP and thioredoxin) have been detected [3, 4]. HepaRG cells seeded at high density show higher mRNA levels for these activities than in those seeded at low density and the highest values are observed in differentiated cells exposed to 2% DMSO [3, 4]. With the exception of CYP2D6, the measured activities can be close or higher (CYP2B6 and CYP3A4) than

those measured in primary human hepatocytes under similar culture conditions, and this capacity can be maintained in cultures over time (3-4 weeks) [3-7]. Therefore, HepaRG cells are thought to potentially possess both the metabolic performances of primary hepatocytes and growth capacity of hepatic cell lines [4, 8].

Basic Procedure:

Protocols

Cryopreserved HepaRG™ (LT catalog no. HPRGC10) in collagen (type I) coated 96-well plates were prepared according to standard methods and maintained in HepaRG™ 770 media for 48 hours post-plating. Prior to chemical additions, culture media was exchanged for serum-free 750 induction media and DMSO was back-added to stock chemicals to a final concentration of 0.5% for all dosing media. 8-point concentration-response curves were generated in half-log dose spacing ($10^{0.5}$-fold increments, maximum concentration 100 µM) for all chemicals. 48 hours after plating test cultures were dosed in duplicates and maintained in CO_2 incubator at 37 °C. Treated cells were imaged with an Essen IncuCyte™ FLR automated phase-contrast microscope with image analysis software at 24 and 48 hours post-exposure to assess morphological changes. The microscope was located inside the incubator to reduce cell disruption due to changes in environmental conditions. Each plate contained two total lysis control wells and four vehicle control wells (0.5% DMSO) as well as 1mM phenobarbital (induction positive control) and 100µM aflatoxin B1 (cytotoxicity positive control) in triplicate concentration response series. Total lysis controls were generated by removing spent culture media and replacing with 0.1% Triton X. Following 48-hour test exposures, 75µL of spent media from each assay plate was transferred to black-walled microtiter plates for cytotoxicity assessment using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega) to assess LDH release. LDH activities were determined from each sample supernatant and were compared with mean responses of each total lysis control by calculation of total cell lysis activities for each replicate. Fluorescent emission was detected using a fluorometer set to optimal gain for each plate and raw data was converted from relative fluorescence units (RFU) to percent cytotoxicity, normalized to the vehicle and total lysis controls on each plate, as below:

% cytotoxicity = 100* [(Experimental Value - Culture Medium Background)/(Maximum LDH Release - Culture Medium Background)]

Cells were lysed with 75µL of RLT buffer, sealed, frozen at -80 °C and sent to EA for gene expression analysis using qRT-PCR using Fluidigm™ 96.96 microfluidic technology to determine ΔΔCt relative to DMSO and housekeeping genes. Each plate analyzed for gene expression included universal human reference RNA, a no template control and an enzyme-free control.

Proprietary Elements:

This assay is not proprietary. Cryopreserved HepaRG™ cells and media used are commercially available from ThermoFisher Scientific. Fluidigm 96.96 microfluidic technology is available from Fluidigm Corporation.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to activate endogenous thyroid signaling pathways, and is intended to provide information on a large number of diverse chemicals. Caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of chemical selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Thermo Fisher Scientific (Formerly Life Technologies)
Global Headquarters
168 Third Avenue
Waltham, MA USA 02451

Phone:
781-622-1000
800-678-5599

Expression Analysis/Quintiles
US Headquarters
5827 South Miami Blvd
Morrisville, NC USA 27560

Phone:
919-998-7000

Assay Publication Year:

Assay Publication:

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The LTEA HepaRG thyroid responsive assay screened the ToxCast library of diverse environmental chemicals to probe for xenobiotic activity and potential to modulate expression of thyroid-hormone responsive gene (THRSP) in metabolically active human hepatocarcinoma cell line HepaRG. Bioactivity was monitored through analysis of cytotoxicity and morphological responses to 8-point half-log concentration series (100 to 0.1 μM) of duplicated chemical samples. Morphological changes were examined through phase-contrast microscopy at 24 and 48 hours post-exposure, and cytotoxicity was determined at 48 hours by LDH quantification. Metabolic activity of HepaRG cells was monitored by exposing the system to a 3-replicate 8-point concentration series of the mycotoxicant aflatoxin B1 (AFL), a cytotoxic agent requiring metabolic activation by cytochrome p450 dependent mixed-function oxidase to be converted to the reactive metabolite aflatoxin B1-8,9-epoxide (AFBO). A triplicate 8-point concentration series of the CAR-inducer phenobarbital was also included as a positive control for gene expression. Following the 48 hour concentration response screening, the cells were lysed and shipped to Expression Analysis/Quintiles for toxicogenomics analyses using real-time PCR conducted on Fluidigm 96.96 Dynamic Array IFCs (integrated fluidic circuits). This assay also included analysis of a chemical reference plate for gene expression inducers, including phenobarbital, omeprazole, fenofibric acid and chenodeoxycholic acid.

Scientific Principles:

Thyroid hormone signaling is essential for normal brain development both before and after birth and has profound effects on cellular metabolism in almost all organs. Thyroid hormone is important in lipid and glucose metabolism, lipolysis, and body weight, and variations in thyroid status in humans has been linked to significant changes in metabolic activity. Thyroidal regulation of

metabolic pathways is a function of crosstalk with nuclear receptor signaling and thyroid hormone availability. One signaling pathway implicated in the link between thyroid status and body weight is regulated by THRSP, a primarily nuclear protein induced by TH and carbohydrate intake, which is closely related to lipid metabolism and breast cancer. An important component of an endocrine disruptor screening program should be the inclusion of assays designed to screen TH disrupting chemicals. The HepaRG cell line is a metabolically active hepatic system which was used to evaluate the potential for xenobiotic chemicals to alter THRSP activity. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous thyroid signaling by monitoring the expression of THRSP (fold-change relative to DMSO and housekeeping genes) following 48-hour incubation with test chemicals.

Method Development Reference:

Rotroff DM, Beam AL, Dix DJ, Farmer A, Freeman KM, Houck KA, Judson RS, LeCluyse EL, Martin MT, Reif DM. 2010. Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by ToxCast chemicals. Journal of Toxicology and Environmental Health, Part B 13:329-346.

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Activation of thyroid-hormone responsive gene (THRSP) monitored through qRT-PCR analysis of mRNA expression.

Analytical Elements:

Raw data were converted from raw relative fluorescence units (RFU) to percent cytotoxicity, normalized to the vehicle and total lysis controls on each plate, as in the following formula: % cytotoxicity = 100* [(Experimental - Culture Medium Background)/(Maximum LDH Release - Culture Medium Background)]. Gene expression was measured as ΔΔCt (log2 fold-induction) relative to DMSO and housekeeping genes. Increased (gain-of-signal) levels of mRNA indicate upregulation of THRSP by test chemical. Concentration-response relationships were determined based on a range of 8 chemical concentrations, and all statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up

ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_dn
LTEA_HepaRG_THRSP_up
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Agonist
Tox21_TR_LUC_GH3_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. [9].

4. Assay Documentation

4.1. References

[1] Gripon, P., et al. (2002). PNAS 99(24): 15655-15660. (PMID: 12432097)
[2] Cerec, V., et al. (2007). Hepatology 45(4): 957-967. (PMID: 17393521)
[3] Aninat, C., et al. (2006). Drug Metab Disposition 34(1): 75-83. (PMID: 16204462)
[4] Guillouzo, A., et al. (2007). Chem-Biol Interact 168(1): 66-73. (No PMID)
[5] Anthérieu, S., et al. (2010). Drug Metab Disposition 38(3): 516-525. (PMID: 20019244)
[6] Lübberstedt, M., et al. (2011). J Pharmacol Toxicol Methods 63(1): 59-68. (PMID: 19328226)
[7] Josse, R., et al. (2008). Drug Metab Disposition 36(6): 1111-1118. (PMID: 18347083)
[8] Andersson, T. B. (2010). Hepatocytes: Methods Protocols: 375-387. (PMID: 20645063)
[9] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATCC, American Tissue Culture Collection
CYP, Cytochrome P450s
DMSO, Dimethyl Sulfoxide
EA, Expression Analysis
EDC, Endocrine disrupting chemicals
FBS, Fetal Bovine Serum
GST, Glutathione S-Transferase
LDH, Lactate Dehydrogenase
LT, Life Technologies

NR, Nuclear Receptor
SULT, Sulfotransferases
THRSP, Thyroid-Hormone Responsive
UGT, UDP-glucuronosyltransferase

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

23 August 2016

Date of Revisions:

20 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 708

NVS_NR_bER

Assay Title: NovaScreen Bovine Estrogen Receptor HTS Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenoestrogenic activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the estrogen receptor. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 18 hours in a 96-well plate.

1.2. Assay Definition

Assay Throughput:

This is a biochemical (cell-free) format, using 96-well plates to incubate radiolabeled ligand with estrogen receptor alpha for 18 hours to measure displacement of estradiol by test chemicals in a competitive binding assay.

Experimental System:

ERα nuclear protein, derived from bovine uterine membranes

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: Bovine uterine membranes
Radioligand: [3H] Estradiol
Final ligand concentration - [0.7 nM]
Non-specific Determinant: 17β-Estradiol - [10 nM]
Positive Control: 17β-Estradiol

Methods:

Incubation Conditions: Reactions are carried out in 10 mM TRIS-HCl (pH 7.4 containing 1.5 mM EDTA, 1.0 mM DTT, and 25 mM sodium molybdate at 0-4 °C for 18 hours. The reaction is terminated by the addition of dextran-coated charcoal and incubated for 20 minutes at 0-4 °C. The reaction mixtures are centrifuged and the radioactivity bound in the supernatant is assessed and compared to control values in order to ascertain any interactions of test compound with the estradiol binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective cell membrane and biotransformation capacity expected in in vivo or cell-based systems. The potential for a particular compound to affect changes in estrogen

signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor bovine estrogen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoestrogenic interaction with bovine nuclear receptors. An initial screening run was conducted exposing estrogen receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Estradiol. 17β-Estradiol (E2) was used as a positive control. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with estrogen nuclear receptor alpha (ERα) derived from bovine uterine membranes.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenoestrogenic ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE) leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Haji, M., Kato, K., Nawata, H., & Ibayashi, H. (1981). "Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat". Brain Res 204(2), 373-386. (PMID: 6780133)

O'Keefe, J. A., & Handa, R. J. (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus". Dev Brain Res 57(1), 119-127. (PMID: 2090365)

Assay Quality Statistics:

Neutral control well median response value, by plate:	2637.21
Neutral control median absolute deviation, by plate:	74.86
Positive control well median response value, by plate:	521.88
Positive control well median absolute deviation, by plate:	21.18
Z' (median across all plates, using positive control wells):	0.86
SSMD (median across all plates, using positive control wells):	-26
Signal-to-noise (median across all plates, using positive control wells):	-29.49
Signal-to-background (median across all plates, using positive control wells):	0.19
CV (median across all plates):	0.03

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [3H] Estradiol (positive control) with estrogen receptor α obtained from bovine uterine membranes and measured by radiometric detection.

Analytical Elements:

The NVS_NR_bER assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] 17β-estradiol binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of 17-β Estradiol (positive control) binding capacity. Following initial screening of test compounds at single concentration (25 µM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 2 baseline median average deviations (2BMAD), the chemical was considered active against the estrogen receptor and was tested in a concentration-response assay for ER binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is

considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.02 µM
Standard maximum concentration tested: 50 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.4.03
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 24.17

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes

57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	No
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	No
98-54-4	4-tert-Butylphenol	NA	Active	No
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	No
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	No

50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	No
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	No
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	No
72-33-3	Mestranol	NA	Active	No
72-43-5	Methoxychlor	Very Weak	Active	No
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	No
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	Yes
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	12	22
Inactive	12	6

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	22	15
Inactive	2	13

In Vitro Sensitivity = 35.2%
In Vitro Specificity = 33.3%
Balanced Accuracy = 34.3%

In Vivo Sensitivity = 59.5%
In Vivo Specificity = 86.7%
Balanced Accuracy = 73.1%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to

environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
E2, Estradiol
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Assay Endpoint ID: 710

NVS_NR_cAR

Assay Title: NovaScreen Chimpanzee Androgen Receptor Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenobiotic activity. The NovaScreen NR assays utilized a cell-free platform to screen a diverse chemical library for nuclear receptor activity in a high-throughput (96-well plate) format. This format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the androgen receptor following 72-hour incubation with test chemicals. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity.

1.2. Assay Definition

Assay Throughput:

Recombinant chimpanzee androgen receptor incubated in 96-well microtiter plates for 72 hours prior to measuring displacement of R1881 radiolabeled ligand by test compounds

Experimental System:

Baculovirus (Sf9/Sf21 cell line) derived recombinant chimpanzee AR

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: Recombinant chimpanzee androgen receptor

Radioligand: [^{3}H]Methyltrienolone (R1881)

Non-specific Determinant: Methyltrienolone (R1881)

Reference Compound: Methyltrienolone (R1881)

Positive Control: Methyltrienolone (R1881)

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity expected in in vivo systems. The potential for a particular compound to affect changes in androgen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office

940 Winter St.

Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Hartig, P., Cardon, M., Blystone, C., Gray, L., & Wilson, V. (2008). High throughput adjustable 96-well plate assay for androgen receptor binding: a practical approach for EDC screening using the chimpanzee AR. Toxicology letters, 181(2), 126-131.

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor chimpanzee androgen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenobiotic androgen receptor interaction with chimpanzee-derived nuclear receptors. An initial screening run was conducted exposing androgen receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Methyltrienolone (R1881). R1881 was used as a positive control. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with recombinant chimpanzee androgen receptor.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting androgen signaling pathways. The androgen receptor mediates gene expression in response to androgen exposures, and modulates the activity for a wide variety of physiological processes, particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility.

The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenobiotic androgen receptor ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Hartig, P., Cardon, M., Lambright, C., Bobseine, K., Gray, L., & Wilson, V. (2007). Substitution of synthetic chimpanzee androgen receptor for human androgen receptor in competitive binding and transcriptional activation assays for EDC screening. Toxicology letters, 174(1), 89-97.

Assay Quality Statistics:

Neutral control well median response value, by plate:	5105.21
Neutral control median absolute deviation, by plate:	89.69
Positive control well median response value, by plate:	284.18
Positive control well median absolute deviation, by plate:	21.96
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.89
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-32
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-39.65
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.05
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [^{3}H] Methyltrienolone (R1881) (positive control) with recombinant chimpanzee androgen receptor.

Analytical Elements:

The NVS_NR_cAR assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] Methyltrienolone (R1881) binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of R1881 (positive control; calculated per plate, or as a median of positive control wells from all plates combined)

binding capacity. Following initial screening of test compounds at single concentration (25 µM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 3 baseline median average deviations (3BMAD), the chemical was considered active against the androgen receptor and was tested in a concentration-response assay for AR binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	2.68
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich

chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [2].

4. Assay Documentation

4.1. References

[1] Veldscholte, J., et al. (1992). J Steroid Biochem Mol Biol 41: 665-669. (PMID: 1562539)
[2] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

NVS_NR_hAR

Assay Title: NovaScreen Human Androgen Receptor Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenobiotic activity. The NovaScreen NR assays utilized a cell-free platform to screen a diverse chemical library for nuclear receptor activity in a high-throughput (96-well plate) format. This format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the androgen receptor following 20-hour incubation with test chemicals. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity.

1.2. Assay Definition

Assay Throughput:

Human androgen receptor incubated in 96-well microtiter plates for 20 hours prior to measuring displacement of R1881 radiolabeled ligand by test compounds.

Experimental System:

AR nuclear protein, derived from human prostate adenocarcinoma (LNCaP cells) cell line (see Caveats for further discussion on this cell line).

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: LNCaP cells

Radioligand: [^{3}H]Methyltrienolone (R1881) (70-87 Ci/mmol)

Final Ligand Concentration – [0.5 nM]

Non-specific Determinant: Methyltrienolone (R1881) - [200 nM]

Reference Compound: Methyltrienolone (R1881)

Positive Control: Methyltrienolone (R1881)

Methods:

Incubation Conditions: Reactions are carried out in 25 mM HEPES buffer (pH 7.4) containing 1.0 mM EDTA, 10 mM sodium molybdate, 10% glycerol, and 0.5 mM PMSF at 0-4°C for 18-20 hours. The reaction is terminated by the addition of dextran-coated charcoal and incubated for 7 minutes at 0-4°C. The reaction mixtures are centrifuged and the radioactivity bound in the supernatant is compared to control values in order to ascertain any interactions of test compound with the testosterone binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity expected in in vivo systems. The potential for a particular compound to affect changes in androgen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

LNCaP cells have been shown to contain a single point mutation changing the sense of codon 868 (Thr to Ala) in the ligand binding domain. The androgen receptor in these cells is known to bind to progestagens and estrogens despite the absence of estrogen or progestagen receptors, and binding to androgens, progestagens, estrogens and several antiandrogens to the mutated androgen receptor protein activates the expression of an androgen-regulated reporter gene (GRE-tk-CAT), indicating that the mutation directly affects both binding specificity and the induction of gene expression. Consequently, the androgen receptor in these cells is known to bind steroid hormones other than androgens (Veldscholte et al. 1992).

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor human androgen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenobiotic androgen receptor interaction with human-derived nuclear receptors. An initial screening run was conducted exposing androgen receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Methyltrienolone (R1881). R1881 was used as a positive control. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top

concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with androgen nuclear receptor (AR) derived from a human prostate adenocarcinoma cell line (LNCaP).

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting androgen signaling pathways. The androgen receptor mediates gene expression in response to androgen exposures, and modulates the activity for a wide variety of physiological processes, particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility.

The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenobiotic androgen receptor ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Traish, A. M., Enzio Muller, R., & Wotiz, H. H. (1986). "Binding of 7 α, 17 α-Dimethyl-19-Nortestosterone (Mibolerone) to Androgen and Progesterone Receptors in Human and Animal Tissues". Endocrinology 118(4), 1327-1333.

Zava, D., Landrum, B., Horwitz, K., & McGuire, W. (1979). "Androgen Receptor Assay with [3H] Methyltrienolone (R1881) in the Presence of Progesterone Receptors". Endocrinology 104(4), 1007-1012.

Assay Quality Statistics:

Neutral control well median response value, by plate:	3618.46
Neutral control median absolute deviation, by plate:	163.175
Positive control well median response value, by plate:	197.325
Positive control well median absolute deviation, by plate:	21.690
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.85

Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-23
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-23.16
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.05
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.04

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [^{3}H] Methyltrienolone (R1881) (positive control) with androgen receptor obtained from human prostate adenocarcinoma cell line LNCaP

Analytical Elements:

The NVS_NR_hAR assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] Methyltrienolone (R1881) binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of R1881 (positive control; calculated per plate, or as a median of positive control wells from all plates combined) binding capacity. Following initial screening of test compounds at single concentration (25 µM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 3 baseline median average deviations (3BMAD), the chemical was considered active against the androgen receptor and was tested in a concentration-response assay for AR binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480

OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	3.65
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	21.90

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [2].

4. Assay Documentation

4.1. References

[1] Veldscholte, J., et al. (1992). J Steroid Biochem Mol Biol 41: 665-669. (PMID: 1562539)
[2] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation;

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA

6. Supporting Information (existing annotations):

NVS_NR_hER

Assay Title: NovaScreen Human Estrogen Receptor HTS Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenoestrogenic activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the estrogen receptor. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 18 hours in a 96-well plate.

1.2. Assay Definition

Assay Throughput:

Human ERα nuclear protein incubated in 96-well microtiter plates for 18 hours prior to measuring displacement of radiolabeled 17β-Estradiol by test compounds.

Experimental System:

ERα nuclear protein, derived from human breast adenocarcinoma (MCF-7) cell line.

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: MCF-7 cells
Radioligand: [3H] Estradiol
Final Ligand Concentration – [0.1 nM]
Non-specific Determinant: 17β-Estradiol - [300 nM]
Reference Compound: 17β-Estradiol
Positive Control: 17β-Estradiol

Methods:

Incubation Conditions: Reactions are carried out in 10 mM TRIS-HCl (pH 7.4) containing 1.5 mM EDTA, 1.0 mM DTT, and 25 mM sodium molybdate at 0-4 °C for 18 hours. The reaction is terminated by the addition of dextran-coated charcoal and incubated for 20 minutes at 0-4°C. The reaction mixtures are centrifuged and the radioactivity bound in the supernatant is assessed and compared to control values in order to ascertain any interactions of test compound with the estradiol binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective cell membrane and biotransformation capacity expected in in vivo and cell-based systems. The potential for a particular compound to affect changes in

estrogen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor human estrogen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoestrogenic interaction with estrogen receptors. An initial screening run was conducted exposing human estrogen receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Estradiol. 17β-Estradiol (E2) was used as a positive control. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with estrogen nuclear receptor alpha (ERα) derived from MCF-7 human breast adenocarcinoma lysate.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput

format. This assay is designed to help identify environmental compounds with a capacity for xenoestrogenic ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE) leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Haji, M., Kato, K., Nawata, H., & Ibayashi, H. (1981). "Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat". Brain Res 204(2), 373-386. (PMID: 6780133)

O'Keefe, J. A., & Handa, R. J. (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus". Dev Brain Res 57(1), 119-127. (PMID: 2090365)

Assay Quality Statistics:

Neutral control well median response value, by plate:	4467.25
Neutral control median absolute deviation, by plate:	93.76
Positive control well median response value, by plate:	293.52
Positive control well median absolute deviation, by plate:	20.89
Z' (median across all plates, using positive control wells):	0.9
SSMD (median across all plates, using positive control wells):	-36
Signal-to-noise (median across all plates, using positive control wells):	-40.33
Signal-to-background (median across all plates, using positive control wells):	0.08
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive displacement of [3H] Estradiol (positive control) with estrogen receptor α obtained from human breast adenocarcinoma cell line (MCF-7) as measured by detection of radioligand.

Analytical Elements:

The NVS_NR_hER assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] 17β-estradiol binding. Raw data values were normalized as percent of 17-β Estradiol (positive control) binding capacity. If the chemical interaction was >30% of the solvent control (DMSO) or if the signal varied by more than 3.0 median average deviations (3MAD), the chemical was considered active against the estrogen receptor and was tested in a concentration-response assay for ER binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model

which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.02 µM
Standard maximum concentration tested: 50 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.07
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 24.43

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes

57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	No
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	No
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	No
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes

50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	Yes
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	18	17
Inactive	8	10

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	22	15
Inactive	4	12

In Vitro Sensitivity = 51.4%
In Vitro Specificity = 55.6%
Balanced Accuracy = 53.5%

In Vivo Sensitivity = 59.5%
In Vivo Specificity = 75%
Balanced Accuracy = 67.3%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to

environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
E2, Estradiol
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

NVS_NR_hFXR_Agonist

Assay Title: NovaScreen Human Farnesoid x Receptor Alpha (FXR) Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for bioactivity. The NVS NR human farnesoid x receptor (FXR, NR1H4) agonist assay format allows for an efficient screening of thousands of chemicals for the ability to competitively bind to the ligand-binding domain of a xenobiotic sensing nuclear receptor. This assay was developed to screen the ToxCast chemical library for potential farnesoid x receptor ligand-binding activity using a TR-FRET competitive displacement assay and a known FXR receptor agonist (Chenodeoxycholic Acid, CDCA) as a reference compound. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 1 hour in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

Human FXR ligand-binding domain (LBD) incubated in 384-well microtiter plates for 1 hour prior to measuring ligand dependent binding of cofactor to the receptor using TR-FRET.

Experimental System:

GST tagged Human- Farnesoid X Receptor Ligand Binding Domain

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials :

Receptor Source: Human- Farnesoid X Receptor Ligand Binding Domain, GST tagged
Cofactor: Fluorescein-SRC2
Reference Agonist: Chenodeoxycholic Acid (CDCA)
Reaction: Agonist dependent binding of receptor to cofactor
Method: Time-Resolved Fluorescence Resonance Energy Transfer

Methods:

Incubation Conditions: Reactions containing receptor, fluorescein-labeled coactivator peptide, terbium labeled antibody and test or control compounds are carried out in buffer (pH 7.5) for 60 minutes at room temperature. Agonist binding causes receptor to bind with coactivator. The gain of TRF between coactivator peptide and terbium-labeled anti-GST antibody is assessed and compared to control values in order to ascertain any interactions of test compound with the ligand binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity expected in in vivo systems. The potential for a particular compound to affect changes in FXR signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen hFXR ligand-binding agonist assay used a biochemical (cell-free) platform in high-throughput (384-well microplate) format to screen the ToxCast chemical library for xenobiotic interaction with human-derived FXR nuclear receptors. An initial screening run was conducted exposing receptors to 25 µM of each chemical (in duplicate). FXR ligand binding by test chemicals was analyzed using fluorescence resonance energy transfer to measure ligand-dependent cofactor recruitment by test compounds. Reactions involved GST-tagged ligand-binding domain of human FXR, fluorescein-labeled coactivator and terbium-labeled GST antibody. Activity was reported as percent of CDCA (a known FXR agonist) ligand-binding and coactivator recruitment. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding of nuclear receptor FXR.

Scientific Principles:

Farnesoid-X-receptor (FXR) is a ligand-activated nuclear receptor which regulates the expression of genes involved in bile acid homeostasis and has a role in the regulation of glucose and lipid metabolic pathways. FXR is primarily expressed in the liver, kidney, intestine and adrenal cortex, and regulates the expression of target genes by binding either as a monomer or as a heterodimer

with the retinoid X receptor (RXR). Numerous studies have reported that FXR exerts protective function during cholestasis, diabetes, liver regeneration, and cancer. The FXR-RXR heterodimer, when bound to DNA, can act as transcriptional activators or inhibitors. FXR is activated by bile acids and the main endogenous ligand for FXR is chenodeoxycholic acid (CDCA). FXR reduces bile acid concentration in the liver by repressing genes involved in bile acid synthesis and regulates lipid metabolism. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenobiotic FXR ligand-binding activity.

Method Development Reference:

Urizar, N. L., A. B. Liverman, T. D. D'Nette, F. V. Silva, P. Ordentlich, Y. Yan, F. J. Gonzalez, R. A. Heyman, D. J. Mangelsdorf and D. D. Moore (2002). "A natural product that lowers cholesterol as an antagonist ligand for FXR." Science 296(5573): 1703-1706.

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.875
Neutral control median absolute deviation, by plate:	0.0178
Positive control well median response value, by plate:	1.761
Positive control well median absolute deviation, by plate:	0.0682
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.79
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	19
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	54.3
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	2.22
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Time-resolved Fluorescent Energy Resonance Transfer between terbium-labeled antibody, a fluorescein-labeled coactivator peptide, and human FXR ligand-binding domain

Analytical Elements:

The NVS_NR_hFXR assay results were analyzed as loss-of-signal in TR_FRET assays where the endpoint measured was inhibition of CDCA binding. Raw data values were normalized as percent of CDCA (positive control, 100% binding activity). If the chemical interaction was >30% of the solvent control (DMSO) activity or at least 3*baseline median average deviations (3BMAD), the chemical was considered active against the receptor and was tested in a concentration-response assay for FXR binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC)

value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up
ATG_FXR_TRANS_up
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50.0 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	13.88
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	83.29

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical

inventory used in this assay includes the “e1k” chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
CDCA, Chenodeoxycholic Acid
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
GST, Glutathione S-Transferase
FXR, Farnesoid X Receptor
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 October 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

NVS_NR_hFXR_Antagonist

Assay Title: NovaScreen Human Farnesoid x Receptor Alpha (FXR) Ligand-Binding Antagonist Screening Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for bioactivity. The NVS NR human farnesoid x receptor (FXR, NR1H4) agonist assay format allows for an efficient screening of thousands of chemicals for the ability to competitively bind to the ligand-binding domain of a xenobiotic sensing nuclear receptor. This assay was developed to screen the ToxCast chemical library for potential farnesoid x receptor ligand-binding activity using a TR-FRET competitive displacement assay and a known FXR receptor agonist (Chenodeoxycholic Acid, CDCA) as a reference compound. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 1 hour in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

Human FXR ligand-binding domain (LBD) incubated in 384-well microtiter plates for 1 hour prior to measuring ligand dependent binding of cofactor to the receptor using TR-FRET.

Experimental System:

GST tagged Human- Farnesoid X Receptor Ligand Binding Domain

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials :

Receptor Source: Human-GST tagged Farnesoid X Receptor Ligand Binding Domain
Cofactor: Fluorescein-SRC2
Receptor Source: Human- Farnesoid X Receptor Ligand Binding Domain
Ligand and concentration: Chenodeoxycholic Acid (CDCA), 12.5 µM
Reaction: Ligand dependent binding of cofactor to the receptor-ligand complex.
Method: Time-Resolved Fluorescence Resonance Energy Transfer

Methods:

Incubation Conditions: Reactions containing receptor, agonist, fluorescein-labeled coactivator peptide, terbium labeled antibody and test or control compounds are carried out in buffer (pH 7.5). The reaction is started by addition of agonist and incubated for 60 minutes at room temperature. Agonist binding causes receptor to bind to coactivator. The loss of FRET between coactivator peptide and terbium-labeled antibody is assessed and compared to control values in order to ascertain any interactions of test compound with the ligand binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity expected in in vivo systems. The potential for a particular compound to affect changes in FXR signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen hFXR ligand-binding antagonist assay used a biochemical (cell-free) platform in high-throughput (384-well microplate) format to screen the ToxCast chemical library for xenobiotic interaction with human-derived FXR nuclear receptors. An initial screening run was conducted exposing receptors to 25 µM of each chemical (in duplicate). FXR ligand binding by test chemicals was analyzed using fluorescence resonance energy transfer to measure loss-of-signal competitive displacement of agonist (CDCA) by test compounds. Reactions involved GST-tagged ligand-binding domain of human FXR, fluorescein-labeled coactivator and terbium-labeled GST antibody. Activity was reported as percent of Guggulsterone (a known FXR antagonist) binding. If the response signal differed by over 30% or varied by a minimum of 3.0 baseline median absolute deviations (3BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding of nuclear receptor FXR.

Scientific Principles:

Farnesoid-X-receptor (FXR) is a ligand-activated nuclear receptor which regulates the expression of genes involved in bile acid homeostasis and has a role in the regulation of glucose and lipid metabolic pathways. FXR is primarily expressed in the liver, kidney, intestine and adrenal cortex,

and regulates the expression of target genes by binding either as a monomer or as a heterodimer with the retinoid X receptor (RXR). Numerous studies have reported that FXR exerts protective function during cholestasis, diabetes, liver regeneration, and cancer. The FXR-RXR heterodimer, when bound to DNA, can act as transcriptional activators or inhibitors. FXR is activated by bile acids and the main endogenous ligand for FXR is chenodeoxycholic acid (CDCA). FXR reduces bile acid concentration in the liver by repressing genes involved in bile acid synthesis and regulates lipid metabolism. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenobiotic FXR ligand-binding activity.

Method Development Reference:

Urizar, N. L., A. B. Liverman, T. D. D'Nette, F. V. Silva, P. Ordentlich, Y. Yan, F. J. Gonzalez, R. A. Heyman, D. J. Mangelsdorf and D. D. Moore (2002). "A natural product that lowers cholesterol as an antagonist ligand for FXR." Science 296(5573): 1703-1706.

Assay Quality Statistics:

Neutral control well median response value, by plate:	194004
Neutral control median absolute deviation, by plate:	348.411
Positive control well median response value, by plate:	2908.25
Positive control well median absolute deviation, by plate:	333.956
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.93
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-54
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-110.84
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.04
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.01

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Time-resolved Fluorescent Energy Resonance Transfer reactions containing ligand-binding domain of human FXR, agonist, fluorescein-labeled coactivator peptide, terbium labeled antibody and test or control compounds.

Analytical Elements:

The NVS_NR_hFXR assay results were analyzed as loss-of-signal in TR_FRET assays where the endpoint measured was inhibition of CDCA binding. Raw data values were normalized as percent of Guggulsterone (positive control, 100% binding activity). If the chemical interaction was >30% of the solvent control (DMSO) activity or at least 3*baseline median average deviations (3BMAD), the chemical was considered active against the receptor and was tested in a concentration-response assay for FXR binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-

loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up
ATG_FXR_TRANS_up
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	4.213
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	25.28

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the

latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
CDCA, Chenodeoxycholic Acid
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
GST, Glutathione S-Transferase
FXR, Farnesoid X Receptor
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

29 August 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

NVS_NR_hPPARa

Assay Title: NovaScreen Human Peroxisome Proliferator-activated Receptor Alpha (PPARα) Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for bioactivity. The NVS NR human PPARα assay format allows for an efficient screening of thousands of chemicals for the ability to competitively bind to the ligand-binding domain of a xenobiotic sensing nuclear receptor. This assay was developed to screen the ToxCast chemical library for potential to interfere with peroxisome proliferator-activated receptor alpha (PPARα) ligand-binding activity using a TR-FRET competitive displacement assay and a known PPARα receptor agonist (GW7647) as a reference compound. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 1 hour in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

Human PPARα ligand-binding domain (LBD) incubated in 384-well microtiter plates for 1 hour prior to measuring ligand dependent binding of cofactor to the receptor using TR-FRET.

Experimental System:

Ligand-binding domain of human PPARα

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: Human PPARα ligand-binding domain (LBD)

Ligand: Fluormone PPAR green, 20nM

Reference Compound: GW7647

Reaction: Ligand dependent binding of cofactor to the receptor.

Method: Time-Resolved Fluorescence Resonance Energy Transfer

Methods:

Incubation Conditions: Reactions are carried out in buffer (pH 7.5) for 60 minutes at room temperature. The loss of FRET between fluoromone and Tb-labeled anti-PPAR antibody is assessed and compared to control values in order to ascertain any interactions of test compound with the ligand binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity

expected in in vivo systems. The potential for a particular compound to affect changes in PPAR signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen PPARα ligand-binding assay used a biochemical (cell-free) platform in high-throughput (384-well microplate) format to screen the ToxCast chemical library for xenobiotic interaction with human-derived nuclear receptors. An initial screening run was conducted exposing receptors to 25 µM of each chemical (in duplicate). PPARα ligand binding by test chemicals was analyzed using fluorescence resonance energy transfer to measure displacement of a fluorescent small molecule pan-PPAR ligand (Fluormone™ PPAR Green) and human PPARα ligand-binding domain by test compounds. Activity was reported as percent of GW7647 (a potent PPARα agonist) PPARα ligand-binding. If the response signal differed by over 30% or varied by a minimum of 2.0 baseline median absolute deviations (2BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding of nuclear receptor PPARα.

Scientific Principles:

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-activated nuclear receptor which regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of energy homeostasis. PPARα plays a crucial role in the regulation of proteins involved in fatty acid transport and hepatic uptake, is expressed predominantly in metabolically active tissues, including liver, kidney, skeletal muscle, and brown fat and is a target for hyperlipidemia drugs, fatty acids (and their derivative eicosanoids) and xenobiotics. PPARα has been implicated as the proposed target for the nongenotoxic (epigenetic) carcinogens, e.g., the halogenated olefin solvents

trichloroethylene and perchloroethylene. The NVS_NR_hPPARa assay used TR-FRET visualization of receptor ligand-binding, relative to a known receptor antagonist, to evaluate xenobiotic PPARα interactions. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenobiotic PPARα ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARa) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that PPARa activation in utero is the molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to impaired fertility in males (AOP Under EAGMST Review), and there is some evidence that PPARa activation is the MIE for a putative pathways leading to hepatocellular adenomas and carcinomas and may be involved in increased pancreatic acinar tumors (in mouse and rat models) (AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPARa activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

van Raalte, D. H., Li, M., Pritchard, P. H., & Wasan, K. M. (2004). Peroxisome proliferator-activated receptor (PPAR)-α: a pharmacological target with a promising future. Pharmaceutical Research, 21(9), 1531-1538. PMID: 15497675

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.969
Neutral control median absolute deviation, by plate:	0.040
Positive control well median response value, by plate:	0.143
Positive control well median absolute deviation, by plate:	0.0074
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.77
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-17
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-19.49
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.15
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.04

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPARα ligand-binding; measured using Time-resolved Fluorescent Energy Resonance Transfer (TR-FRET) between terbium-labeled anti-PPARα antibody, a fluorescent small molecule PPAR ligand tracer (Fluormone™ Pan-PPAR Green), and human PPARα ligand-binding domain.

Analytical Elements:

The NVS_NR_hPPARa assay results were analyzed as loss-of-signal in TR_FRET assays where the endpoint measured was inhibition of GW7647 binding. Raw data values were normalized as

percent of GW7647 (positive control, 100% activity). If the chemical interaction was >30% of the solvent control (DMSO) activity or at least 2 baseline median average deviations (2BMAD), the chemical was considered active against the receptor and was tested in a concentration-response assay for PPARα binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR alpha activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPARa_TRANS_up
NVS_NR_hPPARa

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	5.147
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	30.883

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for

chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen
PPAR, Peroxisome Proliferator-Activated Receptor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

29 August 2016

Date of Revisions:

22 December 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation;

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA

6. Supporting Information (existing annotations):

NVS_NR_hPPARg

Assay Title: NovaScreen Human Peroxisome Proliferator-activated Receptor Gamma (PPARg) Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for bioactivity. The NVS NR human PPARγ assay format allows for an efficient screening of thousands of chemicals for the ability to competitively bind to the ligand-binding domain of a xenobiotic sensing nuclear receptor. This assay was developed to screen the ToxCast chemical library for potential to interfere with peroxisome proliferator-activated receptor gamma (PPARγ) ligand-binding activity using a radiometric measurements of fluorescent polarization and a potent PPARγ receptor agonist (Ciglitazone) as a reference compound (representing 100% ligand-binding activity). Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 2 hours in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

Ligand-binding domain of human PPARγ incubated with test chemicals in 384-well microtiter plates for 2 hours prior to measuring fluorescence polarization.

Experimental System:

Ligand-binding domain of human PPARγ

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials :

Receptor Source: Human recombinant PPARγ

Reference Compound: Ciglitazone, 5nM

Method: Fluorescence Polarization

Methods:

Assay Conditions: Reactions are carried out in 50 mM Tris (pH 7.4) for 2 hours at room temperature. The fluorescence polarization signal is assessed and compared to control values in order to ascertain any interactions of test compound with the ligand binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity expected in in vivo systems. The potential for a particular compound to affect changes in PPAR signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a

measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen PPARγ ligand-binding assay used a biochemical (cell-free) platform in high-throughput (384-well microplate) format to screen the ToxCast chemical library for xenobiotic interaction with human-derived nuclear receptors. An initial screening run was conducted exposing receptors to 25 µM of each chemical (in duplicate). PPARγ ligand binding by test chemicals was analyzed using fluorescence polarization to measure ligand binding to human PPARγ by test compounds. Activity was reported as percent of Ciglitazone (a potent PPARγ agonist). If the response signal differed by over 30% or varied by a minimum of 2.0 baseline median absolute deviations (2BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding of nuclear receptor PPARγ.

Scientific Principles:

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor which regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of energy homeostasis. PPARγ is primarily expressed in adipose tissue, macrophages and in the colon where it controls adipocyte differentiation, lipid storage and inflammatory responses. PPARγ agonists, the thiazolidinediones (TZDs), improve insulin sensitivity, lower glucose levels, and lower plasma triglycerides and free fatty acid (FFA) levels by enhancing their uptake into adipocytes. The NVS_NR_hPPARg assay used fluorescent polarization radiometric visualization of receptor ligand-binding, to measure modulation of PPARγ activity relative to a known receptor antagonist, to evaluate xenobiotic PPARγ interactions. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of

biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for PPARγ ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARg) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is some evidence to support a putative AOP linking PPAR gamma receptor activation with increased occurrence of sarcomas in rats, mice, and hamsters (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPAR activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., & Milburn, M. V. (1998). "Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ". Nature 395(6698), 137-143. (PMID: 9744270)

Assay Quality Statistics:

Neutral control well median response value, by plate:	177
Neutral control median absolute deviation, by plate:	4.45
Positive control well median response value, by plate:	56.3
Positive control well median absolute deviation, by plate:	3.78
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.76
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-17
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-24.78
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.38
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPARα ligand-binding; measured using fluorescent polarization

Analytical Elements:

The NVS_NR_hPPARg assay results were analyzed as loss-of-signal in fluorescent polarization assays. Raw data values were normalized as percent of Ciglitazone (positive control, 100% activity). If the chemical interaction was >30% of the solvent control (DMSO) activity or at least 2 baseline median average deviations (2BMAD), the chemical was considered active against the receptor and was tested in a concentration-response assay for PPARγ binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each

chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR gamma activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
ATG_PPARg_TRANS_up
OT_PPARg_PPARgSRC1_0480
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_Agonist_ratio
Tox21_PPARg_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	4.036
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	24.216

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the

latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen
PPAR, Peroxisome Proliferator-Activated Receptor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 724

NVS_NR_hTRa_Antagonist

Assay Title: NovaScreen Human Thyroid Receptor Alpha Ligand-Binding Antagonist Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for thyroidal activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a thyroid hormone (T3) from the ligand-binding domain of the thyroid receptor alpha, using a known thyroid receptor antagonist (Bisphenol A) as a reference compound. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 1 hour in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

Recombinant human thyroid receptor incubated in 384-well microtiter plates for 1 hour prior to measuring displacement of ligand agonist (T3) by test compounds, using AlphaLISA immunoassay to detect ligand-dependent co-factor recruitment and Bisphenol A as a TRα-binding reference compound.

Experimental System:

Recombinant human thyroid receptor nuclear protein

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: Recombinant human thyroid receptor alpha
Ligand: 3,3',5-Triiodo-L-Thyronine (T3)
Final Ligand Concentration: 15 nM
Reference Compound: Bisphenol A
Reaction: Ligand dependent binding of cofactor to the receptor.
Method: Luminescent emission at 520 – 620 nm after excitation at 680 nm.

Methods:

Incubation Conditions: Reaction is incubated for 60 minutes at room temperature in 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.01% Tween-20, 0.1% Bovine Serum Albumin (fatty acid free), and 0.5 mM DTT. Acceptor and donor beads are then added and the assay is read to determine inhibition of agonist-induced binding of receptor to cofactor peptide.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity

expected in in vivo systems. The potential for a particular compound to affect changes in thyroid signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen thyroid receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (384-well microplate) format to screen the ToxCast chemical library for xenobiotic interaction with human-derived nuclear receptors. An initial screening run was conducted exposing thyroid receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using displacement of T3 relative to displacement by Bisphenol A, the positive control. If the response signal differed by over 30% or varied by a minimum of 2.0 baseline median absolute deviations (2BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding and inactivation of thyroid nuclear receptor alpha (TRα).

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting thyroid signaling pathways. TH is essential for normal brain development both before and after birth and has profound effects on cellular metabolism in almost all organs. One potential mechanism by which endocrine disrupting chemicals may produce toxic effects is by interfering with the ability of thyroid hormones (T3, triiodothyronine and T4, thyroxine) to direct normal development and metabolism. Compounds which interfere with thyroid hormone signaling can result in neurological disorders by disrupting normal developmental processes. Thyroid hormones also have important roles in the initiation and

proliferation of central nervous system and cardiovascular tissues. An important component of an endocrine disruptor screening program should be the inclusion of assays designed to screen TH disrupting compounds. The NVS_NR_hTHa_Antagonist assay used displacement of the thyroid hormone T3 (Triiodothyronine) binding to human thyroid receptor, relative to displacement by Bisphenol A, a known thyroid receptor antagonist [1, 2].

The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenobiotic ligand-binding activity.

Method Development Reference:

Moriyama, K. et al. Thyroid Hormone Action Is Disrupted by Bisphenol A as an Antagonist. J. of Clinical Endocrinology and Metabolism 87:5185 (2002)

Assay Quality Statistics:

Neutral control well median response value, by plate:	633402
Neutral control median absolute deviation, by plate:	12644.4
Positive control well median response value, by plate:	11403.5
Positive control well median absolute deviation, by plate:	372.133
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.91
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-40
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-44.6
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.02
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive binding of T3 with human thyroid receptor, relative to displacement by Bisphenol A (positive control).

Analytical Elements:

The NVS_NR_hTRa_Antagonist assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of Bisphenol A binding. Raw data values were normalized as percent of Bisphenol A (positive control, 100% inhibition) activity. If the chemical interaction was >30% of the solvent control (DMSO) activity or at least 3 baseline median average deviations (3BMAD), the chemical was considered active against the thyroid receptor and was tested in a concentration-response assay for TRα binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning

model and used in further analysis as the most appropriate predictor of xenobiotic effects. Thyroid receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up
ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_up
LTEA_HepaRG_THRSP_dn
Tox21_TR_LUC_GH3_Agonist
Tox21_TR_LUC_GH3_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	1.302
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds

recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [3].

4. Assay Documentation

4.1. References

[1] Moriyama, K., et al. (2002). J Clinical Endocrinol Metabol 87(11): 5185-5190. (PMID: 12414890)
[2] Zoeller, R. T., et al. (2005). Endocrinology 146(2): 607-612. (PMID: 15498886)
[3] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
NR, Nuclear Receptor
NVS, NovaScreen
T3, Triiodothyronine
T4, Thyroxine
TH, Thyroid Hormone
TR, Thyroid Receptor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

25 July 2016

Date of Revisions:

21 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Assay Endpoint ID: 725

NVS_NR_mERa

Assay Title: NovaScreen Murine Estrogen Receptor HTS Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenoestrogenic activity. This assay format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the estrogen receptor. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity. This assay was run for a test duration of 18 hours in a 96-well plate.

1.2. Assay Definition

Assay Throughput:

This is a biochemical (cell-free) format, using 96-well plates to incubate radiolabeled ligand with estrogen receptor alpha for 18 hours to measure displacement of estradiol by test chemicals in a competitive binding assay.

Experimental System:

ERα nuclear protein, derived from mouse tissue

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: murine ER alpha

Radioligand: [3H] Estradiol - [1 nM]

Non-specific Determinant: 17β-Estradiol - [1 µM]

Reference Compound: 17β-Estradiol

Positive Control: 17β-Estradiol

Methods:

Incubation Conditions: Reactions are carried out in 25 mM TRIS-HCl (pH 7.4) containing 1.5 mM EDTA, 1 mM DTT, and 25 mM sodium molybdate at 0-4 °C for 18 hours. The reaction is terminated by rapid vacuum filtration onto glass fiber filters soaked in 0.5% PEI. Filters were washed with cold 50 mM NaCl. Radioactivity trapped onto the filters is determined and compared to control values in order to ascertain any interactions of test compound with the estrogen receptor binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective cell membrane and biotransformation capacity expected in in vivo and cell-based systems. The potential for a particular compound to affect changes in estrogen signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is

also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor murine estrogen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoestrogenic interaction with murine estrogen receptors. An initial screening run was conducted exposing estrogen receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Estradiol. 17β-Estradiol (E2) was used as a positive control. If the response signal differed by over 30% from the solvent control (DMSO) or if the signal varied by more than 2.0 median average deviations (2MAD), the chemical was considered active and retested in a concentration response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with estrogen nuclear receptor alpha (ERα) derived from murine tissues.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting estrogen signaling pathways. The estrogen receptor mediates gene expression in response to estrogen exposure, and modulates the activity for a wide variety of physiological processes. The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for xenoestrogenic ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor activity in early life is a molecular initiating event (MIE) leading to breast cancer in both animal and human models and to endometrial carcinoma in the mouse, and ER agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Haji, M., Kato, K., Nawata, H., & Ibayashi, H. (1981). "Age-related changes in the concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of the rat". Brain Res 204(2), 373-386. (PMID: 6780133)

O'Keefe, J. A., & Handa, R. J. (1990). "Transient elevation of estrogen receptors in the neonatal rat hippocampus". Dev Brain Res 57(1), 119-127. (PMID: 2090365)

Assay Quality Statistics:

Neutral control well median response value, by plate:	29473
Neutral control median absolute deviation, by plate:	1040.79
Positive control well median response value, by plate:	601
Positive control well median absolute deviation, by plate:	35.58
Z' (median across all plates, using positive control wells):	0.83
SSMD (median across all plates, using positive control wells):	-19
Signal-to-noise (median across all plates, using positive control wells):	-18.65
Signal-to-background (median across all plates, using positive control wells):	0.02
CV (median across all plates):	0.05

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [3H] Estradiol (positive control) with estrogen receptor α obtained from murine tissue source as measured by radiometric detection.

Analytical Elements:

The NVS_NR_mER assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] 17β-estradiol binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of 17-β Estradiol (positive control) binding capacity. Following initial screening of test compounds at single concentration (25 µM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 2 baseline median average deviations (2BMAD), the chemical was considered active against the estrogen receptor and was tested in a concentration-response assay for ER binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of

xenobiotic effects. Estrogen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 8
Target (nominal) number of replicates: 1
Standard minimum concentration tested: 0.02 µM
Standard maximum concentration tested: 50 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.33
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 25.96

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes

50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	No
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	No
599-64-4	4-Cumylphenol	Weak	Active	No
104-43-8	4-Dodecylphenol	NA	Active	No
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	No
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	No
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	No
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	Yes
56-53-1	Diethylstilbestrol	Strong	Active	No
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes

120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	No
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	No
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	No
72-33-3	Mestranol	NA	Active	No
72-43-5	Methoxychlor	Very Weak	Active	No
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	Yes
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	14	18
Inactive	9	9

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	18	18
Inactive	5	9

In Vitro Sensitivity = 43.8%
In Vitro Specificity = 50%
Balanced Accuracy = 46.9%

In Vivo Sensitivity = 50%
In Vivo Specificity = 64.3%
Balanced Accuracy = 57.2%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and

suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
ER, Estrogen Receptor
E2, Estradiol
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Assay Endpoint ID: 726

NVS_NR_rAR

Assay Title: NovaScreen Rat Androgen Receptor Ligand-Binding Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

High-throughput screening of in vitro chemical-target interactions across a wide variety of compounds through a broad range of biochemical interactions will help describe the chemical-assay bioactivity space for chemicals with limited available information. There exists a large number of environmental chemicals for which there is little information about the potential for xenobiotic activity. The NovaScreen NR assays utilized a cell-free platform to screen a diverse chemical library for nuclear receptor activity in a high-throughput (96-well plate) format. This format allows for an efficient screening of thousands of chemicals for the ability to bind to the receptor and displace a radiolabeled ligand from the ligand-binding domain of the androgen receptor following 18-hour incubation with test chemicals. Biochemical high-throughput screening offers preliminary evidence for chemical targets in a cell or tissues which, when combined with information from literature or targeted in vivo studies, can indicate potential pathways for toxicity.

1.2. Assay Definition

Assay Throughput:

Human androgen receptor incubated in 96-well microtiter plates for 18 hours prior to measuring displacement of R1881 radiolabeled ligand by test compounds.

Experimental System:

Rat androgen receptor recombinant LBD domain

Xenobiotic Biotransformation Potential:

None

Basic Procedure:

Materials:

Receptor Source: Testosterone pre-treated Rat prostate

Radioligand: [^{3}H]Methyltrienolone (R1881) (70-87 Ci/mmol)

Final Ligand Concentration – [1.0 nM]

Non-specific Determinant: Methyltrienolone (R1881) - [1.0 nM]

Reference Compound: Methyltrienolone (R1881)

Positive Control: Methyltrienolone (R1881)

Methods:

Incubation Conditions: Reactions are carried out in 25 mM HEPES buffer (pH 7.4) containing, 10 mM sodium molybdate, 0.5 mM DTT, 250 mM sucrose, 2.5 mM MgCl2, and 1 mM PMSF at 0-4°C for 18 hours. The reaction is terminated by rapid vacuum filtration onto GF/C filters and the radioactivity bound to the filter is compared to control values in order to ascertain any interactions of test compound with the testosterone binding site.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The NovaScreen Nuclear Receptor assays described here are run in a cell-free format, and as such lack the ability to model the protective (membrane) mechanisms and biotransformation capacity expected in in vivo systems. The potential for a particular compound to affect changes in androgen

signaling pathways is not exclusively a function of receptor-ligand binding affinity, but is also a measure of the propensity for the activated receptor-ligand complex to form dimers and recruit co-activators, of the affinity for the activated complex to bind to hormone response element DNA sequences, and of interactions with other signaling pathways.

1.3. Assay References

Assay Source Contact Information:

PerkinElmer Office
940 Winter St.
Waltham, Massachusetts 02451
United States
Tel: (781) 663-6900

Assay Publication Year:

2011

Assay Publication:

Knudsen, T. B., Houck, K. A., Sipes, N. S., Singh, A. V., Judson, R. S., Martin, M. T., Weissman, A., Kleinstreuer, N. C., Mortensen, H. M., Reif, D. M., Rabinowitz, J. R., Setzer, R. W., Richard, A. M., Dix, D. J., & Kavlock, R. J. (2011). "Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets". Toxicology 282(1-2), 1-15. (PMID: 21251949)

Sipes, N. S., Martin, M. T., Kothiya, P., Reif, D. M., Judson, R. S., Richard, A. M., Houck, K. A., Dix, D. J., Kavlock, R. J., & Knudsen, T. B. (2013). "Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays". Chem Res Toxicol 26(6), 878-895. (PMID: 23611293

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The NovaScreen nuclear receptor rat androgen receptor ligand-binding assay used a biochemical (cell-free) platform in high-throughput (96-well microplate) format to screen the ToxCast chemical library for xenoandrogenic interaction with rodent-derived androgen receptors. An initial screening run was conducted exposing androgen receptors to 25 µM of each chemical (in duplicate). Response to chemical perturbation was measured using radioligand detection (via liquid scintillation counting) of displacement of [^{3}H]-Methyltrienolone (R1881). R1881 was used as a positive control and compound activity was measured as loss-of-signal relative to radiolabeled R1881 AR ligand binding. If the response signal differed by over 30% or varied by a minimum of 2.0 baseline median absolute deviations (2BMAD) from the vehicle control (DMSO), the chemical was considered active and retested in a concentration–response format assay using 8 concentrations derived from a serial dilution series using half-log increments and a top concentration of 50 µM. This assay used a cell-free high-throughput format to probe a diverse chemical library for potential ligand-binding activity with androgen nuclear receptor (AR) derived from testosterone-treated rat prostrate.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are compounds which interfere with normal hormone biosynthesis, signaling or metabolism and impact regulatory pathways in humans and wildlife. Many EDCs interfere with normal steroidal activity by impacting androgen signaling pathways. The androgen receptor mediates gene expression in response to androgen exposures, and modulates the activity for a wide variety of physiological processes, particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like

developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility.

The NovaScreen assays are modifications of pre-clinical drug development assays and are designed to examine chemical effects on a broad spectrum of biochemical targets or potential molecular initiating events in a high-throughput format. This assay is designed to help identify environmental compounds with a capacity for androgen receptor ligand-binding activity.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Traish, A. M., Enzio Muller, R., & Wotiz, H. H. (1986). "Binding of 7 α, 17 α-Dimethyl-19-Nortestosterone (Mibolerone) to Androgen and Progesterone Receptors in Human and Animal Tissues". Endocrinology 118(4), 1327-1333.

Zava, D., Landrum, B., Horwitz, K., & McGuire, W. (1979). "Androgen Receptor Assay with [3H] Methyltrienolone (R1881) in the Presence of Progesterone Receptors". Endocrinology 104(4), 1007-1012.

Assay Quality Statistics:

Neutral control well median response value, by plate:	5336
Neutral control median absolute deviation, by plate:	266.87
Positive control well median response value, by plate:	761
Positive control well median absolute deviation, by plate:	37.065
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.76
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-15
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-15.24
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	0.14
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.06

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Competitive radioligand binding of [^{3}H]-Methyltrienolone (R1881) (positive control) with androgen receptor obtained from rat prostrate pre-treated with testosterone.

Analytical Elements:

The NVS_NR_rAR assay results were analyzed as loss-of-signal in competitive displacement assays where the endpoint measured was inhibition of [^{3}H] Methyltrienolone (R1881) binding. Raw data values were normalized to DMSO (neutral control) and reported as percent of R1881 (positive control; calculated per plate, or as a median of positive control wells from all plates combined) binding capacity. Following initial screening of test compounds at single concentration (25 µM), if the chemical response was >30% of the solvent control (DMSO) activity or at least 3 baseline median average deviations (3BMAD), the chemical was considered active against the androgen receptor and was tested in a concentration-response assay for AR binding using 8 concentrations with 3-fold serial dilutions generally beginning at a high concentration of 50 µM. Response was calculated as a percent of positive control activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publically available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	8
Target (nominal) number of replicates:	1
Standard minimum concentration tested:	0.02 µM
Standard maximum concentration tested:	50 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	5.172
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	31.035

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [1].

4. Assay Documentation

4.1. References

[1] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
DMSO, Dimethyl Sulfoxide
EDC, Endocrine disrupting chemicals
MIE, Molecular Initiating Event
NR, Nuclear Receptor
NVS, NovaScreen

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. **Potential Regulatory Applications**

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation;

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA

6. **Supporting Information (existing annotations):**

OT_AR_ARELUC_AG_1440

Assay Name: Odyssey Thera CHO-K1 Androgen Response Element Luciferase 24-hr Agonist Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera AR androgen response element (ARE) luciferase (LUC) agonist assay used CHO-K1 cells stably expressing both full-length human AR transcription factors and an ARE-luciferase reporter construct to screen a diverse chemical library for potential xenobiotic androgen receptor ligand-binding activity. This assay was developed to measure long-term transcriptional changes induced by ligand-binding of androgen receptor alpha (AR) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human AR and an ARE reporter construct driving expression of luciferase. AR interacts with androgenic ligands at the ligand-binding domain and with ARE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with cells in a 384-well plate, xenobiotic agonism of AR is detected by measuring bioluminescent signal produced by AR-moderated transcriptional activity, and xenobiotic compounds with the capacity to interfere with androgenic pathways can be quantified using a luminometer to detect an increase in bioluminescence relative to baseline activity (DMSO control).

1.2. Assay Definition

Assay Throughput:

Stably transfected CHO-K1 cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring luminescence increases resulting from AR transactivation by test compounds.

Experimental System:

CHO-K1 is an immortal mammal ovary fibroblast cell line derived from Chinese hamster cells isolated in 1957 (Puck et al. 1958). CHO-K1 is a widely used cell line with well characterized cell transfection methods frequently utilized for large-scale production of numerous pharmaceutical proteins (including hormones, antibodies, and blood factors) since these cells are capable of folding, assembling and post-translationally modifying proteins in a manner that is more comparable to humans (Kildegaard et al. 2013). The Odyssey Thera AR ARE luciferase assays used CHO-K1 cells with stably transfected full-length AR and ARE-luciferase reporter constructs.

Xenobiotic Biotransformation Potential:

CHO-K1 cells have the capacity to metabolize the anti-androgenic fungicide vinclozolin (Jacobs et al. 2008), however the intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

To identify AR agonists, CHO-K1 cells are seeded into a white-walled/white-bottom 384-well plate, followed by treatment with compounds of interest or controls for 24 hours. A luciferase assay mix containing D-luciferin and ATP in PBS is then added to the cells and luminescence quantified on a Luminoskan (Thermo Scientific) luminometer. Modulation of this assay is quantified as an increase in mean luminescence intensity relative to vehicle controls. To evaluate specificity of the AR /ARE-luc assay, several known AR agonists, AR antagonists, and ER-selective compounds were also tested in 5-pt concentration-response format in a minimum of 7 assay plates. OT determined the reproducibility of DHT-induced AR/ARE-luc transcriptional activation with a total of seven 384-well plates run on 2 different days, for 24-h time frames. A minimum of 3 replicate wells were analyzed

for each sample with 14 replicate wells for vehicle controls. Luminescence data were captured on the Luminoskan luminometer.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

Assay Publication:

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera androgen response element (ARE) luciferase (LUC) agonist assay was developed to measure long-term transcriptional changes induced by ligand-binding of androgen receptor alpha (AR) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human AR and an ARE reporter construct driving expression of luciferase. AR interacts with androgenic ligands at the ligand-binding domain and with ARE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with cells, xenobiotic agonism of AR is detected by introduction of assay mix containing D-luciferin and ATP to the culture serum, and the resultant bioluminescence can be quantified using a luminometer as an increase in mean signal relative to baseline activity (DMSO control). Concentration response models are based on 6-point concentration series (0.3 – 100 µM) run in triplicate, using 6α-Fluorotestosterone as a positive control. To determine the sensitivity of the AR/ARE-luc assay system, the range of EC50 values for DHT was determined in seven 384-well plates run on 2 different days, and values did not vary by more than 4-fold for the 24-hour endpoint.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are chemicals found in the environment or introduced in one's diet that perturb normal hormone biosynthesis, metabolism and downstream gene transcription. A significant subset of EDC's including industrial chemicals, organochlorinated pesticides, and plasticizers have the capacity to bind to the androgen receptor (AR), a member of the nuclear receptor superfamily that is activated by androgens (Luccio-Camelo and Prins 2011, Sultan et al. 2001). Due to the androgen-dependence of male sexual differentiation, exposure to

EDC's can result in reduced sperm counts, increased infertility, and elevated testicular and prostate cancer risks (Luccio-Camelo and Prins 2011).

AR is a ligand-inducible nuclear hormone receptor that mediates transcription through a series of events including ligand binding, DNA binding to androgen response elements, and interaction with various co-activators. The OT_AR_ARE_LUC_Agonist Assay uses CHOK1 cells stably transfected with full-length AR to monitor ARE-driven expression of luciferase reporter activity in response to chemical exposures over 24 hours.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	6.52
Neutral control median absolute deviation, by plate:	1.275
Positive control well median response value, by plate:	92.04
Positive control well median absolute deviation, by plate:	15.75
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.35
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	5
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	59.62
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	14.04
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.2

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Agonism of AR transcription factors and binding to androgen response element sequence located upstream of transfected luciferase reporter gene as measured by monitoring increased luminescent emission relative to DMSO (neutral control) baseline.

Analytical Elements:

OT_AR_ARELUC_agonist_1440 readout data was analyzed in the positive (gain of signal) fitting direction over DMSO controls (baseline), and was reported as a percentage of positive control (6α-fluorotestosterone) activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	2.518
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical

constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Puck, T. T., et al. (1958). J Exper Med 108(6): 945-956. (PMID: 13598821)
[2] Kildegaard, H. F., et al. (2013). Curr Opin Biotechnol 24(6): 1102-1107. (PMID: 23523260)
[3] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[4] Sultan, C., et al. (2001). Mol Cell Endocrinol 178(1): 99-105. (PMID: 11403899)
[5] Luccio-Camelo, D. C. and G. S. Prins (2011). J Steroid Biochem Mol Biol 127(1): 74-82. (PMID: 21515368)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ARE, Androgen Response Element
CHO, Chinese Hamster Ovary
EDC, Endocrine Disrupting Compounds
DBD, DNA Binding Domain
DHT, 4,5 α-Dihydrotestosterone
LBD, Ligand Binding Domain
LUC, Luciferase
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_AR_ARSRC1_0480

Assay Name: Odyssey Thera HEK293T Androgen Receptor / SRC-1 Co-activator 8-hr Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera androgen receptor/steroid receptor co-activator SRC-1 assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the androgen receptor response pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the androgen signaling pathway following 8-hour incubation of test chemical with transformed HEK293T cells in 384-well plates

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 8 hours prior to monitoring fluorescence emission resulting from xenobiotic AR activation and co-factor recruitment.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the human AR (stably expressed in HEK293T) for xenobiotic androgen receptor activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 (Graham et al. 1977). The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid (Bylund et al. 2004). HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) (Dai et al. 2015) and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method (Lin et al. 2014).

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT AR / SRC-1 assay assessed androgen receptor chemical interactions using a rapidly maturing, intensely fluorescent mutant of YFP known as Venus, rationally dissected into two separate fragments. The fragments were obtained as follows: first, fragments coding for YFP[1] and YFP[2] (corresponding to amino acid residues 1–158 and 159–239 of the full length YFP, respectively) were generated by oligonucleotide synthesis (Blue Heron Biotechnology), and then PCR mutagenesis was used to generate the mutant fragments IFP[1] and IFP[2]. Fusion constructs were transfected into HEK293T cells with a $(Gly_4Ser)_2$ linker between the AR/SRC-1 and YFP fragment genes to facilitate complementation when interacting proteins bring fragments into close proximity. The construct is stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free DMEM medium supplemented with 10%

dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells and subcellular compartment boundaries prior to signal detection. Images were acquired on an Evotec Opera at 2 wavelengths (488 and 635nm), and the ratio of fluorescence in the nucleus relative to fluorescence in the cytoplasm (N/C Ratio) in the 488nm channel was calculated for a minimum of 400 cells per image. Both agonists and antagonists of the AR receptor induce nuclear translocation to varying degrees.

Proprietary Elements:

Odyssey Thera assays used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera AR/SRC-1 assay is a protein-complementation assay (PCA) comprised of the full length human AR and the nuclear receptor interacting domain of SRC-1, each fused to an inactive fragment of YFP. Unliganded AR is bound by heat shock/co-chaperone proteins in an inactive state in the cytoplasm (Pratt and Toft 1997) therefore fluorescent signal in the basal or unstimulated state of the assay is predominately present in the cytoplasm. In response to ligand binding, the AR/SRC-1 YFP complex translocates from the cytoplasm to the nucleus, and this assay records compound-AR interactions by measurement of nucleus : cytoplasm (N/C) signal ratios. Each AR protein and its associated coactivator (SRC-1) contain a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the androgen responsive signaling pathway is

impacted by chemical activation or interference, the resulting YFP signal production can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic AR ligand-binding. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track changes at the level of cell functioning which may occur at a number of points along the androgen signaling pathway following an 8 hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and DHT (5α-Dihydrotestosterone) as a positive control and measure of 100% ligand-binding activity in AR. Concentration-response models are based on 6-point concentration series (0.3 – 100 µM) run in triplicate. Preliminary experiments examined the temporal nature of the AR/SRC-1 translocation and determined that maximum S/B was achieved after 8 hours, and while EC50s did not vary considerably over time, a more robust estimation of lower concentrations was achieved in longer duration assays. The OT AR/SRC-1 assay was also run for 16 hours (see description for OT_AR_ARSRC1_0960). OT initially treated the cells with the AR agonist 4-5, dihydrotestosterone (DHT) in 10-point concentration- response format for 8 hours to assess sensitivity of the AR/SRC-1 assay to ligand.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are chemicals found in the environment or introduced in one's diet that perturb normal hormone biosynthesis, metabolism and downstream gene transcription. A significant subset of EDC's including industrial chemicals, organochlorinated pesticides, and plasticizers have the capacity to bind to the androgen receptor (AR), a member of the nuclear receptor superfamily that is activated by androgens (Luccio-Camelo and Prins 2011, Sultan et al. 2001). Due to the androgen-dependence of male sexual differentiation, exposure to EDC's can result in reduced sperm counts, increased infertility, and elevated testicular and prostate cancer risks (Luccio-Camelo and Prins 2011).

AR is a ligand-inducible nuclear hormone receptor that mediates transcription through a series of events including ligand binding, DNA binding to androgen response elements, and interaction with various co-activators. These co-activators are components required for androgen-dependent transcription, and either physically link the AR to the basal transcriptional machinery or modulate chromatin via methylation or acetylation (McKenna et al. 1999). Over 169 proteins have been reported as potential AR co-regulators (Heemers and Tindall 2007) including the prototypical nuclear receptor coactivator, SRC-1. While numerous assays have been described in the literature that assess AR function using transcriptional readouts (Vinggaard et al. 1999), ligand competition binding (Féau et al. 2011) or cellular dynamics of GFP-tagged AR (Sultan et al. 2001, Szafran et al. 2008), the OT AR/SRC-1 assay evaluated EDC-induced AR activity in the context of the receptor's interaction with the steroid receptor co-activator protein, SRC-1. The advantage of this approach is that compounds that favor interaction of AR with SRC-1 (such as ligands), indicating the activated state of the receptor, can be readily detected. In addition, compounds that perturb this interaction by acting upstream in the pathway (e.g. through non-genomic effects) may also be identified. Therefore, this assay represents a novel tool for evaluating endocrine disrupting agents which have potential to interfere with endogenous androgen signaling in a high throughput screening mode.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a

putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.786
Neutral control median absolute deviation, by plate:	0.017
Positive control well median response value, by plate:	1.5075
Positive control well median absolute deviation, by plate:	0.0363
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.77
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	17
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	42.17
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	1.93
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor-mediated signaling pathway stable protein formation in response to AR agonism and SRC-1 co-activator recruitment measured by monitoring increased fluorescence relative to DMSO (neutral control) baseline.

Analytical Elements:

Each data point was formed by taking the log of the ratio of the sample signal to the control signal. A minimum of 8 replicate wells were analyzed each for sample and vehicle controls. Wells located in the outer ring of the plate were omitted due to the potential for edge effects. Data were captured on a Perkin Elmer Opera confocal microscope: 8 images per well in two wavelengths with a minimum of 400 cells per image. Each data point represents the average of 32 images acquired in four wells, normalized to the N/C Ratio calculated for 8 vehicle control wells (64 images). Gain-of-signal data are plotted as percent of activity where max activity corresponds to 1µM DHT, and relative to DMSO, negative control and baseline activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria;

either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	2.456
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds

recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals (Richard et al. 2016).

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Pratt, W. B. and D. O. Toft (1997). Endocr Rev 18(3): 306-360. (PMID: 9183567)
[6] Sultan, C., et al. (2001). Mol Cell Endocrinol 178(1): 99-105. (PMID: 11403899)
[7] Luccio-Camelo, D. C. and G. S. Prins (2011). J Steroid Biochem Mol 127(1): 74-82. (PMID: 21515368)
[8] McKenna, N. J., et al. (1999). J Steroid Biochem Mol Biol 69(1): 3-12. (PMID: 10418975)
[9] Heemers, H. V. and D. J. Tindall (2007). Endocr Rev 28(7): 778-808. (PMID: 17940184)
[10] Vinggaard, A. M., et al. (1999). Toxicol Appl Pharmacol 155(2): 150-160. (PMID: 10053169)
[11] Féau, C., et al. (2011). Androgen Action: Methods and Protocols: 59-68. (PMID: 19171919)
[12] Szafran, A. T., et al. (2008). PLoS One 3(11): e3605. (PMID: 18978937)
[13] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATTC, American Tissue Culture Collection
DBD, DNA Binding Domain
DHT, 4-5, Dihydrotestosterone
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA , Protein-Fragment Complementation
SRC-1, Steroid Receptor Coactivator 1
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

OT_AR_ARSRC1_0960

Assay Name: Odyssey Thera HEK293T Androgen Receptor / SRC-1 Co-activator 16-hr Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera androgen receptor/steroid receptor co-activator SRC-1 assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the androgen receptor response pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the androgen signaling pathway following 16-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring fluorescence emission resulting from xenobiotic AR activation and co-factor recruitment.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the human AR (stably expressed in HEK293T) for xenobiotic androgen receptor activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 (Graham et al. 1977). The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid (Bylund et al. 2004). HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) (Dai et al. 2015) and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method (Lin et al. 2014).

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT AR/SRC-1 assay assessed androgen receptor chemical interactions using a rapidly maturing, intensely fluorescent mutant of YFP known as Venus, rationally dissected into two separate fragments. The fragments were obtained as follows: first, fragments coding for YFP[1] and YFP[2] (corresponding to amino acid residues 1–158 and 159–239 of the full length YFP, respectively) were generated by oligonucleotide synthesis (Blue Heron Biotechnology), and then PCR mutagenesis was used to generate the mutant fragments IFP[1] and IFP[2]. Fusion constructs were transfected into HEK293T cells with a $(Gly_4Ser)_2$ linker between the AR/SRC-1 and YFP fragment genes to facilitate complementation when interacting proteins bring fragments into close proximity. The construct is stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free DMEM medium supplemented with 10% dextran-treated

FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 16 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells and subcellular compartment boundaries prior to signal detection. Images were acquired on an Evotec Opera at 2 wavelengths (488 and 635nm), and the ratio of fluorescence in the nucleus relative to fluorescence in the cytoplasm (N/C Ratio) in the 488nm channel was calculated for a minimum of 400 cells per image. Both agonists and antagonists of the AR receptor induce nuclear translocation to varying degrees.

Proprietary Elements:

Odyssey Thera assays used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera AR/SRC-1 assay is a protein-complementation assay (PCA) comprised of the full length human AR and the nuclear receptor interacting domain of SRC-1, each fused to an inactive fragment of YFP. Unliganded AR is bound by heat shock/co-chaperone proteins in an inactive state in the cytoplasm (Pratt and Toft 1997) therefore fluorescent signal in the basal or unstimulated state of the assay is predominately present in the cytoplasm. In response to ligand binding, the AR/SRC-1 YFP complex translocates from the cytoplasm to the nucleus, and this assay records compound-AR interactions by measurement of nucleus : cytoplasm (N/C) signal ratios. Each AR protein and its associated coactivator (SRC-1) contain a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the androgen responsive signaling pathway is

impacted by chemical activation or interference, the resulting YFP signal production can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic AR ligand-binding. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track changes at the level of cell functioning which may occur at a number of points along the androgen signaling pathway following an 16 hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and DHT (5α-Dihydrotestosterone) as a positive control and measure of 100% ligand-binding activity in AR. Concentration-response models are based on 6-point concentration series (0.3 – 100 µM) run in triplicate. Preliminary experiments examined the temporal nature of the AR/SRC-1 translocation and determined that maximum S/B was achieved after 8 hours, and while EC50s did not vary considerably over time, a more robust estimation of lower concentrations was achieved in longer duration assays. The OT AR/SRC-1 assay was also run for 8 hours (see description for OT_AR_ARSRC1_0480). OT initially treated the cells with the AR agonist 4-5, dihydrotestosterone (DHT) in 10-point concentration- response format for 8 hours to assess sensitivity of the AR/SRC-1 assay to ligand.

Scientific Principles:

Endocrine disrupting chemicals (EDCs) are chemicals found in the environment or introduced in one's diet that perturb normal hormone biosynthesis, metabolism and downstream gene transcription. A significant subset of EDC's including industrial chemicals, organochlorinated pesticides, and plasticizers have the capacity to bind to the androgen receptor (AR), a member of the nuclear receptor superfamily that is activated by androgens (Luccio-Camelo and Prins 2011, Sultan et al. 2001). Due to the androgen-dependence of male sexual differentiation, exposure to EDC's can result in reduced sperm counts, increased infertility, and elevated testicular and prostate cancer risks (Luccio-Camelo and Prins 2011).

AR is a ligand-inducible nuclear hormone receptor that mediates transcription through a series of events including ligand binding, DNA binding to androgen response elements, and interaction with various co-activators. These co-activators are components required for androgen-dependent transcription, and either physically link the AR to the basal transcriptional machinery or modulate chromatin via methylation or acetylation (McKenna et al. 1999). Over 169 proteins have been reported as potential AR co-regulators (Heemers and Tindall 2007) including the prototypical nuclear receptor coactivator, SRC-1. While numerous assays have been described in the literature that assess AR function using transcriptional readouts (Vinggaard et al. 1999), ligand competition binding (Féau et al. 2011) or cellular dynamics of GFP-tagged AR (Sultan et al. 2001, Szafran et al. 2008), the OT AR/SRC-1 assay evaluated EDC-induced AR activity in the context of the receptor's interaction with the steroid receptor co-activator protein, SRC-1. The advantage of this approach is that compounds that favor interaction of AR with SRC-1 (such as ligands), indicating the activated state of the receptor, can be readily detected. In addition, compounds that perturb this interaction by acting upstream in the pathway (e.g. through non-genomic effects) may also be identified. Therefore, this assay represents a novel tool for evaluating endocrine disrupting agents which have potential to interfere with endogenous androgen signaling in a high throughput screening mode.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a

putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.7645
Neutral control median absolute deviation, by plate:	0.0133
Positive control well median response value, by plate:	1.576
Positive control well median absolute deviation, by plate:	0.0341
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.82
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	21
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	60.12
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	2.05
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.02

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor-mediated signaling pathway stable protein formation in response to AR agonism and SRC-1 co-activator recruitment measured by monitoring increased fluorescence relative to DMSO (neutral control) baseline.

Analytical Elements:

Each data point was formed by taking the log of the ratio of the sample signal to the control signal. A minimum of 8 replicate wells were analyzed each for sample and vehicle controls. Wells located in the outer ring of the plate were omitted due to the potential for edge effects. Data were captured on a Perkin Elmer Opera confocal microscope: 8 images per well in two wavelengths with a minimum of 400 cells per image. Each data point represents the average of 32 images acquired in four wells, normalized to the N/C Ratio calculated for 8 vehicle control wells (64 images). Gain-of-signal data are plotted as percent of activity where max activity corresponds to 1µM DHT, and relative to DMSO, negative control and baseline activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria;

either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
Tox21_AR_BLA_Agonist_ratio
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	1.638
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds

recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [13].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Pratt, W. B. and D. O. Toft (1997). Endocr Rev 18(3): 306-360. (PMID: 9183567)
[6] Sultan, C., et al. (2001). Mol Cell Endocrinol 178(1): 99-105. (PMID: 11403899)
[7] Luccio-Camelo, D. C. and G. S. Prins (2011). J Steroid Biochem Mol Biol 127(1): 74-82. (PMID: 21515368)
[8] McKenna, N. J., et al. (1999). J Steroid Biochem Mol Biol 69(1): 3-12. (PMID: 10418975)
[9] Heemers, H. V. and D. J. Tindall (2007). Endocr Rev 28(7): 778-808. (PMID: 17940184)
[10] Vinggaard, A. M., et al. (1999). Toxicol Appl Pharmacol 155(2): 150-160.
[11] Féau, C., et al. (2011). Androgen Action: Meth Prot: 59-68. (PMID: 19171919)
[12] Szafran, A. T., et al. (2008). PLoS One 3(11): e3605. (PMID: 18978937)
[13] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATTC, American Tissue Culture Collection
DBD, DNA Binding Domain
DHT, 4-5, Dihydrotestosterone
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
SRC-1, Steroid Receptor Coactivator 1
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

OT_ER_ERaERa_0480

Assay Name: Odyssey Thera Estrogen Receptor α/α Homodimer 8-hour Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha homodimer (ERα/ERα) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 8-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 8 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERα (stably expressed in HEK293T) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERα assay is a homodimer PCA of the ligand binding domain (amino acids 310-547) of human ERα stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to

a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERα LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 8 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERα LBD assay utilized the ability of the ERα to homodimerize upon ligand-binding with estrogenic compounds [5]. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα protein contains a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the estrogenic pathway is stimulated, separate ERα proteins form homodimers and the resulting YFP signal can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic ligand-binding and ERα activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following an 8-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ERα.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6].

Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation [7]. Thus, a highly sensitive assay that can detect ERα LBD binding in the context of a whole cell would serve as a powerful predictor of human-relevant estrogenic effects. The Odyssey Thera Ligand Binding assays used protein-fragment complementation (PCA) to measure dose-dependent homodimerization of estrogen receptor (ER) α expressed in human embryonic kidney cell line HEK293T. This dimerization and concurrent conformational changes brings into close proximity the fused fragments of split-yellow fluorescent protein (YFP), leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.859
Neutral control median absolute deviation, by plate:	0.256
Positive control well median response value, by plate:	15.56
Positive control well median absolute deviation, by plate:	1.10
Z' (median across all plates, using positive control wells):	0.7
SSMD (median across all plates, using positive control wells):	12
Signal-to-noise (median across all plates, using positive control wells):	53.55
Signal-to-background (median across all plates, using positive control wells):	17.85
CV (median across all plates):	0.3

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen receptor homodimerization in response to ligand-binding as measured with protein complementation assay technology by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERa_0480 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and signal baseline for activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3

Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 1.66
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	No
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes

117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	16
Inactive	14	8

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	29	13
Inactive	6	11

In Vitro Sensitivity = 56.8%

In Vitro Specificity = 36.4%

Balanced Accuracy = 46.6%

In Vivo Sensitivity = 69.0%
In Vivo Specificity = 64.7%
Balanced Accuracy = 66.9%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [8].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie- Inter J Pharma Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Katzenellenbogen, B. S., et al. (1993). J Ster Biochem Mol Biol 47(1): 39-48. (PMID: 8274440)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[8] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ER_ERaERa_1440

Assay Name: Odyssey Thera Estrogen Receptor α/α Homodimer 24-hour Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha homodimer (ERα/ERα) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 24-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERα (stably expressed in HEK293T) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERα assay is a homodimer PCA of the ligand binding domain (amino acids 310-547) of human ERα stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to

a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERα LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 24 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERα LBD assay utilized the ability of the ERα to homodimerize upon ligand-binding with estrogenic compounds [5]. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα protein contains a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the estrogenic pathway is stimulated, separate ERα proteins form homodimers and the resulting YFP signal can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic ligand-binding and ERα activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following an 8-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ERα.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6].

Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation [7]. Thus, a highly sensitive assay that can detect ERα LBD binding in the context of a whole cell would serve as a powerful predictor of human-relevant estrogenic effects. The Odyssey Thera Ligand Binding assays used protein-fragment complementation (PCA) to express a dose-dependent homodimer which binds to estrogen receptor (ER) α expressed in human embryonic kidney cell line HEK293T. This dimizeration and concurrent conformational changes brings into close proximity the fused fragments of split-yellow fluorescent protein (YFP), leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.943
Neutral control median absolute deviation, by plate:	0.250
Positive control well median response value, by plate:	30.36
Positive control well median absolute deviation, by plate:	1.81
Z' (median across all plates, using positive control wells):	0.8
SSMD (median across all plates, using positive control wells):	17
Signal-to-noise (median across all plates, using positive control wells):	137.55
Signal-to-background (median across all plates, using positive control wells):	36.88
CV (median across all plates):	0.25

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor α ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERa_1440 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3

Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.640
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	No
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes

117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	16
Inactive	14	8

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	30	12
Inactive	5	12

In Vitro Sensitivity = 56.8%

In Vitro Specificity = 36.4%

Balanced Accuracy = 46.6%

In Vivo Sensitivity = 71.4%
In Vivo Specificity = 70.6%
Balanced Accuracy =71.0%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [8].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie - Int J of Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Katzenellenbogen, B. S., et al. (1993). J Steroid Biochem Molecul Biol 47(1): 39-48. (PMID: 8274440)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[8] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA , Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ER_ERaERb_0480

Assay Name: Odyssey Thera Estrogen Receptor α/β Homodimer 8-hour Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha heterodimer (ERα/ERβ) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the estrogenic pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 8-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 8 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ER (isoforms α and β, stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines COS-7 and HepG2 cell lines [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERβ assay is a heterodimer PCA of the ligand binding domains (LBD) of ERα (amino acids 310-547) and human ERβ (amino acids 263-489) stably expressed in HEK293T cells. ERα/ERβ LBD cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire

cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 8 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor heterodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERβ LBD assay utilized the ability of ERα and ER to form heterodimers following ligand-binding with estrogenic compounds. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα and ERβ protein contains a fragment of a reporter enzyme (YFP) and when both proteins come in contact to form homo- or heterodimers, the resulting YFP signal can be measured using fluorescence microscopy and used to screen a diverse chemical library for potential xenobiotic ligand-binding and ER activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following an 8 hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and

baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ER α/β.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ which play different roles in mediating the actions of estrogenic compounds. Multiple studies have determined that the two isoforms can form functional homo- and hetero-dimers in vitro and in vivo which are capable of binding DNA [6] and initiating transcription of target genes [7]. Furthermore, ER homo- and heterodimers display ligand-selective activity [8] leading in turn to a unique but overlapping set of dimer-mediated transcriptional changes [9, 10]. Thus, a complete understanding of the potential estrogenic effects of EDCs requires the comprehensive profiling of the three physiological dimers. To assess the activity of the ERα/β heterodimer, this assay was designed to utilize the ability of the coexpressed ERα and ERβ LBDs to heterodimerize upon ligand-binding with estrogenic compounds. This dimerization and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0.875
Neutral control median absolute deviation, by plate:	0.126
Positive control well median response value, by plate:	3.22
Positive control well median absolute deviation, by plate:	0.21
Z' (median across all plates, using positive control wells):	0.55
SSMD (median across all plates, using positive control wells):	9
Signal-to-noise (median across all plates, using positive control wells):	19.43
Signal-to-background (median across all plates, using positive control wells):	3.64
CV (median across all plates):	0.15

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor (α / β) ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERb_0480 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 4.66
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 23.30

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes

50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	25	12
Inactive	15	7

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	32	10
Inactive	8	9

In Vitro Sensitivity = 67.6%

In Vitro Specificity = 31.8%

Balanced Accuracy = 49.7%

In Vivo Sensitivity = 76.2%
In Vivo Specificity = 52.9%
Balanced Accuracy = 58.3%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [11].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogen Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Molecul Endocrin 43(2): 65-72. (PMID: 19376833)
[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS 105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event

NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ER_ERaERb_1440

Assay Name: Odyssey Thera Estrogen Receptor α/β Homodimer 24-hour Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha heterodimer (ERα/ERβ) assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the estrogenic pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following 24-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ER (isoforms α and β, stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERα / ERβ assay is a heterodimer PCA of the ligand binding domains (LBD) of ERα (amino acids 310-547) and human ERβ (amino acids 263-489) stably expressed in HEK293T cells. ERα/ERβ LBD cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire

cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERα/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 24 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor heterodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera ERα/ERβ LBD assay utilized the ability of ERα and ER to form heterodimers following ligand-binding with estrogenic compounds. This activity is monitored via Protein-Fragment Complementation Assays (PCAs) which investigate the biochemical pathways capable of bringing separate protein fragments into close proximity. Each ERα and ERβ protein contains a fragment of a reporter enzyme (YFP) and when both proteins come in contact to form homo- or heterodimers, the resulting YFP signal can be measured using fluorescence microscopy and used to screen a diverse chemical library for potential xenobiotic ligand-binding and ER activation. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the estrogen signaling pathway following a 24 hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and

baseline signal and 17β-estradiol as a positive control and measure of 100% ligand-binding activity in ER α/β.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ which play different roles in mediating the actions of estrogenic compounds. Multiple studies have determined that the two isoforms can form functional homo- and hetero-dimers in vitro and in vivo which are capable of binding DNA [6] and initiating transcription of target genes [7]. Furthermore, ER homo- and heterodimers display ligand-selective activity [8] leading in turn to a unique but overlapping set of dimer-mediated transcriptional changes [9, 10]. Thus, a complete understanding of the potential estrogenic effects of EDCs requires the comprehensive profiling of the three physiological dimers. To assess the activity of the ERα/β heterodimer, this assay was designed to utilize the ability of the coexpressed ERα and ERβ LBDs to heterodimerize upon ligand-binding with estrogenic compounds. This dimerization and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.00
Neutral control median absolute deviation, by plate:	0.140
Positive control well median response value, by plate:	6.79
Positive control well median absolute deviation, by plate:	0.43
Z' (median across all plates, using positive control wells):	0.72
SSMD (median across all plates, using positive control wells):	13
Signal-to-noise (median across all plates, using positive control wells):	43.18
Signal-to-background (median across all plates, using positive control wells):	6.82
CV (median across all plates):	0.14

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor (α / β) ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERaERb_1440 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 1.91
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes

50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	Yes
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	27	10
Inactive	15	7

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	32	10
Inactive	10	7

In Vitro Sensitivity = 73.0%

In Vitro Specificity = 31.8%

Balanced Accuracy = 52.4%

In Vivo Sensitivity = 76.2%
In Vivo Specificity = 41.2%
Balanced Accuracy = 58.7%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [11].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogen Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Molecul Endocrin 43(2): 65-72. (PMID: 19376833)
[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS 105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event

NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA , Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ER_ERbERb_0480

Assay Name: Odyssey Thera Estrogen Receptor β/β Homodimer 8-hour Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor beta (ERβ/ERβ) ligand-binding assay used Protein-Fragment Complementation Assays (PCAs) to probe estrogen receptor beta (ERβ) for xenoestrogenic nuclear receptor binding and subsequent homodimer formation in stably transfected human embryonic kidney cells (cell line HEK293T). This assay format is designed to investigate the biochemical pathways capable of bringing separate, rationally dissected yellow fluorescent protein (YFP) fragments, which are linked in-frame to ERβ genes, into close proximity. When the reporter enzyme fragments are physically adjacent, the result is YFP reassembly and functional signal production. This fluorescence signal is only produced when the estrogen receptor beta pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenoestrogenic impacts on whole cell functioning which may affect functioning at a number of points along the estrogen signaling pathway. This activity was monitored following 8-hour incubation of test chemical or solvent with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 8 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERβ (stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERβ / ERβ assay is a homodimer PCA of the ligand binding domain (LBD; amino acids 310-547) of human ERβ stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 8 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily

punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERβ/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 8 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ, which play different roles in mediating the actions of estrogenic compounds. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation, while ERβ is thought to inhibit estrogen dependent cell growth, especially in breast cancer cells [6-8]. Several studies have associated a loss of ERβ or a decreased ratio of ERβ/ERα with other cancer types, including ovarian and colorectal cancers [9], suggesting a tumor suppressor role for ERβ in several cell types. Several isotype-selective ligands that bind one receptor with higher affinity than the other have been identified [10, 11] that produce distinct physiological effects when tested in animal models [9]. Therefore, a highly sensitive assay that can detect ERβ transcriptional changes in the context of a whole cell would be a useful tool to differentiate EDC's that preferentially activate ERβ-specific pathways. The OT ERβ/ERβ LBD PCA utilizes the ability of the ERβ LBD to homodimerize upon binding to estrogenic compounds. This dimizeration and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.11
Neutral control median absolute deviation, by plate:	0.19
Positive control well median response value, by plate:	6.75
Positive control well median absolute deviation, by plate:	0.37
Z' (median across all plates, using positive control wells):	0.7
SSMD (median across all plates, using positive control wells):	13
Signal-to-noise (median across all plates, using positive control wells):	27.9
Signal-to-background (median across all plates, using positive control wells):	5.81
CV (median across all plates):	0.18

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor beta ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERbERb_0480 assay was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up

NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 3.04
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes

599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	Yes
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes

556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	27	10
Inactive	15	7

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	31	11
Inactive	8	9

In Vitro Sensitivity = 73.0%
In Vitro Specificity = 31.8%
Balanced Accuracy = 52.4%

In Vivo Sensitivity = 73.8%
In Vivo Specificity = 52.9%
Balanced Accuracy = 63.4%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)

[3] Dai, D., et al. (2015). Die Pharmazie- Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Mol Endocrin 43(2): 65-72. (PMID: 19376833)
[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Kraichely, D. M., et al. (2000). Endocrinology 141(10): 3534-3545. (PMID: 11014206)
[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ER_ERbERb_1440

Assay Name: Odyssey Thera Estrogen Receptor β/β Homodimer 24-hour Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor beta (ERβ/ERβ) ligand-binding assay used Protein-Fragment Complementation Assays (PCAs) to probe estrogen receptor beta (ERβ) for xenoestrogenic nuclear receptor binding and subsequent homodimer formation in stably transfected human embryonic kidney cells (cell line HEK293T). This assay format is designed to investigate the biochemical pathways capable of bringing separate, rationally dissected yellow fluorescent protein (YFP) fragments which are linked in-frame to ERβ genes into close proximity. When the reporter enzyme fragments are physically adjacent, the result is YFP reassembly and functional signal production. This fluorescence signal is only produced when the estrogen receptor beta pathway is stimulated. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenoestrogenic impacts on whole cell functioning which may affect functioning at a number of points along the estrogen signaling pathway. This activity was monitored following 24-hour incubation of test chemical or solvent with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the wild type human ERβ (stably expressed in HEK293T cells) for xenoestrogenic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT ERβ / ERβ assay is a homodimer PCA of the ligand binding domain (LBD; amino acids 310-547) of human ERβ stably expressed in HEK293T cells. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium supplemented with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is primarily

punctuate and cytoplasmic. Modulation of this assay is quantified as an increase in mean fluorescence intensity in the nucleus (for agonists) or in the nucleus and cytoplasm (antagonists) relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. The latter instrument is favored for this assay because of the rapid mode of data acquisition, the ability to acquire data from the entire cell population in a well (as opposed to a fraction of the well on the high content devices) and the larger dynamic range typically achieved with this instrument. To assess sensitivity of the ERβ/ERβ LBD assay to ligand, cells were treated with the ER agonist 17-β-estradiol (E2) in 10-point concentration- response format for 24 hours and monitored response on a laser scanning plate cytometer (Acumen eX3; TTP Lab Tech).

Proprietary Elements:

Odyssey Thera assay described here used patented PCA technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor homodimerization, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [5].

The estrogen receptor is expressed in two forms, ERα and ERβ which play different roles in mediating the actions of estrogenic compounds. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation, while ERβ is thought to inhibit estrogen dependent cell growth, especially in breast cancer cells [6-8]. Several studies have associated a loss of ERβ or a decreased ratio of ERβ/ERα with other cancer types, including ovarian and colorectal cancers [9], suggesting a tumor suppressor role for ERβ in several cell types. Several isotype-selective ligands that bind one receptor with higher affinity than the other have been identified [10, 11] that produce distinct physiological effects when tested in animal models [9]. Therefore, a highly sensitive assay that can detect ERβ transcriptional changes in the context of a whole cell would be a useful tool to differentiate EDC's that preferentially activate ERβ-specific pathways. The OT ERβ/ERβ LBD PCA utilizes the ability of the ERβ LBD to homodimerize upon binding to estrogenic compounds. This dimizeration and concurrent conformational changes brings into close proximity the fused fragments of split-YFP, leading to a dramatic increase in YFP intensity. This intensity change is dose-dependent and shows saturation-binding that plateaus differently for agonists versus antagonists.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	1.25
Neutral control median absolute deviation, by plate:	0.19
Positive control well median response value, by plate:	14.01
Positive control well median absolute deviation, by plate:	0.65
Z' (median across all plates, using positive control wells):	0.79
SSMD (median across all plates, using positive control wells):	18
Signal-to-noise (median across all plates, using positive control wells):	67.22
Signal-to-background (median across all plates, using positive control wells):	10.89
CV (median across all plates):	0.15

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Estrogen signaling pathway protein fragment dimerization and enzyme formation in response to estrogen receptor beta ligand-binding as detected by monitoring fluorescence intensity.

Analytical Elements:

OT_ER_ERbERb_1440 was analyzed into in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline signal. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa

OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_0480
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 1.48
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No

104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No

57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	24	13
Inactive	13	9

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	29	13
Inactive	8	9

In Vitro Sensitivity = 64.9%
In Vitro Specificity = 40.9%
Balanced Accuracy = 52.9%

In Vivo Sensitivity = 69.0%
In Vivo Specificity = 52.9%
Balanced Accuracy = 61.0%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [12].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogen Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie-Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[6] Papoutsi, Z., et al. (2009). J Molecul Endocrin 43(2): 65-72. (PMID: 19376833)

[7] Cowley, S. M., et al. (1997). J Biol Chem 272(32): 19858-19862. (PMID: 9242648)
[8] Powell, E. and W. Xu (2008). PNAS 105(48): 19012-19017. (PMID: 19022902)
[9] Monroe, D. G., et al. (2005). Mol Endocrinol 19(6): 1555-1568. (PMID: 15802376)
[10] Li, X., et al. (2004). Mol Cell Biol 24(17): 7681-7694. (PMID: 15314175)
[11] Kraichely, D. M., et al. (2000). Endocrinology 141(10): 3534-3545. (PMID: 11014206)
[12] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
E2, Estradiol
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ERa_EREGFP_0120

Assay Name: Odyssey Thera HeLa cell-based high content GFP:Prolactin 2-hour assay to monitor active Estrogen Receptor (ER) transcriptional units

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha (ERα) GFP estrogen response element (ERE) assay used human cervical adenocarcinoma (HeLa) cells stably expressing both full-length, fluorescently-tagged human ERα transcription factor and multiple tandem prolactin promotor sequences to screen a diverse chemical library for potential xenoestrogenic activity. Sensitive microscopic visualization of prolactin promoter occupancy by a GFP-tagged ERα was capable of measuring agonist- and antagonist- ERα-ligand binding following a 2-hour incubation of test compounds with cells in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

HeLa cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 2 hours.

Experimental System:

GFP_ERα:PRL-HeLa cells are cervical adenocarcinoma epithelial cells, isolated from a 31 year old African-American female in February, 1951 [1], which constitutively express fluorescently-tagged full length human ERα and multiple integrated prolactin promotor sequences. The stable ER:PRL-HeLa cell line was developed by the Mancini lab at Baylor College of Medicine [2].

Xenobiotic Biotransformation Potential:

Constitutive expression of CYP1A1 and CYP1B1 mRNA; CYP1A2 expression was examined but not detected in HeLa cells [3, 4]. Expression of tumor-suppressing p53 and pRB proteins has been reported to be low [5].

Basic Procedure:

The stable ERα:PRL-HeLa line is seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 2 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. All assay endpoints are quantified using high content image analysis algorithms.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource-intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2014

Assay Publication:

Stossi, F., Bolt, M. J., Ashcroft, F. J., Lamerdin, J. E., Melnick, J. S., Powell, R. T., Dandekar, R. D., Mancini, M. G., Walker, C. L., Westwick, J. K., & Mancini, M. A. (2014). "Defining estrogenic mechanisms of bisphenol A analogs through high throughput microscopy-based contextual assays". Chem Biol 21(6), 743-753. (PMID: 24856822)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

This Odyssey Thera estrogen receptor α green fluorescent protein (GFP) estrogen response element (ERE) assay was developed to measure transcriptional changes induced by ligand-binding as detected in a cervical adenocarcinoma cell line stably expressing both full-length human ERα and multiple estrogen responsive prolactin promoter sequences. ERα interacts with estrogenic ligands and following 2-hour incubation of test compound with cells in a 384-well plate, xenoestrogenic activation of a microscopically visible reporter gene is detected as an increase in mean signal relative to baseline activity (DMSO control) using a 10-point concentration-response assay format.

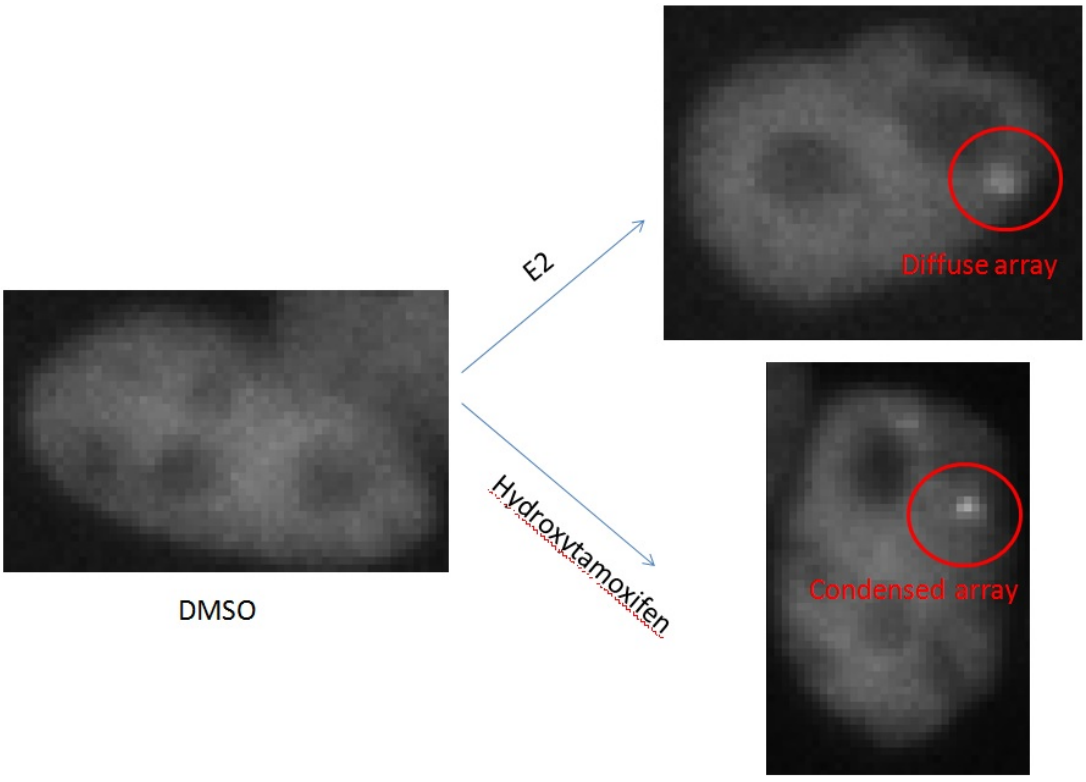


Figure 1. Single-cell views of the ER:PRL-HeLa cell line showing GFP-ERα accumulation on ERE arrays in response to vehicle (DMSO), agonist (E2) or antagonist (4-hydroxytamoxifen). Images were taken on an InCell 2000 with a 20X 0.75NA objective, provided curtesy of Odyssey Thera, Inc.

The ER:PRL-HeLa line constitutively expresses physiologically-relevant levels of fluorescently-tagged, full-length human ERα, and contains multi-copy genomic insertions of the prolactin promoter containg estrogen receptor response elements. When stimulated by agonists, tagged ERα accumulates on the prolactin array in an open (transcriptionally-active) binding mode, leading to a bright, micron-sized spot while antagonist-treated cells lead to tagged-ERα binding in its closed (transcriptionally-repressive) binding-mode, leading to a condensed array that appears as a sub-micron-sized point.

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD's proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6]. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Ashcroft, F. J., Newberg, J. Y., Jones, E. D., Mikic, I., & Mancini, M. A. (2011). "High content imaging-based assay to classify estrogen receptor-α ligands based on defined mechanistic outcomes". Gene 477(1-2), 42-52. (PMID: 21256200)

Sharp, Z. D., Mancini, M. G., Hinojos, C. A., Dai, F., Berno, V., Szafran, A. T., et al. (2006). Estrogen-receptor-α exchange and chromatin dynamics are ligand-and domain-dependent. Journal of cell science, 119(19), 4101-4116. (PMID: 16968748)

Assay Quality Statistics:

Neutral control well median response value, by plate:	8.75
Neutral control median absolute deviation, by plate:	1.48
Positive control well median response value, by plate:	321.5
Positive control well median absolute deviation, by plate:	16.49
Z' (median across all plates, using positive control wells):	0.81
SSMD (median across all plates, using positive control wells):	19
Signal-to-noise (median across all plates, using positive control wells):	129.09
Signal-to-background (median across all plates, using positive control wells):	35.68
CV (median across all plates):	0.17

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Ligand binding of estrogen receptor α and xenoestrogenic effects on transcriptional regulation of a fluorescent reporter gene.

Analytical Elements:

OT_ERa_GFPERaERE_0120 readout data was analyzed in the positive (gain of signal) fitting direction using percent activity 4-Hydroxytamoxifen as positive control (100% activity) over DMSO controls. All statistical analyses were conducted using R programming language, employing tcpl package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor transactivation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM

Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.488
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	No
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes

115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	22	15
Inactive	14	8

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	29	13
Inactive	7	10

In Vitro Sensitivity = 59.5%
In Vitro Specificity = 36.4%
Balanced Accuracy = 48.0%

In Vivo Sensitivity = 69.0%
In Vivo Specificity = 64.7%
Balanced Accuracy = 58.8%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [7].

4. Assay Documentation

4.1. References

[1] Jones, H. W., et al. (1971). Obstetrics & Gynecology 38(6): 945-949. (PMID: 4942173)
[2] Nakajima, M., et al. (2003). Toxicol Lett 144(2): 247-256. (PMID: 12927368)
[3] Iwanari, M., et al. (2002). Arch Toxicol 76(5-6): 287-298. (PMID: 12107646)
[4] Scheffner, M., et al. (1991). PNAS 88(13): 5523-5527. (PMID: 1648218)
[5] Ashcroft, F. J., et al. (2011). Gene 477(1-2): 42-52. (PMID: 21256200)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

4HT, 4-hydroxytamoxifen
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathways
DMSO, Dimethyl Sulfoxide
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
GFP, Green Fluorescent Protein
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ERa_EREGFP_0480

Assay Name: Odyssey Thera HeLa cell-based high content GFP:Prolactin 8-hour assay to monitor active Estrogen Receptor (ER) transcriptional units

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen receptor alpha (ERα) GFP estrogen response element (ERE) assay used human cervical adenocarcinoma (HeLa) cells stably expressing both full-length, fluorescently-tagged human ERα transcription factor and multiple tandem prolactin promotor sequences to screen a diverse chemical library for potential xenoestrogenic activity. Sensitive microscopic visualization of prolactin promoter occupancy by a GFP-tagged ERα was capable of measuring agonist- and antagonist- ERα-ligand binding following a 8-hour incubation of test compounds with cells in a 384-well plate.

1.2. Assay Definition

Assay Throughput:

HeLa cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 8 hours.

Experimental System:

GFP_ERα:PRL-HeLa cells are cervical adenocarcinoma epithelial cells, isolated from a 31 year old African-American female in February, 1951 [1], which constitutively express fluorescently-tagged full length human ERα and multiple integrated prolactin promotor sequences.

Xenobiotic Biotransformation Potential:

Constitutive expression of CYP1A1 and CYP1B1 mRNA; CYP1A2 expression was examined but not detected in HeLa cells [2, 3]. Expression of tumor-suppressing p53 and pRB proteins has been reported to be low [4].

Basic Procedure:

The stable ERα:PRL-HeLa line is seeded into optical quality 384-well poly-D-lysine coated plates in phenol red-free medium with 10% dextran-treated FBS and allowed to adhere for 24 hours prior to treatment with test compounds or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells prior to signal detection. All assay endpoints are quantified using high content image analysis algorithms.

Proprietary Elements:

This assay is not proprietary. ER:PRL-HeLa cell line was developed and provided to OT by the Mancini lab at Baylor [5].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2014

Assay Publication:

Stossi, F., Bolt, M. J., Ashcroft, F. J., Lamerdin, J. E., Melnick, J. S., Powell, R. T., Dandekar, R. D., Mancini, M. G., Walker, C. L., Westwick, J. K., & Mancini, M. A. (2014). "Defining estrogenic mechanisms of bisphenol A analogs through high throughput microscopy-based contextual assays". Chem Biol 21(6), 743-753. (PMID: 24856822)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

This Odyssey Thera estrogen receptor α green fluorescent protein (GFP) estrogen response element (ERE) assay was developed to measure long-term transcriptional changes induced by ligand-binding as detected in a cervical adenocarcinoma cell line stably expressing both full-length human ERα and multiple estrogen responsive prolactin promoter sequences. ERα interacts with estrogenic ligands and following 2-hour incubation of test compound with cells in a 384-well plate, xenoestrogenic activation of a microscopically visible reporter gene is detected as an increase in mean signal relative to baseline activity (DMSO control) using a 10-point concentration-response assay format.

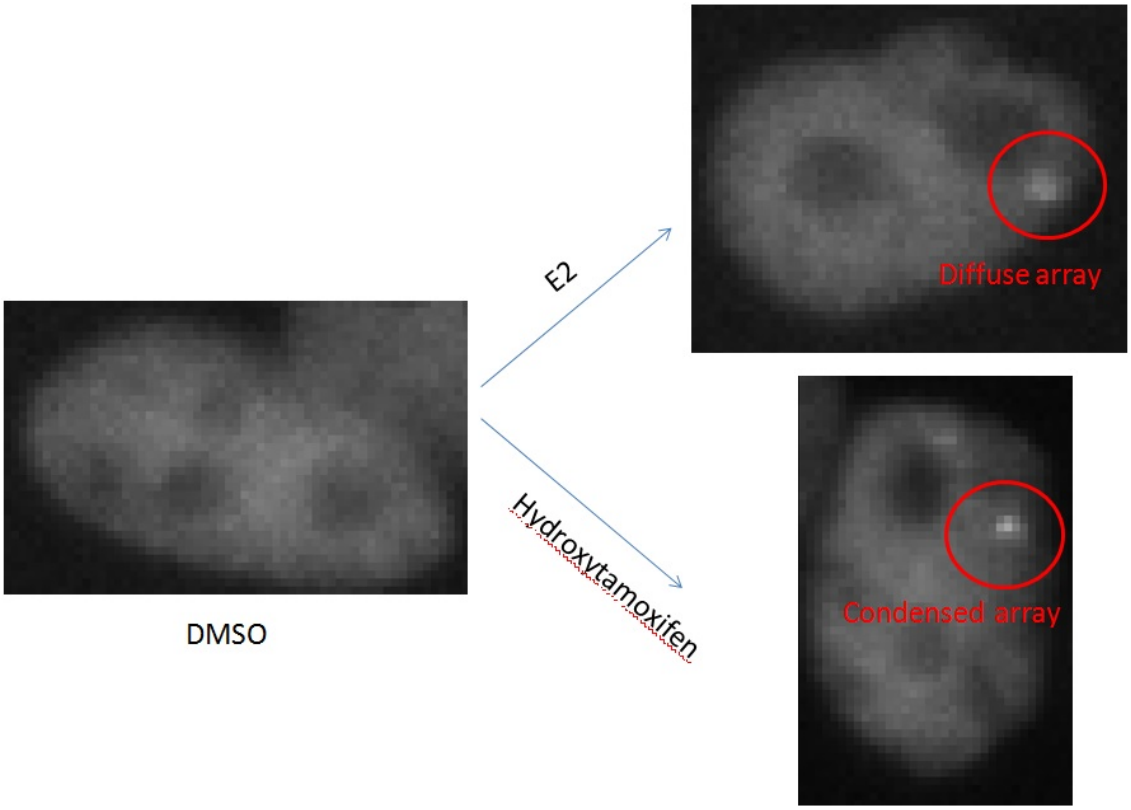


Figure 1. Single-cell views of the ER:PRL-HeLa cell line showing GFP-ERα accumulation on ERE arrays in response to vehicle (DMSO), agonist (E2) or antagonist (4-hydroxytamoxifen). Images were taken on an InCell 2000 with a 20X 0.75NA objective, provided curtesy of Odyssey Thera, Inc.

ER:PRL-HeLa line constitutively expresses physiologically-relevant levels of fluorescently-tagged, full-length human ERα, and contains multi-copy genomic insertions of the prolactin promoter containg estrogen receptor response elements. When stimulated by agonists, tagged ERα accumulates on the prolactin array in an open (transcriptionally-active) binding mode, leading to a bright, micron-sized spot while antagonist-treated cells lead to tagged-ERα binding in its closed (transcriptionally-repressive) binding-mode, leading to a condensed array that appears as a sub-micron-sized point).

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NHRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD's proclivity to bind a

disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [6]. Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the MIE leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative adverse outcome pathways leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Ashcroft, F. J., Newberg, J. Y., Jones, E. D., Mikic, I., & Mancini, M. A. (2011). "High content imaging-based assay to classify estrogen receptor-α ligands based on defined mechanistic outcomes". Gene 477(1-2), 42-52. (PMID: 21256200)

Sharp, Z. D., Mancini, M. G., Hinojos, C. A., Dai, F., Berno, V., Szafran, A. T., et al. (2006). Estrogen-receptor-α exchange and chromatin dynamics are ligand-and domain-dependent. Journal of cell science, 119(19), 4101-4116. (PMID: 16968748)

Assay Quality Statistics:

Neutral control well median response value, by plate:	6.5
Neutral control median absolute deviation, by plate:	0.741
Positive control well median response value, by plate:	351.25
Positive control well median absolute deviation, by plate:	16.68
Z' (median across all plates, using positive control wells):	0.84
SSMD (median across all plates, using positive control wells):	20
Signal-to-noise (median across all plates, using positive control wells):	410.09
Signal-to-background (median across all plates, using positive control wells):	52.13
CV (median across all plates):	0.15

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Ligand binding of estrogen receptor α and xenoestrogenic effects on transcriptional regulation of a fluorescent reporter gene.

Analytical Elements:

OT_ERa_GFPERaERE_0480 readout data was analyzed in the positive (gain of signal) fitting direction using percent activity 4-Hydroxytamoxifen as positive control (100% activity) over DMSO controls. All statistical analyses were conducted using R programming language, employing tcpl package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the

baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 6
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.3 µM
Standard maximum concentration tested: 100 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.227
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	No

131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	No
104-40-5	4-Nonylphenol	Very Weak	Active	No
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	No
61-82-5	Amitrole	NA	Inactive	No
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	No
480-40-0	Chrysin	Very Weak	NA	No
50-22-6	Corticosterone	Inactive	NA	No
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	Yes
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	Yes
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes

51630-58-1	Fenvalerate	NA	Inactive	No
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	No
520-18-3	Kaempferol	Very Weak	Inactive	No
143-50-0	Kepone	Weak	NA	Yes
65277-42-1	Ketoconazole	Inactive	NA	Yes
330-55-2	Linuron	Inactive	NA	No
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	No
72-55-9	p,p'-DDE	Very Weak	Weak	No
87-86-5	Pentachlorophenol	NA	Inactive	No
57-30-7	Phenobarbital sodium	Inactive	NA	No
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	Yes
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	16
Inactive	13	9

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	26	16
Inactive	8	9

In Vitro Sensitivity = 56.8%
In Vitro Specificity = 40.9%
Balanced Accuracy = 48.9%

In Vivo Sensitivity = 61.9%
In Vivo Specificity = 52.9%
Balanced Accuracy = 57.4%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for

chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [7].

4. Assay Documentation

4.1. References

[1] Jones, H. W., et al. (1971). Obstetrics & Gynecology 38(6): 945-949. (PMID: 4942173)
[2] Nakajima, M., et al. (2003). Toxicol Lett 144(2): 247-256. (PMID: 12927368)
[3] Iwanari, M., et al. (2002). Arch Toxicol 76(5-6): 287-298. (PMID: 12107646)
[4] Scheffner, M., et al. (1991). PNAS 88(13): 5523-5527. (PMID: 1648218)
[5] Ashcroft, F. J., et al. (2011). Gene 477(1-2): 42-52. (PMID: 21256200)
[6] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[7] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

4HT, 4-hydroxytamoxifen
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathways
DMSO, Dimethyl Sulfoxide
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
GFP, Green Fluorescent Protein
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ERa_ERELUC_AG_1440

Assay Name: Odyssey Thera CHO-K1 cell-based luciferase assay to measure estrogen receptor alpha / ERE transcriptional activity (agonist mode)

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen response element (ERE) luciferase (LUC) agonist assay was developed to measure long-term transcriptional changes induced by ligand-binding of estrogen receptor alpha (ERα) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human ERα and an ERE reporter construct driving expression of luciferase. ERα interacts with estrogenic ligands at the ligand-binding domain and with ERE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with cells in a 384-well plate, xenobiotic agonism of ERα is detected by measuring bioluminescent signal produced by ER-moderated transcriptional activity, and potential xenoestrogenic compounds can be identified using a luminometer to quantify an increase in mean signal relative to baseline activity (DMSO control).

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

CHO-K1 is an immortal mammal ovary fibroblast cell line derived from Chinese hamster cells isolated in 1957 [1]. CHO-K1 is a widely used cell line with well characterized cell transfection methods frequently utilized for large-scale production of numerous pharmaceutical proteins (including hormones, antibodies, and blood factors) since these cells are capable of folding, assembling and post-translationally modifying proteins in a manner that is more comparable to humans [2]. The Odyssey Thera ERE_luc assays used CHO-K1 cells with stably transfected full-length ERα and ERE-luciferase reporter construct.

Xenobiotic Biotransformation Potential:

CHO-K1 cells have the capacity to metabolize the anti-androgenic fungicide vinclozolin [3], however the intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

To identify ER agonists, CHO-K1 cells are seeded into a white-walled/white-bottom 384-well plate followed by treatment with compounds of interest or controls for 24 hours. A luciferase assay mix containing D-luciferin and ATP in PBS is then added to the cells and luminescence quantified on a Luminoskan (Thermo Scientific) luminometer. Modulation of this assay is quantified as an increase in mean luminescence intensity relative to vehicle controls.

To evaluate specificity of the ERα /ERE-luc assay, several known ER agonists were also tested in 5-pt concentration-response format in a minimum of 7 assay plates.

OT determined the reproducibility of E2-induced ERα/ERE-luc transcriptional activation with a total of seven 384-well plates run on 3 different days, for 24 hours. A minimum of 3 replicate wells were analyzed for each sample with 14 replicate wells for vehicle controls. Luminescence data were

captured on the Luminoskan luminometer. The range of EC50 values for E2 was determined in the ERα/ERE-luc transcriptional assay in 9 384-well plates run on 3 different days.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

Assay Publication:

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera estrogen response element (ERE) luciferase (LUC) agonist assay was developed to measure transcriptional changes induced by ligand-binding of estrogen receptor alpha (ERα) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human ERα and an ERE reporter construct driving expression of luciferase. ERα interacts with estrogenic ligands at the ligand-binding domain and with ERE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with cells in a 384-well plate, xenobiotic agonism of ERα is detected by measuring bioluminescent signal produced by ER-moderated transcriptional activity, and potential xenoestrogenic compounds can be identified using a luminometer to quantify an increase in mean signal relative to baseline activity (DMSO control).

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NHRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [4].

Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation [5]. Thus, a highly sensitive assay that can detect ERα LBD binding in the context of a whole cell would serve as a

powerful predictor of human-relevant estrogenic effects. The OT_ERa_ERE_LUC_Agonist Assay uses CHOK1 cells stably transfected with full-length ERα to monitor ERE-driven expression of luciferase reporter activity in response to chemical exposures over 24 hours.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds which potentially affect endocrine systems in exposed populations by interacting with estrogen receptor mediated signaling pathways. There is evidence that estrogen receptor activation in early life is a molecular initiating event (MIE) in a developing Adverse Outcome Pathways (AOP) leading to endometrial carcinoma in the mouse (currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of ER interference in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Katzenellenbogen, B. S., Bhardwaj, B., Fang, H., Ince, B. A., Pakde, F., Reese, J. C., Schodin, D., & Wrenn, C. K. (1993). "Hormone binding and transcription activation by estrogen receptors: analyses using mammalian and yeast systems". The Journal of Steroid Biochemistry and Molecular Biology 47(1), 39-48. (PMID: 8274440)

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Agonism of ERα transcription factor and DNA-binding to ERE sequence located upstream of transfected luciferase reporter gene measured by monitoring increased luminescence.

Analytical Elements:

OT_ERa_ERE_LUC_agonist_1440 readout data was analyzed in the positive fitting direction (receptor gain-of-signal activity) as a percent of 17b-Estradiol (positive control, 100% activation) and relative to DMSO, negative control and baseline activity. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up

ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Puck, T. T., et al. (1958). The J Exper Medicine 108(6): 945-956. (PMID: 13598821)
[2] Kildegaard, H. F., et al. (2013). Curr Opin Biotechnol 24(6): 1102-1107. (PMID: 23523260)
[3] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[4] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)

[5] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
CHO, Chinese Hamster Ovary
DMSO, Dimethyl Sulfoxide
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
LUC, Luciferase
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

25 November 2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ERa_ERELUC_ANT_1440

Assay Name: Odyssey Thera CHO-K1 cell-based luciferase assay to measure estrogen receptor alpha / ERE transcriptional activity (antagonist mode)

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen response element (ERE) luciferase (LUC) antagonist assay was developed to measure transcriptional changes induced by ligand-binding of estrogen receptor alpha (ERα) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human ERα and an ERE reporter construct driving expression of luciferase. ERα interacts with estrogenic ligands at the ligand-binding domain and with ERE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with cells in a 384-well plate, xenobiotic antagonism of ERα is detected by measuring a reduction in bioluminescent signal produced by ER-moderated transcriptional activity induced by a known ER agonist (17beta-estradiol), and potential estrogenic compounds can be identified using a luminometer to quantify an increase in activity relative to vehicle controls (DMSO).

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

CHO-K1 is an immortal mammal ovary fibroblast cell line derived from Chinese hamster cells isolated in 1957 [1]. CHO-K1 is a widely used cell line with well characterized cell transfection methods frequently utilized for large-scale production of numerous pharmaceutical proteins (including hormones, antibodies, and blood factors) since these cells are capable of folding, assembling and post-translationally modifying proteins in a manner that is more comparable to humans [2]. The Odyssey Thera ERE_luc assays used CHO-K1 cells with stably transfected full-length ERα and ERE-luciferase reporter construct.

Xenobiotic Biotransformation Potential:

CHO-K1 cells have the capacity to metabolize the anti-androgenic fungicide vinclozolin [3], however the intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

To identify ER antagonists, the ERα/ERE-luc assay is run in the presence of low amounts of ER ligand. The cells are seeded into a white-walled/white-bottom 384-well plate in the presence of agonist (1nM 17β-Estradiol), followed by treatment with compounds of interest or controls for 24 hours. A luciferase assay mix containing D-luciferin and ATP in PBS is then added to the cells and luminescence quantified on a Luminoskan (Thermo Scientific) luminometer. Modulation of this assay is quantified as an increase in mean luminescence intensity relative to vehicle controls. To demonstrate sensitivity of the ERα /ERE-luc assay to ER antagonists, OT treated the cells with 4-hydroxytamoxifen (4HT) in concentration-response format for 24 hours in the presence of E2. To evaluate specificity of the ERα /ERE-luc assay, several known ER agonists, ER antagonists, and AR-selective compounds were also tested in 5-pt concentration-response format in a minimum of 7 assay plates. OT determined the reproducibility of 4HT-mediated inhibition of E2-stimulated ERα/ERE-Luciferase transcriptional activation with a total of nine 384-well plates run on 3 different

days, and EC50 values for 4HT were determined at the 24h time point. A minimum of 3 replicate wells were analyzed for each sample with 14 replicate wells for vehicle controls. Luminescence data were captured on the Luminoskan luminometer.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

Assay Publication:

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera estrogen response element (ERE) luciferase (LUC) antagonist assay was developed to measure transcriptional changes induced by ligand-binding of estrogen receptor alpha (ERα) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human ERα and an ERE reporter construct driving expression of luciferase. ERα interacts with estrogenic ligands at the ligand-binding domain and with ERE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with agonist-stimulated cells in a 384-well plate, xenobiotic antagonism of ERα is detected by measuring a reduction in bioluminescent signal induced by a known ER agonist (17beta-estradiol) relative to vehicle controls (DMSO).

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [4].

Cell-based and in vivo experiments suggest that the ERα isoform is the primary mediator of estrogenic effects by EDCs on reproductive development and on cell proliferation [5]. Thus, a sensitive assay that can detect ERα LBD binding in the context of a whole cell would serve as a

powerful predictor of human-relevant estrogenic effects. The OT_ERa_ERE_LUC_Antagonist Assay uses CHO-K1 cells stably transfected with full-length ERα to monitor xenobiotic interference with ERE-driven expression of luciferase reporter activity in response to chemical exposures over 24 hours.

Method Development Reference:

Katzenellenbogen, B. S., Bhardwaj, B., Fang, H., Ince, B. A., Pakde, F., Reese, J. C., Schodin, D., & Wrenn, C. K. (1993). "Hormone binding and transcription activation by estrogen receptors: analyses using mammalian and yeast systems". The Journal of steroid biochemistry and molecular biology 47(1), 39-48. (PMID: 8274440)

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Antagonism of ERα transcription factor and repression of DNA-binding to ERE sequence located upstream of transfected luciferase reporter gene measured by monitoring increased luminescence.

Analytical Elements:

OT_ERa_ERE_LUC_Antagonist_1440 readout data was analyzed in the positive fitting direction using log2 fold-induction over DMSO controls. Activity was monitored as loss-of-signal relative to the positive control of (Z)-4-Hydroxytamoxifen. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480

OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERa_ERELUC_AG_1440
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Puck, T. T., et al. (1958). The J Exper Medicine 108(6): 945-956. (PMID: 13598821)
[2] Kildegaard, H. F., et al. (2013). Curr Opin Biotechnol 24(6): 1102-1107. (PMID: 23523260)
[3] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[4] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[5] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

4HT, 4-hydroxytamoxifen
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
CHO, Chinese Hamster Ovary
DMSO, Dimethyl Sulfoxide

E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
LUC, Luciferase
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_ERb_ERELUC_ANT_1440

Assay Name: Odyssey Thera CHO-K1 cell-based luciferase assay to measure estrogen receptor beta / ERE transcriptional activity (antagonist mode)

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera estrogen response element (ERE) luciferase (LUC) antagonist assay was developed to measure transcriptional changes induced by ligand-binding of estrogen receptor beta (ERβ) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human ERβ and an ERE reporter construct driving expression of luciferase. ERβ interacts with estrogenic ligands at the ligand-binding domain and with ERE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with cells in a 384-well plate, xenobiotic antagonism of ERβ is detected by measuring a reduction in bioluminescent signal produced by ER-moderated transcriptional activity induced by a known ER agonist (17beta-estradiol), and potential estrogenic compounds can be identified using a luminometer to quantify an increase in activity relative to vehicle controls (DMSO).

1.2. Assay Definition

Assay Throughput:

HEK-293T cells are seeded into 384-well microtiter plates and allowed to adhere for 24 hours prior to 24 hour chemical exposures.

Experimental System:

CHO-K1 is an immortal mammal ovary fibroblast cell line derived from Chinese hamster cells isolated in 1957 [1]. CHO-K1 is a widely used cell line with well characterized cell transfection methods frequently utilized for large-scale production of numerous pharmaceutical proteins (including hormones, antibodies, and blood factors) since these cells are capable of folding, assembling and post-translationally modifying proteins in a manner that is more comparable to humans [2]. The Odyssey Thera ERE_luc assays used CHO-K1 cells with stably transfected full-length ER and ERE-luciferase reporter construct.

Xenobiotic Biotransformation Potential:

CHO-K1 cells have the capacity to metabolize the anti-androgenic fungicide vinclozolin [3], however the intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

To identify ER antagonists, the ERβ/ERE-luc assay is run in the presence of low amounts of ER ligand. The cells are seeded into a white-walled/white-bottom 384-well plate in the presence of agonist (1nM 17β-Estradiol), followed by treatment with compounds of interest or controls for 24 hours. A luciferase assay mix containing D-luciferin and ATP in PBS is then added to the cells and luminescence quantified on a Luminoskan (Thermo Scientific) luminometer. Modulation of this assay is quantified as an increase in mean luminescence intensity relative to vehicle controls. To demonstrate sensitivity of the ERβ/ERE-luc assay to ER antagonists, cells were treated with 4-hydroxytamoxifen (4HT) in concentration-response format for 24 hours in the presence of E2. OT determined the reproducibility of 4HT-mediated inhibition of E2-stimulated ERβ/ERE-Luciferase transcriptional activation with a total of three 384-well plates run on a single day, and EC_{50} values for 4HT were determined from those plates at the 24h time point. A minimum of 3 replicate wells

were analyzed for each sample with 14 replicate wells for vehicle controls. Luminescence data were captured on the Luminoskan luminometer.

Proprietary Elements:

This assay is not proprietary.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

Assay Publication:

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Odyssey Thera estrogen response element (ERE) luciferase (LUC) antagonist assay was developed to measure transcriptional changes induced by ligand-binding of estrogen receptor alpha (ER□) detected in a mammalian (Chinese hamster ovary; CHO-K1) cell line stably expressing both full-length human ER□ and an ERE reporter construct driving expression of luciferase. ER□ interacts with estrogenic ligands at the ligand-binding domain and with ERE sequences at the DNA-binding domain. Following 24-hour incubation of compound of interest with agonist-stimulated cells in a 384-well plate, xenobiotic antagonism of ER□ is detected by measuring a reduction in bioluminescent signal induced by a known ER agonist (17beta-estradiol) relative to vehicle controls (DMSO).

Scientific Principles:

Endocrine disrupting compounds bind to nuclear hormone receptors, leading to a diverse array of transcriptional and signaling pathway alterations. These cell- and tissue-type specific changes affect many aspects of human physiology, including those involved in inflammation, neonatal development and oncogenesis. Among the human NHRs, the estrogen receptor family is particularly susceptible to perturbation by EDCs because of the ER LBD proclivity to bind a disparate set of small hydrophobic molecules commonly found in nature and in man-made materials [4].

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds which potentially affect endocrine systems in exposed populations by interacting with estrogen receptor mediated signaling pathways. Chemical-activity profiles derived from this assay can inform prioritization decisions for compound

selection in more resource intensive *in vivo* studies to further investigate the involvement of ER interference in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Katzenellenbogen, B. S., Bhardwaj, B., Fang, H., Ince, B. A., Pakde, F., Reese, J. C., Schodin, D., & Wrenn, C. K. (1993). "Hormone binding and transcription activation by estrogen receptors: analyses using mammalian and yeast systems". The Journal of Steroid Biochemistry and Molecular Biology 47(1), 39-48. (PMID: 8274440)

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Antagonism of ERβ transcription factor and DNA-binding to ERE sequence located upstream of transfected luciferase reporter gene measured by monitoring increased luminescence.

Analytical Elements:

OT_ERβ_ERE_LUC_Antagonist_1440 readout data was analyzed in the positive fitting direction using log2 fold-induction over DMSO controls. Activity was monitored as loss-of-signal relative to the positive control of (Z)-4-Hydroxytamoxifen (100% inhibition). All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_1440
OT_ER_ERaERb_0480
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440

OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERa_ERELUC_ANT_1440
OT_ERa_ERELUC_AG_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	ZZZBB
Target (nominal) number of replicates:	ZZZCB
Standard minimum concentration tested:	ZZZDB µM
Standard maximum concentration tested:	ZZZEB µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	ZZZFB
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	ZZZGB

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Puck, T. T., et al. (1958). J Exper Med 108(6): 945-956. (PMID: 13598821)
[2] Kildegaard, H. F., et al. (2013). Curr Opin Biotechnol 24(6): 1102-1107. (PMID: 23523260)
[3] Jacobs, M., et al. (2008). Curr Drug Metab 9(8): 796-826. (PMID: 18855613)
[4] Shanle, E. K. and W. Xu (2010). Chem Res Toxicol 24(1): 6-19. (PMID: 21053929)
[5] Helguero, L. A., et al. (2005). Oncogene 24(44): 6605-6616. (PMID: 16007178)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
CHO, Chinese Hamster Ovary
DMSO, Dimethyl Sulfoxide
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
DBD, DNA Binding Domain
LBD, Ligand Binding Domain
LUC, Luciferase
MIE, Molecular Initiating Event
MTC, Maximum Tolerated Concentrations
NR, Nuclear Receptors
NHR, Nuclear Hormone Receptors
OT, Odyssey Thera
PBS, Phosphate Buffered Saline
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

26 May 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

OT_FXR_FXRSRC1_0480

Assay Name: Odyssey Thera Farnesoid X Receptor (FXR) / Steroid Receptor Co-Activator 8-hr Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera Farnesoid X Receptor/Steroid Receptor Co-Activator SRC-1 assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the farnesoid X receptor (FXR; NR1H4) response pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the FXR signaling pathway. The Odyssey Thera FXR/SRC-1 assay is a transient PCA performed in HEK293T cells expressing the full length human FXR and the nuclear receptor interacting domain (NID) of SRC-1, each fused to a fragment of YFP. The plasmids are co-expressed for 24 hours prior to 8-hour incubation with test compounds in 384-well plates.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 8 hours prior to monitoring fluorescence emission resulting from xenobiotic FXR activation and co-factor recruitment.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the human FXR protein (transiently expressed in HEK293T) for xenobiotic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT FXR/SRC-1 assay assessed receptor-chemical interactions using a rapidly maturing, intensely fluorescent mutant of YFP known as Venus, rationally dissected into two separate fragments. The fragments were obtained as follows: first, fragments coding for YFP[1] and YFP[2] (corresponding to amino acid residues 1–158 and 159–239 of the full length YFP, respectively) were generated by oligonucleotide synthesis (Blue Heron Biotechnology), and then PCR mutagenesis was used to generate the mutant fragments IFP[1] and IFP[2]. Fusion constructs were transfected into HEK293T cells with a $(Gly_4Ser)_2$ linker between the FXR/SRC-1 and YFP fragment genes to

facilitate complementation when interacting proteins bring fragments into close proximity. The construct is transiently transfected in HEK293T cells 24 hours prior to treatment with test compounds. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in MEM alpha medium (Invitrogen) supplemented with 10% FBS (Gemini Bio-Products) 1% penicillin and 1% streptomycin, and grown in 37 °C incubator equilibrated to 5% CO2. Cells are allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells and subcellular compartment boundaries prior to signal detection. Fluorescent signal in the basal state of the assay is restricted to the nucleus. Modulation of this assay is quantified as an increase in mean fluorescence in the nucleus relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. 8 Images per well were acquired on an Evotec Opera at 2 wavelengths (488 and 635nm), and the mean nuclear fluorescence in the 488nm channel was calculated for a minimum of 400 cells per image.

Proprietary Elements:

Odyssey Thera PCA assays used patented technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote FXR mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The OT FXR/SRC-1 assay is a PCA expressed transiently in HEK293T cells for 24h prior to treatment with test compound for 8 hours. Cells are fixed with 4% formaldehyde and stained with Draq5 to identify cells and subcellular compartment boundaries prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is restricted to the nucleus. Modulation of this assay

is quantified as an increase in mean fluorescence in the nucleus relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. Stimulation of the assay was assessed in a 10-point dose response with the selective FXR agonist GW4064 with a concentration range of 10 - 0.0003µM. Each FXR protein and its associated coactivator (SRC-1) contain a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the FXR signaling pathway is impacted by chemical activation or interference, the resulting YFP signal production can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic ligand-binding. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track changes at the level of cell functioning which may occur at a number of points along the signaling pathway following an 8-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and GW4064 as a positive control and measure of 100% FXR/SRC-1 activation.

Scientific Principles:

The farnesoid X receptor (FXR) is a nuclear receptor whose primary function is to act as a bile acid sensor, protecting the liver from bile acid toxicity by regulating the transcription of genes involved in bile acid homeostasis [5]. Recent studies have also established that FXR is a master regulator of lipid and glucose homeostasis [6] and disruption of FXR in mouse models leads to phenotypes associated with metabolic disease, including diabetes and hypercholesterolemia [6]. FXR also plays roles in liver regeneration [7]. FXR is a non-steroidal nuclear receptor that binds to DNA as a heterodimer with the retinoic acid receptor, RXRα. The FXR/RXR heterodimer is "permissive" in that the pair becomes transcriptionally active in the presence of either an RXR-selective ligand or a FXR ligand [8]. This heterodimer is thought to be pre-bound to the target response element on DNA prior to ligand binding, existing in a complex with corepressor proteins. Upon ligand binding, changes in the conformation of FXR result in release of the corepressors and subsequent binding to transcriptional coactivator proteins. FXR binds to a number of co-activator proteins, including the prototypical nuclear receptor coactivator, SRC-1 [9]. Since FXR/RXRα complexes are not expected to be selective for FXR activation while ligand binding favors interaction of FXR with SRC-1, this assay is designed to monitor the interaction between FXR and SRC-1 to detect xenobiotic activation of FXR by test compounds.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	36.423
Neutral control median absolute deviation, by plate:	3.87
Positive control well median response value, by plate:	104.77
Positive control well median absolute deviation, by plate:	8.521
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.46
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	7
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	17.81
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	2.81

Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.1

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

farnesoid X receptor signaling pathway protein formation in response FXR ligand-binding and FXR/SRC-1 protein complex formation.

Analytical Elements:

Each data point was formed by taking the log of the ratio of the sample signal to the vehicle control (DMSO) signal. A minimum of 8 replicate wells were analyzed each for sample and vehicle controls. Wells located in the outer ring of the plate were omitted due to the potential for edge effects. Data were captured on a confocal microscope: the fold increase in mean fluorescence intensity was calculated relative to the vehicle controls from 16 images, each containing a minimum of 350 cells. Gain-of-signal activity indicates FXR/SRC-1 complex formation and data are plotted as percent of GW4064 activity, and are plotted relative to DMSO, negative control and signal baseline. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up
ATG_FXR_TRANS_up
NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad): 5.013
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad): 25.064

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharm Intern J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Commun 5. (PMID: 25182477)
[5] Kim, I., et al. (2007). J Lipid Res 48(12): 2664-2672. (PMID: 17720959)
[6] Ma, K., et al. (2006). The Journal of Clin Invest 116(4): 1102-1109. (PMID: 16557297)
[7] Huang, W., et al. (2006). Science 312(5771): 233-236. (PMID: 16614213)
[8] Pérez, E., et al. (2012). Biochimica et Biophysica Acta (1): 57-69. (PMID: 21515403)
[9] Wang, Z., et al. (2006). Cell Metabolism 3(2): 111-122. (PMID: 16459312)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATTC, American Tissue Culture Collection
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
FXR, Farnesoid X Receptor
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
SRC-1, Steroid Receptor Coactivator 1

YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 October 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

OT_FXR_FXRSRC1_1440

Assay Name: Odyssey Thera Farnesoid X Receptor (FXR) / Steroid Receptor Co-Activator 24-hr Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera Farnesoid X Receptor/Steroid Receptor Co-activator SRC-1 assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the farnesoid X receptor (FXR; NR1H4) response pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the FXR signaling pathway. The Odyssey Thera FXR/SRC-1 assay is a transient PCA performed in HEK293T cells expressing the full length human FXR and the nuclear receptor interacting domain (NID) of SRC-1, each fused to a fragment of YFP. The plasmids are co-expressed for 24 hours prior to 24 hour incubation with test compounds in 384-well plates.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 8 hours prior to monitoring fluorescence emission resulting from xenobiotic FXR activation and co-factor recruitment.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the human FXR protein (transiently expressed in HEK293T) for xenobiotic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT FXR/SRC-1 assay assessed receptor-chemical interactions using a rapidly maturing, intensely fluorescent mutant of YFP known as Venus, rationally dissected into two separate fragments. The fragments were obtained as follows: first, fragments coding for YFP[1] and YFP[2] (corresponding to amino acid residues 1–158 and 159–239 of the full length YFP, respectively) were generated by oligonucleotide synthesis (Blue Heron Biotechnology), and then PCR mutagenesis was used to generate the mutant fragments IFP[1] and IFP[2]. Fusion constructs were transfected into HEK293T cells with a $(Gly_4Ser)_2$ linker between the FXR/SRC-1 and YFP fragment genes to

facilitate complementation when interacting proteins bring fragments into close proximity. The construct is transiently transfected in HEK293T cells 24 hours prior to treatment with test compounds. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in MEM alpha medium (Invitrogen) supplemented with 10% FBS (Gemini Bio-Products) 1% penicillin and 1% streptomycin, and grown in 37 °C incubator equilibrated to 5% CO2. Cells are allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells and subcellular compartment boundaries prior to signal detection. Fluorescent signal in the basal state of the assay is restricted to the nucleus. Modulation of this assay is quantified as an increase in mean fluorescence in the nucleus relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. 8 Images per well were acquired on an Evotec Opera at 2 wavelengths (488 and 635nm), and the mean nuclear fluorescence in the 488nm channel was calculated for a minimum of 400 cells per image.

Proprietary Elements:

Odyssey Thera PCA assays used patented technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote FXR mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None Reported

2. Assay Component Descriptions

Assay Objectives:

The OT FXR/SRC-1 assay is a PCA expressed transiently in HEK293T cells for 24h prior to treatment with test compound for 8 hours. Cells are fixed with 4% formaldehyde and stained with Draq5 to identify cells and subcellular compartment boundaries prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is restricted to the nucleus. Modulation of this assay

is quantified as an increase in mean fluorescence in the nucleus relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. Stimulation of the assay was assessed in a 10-point dose response with the selective FXR agonist GW4064 with a concentration range of 10 - 0.0003µM. Each FXR protein and its associated coactivator (SRC-1) contain a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the FXR signaling pathway is impacted by chemical activation or interference, the resulting YFP signal production can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic ligand-binding. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track changes at the level of cell functioning which may occur at a number of points along the signaling pathway following a 24-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and GW4064 as a positive control and measure of 100% FXR/SRC-1 activation.

Scientific Principles:

The farnesoid X receptor (FXR) is a nuclear receptor whose primary function is to act as a bile acid sensor, protecting the liver from bile acid toxicity by regulating the transcription of genes involved in bile acid homeostasis [5]. Recent studies have also established that FXR is a master regulator of lipid and glucose homeostasis [6] and disruption of FXR in mouse models leads to phenotypes associated with metabolic disease, including diabetes and hypercholesterolemia [6]. FXR also plays roles in liver regeneration [7]. FXR is a non-steroidal nuclear receptor that binds to DNA as a heterodimer with the retinoic acid receptor, RXRα. The FXR/RXR heterodimer is "permissive" in that the pair becomes transcriptionally active in the presence of either an RXR-selective ligand or a FXR ligand [8]. This heterodimer is thought to be pre-bound to the target response element on DNA prior to ligand binding, existing in a complex with corepressor proteins. Upon ligand binding, changes in the conformation of FXR result in release of the corepressors and subsequent binding to transcriptional coactivator proteins. FXR binds to a number of co-activator proteins, including the prototypical nuclear receptor coactivator, SRC-1 [9]. Since FXR/RXRα complexes are not expected to be selective for FXR activation while ligand binding favors interaction of FXR with SRC-1, this assay is designed to monitor the interaction between FXR and SRC-1 to detect xenobiotic activation of FXR by test compounds.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	72.869
Neutral control median absolute deviation, by plate:	8.851
Positive control well median response value, by plate:	179.36
Positive control well median absolute deviation, by plate:	12.127
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z’ (median across all plates, using positive control wells):	0.33
Z’ (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	6
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	11.05
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	2.36

Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.13

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Farnesoid X receptor signaling pathway protein formation in response to FXR/SRC-1 protein complex formation following ligand-binding.

Analytical Elements:

Each data point was formed by taking the log of the ratio of the sample signal to the vehicle control (DMSO) signal. A minimum of 8 replicate wells were analyzed each for sample and vehicle controls. Wells located in the outer ring of the plate were omitted due to the potential for edge effects. Data were captured on a confocal microscope: the fold increase in mean fluorescence intensity was calculated relative to the vehicle controls from 16 images, each containing a minimum of 350 cells. Gain-of-signal activity indicates FXR/SRC-1 complex formation and data are plotted as percent of GW4064 activity, and are plotted relative to DMSO, negative control and signal baseline. package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up
ATG_FXR_TRANS_up
NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	8.324
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	41.62

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [10].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharm Intern J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Commun 5. (PMID: 25182477)
[5] Kim, I., et al. (2007). J Lipid Res 48(12): 2664-2672. (PMID: 17720959)
[6] Ma, K., et al. (2006). The Journal of Clin Invest 116(4): 1102-1109. (PMID: 16557297)
[7] Huang, W., et al. (2006). Science 312(5771): 233-236. (PMID: 16614213)
[8] Pérez, E., et al. (2012). Biochimica et Biophysica Acta (1): 57-69. (PMID: 21515403)
[9] Wang, Z., et al. (2006). Cell Metabolism 3(2): 111-122. (PMID: 16459312)
[10] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
ATTC, American Tissue Culture Collection
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
FXR, Farnesoid X Receptor
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
OT, Odyssey Thera
PCA, Protein-Fragment Complementation
SRC-1, Steroid Receptor Coactivator 1

YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2 October 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

OT_PPARg_PPARgSRC1_0480

Assay Name: Odyssey Thera Peroxisome Proliferator-activated Receptor Gamma (PPARg) / SRC-1 Co-activator 8-hr Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera peroxisome proliferator-activated receptor gamma/steroid receptor co-activator SRC-1 assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the peroxisome proliferator-activated receptors (PPAR) response pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the PPAR signaling pathway following 8-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 8 hours prior to monitoring fluorescence emission resulting from xenobiotic PPAR gamma activation and co-factor recruitment.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the human PPARγ protein (transiently expressed in HEK293T) for xenobiotic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT PPARγ/SRC-1 assay assessed receptor-chemical interactions using a rapidly maturing, intensely fluorescent mutant of YFP known as Venus, rationally dissected into two separate fragments. The fragments were obtained as follows: first, fragments coding for YFP[1] and YFP[2] (corresponding to amino acid residues 1–158 and 159–239 of the full length YFP, respectively) were generated by oligonucleotide synthesis (Blue Heron Biotechnology), and then PCR mutagenesis was used to generate the mutant fragments IFP[1] and IFP[2]. Fusion constructs were transfected into HEK293T cells with a $(Gly_4Ser)_2$ linker between the PPARγ/SRC-1 and YFP fragment genes to facilitate complementation when interacting proteins bring fragments into close proximity. The construct is transiently transfected in HEK293T cells 48 hours prior to treatment with test

compounds. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in MEM alpha medium (Invitrogen) supplemented with 10% FBS (Gemini Bio-Products) 1% penicillin and 1% streptomycin, and grown in 37°C incubator equilibrated to 5% CO2. Cells are allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 8 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells and subcellular compartment boundaries prior to signal detection. 8 Images per well were acquired on an Evotec Opera at 2 wavelengths (488 and 635nm), and the ratio of fluorescence in the nucleus relative to fluorescence in the cytoplasm (N/C Ratio) in the 488nm channel was calculated for a minimum of 350 cells per image.

Proprietary Elements:

Odyssey Thera PCA assays used patented technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote PPAR gamma mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The OT PPARγ/SRC-1 assay is a PCA expressed transiently in HEK293T cells for 48h prior to treatment with test compound for 8 hours. Cells are fixed with 4% formaldehyde and stained with Draq5 to identify cells and subcellular compartment boundaries prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is restricted to the nucleus. Modulation of this assay is quantified as an increase in mean fluorescence in the nucleus relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. Stimulation of the assay was assessed in a 10-point dose response with the selective

PPARγ agonist GW1929. Each PPARγ protein and its associated coactivator (SRC-1) contain a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the PPARγ responsive signaling pathway is impacted by chemical activation or interference, the resulting YFP signal production can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic PPARγ ligand-binding. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track changes at the level of cell functioning which may occur at a number of points along the signaling pathway following an 8-hour incubation of cells with test compound in 384-well plate, using DMSO as a negative control and baseline signal and GW1929 as a positive control and measure of 100% PPARγ/SRC1 activation.

Scientific Principles:

The peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor that plays a key role in mediating differentiation of adipocytes and regulating fat metabolism. PPARγ has been implicated in the pathophysiology of atherosclerosis, inflammation, obesity, diabetes, immune response, and ageing. The PPARγ nuclear receptor functions as a transcription factor as part of a large protein complex through interactions with transcriptional co-repressors and co-activators. PPARs form heterodimers with the retinoid X receptor (RXR) and bind to PPAR response elements (PPREs) in enhancer sites of regulated genes. In the absence of ligand, nuclear receptor co-repressors bind to these heterodimers and recruit histone deactylases (HDACs) to repress transcription. Ligand binding to the C-terminal activation function (AF-2) domain induces a conformational change in the receptor dimer which excludes co-repressors from the complex. Ligand binding also increases PPAR's affinity for a number of co-activators, including SRC-1, whose binding facilitates chromatin remodeling by histone modification and nucleosome mobilization, leading to the recruitment of the basal transcription machinery to PPAR target genes. Therefore, measuring stimulation of the PPARγ/SRC-1 complex represents ligand-dependent activation of the receptor.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARg) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is some evidence to support a putative AOP linking PPAR gamma receptor activation with increased occurrence of sarcomas in rats, mice, and hamsters (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPAR activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	11.16
Neutral control median absolute deviation, by plate:	1.825
Positive control well median response value, by plate:	107.941
Positive control well median absolute deviation, by plate:	7.784
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z’ (median across all plates, using positive control wells):	0.69

Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	60.83
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	10.57
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	0.18

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Peroxisome proliferator-activated receptor gamma signaling pathway protein formation in response to PPARγ ligand-binding /SRC-1 co-factor recruitment.

Analytical Elements:

Each data point was formed by taking the log of the ratio of the sample signal to the vehicle control (DMSO) signal. A minimum of 8 replicate wells were analyzed each for sample and vehicle controls. Wells located in the outer ring of the plate were omitted due to the potential for edge effects. Data were captured on a confocal microscope: the fold increase in mean fluorescence intensity was calculated relative to the vehicle controls from 16 images, each containing a minimum of 350 cells. Gain-of-signal activity indicates PPARγ/SRC-1 complex formation and data are plotted as percent of GW1929 activity, and are plotted relative to DMSO, negative control and signal baseline. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR gamma activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
ATG_PPARg_TRANS_up
NVS_NR_hPPARg
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_Agonist_ratio
Tox21_PPARg_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	1.192
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATTC, American Tissue Culture Collection
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event

OT, Odyssey Thera
PCA, Protein-Fragment Complementation
PPAR, Peroxisome Proliferator-Activated Receptor
SRC-1, Steroid Receptor Coactivator 1
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

22 December 2016

Author of Revisions:

EPA NCCT

5. Supporting Information

OT_PPARg_PPARgSRC1_1440

Assay Name: Odyssey Thera Peroxisome Proliferator-activated Receptor Gamma (PPARg) / SRC-1 Co-activator 24-hr Protein-Complementation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The Odyssey Thera peroxisome proliferator-activated receptor gamma/steroid receptor co-activator SRC-1 assay used Protein-Fragment Complementation Assays (PCAs) to investigate the biochemical pathways which bring separate protein fragments into close proximity and result in functional reporter enzyme (yellow fluorescent protein, YFP) signal production when the peroxisome proliferator-activated receptors (PPAR) response pathway is unimpeded. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track xenobiotic changes at the level of cell functioning which may occur at a number of points along the PPAR signaling pathway following 24-hour incubation of test chemical with transformed HEK293T cells in 384-well plates.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 384-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from xenobiotic PPAR gamma activation and co-factor recruitment.

Experimental System:

This assay monitors the ligand binding domain (LBD) of the human PPARγ protein (transiently expressed in HEK293T) for xenobiotic activation. The HEK-293 cell line are human embryonic epithelial kidney cells (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK-293T cells are derived from the HEK293 cell line by the addition of the SV40 large T antigen that has been shown to increase vector production of some viral vectors. HEK293T are reported to have relatively high transfection efficiencies when compared to other cell lines (COS-7 and HepG2) [3] and it is among the most frequently utilized cell lines for in small-scale protein production and in viral vector propagation using the transient transfection method [4].

Xenobiotic Biotransformation Potential:

Constitutive expression of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

The OT PPARγ/SRC-1 assay assessed receptor-chemical interactions using a rapidly maturing, intensely fluorescent mutant of YFP known as Venus, rationally dissected into two separate fragments. The fragments were obtained as follows: first, fragments coding for YFP[1] and YFP[2] (corresponding to amino acid residues 1–158 and 159–239 of the full length YFP, respectively) were generated by oligonucleotide synthesis (Blue Heron Biotechnology), and then PCR mutagenesis was used to generate the mutant fragments IFP[1] and IFP[2]. Fusion constructs were transfected into HEK293T cells with a $(Gly_4Ser)_2$ linker between the PPARγ/SRC-1 and YFP fragment genes to facilitate complementation when interacting proteins bring fragments into close proximity. The construct is transiently transfected in HEK293T cells 48 hours prior to treatment with test

compounds. Cells are seeded into optical quality 384-well poly-D-lysine coated plates in MEM alpha medium (Invitrogen) supplemented with 10% FBS (Gemini Bio-Products) 1% penicillin and 1% streptomycin, and grown in 37°C incubator equilibrated to 5% CO2. Cells are allowed to adhere for 24 hours prior to treatment with compounds of interest or controls for 24 hours. Cells are fixed in 4% formaldehyde and stained with Draq5 (BioStatus) to identify cells and subcellular compartment boundaries prior to signal detection. 8 Images per well were acquired on an Evotec Opera at 2 wavelengths (488 and 635nm), and the ratio of fluorescence in the nucleus relative to fluorescence in the cytoplasm (N/C Ratio) in the 488nm channel was calculated for a minimum of 350 cells per image.

Proprietary Elements:

Odyssey Thera PCA assays used patented technology.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote PPAR gamma mediated DNA transcription, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

Odyssey Thera Inc.
4550 Norris Canyon Road, Suite 140
San Ramon, CA 94583
Tel: (925) 242-5000

Assay Publication Year:

2003

Assay Publication:

MacDonald, M. L., Lamerdin, J., Owens, S., Keon, B. H., Bilter, G. K., Shang, Z., Huang, Z., Yu, H., Dias, J., Minami, T., Michnick, S. W., & Westwick, J. K. (2006). "Identifying off-target effects and hidden phenotypes of drugs in human cells". Nat Chem Biol 2(6), 329-337. (PMID: 16680159)

Yu, H., West, M., Keon, B. H., Bilter, G. K., Owens, S., Lamerdin, J., & Westwick, J. K. (2003). "Measuring drug action in the cellular context using protein-fragment complementation assays". Assay Drug Dev Technol 1(6), 811-822. (PMID: 15090227)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The OT PPARγ/SRC-1 assay is a PCA expressed transiently in HEK293T cells for 48h prior to treatment with test compounds for 24 hours. Cells are fixed with 4% formaldehyde and stained with Draq5 to identify cells and subcellular compartment boundaries prior to signal detection. Fluorescent signal in the basal state of the assay is very low and is restricted to the nucleus. Modulation of this assay is quantified as an increase in mean fluorescence in the nucleus relative to vehicle controls, and can be quantified on a high content imaging device or a laser scanning cytometer. Stimulation of the assay was assessed in a 10-point dose response with the selective

PPARγ agonist GW1929. Each PPARγ protein and its associated coactivator (SRC-1) contain a rationally dissected fragment of a yellow-fluorescent protein (YFP) reporter enzyme. When the PPARγ responsive signaling pathway is impacted by chemical activation or interference, the resulting YFP signal production can be measured using fluorescence microscopy to screen a diverse chemical library for potential xenobiotic PPARγ ligand-binding. Changes in protein complex interactions can be impacted by a variety of biochemical events within a pathway, and this assay is designed to track changes at the level of cell functioning which may occur at a number of points along the signaling pathway following a 24-hour incubation of cells with test chemicals in 384-well plate, using DMSO as a negative control and baseline signal and GW1929 as a positive control and measure of 100% PPARγ/SRC1 activation.

Scientific Principles:

The peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor that plays a key role in mediating differentiation of adipocytes and regulating fat metabolism. PPARγ has been implicated in the pathophysiology of atherosclerosis, inflammation, obesity, diabetes, immune response, and ageing. The PPARγ nuclear receptor functions as a transcription factor as part of a large protein complex through interactions with transcriptional co-repressors and co-activators. PPARs form heterodimers with the retinoid X receptor (RXR) and bind to PPAR response elements (PPREs) in enhancer sites of regulated genes. In the absence of ligand, nuclear receptor co-repressors bind to these heterodimers and recruit histone deactylases (HDACs) to repress transcription. Ligand binding to the C-terminal activation function (AF-2) domain induces a conformational change in the receptor dimer which excludes co-repressors from the complex. Ligand binding also increases PPAR's affinity for a number of co-activators, including SRC-1, whose binding facilitates chromatin remodeling by histone modification and nucleosome mobilization, leading to the recruitment of the basal transcription machinery to PPAR target genes. Therefore, measuring stimulation of the PPARγ/SRC-1 complex represents ligand-dependent activation of the receptor.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with peroxisome proliferator-activated receptor alpha (PPARg) receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is some evidence to support a putative AOP linking PPAR gamma receptor activation with increased occurrence of sarcomas in rats, mice, and hamsters (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of PPAR activation in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A., & Pelletier, J. N. (2000). "Detection of protein-protein interactions by protein fragment complementation strategies". Methods Enzymol 328, 208-230. (PMID: 11075347)

Assay Quality Statistics:

Neutral control well median response value, by plate:	10.104
Neutral control median absolute deviation, by plate:	1.483
Positive control well median response value, by plate:	133.452
Positive control well median absolute deviation, by plate:	11.684
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z’ (median across all plates, using positive control wells):	0.68

Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	90.3
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	14.2
Signal-to-background (median across all plates, using positive control wells):	NA
CV (median across all plates):	0.15

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Peroxisome proliferator-activated receptor gamma signaling pathway protein formation in response to PPARγ ligand-binding /SRC-1 co-factor recruitment.

Analytical Elements:

Each data point was formed by taking the log of the ratio of the sample signal to the vehicle control (DMSO) signal. A minimum of 8 replicate wells were analyzed each for sample and vehicle controls. Wells located in the outer ring of the plate were omitted due to the potential for edge effects. Data were captured on a confocal microscope: the fold increase in mean fluorescence intensity was calculated relative to the vehicle controls from 16 images, each containing a minimum of 350 cells. Gain-of-signal activity indicates PPARγ/SRC-1 complex formation and data are plotted as percent of GW1929 activity, and are plotted relative to DMSO, negative control and signal baseline. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR activity was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 30% of neutral controls or 3 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
ATG_PPARg_TRANS_up
NVS_NR_hPPARg
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_Agonist_ratio
Tox21_PPARg_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.938
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Dai, D., et al. (2015). Die Pharmazie Int J Pharm Sci 70(1): 33-37. (PMID: 25975096)
[4] Lin, Y.-C., et al. (2014). Nature Comm 5. (PMID: 25182477)
[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
ATTC, American Tissue Culture Collection
DBD, DNA Binding Domain
DMSO, Dimethyl Sulfoxide
HEK, Human Embryonic Kidney
LBD, Ligand Binding Domain
MIE, Molecular Initiating Event

OT, Odyssey Thera
PCA, Protein-Fragment Complementation
PPAR, Peroxisome Proliferator-Activated Receptor
SRC-1, Steroid Receptor Coactivator 1
YFP, Yellow Fluorescent Protein

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information:

Tox21_AR_BLA_Agonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Androgen Receptor Agonist Transactivation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

Androgen receptor (AR), a nuclear hormone receptor, plays critical roles in the development and differentiation of male embryos, in the initiation and maintenance of spermatogenesis and in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that inhibit AR signaling, an AR-UAS-bla GripTite™ cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS) stably integrated into HEK293 cells was used to screen the Tox21 chemical library. This experimental system expresses a fusion protein of a rodent androgen receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic androgen binding compounds stimulates β-lactamase reporter gene expression.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring fluorescence emission resulting from xenobiotic AR gene expression.

Experimental System:

GeneBLAzer® AR-UAS-bla GripTite™ 293 cells contain the ligand-binding domain (LBD) of the rat androgen receptor (AR) fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer® UAS-bla GripTite™ 293 cell line. This portion of the rat AR is identical to the human AR in the conserved LBD and differs from the human sequence at five residues in the hinge region. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4 (DBD)-AR (LBD) fusion protein, the protein binds to the UAS, resulting in expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM, high glucose	Invitrogen	Invitrogen/11965

Opti-MEM	Invitrogen	Invitrogen/11058
Dialyzed FBS	Invitrogen	Invitrogen/26400
HEPES	Invitrogen	Invitrogen/15630
NEAA	Invitrogen	Invitrogen/11140
Sodium pyruvate	Invitrogen	Invitrogen/11360
Penicillin and Streptomycin	Invitrogen	Invitrogen/15140
Hygromycin	Invitrogen	Invitrogen/10687
Zeocin	Invitrogen	Invitrogen/R250-01
0.05% Trypsin-EDTA	Invitrogen	Invitrogen/25300
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen/12648
Black-clear bottom 1536 well plates	Greiner	Greiner/789092F
LiveBLAzer B/G FRET substrate	Invitrogen	Invitrogen/K1028
Multidrop COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD dispenser	Beckman Coulter	Beckman Coulter
Envision Plate Reader	Perkin Elmer	Perkin Elmer
R1881 or Methyltrienolone	Perkin Elmer	Perkin Elmer/ NLP005005MG

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
DMEM, high glucose	90%	-	90%	-
Opti-MEM	-	90%	-	-
Dialyzed FBS	10%	10%	10%	-
HEPES	25mM	-	25mM	-
NEAA	0.1mM	0.1mM	0.1mM	-
Sodium pyruvate	1mM	1mM	1mM	-
Penicillin and Streptomycin	100U/ml and 100 µg/ml	100U/ml and 100 µg/ml	100U/ml and 100 µg/ml	-
Hygromycin	80 µg/ml	-	-	-
Zeocin	80 µg/ml	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 -1ml frozen cells of AR-bla were taken in pre-warmed 10ml of thaw medium for centrifuging

1.2.2 -Thaw medium is used to re-suspend the pellet

1.2.3 -Seed the cells at 2 million per T-75 flask with thaw medium

1.3. Propagation method

1.3.1 -Detach the cells from the flask using 0.05% Trypsin
1.3.2 -The cells are re-seeded in T-225 flask at 3-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing
2.2 -Resuspend the pellet with assay medium
2.3 -Plate the cells in black-clear bottom 1536 well plate at 2000/well/6 µL through 8 tip Multidrop plate dispenser
2.4 -Incubate at 37°C for 5hrs
2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool
2.6 -Incubate at 37°C for 16hrs
2.7 -Add 1 µL of CCF4 (FRET Substrate) dye using a single tip plate dispenser (Bioraptr)
2.8 -Incubate at room temperature for 2hrs
2.9 -Read the fluorescence intensity through Envision plate reader

AR-bla cells were dispensed at 2,000 cells/6 µL/well of assay medium into black wall/clear-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37°C and 5% CO_2 for 4 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37°C and 5% CO_2 for 16 h. Then 1 µL of LiveBLAzer™ B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The plates were incubated at room temperature for 2 h, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [3]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang

NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 androgen receptor beta lactamase agonist assay screened a library of diverse environmental chemicals to probe for xenobiotic androgen receptor ligand-binding and potential to induce androgen-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 37 hour incubation of cells with test compounds a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for 2 hours. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known AR agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation. Concentration-response relationships were determined by monitoring luminescent signals relative to DMSO-only exposures which provided a signal baseline, and to a known androgen receptor agonist (Methyltrienolone) as a positive control, which provided an indication of 100% androgen receptor activation.

Scientific Principles:

Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological

processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous androgenic signaling pathways.

The Tox21 AR bla assays are qHTS format assays which measured the ability of a chemical to interact with AR by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses rat AR in a one-hybrid GAL4 system to quantify xenobiotic androgen receptor agonism**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.198
Neutral control median absolute deviation, by plate:	6.374
Positive control well median response value, by plate:	99.356
Positive control well median absolute deviation, by plate:	21.535
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.15
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	4
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	15.51
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-412
Signal-to-background (median across all plates, using negative control wells):	NA

CV (median across all plates):	-28.54

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor agonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

Beta lactamase expression in the AR_BLA_Agonist_ratio assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (Methyltrienolone; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of Methyltrienolone (R1881) activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_LUC_MDAKB2_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	3.303
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Tox21_AR_BLA_Antagonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Androgen Receptor Antagonist Transactivation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

Androgen receptor (AR), a nuclear hormone receptor, plays critical roles in the development and differentiation of male embryos, in the initiation and maintenance of spermatogenesis and in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that inhibit AR signaling, an AR-UAS-bla GripTite™ cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS) stably integrated into HEK293 cells was used to screen the Tox21 chemical library. This experimental system expresses a fusion protein of a rodent androgen receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds stimulates β-lactamase reporter gene expression, and AR antagonism by test compounds results in decreased signal production relative to cyproterone acetate in the presence of a known AR agonist (R1881). To detect loss of signal due to compound cytotoxicity, a CellTiter-Glo fluorescence assay to measure ATP production was run concurrently in all wells using tetraoctylammonium bromide as a positive control for cell death.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring fluorescence emission resulting from xenobiotic AR gene expression.

Experimental System:

GeneBLAzer® AR-UAS-bla GripTite™ 293 cells contain the ligand-binding domain (LBD) of the rat androgen receptor (AR) fused to the DNA-binding domain of GAL4 stably integrated in the cell line. This portion of the rat AR is identical to the human AR in the conserved LBD and differs from the human sequence at five residues in the hinge region. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). This assay is performed with small amounts of a known AR agonist (R1881) added to each well, and when an antagonistic compound binds to the LBD of the GAL4-AR fusion protein binding to the UAS is inhibited, interfering with expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 (Dohr et al. 1995). The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid (Bylund et al. 2004). HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

QUALITY CONTROL PRECAUTIONS:

1. Cell culture is maintained by passaging twice a week and should not reach more than 90% confluence
2. The assay should be performed in black-clear bottom 1536 well plates, so the bottom of the plates should not be touched

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM, high glucose	Invitrogen	Invitrogen/11965
Opti-MEM	Invitrogen	Invitrogen/11058
Dialyzed FBS	Invitrogen	Invitrogen/26400
HEPES	Invitrogen	Invitrogen/15630
NEAA	Invitrogen	Invitrogen/11140
Sodium pyruvate	Invitrogen	Invitrogen/11360
Penicillin and Streptomycin	Invitrogen	Invitrogen/15140
Hygromycin	Invitrogen	Invitrogen/10687
Zeocin	Invitrogen	Invitrogen/R250-01
0.05% Trypsin-EDTA	Invitrogen	Invitrogen/25300
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen/12648
Black-clear bottom 1536 well plates	Greiner	Greiner/789092F
LiveBLAzer B/G FRET substrate	Invitrogen	Invitrogen/K1028
CellTiter-Glo Assay Custom Solution	Promega	Promega/X2371
Multidrop COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD dispenser	Beckman Coulter	Beckman Coulter
Envision Plate Reader	Perkin Elmer	Perkin Elmer
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
Cyproterone acetate (Antagonist control compound)	Sigma Aldrich	Sigma Aldrich/C3412

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
DMEM, high glucose	90%	-	90%	-
Opti-MEM	-	90%	-	-
Dialyzed FBS	10%	10%	10%	-
HEPES	25mM	-	25mM	-
NEAA	0.1mM	0.1mM	0.1mM	-
Sodium pyruvate	1mM	1mM	1mM	-

Penicillin and Streptomycin	100U/ml and 100 µg/ml	100U/ml and 100 µg/ml	100U/ml and 100 µg/ml	-
Hygromycin	80 µg/ml	-	-	-
Zeocin	80 µg/ml	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 -1ml frozen cells of AR-bla were taken in pre-warmed 10ml of thaw medium for centrifuging

1.2.2 -Thaw medium is used to re-suspend the pellet

1.2.3 -Seed the cells at 2 million per T-75 flask with thaw medium

1.3. Propagation method

1.3.1 -Detach the cells from the flask using 0.05% Trypsin

1.3.2 -The cells are re-seeded in T-225 flask at 3-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing

2.2 -Resuspend the pellet with assay medium

2.3 -Plate the cells in black-clear bottom 1536 well plate at 2000/well/6 µL through 8 tip Multidrop plate dispenser

2.4 -Incubate at 37 °C for 5hrs

2.5 -Add 1uL of assay buffer by using single tip of a plate dispenser (Bioraptr) into bottom 1/3rd part of 2 and 3 columns

2.6 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool

2.7 -Add 1µL of 10nM (final) R1881 by using single tip of a plate dispenser (Bioraptr) into all the wells except the buffer dispensed wells of bottom 1/3rd part of 2 and 3 columns

2.8 -Incubate at 37 °C for 16hrs

2.9 -Add 1 µL of CCF4 (FRET Substrate) dye using a single tip plate dispenser (Bioraptr)

2.10 -Incubate at room temperature for 2hrs

2.11 -Read the fluorescence intensity through Envision plate reader

2.12 -Add 4µL of CellTiter-Glo assay reagent using a single tip of a plate dispenser (Bioraptr)

2.13 -Incubate at room temperature for 30min

2.14 -Read the luminescence through ViewLux plate reader

AR-bla cells were dispensed at 2,000 cells/5 µL of assay medium per well into black wall/clear-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37 °C and 5% CO2 for 4 hr, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1 µL of agonist (R1881) in culture medium using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The plates were incubated at a 37 °C and 5% CO2 for 16 h. After 1 µL of LiveBLAzerTM B/G FRET substrate was added using a Bioraptr FRD, the plates were incubated at room temperature for 2 h and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 4 µL/well of CellTiter-Glo reagent was added into the assay plates using a Bioraptr FRD. After 30 min incubation

at room temperature, the luminescence intensity in the plates was measured using a ViewLux (PerkinElmer) plate reader.

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) (Michael et al. 2008). GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None Reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 androgen receptor beta lactamase antagonist assay screened a library of diverse environmental compounds to probe for xenobiotic androgen receptor ligand-binding and potential to suppress androgen-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 16 hour incubation of cells with test compounds a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for 2 hours. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation. Concentration-response relationships were determined by monitoring FRET signals relative to DMSO-only exposures which provided a signal baseline, and to a known androgen receptor antagonist (Cyproterone acetate) as a positive control which provided a reference for 100% androgen receptor inhibition, as assessed in the presence of 0.01 µM R1881, a known AR agonist.

Scientific Principles:

Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous androgenic signaling by monitoring the decrease in luminescent signals relative to a known androgen receptor antagonist (Cyproterone acetate), an indicator of androgenic interference.

The Tox21 AR bla assays are qHTS format assays which measured the ability of a chemical to interact with AR by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses rat AR and a one-hybrid GAL4 system to quantify xenobiotic androgen receptor antagonism**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a

putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.188
Neutral control median absolute deviation, by plate:	6.931
Positive control well median response value, by plate:	-100.309
Positive control well median absolute deviation, by plate:	3.043
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.71
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-13
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-14.97
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	510.39
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-36.98

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor antagonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

Beta lactamase expression in the AR_BLA_Antagonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized to positive controls (Cyproterone acetate; 100% inhibition) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of cyproterone acetate (a known AR inhibitor) activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both

increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

Tox21_AR_LUC_MDAKB2_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	4.349
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	26.092

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Tox21_AR_LUC_MDAKB2_Agonist

Assay Name: Tox21 Luciferase MDA-KB2 Androgen Receptor Agonist Transactivation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

Androgen receptor (AR), a nuclear hormone receptor, plays critical roles in the development and differentiation of male embryos, in the initiation and maintenance of spermatogenesis and in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that activate AR signaling, MDA-kb2 AR-luc cell line (ATCC; [1]) was used to screen the Tox21 10K compound library by monitoring the increase in luminescent signals produced following androgen receptor ligand-binding and luciferase reporter gene transcription. MDA-kb2 AR-luc cell line is human breast carcinoma cell line that was stably transfected with a luciferase reporter gene under control of the MMTV promoter containing response elements for both androgen receptor (AR) and glucocorticoid receptor (GR).

1.2. Assay Definition

Assay Throughput:

Stably transfected MDA-kb2 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring luminescence resulting from AR gene expression.

Experimental System:

The MDA-kb2 AR-luc cell line was derived from epithelial breast cancer cell line, MDA-MB-453 (originally obtained in 1976 from pleural effusion of metastatic carcinoma from 48-yo Caucasian female) by stable transfection with a mouse mammary tumor virus (MMTV) neomycin-resistant luciferase reporter gene construct. MDA-MB-453 cells have fibroblastic morphology and were selected for transformation due to high levels of functional, endogenous androgen and glucocorticoid receptors [while estrogen receptor (ER) α and progesterone receptor (PR) mRNA are not detectable, and ERβ is apparently expressed at very low levels]. This cell line expresses firefly luciferase under control of a MMTV promoter that contains response elements for both GR and AR. MDA-kb2 may be used in an in vitro assay to screen androgen agonist and antagonists and to characterize its specificity and sensitivity to endocrine disrupting chemicals [1].

Xenobiotic Biotransformation Potential:

Metabolic activity has been examined for the parental MDA-MB-453 cells, and CYP1A1 and CYP1B1 have been shown to be inducible following TCDD exposures, with cells shown to respond to exposure to AhR agonists with highly preferential induction of CYP1B1 as opposed to CYP1A1 [2-5].

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Leibovitz's L-15 Medium	ATCC	ATCC / 30-2008
Fetal Bovine Serum	Hyclone	Hyclone / SH30071.03
Penicillin/Streptomycin	Invitrogen	Invitrogen / 15140

Recovery Cell culture Freezing Medium	Invitrogen	Invitrogen / 12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen / 25300
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo(TM) Luciferase Assay System	Promega	Promega / E6120
R1881 or Methyltrienolone (Agonist control compound)	Perkin Elmer	Perkin Elmer/ NLP005005MG

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Leibovitz's L-15 Medium	100%	100%	100%	-
Fetal Bovine Serum	10%	10%	10%	-
Penicillin & Streptomycin	100U/ml & 100ug/ml	100U/ml & 100ug/ml	100U/ml & 100ug/ml	-
Recovery Cell culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 -Thaw a vial of cells in 9ml of pre-warmed thaw/culture medium and then centrifuge

1.2.2 -Resuspend the pellet with the thaw/culture medium and seed at 2 million cells per T-75 flask

1.3. Propagation method

1.3.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture medium

1.3.2 -Passage cells at 6-7 million per T-225 flask

2. Assay Protocol

2.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture/assay medium

2.2 -Dispense 3000 cells/5µL/well (for agonist mode) into 1536-well tissue treated white/solid bottom plates using a 8 tip dispenser (Multidrop)

2.3 -Incubate the plates for 5hrs at 37°C and 0% CO_2

2.4 -Transfer 23nL of compounds from the library collection (0.59nM to 92µM) and positive control

2.5 -Incubate the plates for 16hrs at 37°C and 0% CO_2

2.6 -Then add 5µl of ONE-Glo™ Luciferase reagent using a single tip dispenser (Bioraptr)

2.7 -Incubate the plates at room temperature for 30min

2.8 -Measure luminescence by ViewLux plate reader

MDA-kb2 AR-luc cells were dispensed at 3,000 cells/5µL/well of culture medium into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 °C and 0% CO_2 for 5h, 23nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37 °C and 0% CO_2 for 16h. Then 5µL of ONE-Glo reagent (Promega, Madison, WI) was added to each plate using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA) and luminescence was quantified on a ViewLux (PerkinElmer, Shelton, CT) plate reader after 30 min incubation at room temperature.

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [6].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 androgen receptor luciferase agonist assay screened a large library of diverse environmental compounds to probe for xenobiotic androgenic activity and potential to induce

androgen-dependent transcription, monitored through luciferase reporter gene signal activation using an AR-luciferase reporter gene construct. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 16 hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase ATP detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous androgenic signaling by monitoring the increase in luminescent signals relative to a known androgen receptor agonist (Methyltrienolone) as a positive control, and indicator of androgenic activity.

The Tox21 MDA-kb2 AR luciferase assays are qHTS format assays which measured the ability of a chemical to interact with AR by monitoring modulation of luminescent reporter gene signals. This assay utilized an epithelial breast cancer cell line which expresses firefly luciferase under control of a MMTV promoter that contains androgen response elements to quantify xenobiotic androgen receptor agonism**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Wilson, V. S., Bobseine, K., Lambright, C. R., & Gray, L. (2002). "A novel cell line, MDA-kb2, that stably expresses an androgen-and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists". Toxicol Sci 66(1), 69-81. (PMID: 11861974)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.111
Neutral control median absolute deviation, by plate:	3.344
Positive control well median response value, by plate:	99.51
Positive control well median absolute deviation, by plate:	7.91
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.66
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	29.73
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-893.63
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-29.54

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor agonism and gene expression, as measured by monitoring luminescent production by luciferase reporter gene under control of androgen response element promoters.

Analytical Elements:

The Tox21 MDA-KB2AR-luciferase agonist assay was monitored for increased luminescence (gain-of-signal) relative to methyltrienolone (positive control) signal, using DMSO (negative control) as a baseline for chemical-AR activity, and response was reported as a percent of positive control activity. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	1.010
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Wilson, V. S., et al. (2002). Toxicol Sci 66(1): 69-81. (PMID: 11861974)
[2] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[3] Spink, D. C., et al. (1998). Carcinogenesis 19(2): 291-298. (PMID: 9498279)
[4] Angus, W. G., et al. (1999). Carcinogenesis 20(6): 947-955. (PMID: 10357772)
[5] Spink, B., et al. (2002). Toxicol In Vitro 16(6): 695-704. (PMID: 12423652)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information

Tox21_AR_LUC_MDAKB2_Agonist_3uM_Nilutamide

Assay Name: Tox21 Luciferase MDA-KB2 Androgen Receptor Agonist Transactivation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

Androgen receptor (AR), a nuclear hormone receptor, plays critical roles in the development and differentiation of male embryos, in the initiation and maintenance of spermatogenesis and in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that activate AR signaling, MDA-kb2 AR-luc cell line (ATCC; [1]) was used to screen the Tox21 10K compound library by monitoring the increase in luminescent signals produced following androgen receptor ligand-binding and luciferase reporter gene transcription. MDA-kb2 AR-luc cell line is human breast carcinoma cell line that was stably transfected with a luciferase reporter gene under control of the MMTV promoter containing response elements for both androgen receptor (AR) and glucocorticoid receptor (GR).

1.2. Assay Definition

Assay Throughput:

Stably transfected MDA-kb2 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring luminescence resulting from AR gene expression.

Experimental System:

The MDA-kb2 AR-luc cell line was derived from epithelial breast cancer cell line, MDA-MB-453 (originally obtained in 1976 from pleural effusion of metastatic carcinoma from 48-yo Caucasian female) by stable transfection with a mouse mammary tumor virus (MMTV) neomycin-resistant luciferase reporter gene construct. MDA-MB-453 cells have fibroblastic morphology and were selected for transformation due to high levels of functional, endogenous androgen and glucocorticoid receptors [while estrogen receptor (ER) α and progesterone receptor (PR) mRNA are not detectable, and ERβ is apparently expressed at very low levels]. This cell line expresses firefly luciferase under control of a MMTV promoter that contains response elements for both GR and AR. MDA-kb2 may be used in an in vitro assay to screen androgen agonist and antagonists and to characterize its specificity and sensitivity to endocrine disrupting chemicals [1].

Xenobiotic Biotransformation Potential:

Metabolic activity has been examined for the parental MDA-MB-453 cells, and CYP1A1 and CYP1B1 have been shown to be inducible following TCDD exposures, with cells shown to respond to exposure to AhR agonists with highly preferential induction of CYP1B1 as opposed to CYP1A1 [2-5].

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Leibovitz's L-15 Medium	ATCC	ATCC / 30-2008

Fetal Bovine Serum	Hyclone	Hyclone / SH30071.03
Penicillin/Streptomycin	Invitrogen	Invitrogen / 15140
Recovery Cell culture Freezing Medium	Invitrogen	Invitrogen / 12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen / 25300
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo(TM) Luciferase Assay System	Promega	Promega / E6120
R1881 or Methyltrienolone (Agonist control compound)	Perkin Elmer	Perkin Elmer/ NLP005005MG
Nilutamide	Sigma-Aldrich	Sigma-Aldrich N8534

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Leibovitz's L-15 Medium	100%	100%	100%	-
Fetal Bovine Serum	10%	10%	10%	-
Penicillin & Streptomycin	100U/ml & 100ug/ml	100U/ml & 100ug/ml	100U/ml & 100ug/ml	-
Recovery Cell culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 -Thaw a vial of cells in 9ml of pre-warmed thaw/culture medium and then centrifuge

1.2.2 -Resuspend the pellet with the thaw/culture medium and seed at 2 million cells per T-75 flask

1.3. Propagation method

1.3.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture medium

1.3.2 -Passage cells at 6-7 million per T-225 flask

2. Assay Protocol

2.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture/assay medium

2.2 -Dispense 3000 cells/4µL/well (for agonist mode) into 1536-well tissue treated white/solid bottom plates using a 8 tip dispenser (Multidrop)

2.3 -Incubate the plates for 5hrs at 37°C and 0% CO_2

2.4 - Add 1 µL of 15.0 µM Nilutamide/assay medium (3 µM final concentration) was added using a dispenser.

2.4 -Transfer 23nL of compounds from the library collection (0.59nM to 92µM final assay concentrations) and positive control
2.5 -Incubate the plates for 16hrs at 37°C and 0% CO_2
2.6 -Then add 5µl of ONE-Glo™ Luciferase reagent using a single tip dispenser (Bioraptr)
2.7 -Incubate the plates at room temperature for 30min
2.8 -Measure luminescence by ViewLux plate reader

MDA-kb2 AR-luc cells were dispensed at 3,000 cells/4µL/well of culture medium into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 °C and 0% CO_2 for 5h, 1 µL of 15.0 µM Nilutamide/assay medium was added using a dispenser followed by the compound transfer. 23nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37 °C and 0% CO_2 for 16h. Then 5µL of ONE-Glo reagent (Promega, Madison, WI) was added to each plate using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA) and luminescence was quantified on a ViewLux (PerkinElmer, Shelton, CT) plate reader after 30 min incubation at room temperature.

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [6].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated gene expression and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity

prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 androgen receptor luciferase agonist assay screened a large library of diverse environmental compounds to probe for xenobiotic androgenic activity and potential to induce androgen-dependent transcription, monitored through luciferase reporter gene signal activation using an AR-luciferase reporter gene construct. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 16 hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase ATP detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous androgenic signaling by monitoring the increase in luminescent signals relative to a known androgen receptor agonist (Methyltrienolone) as a positive control, and indicator of androgenic activity.

The Tox21 MDA-kb2 AR luciferase assays are qHTS format assays which measured the ability of a chemical to interact with AR by monitoring modulation of luminescent reporter gene signals. This assay utilized an epithelial breast cancer cell line which expresses firefly luciferase under control of a MMTV promoter that contains androgen response elements to quantify xenobiotic androgen receptor agonism**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the

MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Wilson, V. S., Bobseine, K., Lambright, C. R., & Gray, L. (2002). "A novel cell line, MDA-kb2, that stably expresses an androgen-and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists". Toxicol Sci 66(1), 69-81. (PMID: 11861974)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0
Neutral control median absolute deviation, by plate:	1.191
Positive control well median response value, by plate:	100
Positive control well median absolute deviation, by plate:	7.51
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.74
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	13
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	83.93
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	Inf
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	Inf

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor agonism and gene expression, as measured by monitoring luminescent production by luciferase reporter gene under control of androgen response element promoters.

Analytical Elements:

This assay is a counter-screen that is run in agonist mode but with the inclusion of an AR antagonist, nilutamide, to block agonist activity. Results should be compared to TOX21_AR_LUC_MDAKB2_Agonist (AEID 764) which was run in the absence of nilutamide. Chemicals with activity in AEID 764 but not in AEID 1822 may be interpreted as competitive AR agonists. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of

the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

TOX21_AR_LUC_MDAKB2_Agonist
ATG_AR_TRANS_up
NVS_NR_cAR
NVS_NR_hAR
NVS_NR_rAR
OT_AR_ARELUC_AG_1440
OT_AR_ARSRC1_0480
OT_AR_ARSRC1_0960
Tox21_AR_BLA_Agonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.768
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Wilson, V. S., et al. (2002). Toxicol Sci 66(1): 69-81. (PMID: 11861974)
[2] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[3] Spink, D. C., et al. (1998). Carcinogenesis 19(2): 291-298. (PMID: 9498279)
[4] Angus, W. G., et al. (1999). Carcinogenesis 20(6): 947-955. (PMID: 10357772)
[5] Spink, B., et al. (2002). Toxicol In Vitro 16(6): 695-704. (PMID: 12423652)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information

Tox21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881

Assay Name: Tox21 Luciferase MDA-KB2 Androgen Receptor Antagonist Transactivation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

Androgen receptor (AR), a nuclear hormone receptor, plays critical roles in the development and differentiation of male embryos, in the initiation and maintenance of spermatogenesis and in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that inhibit AR signaling, MDA-kb2 AR-luc cell line (ATCC; (Wilson et al. 2002)) was used to screen the Tox21 compound library using luciferase reporter gene construct downstream of endogenous androgen receptor and exposing the cells to test compounds and a small amount of AR agonist. The cytotoxicity of the Tox21 compounds against the MDA-kb2 AR-luc cell line was tested in parallel by measuring the cell viability using CellTiter-Fluor assay (see Assay Definition) in the same wells. The antagonist assay was also run with a higher concentration of agonist (AEID765) as a specificity control in which competitive antagonists should show a right-shift in potency relative to the assay with lower agonist concentration.

1.2. Assay Definition

Assay Throughput:

Stably transfected MDA-kb2 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring luminescence resulting from AR gene expression.

Experimental System:

The MDA-kb2 AR-luc cell line was derived from epithelial breast cancer cell line, MDA-MB-453 (originally obtained in 1976 from pleural effusion of metastatic carcinoma from 48-yo Caucasian female) by stable transfection with a mouse mammary tumor virus (MMTV) neomycin-resistant luciferase reporter gene construct. MDA-MB-453 cells have fibroblastic morphology and were selected for transformation due to high levels of functional, endogenous androgen and glucocorticoid receptors (while estrogen receptor (ER) α and progesterone receptor (PR) mRNA are not detectable, and ERβ is apparently expressed at very low levels). This cell line expresses firefly luciferase under control of a MMTV promoter that contains response elements for both GR and AR. MDA-kb2 may be used in an in vitro assay to screen androgen agonist and antagonists and to characterize its specificity and sensitivity to endocrine disrupting chemicals (Wilson et al. 2002).

Xenobiotic Biotransformation Potential:

Metabolic activity has been examined for the parental MDA-MB-453 cells, and CYP1A1 and CYP1B1 have been shown to be inducible following TCDD exposures, with cells shown to respond to exposure to AhR agonists with highly preferential induction of CYP1B1 as opposed to CYP1A1 (Angus et al. 1999, Dohr et al. 1995, Spink BC et al. 2002, Spink David C et al. 1998).

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Leibovitz's L-15 Medium	ATCC	ATCC / 30-2008
Fetal Bovine Serum	Hyclone	Hyclone / SH30071.03
Penicillin/Streptomycin	Invitrogen	Invitrogen / 15140
Recovery Cell culture Freezing Medium	Invitrogen	Invitrogen / 12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen / 25300
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo(TM) Luciferase Assay System	Promega	Promega / E6120
CellTiter-Fluor (TM) Cell Viability Assay	Promega	Promega / G6082

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Leibovitz's L-15 Medium	100%	100%	100%	-
Fetal Bovine Serum	10%	10%	10%	-
Penicillin & Streptomycin	100U/ml & 100ug/ml	100U/ml & 100ug/ml	100U/ml & 100ug/ml	-
Recovery Cell culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 -Thaw a vial of cells in 9ml of pre-warmed thaw/culture medium and then centrifuge

1.2.2 -Resuspend the pellet with the thaw/culture medium and seed at 2 million cells per T-75 flask

1.3. Propagation method

1.3.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture medium

1.3.2 -Passage cells at 6-7 million per T-225 flask

2. Assay Protocol

2.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture/assay medium

2.2 -Dispense 3000 cells/4 µL/well into 1536-well tissue treated white/solid bottom plates using an 8 tip dispenser (Multidrop)

2.3 - Incubate the plates for 5 hrs at 37C and 0% CO_2

2.4 - Transfer 23nL of compounds from the library collection (0.59nM to 92µM) and positive control
2.5 - Add 1 µL/well of 2.55 nM R1881agonist (final concentration of 0.5 nM,) in culture medium using Bioraptr Flying Reagent Dispenser workstation (Beckman Coulter, Indianapolis, IN, USA).
2.6 -Incubate the plates for 15.5hr at 37°C and 0% CO_2
2.7 -Add 1µl of CellTiter-Fluor™ Cell Viability Assay reagent using a single tip dispenser (Bioraptr)
2.8 -Incubate the plates at room temperature or 37°C for 30min
2.9 -Measure fluorescence by ViewLux plate reader
2.10 -Then add 4µl of ONE-Glo™ Luciferase reagent using a single tip dispenser (Bioraptr)
2.11 -Incubate the plates at room temperature for 30min
2.12 -Measure luminescence by ViewLux plate reader

MDA-kb2 cells were dispensed at 3,000 cells/4 µL/well of culture medium into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37°C and 0% CO_2 for 5 hr, 23nL of compounds dissolved in DMSO, positive controls or DMSO-only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition 1 µL of agonist (2.5 nM R1881) in culture medium using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The plates were incubated at a 37°C and 0% CO_2 for 16 h. After 15.5 h incubation, 1µL of CellTiter-Fluor reagent (Promega, Madison, WI) was added using a Bioraptr FRD to each plate for measuring cytotoxicity. The fluorescence intensity was quantified on a ViewLux plate reader (PerkinElmer, Shelton, CT) following 30 min incubation at 37°C and 0% CO_2. For measuring luciferase reporter gene activity, 4 µL of ONE-Glo reagent (Promega) was added to each plate using a Bioraptr FRD and luminescence was quantified on a ViewLux (PerkinElmer) plate reader after 30 min incubation at room temperature.

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) (Michael et al. 2008).

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 androgen receptor luciferase antagonist assay screened a large library of diverse environmental compounds to probe for xenobiotic androgenic activity and potential to inhibit androgen-dependent transcription, monitored through luciferase reporter gene signal activity using an AR-luciferase reporter gene construct. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 15.5 hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in antagonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. To help distinguish true antagonistic activity from cytotoxic effects, this assay was multiplexed with a fluorescence-based cell viability assay which measured conserved and constitutive protease activity within live cells (Promega). Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation. Concentration-response relationships were determined by monitoring luminescent signals relative to DMSO-only exposures which provided a signal baseline, and to a known androgen receptor antagonist (Nilutamide) as a positive control which provided a reference for 100% androgen receptor inhibition, as assessed in the presence of 0.5 nM R1881, a known AR agonist.

Scientific Principles:

Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds

capable of interference with endogenous androgenic signaling by monitoring the relative decrease in production of luminescent signals using a known androgen receptor antagonist (nilutamide) as a positive control, and indicator of AR inhibition.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Wilson, V. S., Bobseine, K., Lambright, C. R., & Gray, L. (2002). "A novel cell line, MDA-kb2, that stably expresses an androgen-and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists". Toxicol Sci 66(1), 69-81. (PMID: 11861974)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0
Neutral control median absolute deviation, by plate:	8.68
Positive control well median response value, by plate:	-100
Positive control well median absolute deviation, by plate:	1.896
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.68
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-11.52
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-Inf
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	Inf

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor antagonism and inhibited gene expression, as measured by monitoring production of luminescence resulting from luciferase reporter gene under the regulation androgen response element promoters. This assay was also run with 10 nM R1881 (AEID 765) which can be used to distinguish competitive antagonists from non-competitive or cytotoxic chemicals.

Analytical Elements:

The Tox21 MDA-KB2AR-luciferase agonist assay was monitored for decreased activity (loss-of-signal) relative to DMSO (negative control) and nilutamide signal (positive control, 100% antagonist activity) measured in the presence of small amounts (0.5 nM) of a known agonist (R1881). Relationships are modeled. Concentration-response relationships were determined based on a

range of 15 chemical concentrations using DMSO as a signal baseline, and response was reported as a percent of positive control activity (100% inhibition). All statistical analyses were conducted using R programming language, employing tcpl package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

Tox21_AR_BLA_Antagonist_ratio

Tox21_AR_LUC_MDAKB2_Antagonist_10nM_R1881

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	5.35
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	32.13

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and

ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Wilson, V. S., et al. (2002). Toxicol Sci 66(1): 69-81. (PMID: 11861974)
[2] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[3] Spink, D. C., et al. (1998). Carcinogenesis 19(2): 291-298. (PMID: 9498279)
[4] Angus, W. G., et al. (1999). Carcinogenesis 20(6): 947-955. (PMID: 10357772)
[5] Spink, B., et al. (2002). Toxicol In Vitro 16(6): 695-704. (PMID: 12423652)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

Tox21_AR_LUC_MDAKB2_Antagonist_10nM_R1881

Assay Name: Tox21 Luciferase MDA-KB2 Androgen Receptor Antagonist Transactivation Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

Androgen receptor (AR), a nuclear hormone receptor, plays critical roles in the development and differentiation of male embryos, in the initiation and maintenance of spermatogenesis and in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that inhibit AR signaling, MDA-kb2 AR-luc cell line (ATCC; (Wilson et al. 2002)) was used to screen the Tox21 compound library using luciferase reporter gene construct downstream of endogenous androgen receptor and exposing the cells to test compounds and a small amount of AR agonist. The cytotoxicity of the Tox21 compounds against the MDA-kb2 AR-luc cell line was tested in parallel by measuring the cell viability using CellTiter-Fluor assay (see Assay Definition) in the same wells. This assay serves as a specificity control for AEID 1816 through use of a higher agonist concentration which should result in a right shift in potency for competitive AR antagonists relative to the lower agonist concentration used in AEID 1816.

1.2. Assay Definition

Assay Throughput:

Stably transfected MDA-kb2 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring luminescence resulting from AR gene expression.

Experimental System:

The MDA-kb2 AR-luc cell line was derived from epithelial breast cancer cell line, MDA-MB-453 (originally obtained in 1976 from pleural effusion of metastatic carcinoma from 48-yo Caucasian female) by stable transfection with a mouse mammary tumor virus (MMTV) neomycin-resistant luciferase reporter gene construct. MDA-MB-453 cells have fibroblastic morphology and were selected for transformation due to high levels of functional, endogenous androgen and glucocorticoid receptors (while estrogen receptor (ER) α and progesterone receptor (PR) mRNA are not detectable, and ERβ is apparently expressed at very low levels). This cell line expresses firefly luciferase under control of a MMTV promoter that contains response elements for both GR and AR. MDA-kb2 may be used in an in vitro assay to screen androgen agonist and antagonists and to characterize its specificity and sensitivity to endocrine disrupting chemicals (Wilson et al. 2002).

Xenobiotic Biotransformation Potential:

Metabolic activity has been examined for the parental MDA-MB-453 cells, and CYP1A1 and CYP1B1 have been shown to be inducible following TCDD exposures, with cells shown to respond to exposure to AhR agonists with highly preferential induction of CYP1B1 as opposed to CYP1A1 (Angus et al. 1999, Dohr et al. 1995, Spink BC et al. 2002, Spink David C et al. 1998).

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Leibovitz's L-15 Medium	ATCC	ATCC / 30-2008
Fetal Bovine Serum	Hyclone	Hyclone / SH30071.03
Penicillin/Streptomycin	Invitrogen	Invitrogen / 15140
Recovery Cell culture Freezing Medium	Invitrogen	Invitrogen / 12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen / 25300
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo(TM) Luciferase Assay System	Promega	Promega / E6120
CellTiter-Fluor (TM) Cell Viability Assay	Promega	Promega / G6082

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Leibovitz's L-15 Medium	100%	100%	100%	-
Fetal Bovine Serum	10%	10%	10%	-
Penicillin & Streptomycin	100U/ml & 100ug/ml	100U/ml & 100ug/ml	100U/ml & 100ug/ml	-
Recovery Cell culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 -Thaw a vial of cells in 9ml of pre-warmed thaw/culture medium and then centrifuge

1.2.2 -Resuspend the pellet with the thaw/culture medium and seed at 2 million cells per T-75 flask

1.3. Propagation method

1.3.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture medium

1.3.2 -Passage cells at 6-7 million per T-225 flask

2. Assay Protocol

2.1 -Trypsinize cells from the culturing flask and centrifuge and then resuspend cells in culture/assay medium

2.2 -Dispense 3000 cells/4 µL/well into 1536-well tissue treated white/solid bottom plates using an 8 tip dispenser (Multidrop)

2.3 - Incubate the plates for 5 hrs at 37C and 0% CO_2

2.4 - Transfer 23nL of compounds from the library collection (0.59nM to 92µM) and positive control
2.5 - Add 1 µL/well of 50 nM R1881 agonist (final concentration 10 nM) in culture medium using Bioraptr Flying Reagent Dispenser workstation (Beckman Coulter, Indianapolis, IN, USA).
2.6 -Incubate the plates for 15.5hr at 37°C and 0% CO_2
2.7 -Add 1µl of CellTiter-Fluor™ Cell Viability Assay reagent using a single tip dispenser (Bioraptr)
2.8 -Incubate the plates at room temperature or 37°C for 30min
2.9 -Measure fluorescence by ViewLux plate reader
2.10 -Then add 4µl of ONE-Glo™ Luciferase reagent using a single tip dispenser (Bioraptr)
2.11 -Incubate the plates at room temperature for 30min
2.12 -Measure luminescence by ViewLux plate reader

MDA-kb2 cells were dispensed at 3,000 cells/4 µL/well of culture medium into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37°C and 0% CO_2 for 5 hr, 23nL of compounds dissolved in DMSO, positive controls or DMSO-only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1 µL of agonist (50 nM R1881) in culture medium using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The plates were incubated at a 37°C and 0% CO_2 for 16 h. After 15.5 h incubation, 1µL of CellTiter-Fluor reagent (Promega, Madison, WI) was added using a Bioraptr FRD to each plate for measuring cytotoxicity. The fluorescence intensity was quantified on a ViewLux plate reader (PerkinElmer, Shelton, CT) following 30 min incubation at 37°C and 0% CO_2. For measuring luciferase reporter gene activity, 4 µL of ONE-Glo reagent (Promega) was added to each plate using a Bioraptr FRD and luminescence was quantified on a ViewLux (PerkinElmer) plate reader after 30 min incubation at room temperature.

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) (Michael et al. 2008).

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote androgen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 androgen receptor luciferase antagonist assay screened a large library of diverse environmental compounds to probe for xenobiotic androgenic activity and potential to inhibit androgen-dependent transcription, monitored through luciferase reporter gene signal activity using an AR-luciferase reporter gene construct. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 15.5 hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in antagonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. To help distinguish true antagonistic activity from cytotoxic effects, this assay was multiplexed with a fluorescence-based cell viability assay which measured conserved and constitutive protease activity within live cells (Promega). Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation. Concentration-response relationships were determined by monitoring luminescent signals relative to DMSO-only exposures which provided a signal baseline, and to a known androgen receptor antagonist (Nilutamide) as a positive control which provided a reference for 100% androgen receptor inhibition, as assessed in the presence of 0.01 µM R1881, a known AR agonist. The assay serves as a specificity control for AEID 1816 as competitive antagonists for the androgen receptor should show a right shift in potency in this assay compared to AEID 1816 due to the use of higher concentration of agonist stimulation.

Scientific Principles:

Androgen receptors have pleiotropic regulatory roles in a diverse range of tissues; particularly in mediating the activity of endogenous androgens in the hypothalamus, pituitary, liver, prostate, and testicular tissues. Endogenous androgens are important for target gene expression in physiological processes like developmental differentiation of the male embryo, initiation and maintenance of spermatogenesis and in neuroendocrine system functioning. Endogenous androgens also influence male pubertal maturation and maintenance of secondary sexual characteristics in adults. Disruption of androgen signaling following exposure to endocrine-mimicking environmental

chemicals can result in hormonal cancers, impaired reproductive development and infertility. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous androgenic signaling by monitoring the relative decrease in production of luminescent signals using a known androgen receptor antagonist (nilutamide) as a positive control, and indicator of AR inhibition.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with androgen receptor mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that androgen receptor agonism is a molecular initiating event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in fish populations (EAGMST Approved AOP), and there is some evidence that androgen receptor activation is the MIE for a putative pathway leading to hepatocellular adenomas and carcinomas (in mouse and rat models) (AOP currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive *in vivo* studies to further investigate the involvement of AR agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Wilson, V. S., Bobseine, K., Lambright, C. R., & Gray, L. (2002). "A novel cell line, MDA-kb2, that stably expresses an androgen-and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists". Toxicol Sci 66(1), 69-81. (PMID: 11861974)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.247
Neutral control median absolute deviation, by plate:	8.706
Positive control well median response value, by plate:	-100
Positive control well median absolute deviation, by plate:	2.403
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.66
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-11.43
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	403.07
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-38.35

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Androgen receptor antagonism and inhibited gene expression, as measured by monitoring production of luminescence resulting from luciferase reporter gene under the regulation androgen response element promoters. Use of the 10 nM R1881 should result in a right-shifting of AC50s for competitive AR antagonists relative to the results of the TOX21_AR_LUC_MDAKB2_Antagonist2 assay, which used 0.5 nM R1881. This is useful for distinguishing competitive antagonists from non-competitive or cytotoxic compounds.

Analytical Elements:

The Tox21 MDA-KB2AR-luciferase antagonist assay was monitored for decreased activity (loss-of-signal) relative to DMSO (negative control) and nilutamide signal (positive control, 100% antagonist activity) measured in the presence of a known agonist (R1881, 10 nM). This assay was run as a counter-screen to TOX21_AR_LUC_MDAKB2_Antagonist_0.5 nM_R1881 (AEID 1816) Results should be compared and competitive antagonists should show a right-shift in potency in AEID 765 relative to AEID 1816. Relationships are modeled. Concentration-response relationships were determined based on a range of 15 chemical concentrations using DMSO (negative control) as a signal baseline, and response was reported as a percent of positive control activity (100% inhibition). All statistical analyses were conducted using R programming language, employing tcpl package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Androgen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

TOX21_AR_BLA_Antagonist_ratio

TOX21_AR_LUC_MDAKB2_Antagonist_0.5nM_R1881

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	4.981
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical

constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Wilson, V. S., et al. (2002). Toxicol Sci 66(1): 69-81. (PMID: 11861974)
[2] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[3] Spink, D. C., et al. (1998). Carcinogenesis 19(2): 291-298. (PMID: 9498279)
[4] Angus, W. G., et al. (1999). Carcinogenesis 20(6): 947-955. (PMID: 10357772)
[5] Spink, B., et al. (2002). Toxicol In Vitro 16(6): 695-704. (PMID: 12423652)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
AR, Androgen Receptor
ATCC, American Tissue Culture Collection
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 July 2016

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

Tox21_ERa_BLA_Agonist_ratio

Assay Name: Tox21 Beta-Lactamase-HEK293T Cell-Based qHTS Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

1. Assay Descriptions

1.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify the compounds that inhibit ER signaling, an ER-alpha-UAS-bla GripTite™ cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS) responsive to a GAL4-ER fusion protein stably integrated into HEK293 cells was used to screen the Tox21 chemical library. This experimental system constitutively co-expresses the fusion protein, thel human estrogen receptor alpha (ERα) ligand-binding domain coupled to GAL4 DNA-binding domain which, when activated by xenoestrogenic compounds, stimulates β-lactamase reporter gene expression.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates, incubated with test compounds for 16 hours, and reporter gene substrate added prior to monitoring fluorescence emission resulting from substrate cleavage by ERα-stimulated reporter gene expression.

Experimental System:

GeneBLAzer® ER alpha-UAS-bla GripTite™ cells contain the ligand-binding domain of the human estrogen receptor alpha (ERα) fused to the DNA-binding domain of GAL4 stably integrated into the cell line, commonly called a mammalian one-hybrid assay. These cells stably express a β-lactamase reporter gene (*bla*) under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4-ERα fusion protein, the protein binds to the UAS, resulting in expression of β-lactamase. The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Phenol red-free DMEM	Invitrogen	Invitrogen/21063
DMEM	Invitrogen	Invitrogen/11965

Dialyzed FBS	Invitrogen	Invitrogen/26400
Charcoal stripped FBS	Invitrogen	Invitrogen/12676
NEAA	Invitrogen	Invitrogen/11140
Sodium pyruvate	Invitrogen	Invitrogen/11360
Penicillin and Streptomycin	Invitrogen	Invitrogen/15140
Hygromycin B	Invitrogen	Invitrogen/10687
Zeocin	Invitrogen	Invitrogen/R25001
Black-clear bottom 1536 well plates	Greiner	Greiner/789092F
LiveBLAzer B/G FRET substrate	Invitrogen	Invitrogen/K1028
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen/12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen/25300
Envision Plate Reader	Perkin Elmer	Perkin Elmer
BioRAPTR FRD dispenser	Beckman Coulter	Beckman Coulter
Multidrop COMBI	Thermo Electron Corporation	Thermo Electron Corporation
Beta-Estradiol (Agonist control compound)	Sigma	Sigma/E8875

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Phenol red-free DMEM	-	98%	-	-
DMEM	90%	-	90%	-
Dialyzed FBS	10%	-	10%	-
Charcoal stripped FBS	-	2%	-	-
NEAA	0.1mM	0.1mM	0.1mM	-
Sodium pyruvate	1mM	1mM	1mM	-
Penicillin and Streptomycin	100U/ml-100µg/ml	100U/ml-100µg/ml	100U/ml-100µg/ml	-
Hygromycin B	80µg/ml	-	-	-
Zeocin	100µg/ml	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 1ml frozen cells of ERalpha-bla were taken in pre-warmed 10ml of thaw medium for centrifuging.

1.2.2 2-3ml of the thaw medium is taken to resuspend the pellet
1.2.3 The cells were seeded in T-75 flask at 2 million cells
1.3. Propagation method
1.3.1 The cells are detached using 0.05% Trypsin
1.3.2 Cells are further passaged at a density of 4-5 million cells per T-225 flask

2. Assay Protocol
2.1 Rinse the cells twice with DPBS and detach them using 0.05% Trypsin and centrifuge
2.2 Resuspend the pellet with assay buffer
2.3 Plate the cells in black-clear bottom 1536 well plate at 5000/well/5µL through 8 tip plate dispenser (Multi drop)
2.4 Incubate at 37°C for 5hrs
2.5 Transfer 23nL of the compounds from the library collection and positive control through Pintool
2.6 Incubate at 37°C for 18hrs
2.7 Add 1µL of CCF4 dye using a single tip of a plate dispenser (Bioraptr)
2.8 Incubate at room temperature for 2hrs
2.9 Read the fluorescence intensity through Envision plate reader

ER-alpha-bla cells were cultured in assay medium containing 2% charcoal stripped FBS overnight in the culture flasks before the assay was performed. The cells were dispensed at 5,000 cells/6 uL of assay medium per well into black wall/clear-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37 °C and 5% CO2 for 4 hr, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37C and 5% CO2 for 18 hr. Then 1 uL of LiveBLAzer™ B/G FRET substrate was added using a Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). After the plates were incubated at room temperature for 2 hours, fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, etc.) and signal recording (plate readout) [3]. GeneBLAzer® System is commercially available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]

U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor-□ □-lactamase agonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to induce estrogen-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. Following 18 hour incubation of cells with test compounds, a cell-permeable, FRET-based substrate, CCF4-AM, is introduced and incubated for 2 hours. Once in the cell, cytoplasmic esterases hydrolyze and trap the negatively charged CCF4 substrate in the cytosol where it can be cleaved by the *bla* reporter gene product. Activity is quantified by measuring the ratio of blue (product) to green (substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader and CellTiter-Glo assay reagent (Promega) is also incubated with test system for 30 minutes before readout to detect cell viability.

The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-

fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, GeneBLAzer® ERα-UAS-bla GripTite™ cell line (Invitrogen) has been used to screen the Tox21 library of diverse environmental compounds. ERα-UAS-bla cell line expresses a partial ERα one-hybrid GAL4 system and is stably transfected with a β-lactamase reporter gene.

The Tox21 ERα bla assays are qHTS format assays which measured the ability of a chemical to interact with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses a partial ERα and a one-hybrid GAL4 system to quantify xenoestrogenic agonism**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci U S A 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.04
Neutral control median absolute deviation, by plate:	1.12
Positive control well median response value, by plate:	99.18
Positive control well median absolute deviation, by plate:	20.81
Z' (median across all plates, using positive control wells):	0.33
SSMD (median across all plates, using positive control wells):	5
Signal-to-noise (median across all plates, using positive control wells):	87.86
Signal-to-background (median across all plates, using positive control wells):	-2485.43
CV (median across all plates):	-27.68

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα agonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the ERa_BLA_Agonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (17 beta-estradiol; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of 17 beta-estradiol activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Antagonist_ratio

Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 15
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.001 µM
Standard maximum concentration tested: 90 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.73
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	No
80-05-7	Bisphenol A	Weak	Active	Yes

1478-61-1	Bisphenol AF	NA	Active	No
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	No
84-61-7	Dicyclohexyl phthalate	NA	Inactive	No
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	No
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	Yes
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	No
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	No
789-02-6	o,p'-DDT	Weak	Active	No
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	16	13
Inactive	4	4

In Vivo Activity	ToxCast Active	ToxCast Inactive
Active	17	13
Inactive	6	5

In Vitro Sensitivity = 55.2%
In Vitro Specificity = 50.0%
Balanced Accuracy = 52.6%

In Vivo Sensitivity = 56.7%
In Vivo Specificity = 45.5%
Balanced Accuracy = 51.1%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors
qHTS, quantitative high-throughput screening
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

7 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:
Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Tox21_ERa_BLA_Antagonist_ratio

Assay Name: Tox21 Beta-Lactamase-HEK293T Cell-Based qHTS Assay to Identify Small Molecule Antagonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

1. Assay Descriptions

1.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify the compounds that inhibit ER signaling, an ER-alpha-UAS-bla GripTite™ cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence stably integrated into HEK293 cells was used to screen the Tox21 chemical library. This experimental system constitutively co-expresses a fusion protein of a partial human estrogen receptor alpha (ERα) ligand-binding domain coupled to GAL4 DNA-binding domain which when inhibited by xenoestrogenic compounds suppresses β-lactamase reporter gene expression. To detect loss of signal due to compound cytotoxicity, a CellTiter-Glo fluorescence assay to quantify cellular ATP content was run concurrently in all wells using tetraoctylammonium bromide as a positive control for cell death.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 16 hours prior to monitoring fluorescence emission resulting from xenobiotic ERα gene expression.

Experimental System:

GeneBLAzer® ER alpha-UAS-bla GripTite™ cells contain the ligand-binding domain of the human estrogen receptor alpha (ERα) fused to the DNA-binding domain of GAL4 stably integrated into the cell line. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). This assay is performed with small amounts of a known ERα agonist (E2) added to each well, and when an antagonistic compound binds to the LBD of the GAL4-ERα fusion protein binding to the UAS is inhibited, interfering with expression of β-lactamase. The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 (Graham et al. 1977). The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid (Bylund et al. 2004). HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
Phenol red-free DMEM	Invitrogen	Invitrogen/21063
DMEM	Invitrogen	Invitrogen/11965
Dialyzed FBS	Invitrogen	Invitrogen/26400
Charcoal stripped FBS	Invitrogen	Invitrogen/12676
NEAA	Invitrogen	Invitrogen/11140
Sodium pyruvate	Invitrogen	Invitrogen/11360
Penicillin and Streptomycin	Invitrogen	Invitrogen/15140
Hygromycin B	Invitrogen	Invitrogen/10687
Zeocin	Invitrogen	Invitrogen/R25001
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen/12648
0.05% Trypsin-EDTA	Invitrogen	Invitrogen/25300
LiveBLAzer B/G FRET substrate	Invitrogen	Invitrogen/K1028
CellTiter-Glo Assay Custom Solution	Promega	Promega / X2371
Black-clear bottom 1536 well plates	Greiner	Greiner/789092F
BioRAPTR FRD dispenser	Beckman Coulter	Beckman Coulter
Multidrop COMBI	Thermo Electron Corporation	Thermo Electron Corporation
Envision Plate Reader	Perkin Elmer	Perkin Elmer
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
4-Hydroxytamoxifen (Antagonist control compound)	Sigma	Sigma/H7904

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Phenol red-free DMEM	-	98%	-	-
DMEM	90%	-	90%	-
Dialyzed FBS	10%	-	10%	-
Charcoal stripped FBS	-	2%	-	-
NEAA	0.1mM	0.1mM	0.1mM	-
Sodium pyruvate	1mM	1mM	1mM	-
Penn-strep	100U/ml-100µg/ml	100U/ml-100µg/ml	100U/ml-100µg/ml	-

Hygromycin B	80µg/ml	-	-	-
Zeocin	100µg/ml	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 1ml frozen cells of ERalpha-bla were taken in pre-warmed 10ml of thaw medium for centrifuging.

1.2.2 2-3ml of the thaw medium is taken to resuspend the pellet

1.2.3 The cells were seeded in T-75 flask at 2 million cells

1.3. Propagation method

1.3.1 The cells are detached using 0.05% Trypsin

1.3.2 Cells are further passaged at a density of 4-5 million cells per T-225 flask

2. Assay Protocol

2.1 Rinse the cells twice with DPBS and detach them using 0.05% Trypsin and centrifuge

2.2 Resuspend the pellet with assay buffer

2.3 Plate the cells in black-clear bottom 1536 well plate at 5000/well/5µL through 8 tip plate dispenser (Multi drop)

2.4 Incubate at 37°C for 5hrs

2.5 Transfer 23nL of the compounds from the library collection and positive control through Pintool

2.6 Add 1µL of assay buffer with or without 0.5nM (final) Beta-estradiol

2.7 Incubate at 37°C for 18hrs

2.8 Add 1µL of CCF4 dye using a single tip of a plate dispenser (Bioraptr)

2.9 Incubate at room temperature for 2hrs

2.10 Read the fluorescence intensity through Envision plate reader

2.11 Add 4µL of CellTiter-Glo reagent using a single tip of a plate dispenser (Bioraptr)

2.12 Incubate at room temperature for 30min

2.13 Read the luminescence through ViewLux plate reader

The ER-alpha-bla cells were dispensed at 5,000 cells/5 µL of assay medium per well into black wall/clear-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37°C and 5% CO2 for 4 hr, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1 µL of 17β-estradiol in assay medium using a Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). The plates were incubated at 37°C and 5% CO2 for 18 hr. Then 1 µL of LiveBLAzerTM B/G FRET substrate was added using a Flying Reagent Dispenser, the plates were incubated at room temperature for 2 hours, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 3 µL/well of CellTiter-Glo reagent was added into the assay plates using a FRD. After 30 min incubation at room temperature, the luminescence intensity in the plates was measured using a ViewLux (PerkinElmer) plate reader.

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration,

plate centrifugation and incubation, et cetera) and signal recording (plate readout) (Michael et al. 2008). GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor alpha beta lactamase antagonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to suppress estrogen-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. Each well contained 0.5nM β-estradiol as an ER stimulator

and measured the loss-of-signal as compared to positive control of 4-Hydroxytamoxifen (100% inhibition). The assay is run in triplicate on 1536-well microplates. Following 18 hour incubation of cells with test compounds, a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for 2 hours. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (product) to green (substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader and CellTiter-Glo assay reagent (Promega) is also incubated with test system for 30 minutes before readout to detect cell viability.

The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER antagonists, GeneBLAzer® ERα-UAS-bla GripTite™ cell line (Invitrogen) has been used to screen the Tox21 library of diverse environmental compounds. ERα-UAS-bla cell line expresses a partial ERα one-hybrid GAL4 system and is stably transfected with a β-lactamase reporter gene.

The Tox21 ERα bla assays are qHTS format assays which measured the ability of a chemical to inhibit estrogen receptor alpha (ERα) signaling pathways by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses a partial ERα and a one-hybrid GAL4 system to quantify xenoestrogenic activity**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that

efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα antagonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (4-hydroxytamoxifen; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of 4-hydroxytamoxifen activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440

OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_LUC_BG1_Agonist
Tox21_ERa_LUC_BG1_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	3.329
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.861
Neutral control median absolute deviation, by plate:	19.331
Positive control well median response value, by plate:	-100.09
Positive control well median absolute deviation, by plate:	2.96
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.31
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-5
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-5.13
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	115.14
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-21.07

Reference Chemicals / Predictive Capacity:

3.3. Assay Scope and Limitations

Chemical Library:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate

LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

4. Assay Documentation

4.1. References

[1] Graham, F., et al. (1977). J Gen Virol 36(1): 59-72. (PMID: 886304)

[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)

[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)

[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

E2, Estradiol

EDC, Endocrine Disrupting Compounds

ER, Estrogen Receptor

ERE, Estrogen Response Element

FBS, Fetal Bovine Serum

FRD, Flying Reagent Dispenser

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

MIE, Molecular Initiating Event

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors

qHTS, quantitative high-throughput screening

TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

7 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

Tox21_ERa_LUC_VM7_Agonist

Assay Name: Tox21 VM7 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

1. Assay Descriptions

1.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, VM7-Luc-4E2 cell line has been used to screen the Tox21 library of environmental compounds. The VM7Luc4E2 cell line endogenously expresses full-length ERα and is stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere). This test system was plated into 1536-well microplates and cells were exposed to test chemicals or controls for 24 hours and scanned with a microplate reader to detect bioluminescent signals which result from the enzymatic reaction catalyzed by ONE-Glo™ assay substrate and the induction of an ERα-linked luciferase reporter gene.

1.2. Assay Definition

Assay Throughput:

Stably transfected VM7 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring luminescence resulting from ER gene expression.

Experimental System:

MCF-7 (Michigan Cancer Foundation-7) is a human breast carcinoma cell line originating from tumor tissue taken in 1970 from a 69-year old Caucasian woman. This is an immortalized cell line which endogenously expresses receptors for estrogen (α and β) and progesterone [1] as well as growth factors EGF and IGF-1 [2], and so provides an alternative to breast cell lines for estrogen-sensitive proliferation assays. VM7-Luc-4E2 cells are VMCF7 cells which are stably transfected with plasmids containing four estrogen responsive elements upstream of a luciferase reporter gene [3] to measure transactivation activity occurring along estrogen signaling pathways. The VM7luc estrogen receptor transactivation test method for identifying estrogen receptor activation has been validated by NICEATM and ICCVAM as an appropriate assay for detecting ER antagonism [4].

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
MEM α medium	Invitrogen	Invitrogen, 12561
10% Premium Fetal Bovine Serum	Atlanta Biologicals	Atlanta Biologicals, S11150
Penicillin/Streptomycin	Invitrogen	Invitrogen, 15140
400mg/l G418 (Geneticin)	Invitrogen	Invitrogen, 10131

DMEM phenol red free - low glucose medium	Invitrogen	Sigma, D5921
10% Charcoal/dextran treated Fetal Bovine Serum	Invitrogen	Invitrogen, 12676
L-Glutamine	Invitrogen	Invitrogen, 25030
0.25% Trypsin-EDTA	Invitrogen	Invitrogen / 25200
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo Luciferase Assay system	Promega	Promega / E6130
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen / 12648

Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
MEM α medium	90%	-	90%	-
DMEM phenol red free - low glucose medium	-	90%	-	-
Premium Fetal Bovine Serum	10%	-	10%	-
Charcoal/dextran treated Fetal Bovine Serum	-	10%	-	-
Penicillin/Streptomycin	100U/ml & 100µg/ml	100U/ml & 100µg/ml	100U/ml & 100µg/ml	-
L-Glutamine	-	2mM	-	-
G418 (Geneticin)	400mg/l	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 Thaw a frozen vial of cells in 9ml of pre-warmed medium and seed them in T175 flask at 2 million cells

1.3. Propagation method

1.3.1 Trypsinize cells from the flask and centrifuge

1.3.2 Add culture medium to the pellet and passage at 3-4 million per T-225 flask

2.0 Assay Protocol

2.1 Harvest from the 5-day culture in assay medium and re-suspend cells in assay medium
2.2 Dispense 4000 cells/5µL/well into 1536-well tissue treated white/solid bottom plates
2.3 Incubate the plates for 24hrs (22-24hrs) at 37°C and 5% CO2
2.4 Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool
2.5 Incubate the plates for 22hrs (22-24hrs) at 37°C and 5% CO2
2.6 Add 5µl of ONE-Glo reagent
2.7 Incubate the plates at room temperature for 30min
2.8 Measure luminescence by ViewLux plate reader

Protocol Summary:

VM7-Luc-4E2 cells were cultured in phenol red-free assay medium containing 10% charcoal stripped FBS for 5 days before the assay was performed. The cells were dispensed at 4,000 cells/5 µL of assay medium per well into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37C and 5% CO2 for 24 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37C and 5% CO2 for 22 h, followed by addition of 5 µL of ONE-Glo reagent (Promega, Madison, WI) to each well using Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). The assay plates were incubated at room temperature and luminescence was measured by a ViewLux plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; VM7-Luc-4E2 cell line was provided by Dr. Michael Denison from University of California. The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [5].

Caveats:

It was recently reported that the BG1-Luc-4E2 cell line was derived from MCF7 human breast cancer cells rather than BG-1 cells. Hence, the cell line has been renamed VM7Luc4E2 (https://ntp.niehs.nih.gov/iccvam/methods/endocrine/bg1luc/bg1luc-vm7luc-june2016-508.pdf). The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor alpha VM7 luciferase agonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to induce estrogen-dependent transcription, monitored through luciferase reporter gene signal activation using an endogenous full-length ERα cell line of human breast cancer cell origin (VM7). The assay is run in triplicate on 1536-well microplate and bioluminescence was measured following 24-hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 μM.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, VM7-Luc-4E2 cell line (provided by Dr. Michael Denison from University of California) has been used to screen the Tox21 library of diverse environmental compounds. VM7Luc4E2 cell line endogenously expresses full-length ER-alpha and is stably transfected with a plasmid containing four estrogen responsive elements (ERE) upstream of a luciferase reporter gene.

The ERα_LUC_VM7 assays are qHTS format assays which measured the ability of a chemical to interact with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human breast (VM7) cell line which expresses endogenous full-length ERα and a luciferase reporter gene (ER-luc) to quantify xenoestrogenic activity**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Rogers, J., & Denison, M. (2000). "Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals". In Vitro Mol Toxicol 13(1), 67-82. (PMID: 10900408)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.18
Neutral control median absolute deviation, by plate:	6.87
Positive control well median response value, by plate:	99.58
Positive control well median absolute deviation, by plate:	8.91
Z' (median across all plates, using positive control wells):	0.53
SSMD (median across all plates, using positive control wells):	9
Signal-to-noise (median across all plates, using positive control wells):	14.55
Signal-to-background (median across all plates, using positive control wells):	-548.47
CV (median across all plates):	-39.74

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα agonism as monitored by measuring changes in luminescence resulting from activation of an estrogen-responsive luciferase reporter gene.

Analytical Elements:

The Tox21 VM7-luciferase ERα agonist assay was monitored for signal increase over the DMSO (negative control) baseline, and activity and was calculated as a percentage of 17β-estradiol (positive control) activity. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any

response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_VM7_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 15
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.001 µM
Standard maximum concentration tested: 90 µM
Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 2.78
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes

131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No
84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	No
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	No
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes

51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	8
Inactive	5	3

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	9
Inactive	7	4

In Vitro Sensitivity = 72.4%
In Vitro Specificity = 37.5%
Balanced Accuracy = 55.0%

In Vivo Sensitivity = 70.0%
In Vivo Specificity = 36.4%
Balanced Accuracy = 53.2%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate

LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

4. Assay Documentation

4.1. References

[1] Geisinger, K. R., et al. (1989). Cancer 63(2): 280-288. (PMID: 2910432)

[2] Baldwin, W. S., et al. (1998). In Vitro Cell Develop Biol -Animal 34(8): 649-654. (PMID: 9769151)

[3] Rogers, J. and M. Denison (2000). In Vitro Mol Toxicol 13(1): 67-82. (PMID: 10900408)

[4] OECD (2012). Test No. 457: Bg1luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Publishing.

[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

E2, Estradiol

EDC, Endocrine Disrupting Compounds

ER, Estrogen Receptor

ERE, Estrogen Response Element

FBS, Fetal Bovine Serum

FRD, Flying Reagent Dispenser

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

MIE, Molecular Initiating Event

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors

qHTS, quantitative high-throughput screening

TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

3 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

Tox21_ERa_LUC_VM7_Agonist_ 10nM_ICI182780

Assay Name: Tox21 VM7 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

1. Assay Descriptions

1.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, VM7-Luc-4E2 cell line has been used to screen the Tox21 library of environmental compounds. The VM7Luc4E2 cell line endogenously expresses full-length ERα and is stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere). This test system was plated into 1536-well microplates and cells were exposed to test chemicals or controls for 24 hours and scanned with a microplate reader to detect bioluminescent signals which result from the enzymatic reaction catalyzed by ONE-Glo™ assay substrate and the induction of an ERα-linked luciferase reporter gene.

1.2. Assay Definition

Assay Throughput:

Stably transfected VMCF7 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring luminescence resulting from ER gene expression.

Experimental System:

MCF-7 (Michigan Cancer Foundation-7) is a human breast carcinoma cell line originating from tumor tissue taken in 1970 from a 69-year old Caucasian woman. This is an immortalized cell line which endogenously expresses receptors for estrogen (α and β) and progesterone [1] as well as growth factors EGF and IGF-1 [2], and so provides an alternative to breast cell lines for estrogen-sensitive proliferation assays. VM7-Luc-4E2 cells are VMCF7 cells which are stably transfected with plasmids containing four estrogen responsive elements upstream of a luciferase reporter gene [3] to measure transactivation activity occurring along estrogen signaling pathways. The VM7luc estrogen receptor transactivation test method for identifying estrogen receptor activation has been validated by NICEATM and ICCVAM as an appropriate assay for detecting ER antagonism [4].

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
MEM α medium	Invitrogen	Invitrogen, 12561
10% Premium Fetal Bovine Serum	Atlanta Biologicals	Atlanta Biologicals, S11150

Penicillin/Streptomycin	Invitrogen	Invitrogen, 15140
400mg/l G418 (Geneticin)	Invitrogen	Invitrogen, 10131
DMEM phenol red free - low glucose medium	Invitrogen	Sigma, D5921
10% Charcoal/dextran treated Fetal Bovine Serum	Invitrogen	Invitrogen, 12676
L-Glutamine	Invitrogen	Invitrogen, 25030
0.25% Trypsin-EDTA	Invitrogen	Invitrogen / 25200
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo Luciferase Assay system	Promega	Promega / E6130
Recovery Cell Culture Freezing Medium	Invitrogen	Invitrogen / 12648
ICI 182,780	Sigma-Aldrich	Sigma-Aldrich/I4409

Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
MEM α medium	90%	-	90%	-
DMEM phenol red free - low glucose medium	-	90%	-	-
Premium Fetal Bovine Serum	10%	-	10%	-
Charcoal/dextran treated Fetal Bovine Serum	-	10%	-	-
Penicillin/Streptomycin	100U/ml & 100µg/ml	100U/ml & 100µg/ml	100U/ml & 100µg/ml	-
L-Glutamine	-	2mM	-	-
G418 (Geneticin)	400mg/l	-	-	-
Recovery Cell Culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 Thaw a frozen vial of cells in 9ml of pre-warmed medium and seed them in T175 flask at 2 million cells

1.3. Propagation method

1.3.1 Trypsinize cells from the flask and centrifuge

1.3.2 Add culture medium to the pellet and passage at 3-4 million per T-225 flask

2.0 Assay Protocol

2.1 Harvest from the 5-day culture in assay medium and re-suspend cells in assay medium

2.2 Dispense 4000 cells/4µL/well into 1536-well tissue treated white/solid bottom plates

2.3 Incubate the plates for 24hrs (22-24hrs) at 37°C and 5% CO2

2.4 1 µL of 50.0 nM ICI 182,780/assay medium (10 nM final concentration) was added using a dispenser.

2.5 Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool

2.6 Incubate the plates for 22hrs (22-24hrs) at 37°C and 5% CO2

2.7 Add 5µl of ONE-Glo reagent

2.8 Incubate the plates at room temperature for 30min

2.9 Measure luminescence by ViewLux plate reader

Protocol Summary:

VM7-Luc-4E2 cells were cultured in phenol red-free assay medium containing 10% charcoal stripped FBS for 5 days before the assay was performed. The cells were dispensed at 4,000 cells/4 µL of assay medium per well into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at a 37C and 5% CO2 for 24 h, 1 µL of 50.0 nM ICI 182,780/assay medium was added using a dispenser. Next, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The plates were incubated at 37C and 5% CO2 for 22 h, followed by addition of 5 µL of ONE-Glo reagent (Promega, Madison, WI) to each well using Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). The assay plates were incubated at room temperature and luminescence was measured by a ViewLux plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; VM7-Luc-4E2 cell line was provided by Dr. Michael Denison from University of California. The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [5].

Caveats:

It was recently reported that the VM7-Luc-4E2 cell line was derived from MCF7 human breast cancer cells rather than VMCF7 cells. Hence, the cell line has been renamed VM7Luc4E2 (https://ntp.niehs.nih.gov/iccvam/methods/endocrine/VM7luc/VM7luc-vm7luc-june2016-508.pdf). The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor alpha VM7 luciferase agonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to induce estrogen-dependent transcription, monitored through luciferase reporter gene signal activation using an endogenous full-length ERα cell line of human ovary origin (VM7). The assay is run in triplicate on 1536-well microplate and bioluminescence was measured following 24-hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 μM.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine

function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER agonists, VM7-Luc-4E2 cell line (provided by Dr. Michael Denison from University of California) has been used to screen the Tox21 library of diverse environmental compounds. VM7Luc4E2 cell line endogenously expresses full-length ER-alpha and is stably transfected with a plasmid containing four estrogen responsive elements (ERE) upstream of a luciferase reporter gene.

The ERα_LUC_VM7 assays are qHTS format assays which measured the ability of a chemical to interact with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human ovary (VM7) cell line which expresses endogenous full-length ERα and a luciferase reporter gene (ER-luc) to quantify xenoestrogenic activity**.**

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Rogers, J., & Denison, M. (2000). "Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals". In Vitro Mol Toxicol 13(1), 67-82. (PMID: 10900408)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0
Neutral control median absolute deviation, by plate:	0.928
Positive control well median response value, by plate:	100
Positive control well median absolute deviation, by plate:	5.139
Z' (median across all plates, using positive control wells):	0.82
SSMD (median across all plates, using positive control wells):	19
Signal-to-noise (median across all plates, using positive control wells):	107.72
Signal-to-background (median across all plates, using positive control wells):	Inf
CV (median across all plates):	Inf

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα agonism as monitored by measuring changes in luminescence resulting from activation of an estrogen-responsive luciferase reporter gene.

Analytical Elements:

The Tox21 VM7-luciferase ERα agonist assay was run as counter-screen. This assay is a control that is run in agonist mode but with the inclusion of a potent ER antagonist, ICI 182,780, to block agonist activity. Results should be compared to TOX21_ERa_LUC_VM7_Agonist (AEID 788) which was run in the absence of ICI 182,780. Chemicals active in AEID 788 but not in AEID 2211 may be interpreted as competitive ER agonists. Concentration-response relationships were determined based on a

range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_VM7_Antagonist_0.1nM_E2
Tox21_ERa_LUC_VM7_Antagonist_0.5nM_E2
Tox21_ERa_LUC_VM7_Agonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations: 15
Target (nominal) number of replicates: 3
Standard minimum concentration tested: 0.001 µM
Standard maximum concentration tested: 90 µM

Baseline median absolute deviation for the assay - based on the response values at the 2 lowest tested concentrations (bmad): 0.534
The response cutoff used to derive the hit calls (e.g., 5*bmad, 10*bmad): 20

Reference Chemicals / Predictive Capacity:

CASRN	Chemical Name	*In Vitro* Activity	*In Vivo* Activity	Activity in Assay
57-91-0	17alpha-Estradiol	Moderate	Active	Yes
57-63-6	17alpha-Ethinylestradiol	Strong	Active	Yes
50-28-2	17beta-Estradiol	Strong	Active	Yes
58-18-4	17-Methyltestosterone	Very Weak	Active	Yes
131-55-5	2,2',4,4'-Tetrahydroxybenzophenone	NA	Active	Yes
131-56-6	2,4-Dihydroxybenzophenone	NA	Active	Yes
5153-25-3	2-Ethylhexylparaben	NA	Active	Yes
140-66-9	4-(1,1,3,3-Tetramethylbutyl)phenol	Moderate	Active	Yes
80-46-6	4-(2-Methylbutan-2-yl)phenol	NA	Active	Yes
80-09-1	4,4'-Sulfonyldiphenol	NA	Active	Yes
599-64-4	4-Cumylphenol	Weak	Active	Yes
104-43-8	4-Dodecylphenol	NA	Active	Yes
99-96-7	4-Hydroxybenzoic acid	acid	Inactive	Yes
104-40-5	4-Nonylphenol	Very Weak	Active	Yes
98-54-4	4-tert-Butylphenol	NA	Active	Yes
521-18-6	5alpha-Dihydrotestosterone	Weak	Active	Yes
61-82-5	Amitrole	NA	Inactive	Yes
520-36-5	Apigenin	Very Weak	NA	Yes
85-68-7	Benzyl butyl phthalate	Very Weak	NA	Yes
103-23-1	Bis(2-ethylhexyl)hexanedioate	NA	Inactive	Yes
80-05-7	Bisphenol A	Weak	Active	Yes
1478-61-1	Bisphenol AF	NA	Active	Yes
77-40-7	Bisphenol B	Weak	Active	Yes
94-26-8	Butylparaben	NA	Active	Yes
480-40-0	Chrysin	Very Weak	NA	Yes
50-22-6	Corticosterone	Inactive	NA	Yes
486-66-8	Daidzein	Weak	NA	Yes
117-81-7	Di(2-ethylhexyl) phthalate	Very Weak	Inactive	No

84-74-2	Dibutyl phthalate	Very Weak	Inactive	No
115-32-2	Dicofol	Very Weak	NA	Yes
84-61-7	Dicyclohexyl phthalate	NA	Inactive	Yes
84-66-2	Diethyl phthalate	NA	Inactive	No
56-53-1	Diethylstilbestrol	Strong	Active	No
84-75-3	Dihexyl phthalate	NA	Inactive	No
474-86-2	Equilin	NA	Active	Yes
50-27-1	Estriol	NA	Active	Yes
53-16-7	Estrone	Moderate	Active	No
120-47-8	Ethylparaben	Very Weak	NA	No
60168-88-9	Fenarimol	Very Weak	NA	Yes
51630-58-1	Fenvalerate	NA	Inactive	Yes
446-72-0	Genistein	Weak	Active	Yes
52-86-8	Haloperidol	Inactive	NA	Yes
520-18-3	Kaempferol	Very Weak	Inactive	Yes
143-50-0	Kepone	Weak	NA	No
65277-42-1	Ketoconazole	Inactive	NA	No
330-55-2	Linuron	Inactive	NA	Yes
84-16-2	meso-Hexestrol	Strong	NA	Yes
72-33-3	Mestranol	NA	Active	Yes
72-43-5	Methoxychlor	Very Weak	Active	Yes
68-22-4	Norethindrone	NA	Active	Yes
789-02-6	o,p'-DDT	Weak	Active	Yes
556-67-2	Octamethylcyclotetrasiloxane	NA	Active	Yes
72-55-9	p,p'-DDE	Very Weak	Weak	Yes
87-86-5	Pentachlorophenol	NA	Inactive	Yes
57-30-7	Phenobarbital sodium	Inactive	NA	Yes
32809-16-8	Procymidone	Inactive	NA	No
50-55-5	Reserpine	Inactive	NA	No
52-01-7	Spironolactone	Inactive	NA	No
17924-92-4	Zearalenone	NA	Active	Yes

In Vitro Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	8
Inactive	5	3

In Vivo Activity	**ToxCast Active**	**ToxCast Inactive**
Active	21	9
Inactive	7	4

In Vitro Sensitivity = 72.4%
In Vitro Specificity = 37.5%
Balanced Accuracy = 55.0%

In Vivo Sensitivity = 70.0%

In Vivo Specificity = 36.4%
Balanced Accuracy = 53.2%

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

4. Assay Documentation

4.1. References

[1] Geisinger, K. R., et al. (1989). Cancer 63(2): 280-288. (PMID: 2910432)

[2] Baldwin, W. S., et al. (1998). In Vitro Cell Develop Biol -Animal 34(8): 649-654. (PMID: 9769151)

[3] Rogers, J. and M. Denison (2000). In Vitro Mol Toxicol 13(1): 67-82. (PMID: 10900408)

[4] OECD (2012). Test No. 457: VM7luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Publishing.

[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
E2, Estradiol
EDC, Endocrine Disrupting Compounds
ER, Estrogen Receptor
ERE, Estrogen Response Element
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, quantitative high-throughput screening
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

3 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Tox21_ERa_LUC_VM7_Antagonist_0.1nM_E2

Assay Name: Tox21 VM7 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

1. Assay Descriptions

1.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER antagonists, VM7-Luc-4E2 cell line has been used to screen the Tox21 library of environmental compounds. The VM7Luc4E2 cell line endogenously expresses full-length ERα and is stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere). To measure antagonistic activity, this assay is performed with small amounts of an ERα stimulator (β-estradiol) added to each well and each compound is evaluated against a known ERα antagonist (4-Hydroxytamoxifen) as a positive control (100% inhibition). This test system was plated into 1536-well microplates and cells were exposed to test chemicals or controls for 24 hours and scanned with a microplate reader to detect bioluminescent signals which result from the enzymatic reaction catalyzed by ONE-Glo™ assay substrate and the induction of an ERα-linked luciferase reporter gene. To detect loss of signal due to compound cytotoxicity, a CellTiter-Glo fluorescence assay to measure ATP production was run concurrently in all wells using tetraoctylammonium bromide as a positive control for cell death. The antagonist assay was also run with a higher concentration of agonist (AEID789) as a specificity control in which competitive antagonists should show a right-shift in potency relative to the assay with lower agonist concentration.

1.2. Assay Definition

Assay Throughput:

Stably transfected VMCF7 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring luminescence resulting from ER gene expression.

Experimental System:

MCF-7 (Michigan Cancer Foundation-7) is a human breast carcinoma cell line originating from tumor tissue taken in 1970 from a 69-year old Caucasian woman. This is an immortalized cell line which endogenously expresses receptors for estrogen (α and β) and progesterone [1] as well as growth factors EGF and IGF-1 [2], and so provides an alternative to breast cell lines for estrogen-sensitive proliferation assays. VM7-Luc-4E2 cells are VMCF7 cells which are stably transfected with plasmids containing four estrogen responsive elements upstream of a luciferase reporter gene [3] to measure transactivation activity occurring along estrogen signaling pathways. The VM7luc estrogen receptor transactivation test method for identifying estrogen receptor activation has been validated by NICEATM and ICCVAM as an appropriate assay for detecting ER antagonism [4].

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

QUALITY CONTROL PRECAUTIONS:

1. -Maintain cells below 85-90% confluence in culture medium
2. -For assay purpose, the cells should be cultured in assay medium with 10% charcoal treated FBS for 5 days with alternate day medium changed to fresh medium
3. -Especially while in assay culture, the cells should not reach more than 85% confluence as they would become harder to detach if they reach over confluence

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
MEM α medium	Invitrogen	Invitrogen, 12561
10% Premium Fetal Bovine Serum	Atlanta Biologicals	Atlanta Biologicals, S11150
Penicillin/Streptomycin	Invitrogen	Invitrogen, 15140
400mg/l G418 (Geneticin)	Invitrogen	Invitrogen, 10131
DMEM phenol red free - low glucose medium	Invitrogen	Sigma, D5921
10% Charcoal/dextran treated Fetal Bovine Serum	Invitrogen	Invitrogen, 12676
L-Glutamine	Invitrogen	Invitrogen, 25030
0.25% Trypsin-EDTA	Invitrogen	Invitrogen / 25200
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo Luciferase Assay system	Promega	Promega / E6130
Recovery Cell culture Freezing Medium	Invitrogen	Invitrogen / 12648
CellTiter-Fluor Cell Viability Assay	Promega	Promega / G6082

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
MEM α medium	90%	-	90%	-
DMEM phenol red free - low glucose medium	-	90%	-	-
Premium Fetal Bovine Serum	10%	-	10%	-
Charcoal/dextran treated Fetal Bovine Serum	-	10%	-	-

Penicillin/Streptomycin	100U/ml & 100µg/ml	100U/ml & 100µg/ml	100U/ml & 100µg/ml	-
L-Glutamine	-	2mM	-	-
G418 (Geneticin)	400mg/l	-	-	-
Recovery Cell culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 Thaw a frozen vial of cells in 9ml of pre-warmed medium and seed them in T175 flask at 2 million cells

1.3. Propagation method

1.3.1 Trypsinize cells from the flask and centrifuge

1.3.2 Add culture medium to the pellet and passage at 3-4 million per T-225 flask

2.0 Assay Protocol

2.1 Harvest cells from the 5-day culture in assay medium and resuspend cells in assay medium

2.2 Dispense 4000 cells/5µL/well into 1536-well tissue treated white/solid bottom plates

2.3 Incubate the plates for 24hrs (22-24hrs) at 37°C and 5% CO2

2.4 Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool

2.5 Add 1µL of 0.5 nM (0.1 nM final) beta-Estradiol (E2, agonist) or assay buffer

2.6 Incubate the plates for 22-24hrs at 37°C and 5% CO2

2.7 Add 1ul of CellTiter-Fluor reagent

2.8 Incubate the plates at 37°C for 30min

2.9 Measure fluorescence by ViewLux plate reader

2.10 Add 4ul of ONE-Glo reagent

2.11 Incubate the plates at room temperature for 30min

2.12 Measure luminescence by ViewLux plate reader

Protocol Summary:

VM7-Luc-4E2 cells were cultured in phenol red free assay medium containing 10% charcoal stripped FBS for 5 days before the assay was performed. The cells were dispensed at 4,000 cells/4 µL of assay medium per well into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. The assay plates were incubated at a 37°C and 5% CO2 for 24 h, and then 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate using a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1 µL of 17β-estradiol in assay medium using a Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). After the plates were incubated at 37°C and 5% CO2 for 21.5 h, 1 µL of CellTiter-Fluor reagent (Promega, Madison, WI) of measuring cytotoxicity was added using Flying Reagent Dispenser (FRD) to each well. The assay plates were incubated at 37°C and 5% CO2 for additional 30 min and the fluorescence intensity was quantified by a ViewLux plate reader (PerkinElmer, Shelton, CT). For measuring luciferase reporter gene activity, 4 µL of ONE-Glo reagent (Promega) was added to each plate using an FRD and luminescence was quantified by a ViewLux plate reader after 30 min incubation at room temperature.

Proprietary Elements:

This assay is not proprietary; VM7-Luc-4E2 cell line was provided by Dr. Michael Denison from University of California. The Tox21 qHTS robotic platform has a 1536-well per run capacity and is

capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [5].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor alpha VM7 luciferase antagonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to inhibit estrogen-dependent transcription, monitored through luciferase reporter gene signal activity using an endogenous full-length ERα cell line of human ovary origin (VM7). The assay is run in triplicate on 1536-well microplate and bioluminescence was measured following 24 hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were assayed for cytotoxicity by measuring protease activity with Promega CellTiter-

Fluor with tetraoctylammonium bromide as a positive control for cell death. The compound library was selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. To help distinguish true antagonistic activity from cytotoxic effects, this assay was multiplexed with a fluorescence-based cell viability assay which measured conserved and constitutive protease activity within live cells (Promega). Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER antagonists, VM7-Luc-4E2 cell line (provided by Dr. Michael Denison from University of California) has been used to screen the Tox21 library of diverse environmental compounds. VM7Luc4E2 cell line endogenously expresses full-length ER-alpha and is stably transfected with a plasmid containing four estrogen responsive elements (ERE) upstream of a luciferase reporter gene.

The ERα_LUC_VM7 assays are qHTS format assays which measured the ability of a chemical to interfere with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human breast carcinoma (vMCF7) cell line which expresses endogenous full-length ERα and a luciferase reporter gene (ER-luc) to quantify xenoestrogenic activity**.** The cell line was previously thought to be an ovarian cancer line (BG-1). This change does not alter published validation studies. Additional documentation on this change can be found here: https://ntp.niehs.nih.gov/iccvam/methods/endocrine/VM7luc/VM7luc-vm7luc-june2016-508.pdf.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Rogers, J., & Denison, M. (2000). "Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals". In Vitro Mol Toxicol 13(1), 67-82. (PMID: 10900408)

Assay Quality Statistics:

Neutral control well median response value, by plate:	0
Neutral control median absolute deviation, by plate:	6.815
Positive control well median response value, by plate:	-100
Positive control well median absolute deviation, by plate:	1.344
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.75
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-14
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-14.67
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-Inf
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	Inf

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα antagonism as monitored by measuring changes in luminescence resulting from activation of an estrogen-responsive luciferase reporter gene.

Analytical Elements:

The Tox21 VM7-luciferase ERα antagonist assay was monitored for decreased activity (loss-of-signal) relative to DMSO (negative control) and 4-hydroxytamoxifen (positive control, 100% antagonist activity) measured in the presence of 0.1 nM estradiol agonist stimulation. This assay was also run with 0.5 nM E2 (AEID 789) which can be used to distinguish competitive antagonists from non-competitive or cytotoxic chemicals. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
Tox21_ERa_BLA_Agonist_ratio
Tox21_ERa_BLA_Antagonist_ratio
Tox21_ERa_LUC_VM7_Agonist
Tox21_ERa_LUC_VM7_Agonist_10nM_ICI182780
Tox21_ERa_LUC_VM7_Antagonist_0.5nM_E2

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	3.927
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	23.56

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure

potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

4. Assay Documentation

4.1. References

[1] Geisinger, K. R., et al. (1989). Cancer 63(2): 280-288. (PMID: 2910432)

[2] Baldwin, W. S., et al. (1998). In Vitro Cell Develop Biol -Animal 34(8): 649-654. (PMID: 9769151)

[3] Rogers, J. and M. Denison (2000). In Vitro Mol Toxicol 13(1): 67-82. (PMID: 10900408)

[4] OECD (2012). Test No. 457: VM7luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Publishing.

[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

E2, Estradiol

EDC, Endocrine Disrupting Compounds

ER, Estrogen Receptor

ERE, Estrogen Response Element

FBS, Fetal Bovine Serum

FRD, Flying Reagent Dispenser

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

MIE, Molecular Initiating Event

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors

qHTS, quantitative high-throughput screening

TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

3 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (see existing annotations)

Tox21_ERa_LUC_VM7_Antagonist_0.5nM_E2

Assay Name: Tox21 VM7 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Agonists of the Estrogen Receptor Alpha (ER-alpha) Signaling Pathway

1. Assay Descriptions

1.1. Overview

Assay Summary:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER antagonists, VM7-Luc-4E2 cell line has been used to screen the Tox21 library of environmental compounds. The VM7Luc4E2 cell line endogenously expresses full-length ERα and is stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere). To measure antagonistic activity, this assay is performed with small amounts of an ERα stimulator (β-estradiol) added to each well and each compound is evaluated against a known ERα antagonist (4-Hydroxytamoxifen) as a positive control (100% inhibition). This test system was plated into 1536-well microplates and cells were exposed to test chemicals or controls for 24 hours and scanned with a microplate reader to detect bioluminescent signals which result from the enzymatic reaction catalyzed by ONE-Glo™ assay substrate and the induction of an ERα-linked luciferase reporter gene. To detect loss of signal due to compound cytotoxicity, a CellTiter-Glo fluorescence assay to measure ATP production was run concurrently in all wells using tetraoctylammonium bromide as a positive control for cell death. This assay serves as a specificity control for AEID 2053 through use of a higher agonist concentration which should result in a right shift in potency for competitive AR antagonists relative to the lower agonist concentration used in AEID 2053.

1.2. Assay Definition

Assay Throughput:

Stably transfected VMCF7 cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring luminescence resulting from ER gene expression.

Experimental System:

MCF-7 (Michigan Cancer Foundation-7) is a human breast carcinoma cell line originating from tumor tissue taken in 1970 from a 69-year old Caucasian woman. This is an immortalized cell line which endogenously expresses receptors for estrogen (α and β) and progesterone [1] as well as growth factors EGF and IGF-1 [2], and so provides an alternative to breast cell lines for estrogen-sensitive proliferation assays. VM7-Luc-4E2 cells are vMCF7 cells which are stably transfected with plasmids containing four estrogen responsive elements upstream of a luciferase reporter gene [3] to measure transactivation activity occurring along estrogen signaling pathways. The VM7luc estrogen receptor transactivation test method for identifying estrogen receptor activation has been validated by NICEATM and ICCVAM as an appropriate assay for detecting ER antagonism [4].

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

QUALITY CONTROL PRECAUTIONS:

1. -Maintain cells below 85-90% confluence in culture medium
2. -For assay purpose, the cells should be cultured in assay medium with 10% charcoal treated FBS for 5 days with alternate day medium changed to fresh medium
3. -Especially while in assay culture, the cells should not reach more than 85% confluence as they would become harder to detach if they reach over confluence

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
MEM α medium	Invitrogen	Invitrogen, 12561
10% Premium Fetal Bovine Serum	Atlanta Biologicals	Atlanta Biologicals, S11150
Penicillin/Streptomycin	Invitrogen	Invitrogen, 15140
400mg/l G418 (Geneticin)	Invitrogen	Invitrogen, 10131
DMEM phenol red free - low glucose medium	Invitrogen	Sigma, D5921
10% Charcoal/dextran treated Fetal Bovine Serum	Invitrogen	Invitrogen, 12676
L-Glutamine	Invitrogen	Invitrogen, 25030
0.25% Trypsin-EDTA	Invitrogen	Invitrogen / 25200
1536-well white solid plates	Greiner Bio-One	Greiner Bio-One / 789173-F
MULTIDROP COMBI	Thermo Electron Corporation	Thermo Electron Corporation
BioRAPTR FRD	Beckman Coulter	Beckman Coulter
ViewLux Plate Reader	Perkin Elmer	Perkin Elmer
ONE-Glo Luciferase Assay system	Promega	Promega / E6130
Recovery Cell culture Freezing Medium	Invitrogen	Invitrogen / 12648
CellTiter-Fluor Cell Viability Assay	Promega	Promega / G6082

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
MEM α medium	90%	-	90%	-
DMEM phenol red free - low glucose medium	-	90%	-	-
Premium Fetal Bovine Serum	10%	-	10%	-
Charcoal/dextran treated Fetal Bovine Serum	-	10%	-	-

Penicillin/Streptomycin	100U/ml & 100µg/ml	100U/ml & 100µg/ml	100U/ml & 100µg/ml	-
L-Glutamine	-	2mM	-	-
G418 (Geneticin)	400mg/l	-	-	-
Recovery Cell culture Freezing Medium	-	-	-	100%

1.2. Thawing method

1.2.1 Thaw a frozen vial of cells in 9ml of pre-warmed medium and seed them in T175 flask at 2 million cells

1.3. Propagation method

1.3.1 Trypsinize cells from the flask and centrifuge

1.3.2 Add culture medium to the pellet and passage at 3-4 million per T-225 flask

2.0 Assay Protocol

2.1 Harvest cells from the 5-day culture in assay medium and resuspend cells in assay medium

2.2 Dispense 4000 cells/5µL/well into 1536-well tissue treated white/solid bottom plates

2.3 Incubate the plates for 24hrs (22-24hrs) at 37°C and 5% CO2

2.4 Transfer 23nL of compounds from the library collection and positive control to the assay plates through pintool

2.5 Add 1µL of 2.5nM (0.5 nM final) Beta-Estradiol (E2, agonist) or assay buffer

2.6 Incubate the plates for 21.1/2hrs (22-24hrs) at 37°C and 5% CO2

2.7 Add 1ul of CellTiter-Fluor reagent

2.8 Incubate the plates at 37°C for 30min

2.9 Measure fluorescence by ViewLux plate reader

2.10 Add 4ul of ONE-Glo reagent

2.11 Incubate the plates at room temperature for 30min

2.12 Measure luminescence by ViewLux plate reader

Protocol Summary:

VM7-Luc-4E2 cells were cultured in phenol red free assay medium containing 10% charcoal stripped FBS for 5 days before the assay was performed. The cells were dispensed at 4,000 cells/4 µL of assay medium per well into white wall/solid-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. The assay plates were incubated at a 37°C and 5% CO2 for 24 h, and then 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate using a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1 µL of 17β-estradiol in assay medium using a Flying Reagent Dispenser (Aurora Discovery, San Diego, CA). After the plates were incubated at 37°C and 5% CO2 for 21.5 h, 1 µL of CellTiter-Fluor reagent (Promega, Madison, WI) of measuring cytotoxicity was added using Flying Reagent Dispenser (FRD) to each well. The assay plates were incubated at 37°C and 5% CO2 for additional 30 min and the fluorescence intensity was quantified by a ViewLux plate reader (PerkinElmer, Shelton, CT). For measuring luciferase reporter gene activity, 4 µL of ONE-Glo reagent (Promega) was added to each plate using an FRD and luminescence was quantified by a ViewLux plate reader after 30 min incubation at room temperature.

Proprietary Elements:

This assay is not proprietary; VM7-Luc-4E2 cell line was provided by Dr. Michael Denison from University of California. The Tox21 qHTS robotic platform has a 1536-well per run capacity and is

capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [5].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote estrogen receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Huang, R., Sakamuru, S., Martin, M. T., Reif, D. M., Judson, R. S., Houck, K. A., Casey, W., Hsieh, J. H., Shockley, K. R., Ceger, P., Fostel, J., Witt, K. L., Tong, W., Rotroff, D. M., Zhao, T., Shinn, P., Simeonov, A., Dix, D. J., Austin, C. P., Kavlock, R. J., Tice, R. R., & Xia, M. (2014). "Profiling of the Tox21 10K compound library for agonists and antagonists of the estrogen receptor alpha signaling pathway". Scientific Reports 4, 5664. (PMID: 25012808)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 estrogen receptor alpha VM7 luciferase antagonist assay screened a library of diverse environmental compounds to probe for xenoestrogenic ligand-binding and potential to inhibit estrogen-dependent transcription, monitored through luciferase reporter gene signal activity using an endogenous full-length ERα cell line of human ovary origin (VM7). This assay was run as a counter-screen to TOX21_ERa_LUC_VM7_Antagonist_0.1nM_E2 (AEID 2053). Results should be compared and competitive antagonists should show a right-shift in potency in AEID 789 relative to AEID 2053. The assay is run in triplicate on 1536-well microplate and bioluminescence was measured following 24 hour incubation of cells with test compounds and 30 min incubation of test

system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were assayed for cytotoxicity by measuring protease activity with Promega CellTiter-Fluor with tetraoctylammonium bromide as a positive control for cell death. The compound library was selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. To help distinguish true antagonistic activity from cytotoxic effects, this assay was multiplexed with a fluorescence-based cell viability assay which measured conserved and constitutive protease activity within live cells (Promega). The assay serves as a specificity control for AEID 2053 as competitive antagonists for the androgen receptor should show a right shift in potency in this assay compared to AEID 2053 due to the use of higher concentration of agonist stimulation.

Scientific Principles:

Estrogen receptor (ER), a nuclear hormone receptor, plays an important role in development, metabolic homeostasis and reproduction. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone receptors like ER causes disruption of normal endocrine function. Therefore, it is important to understand the effect of environmental chemicals on the ER signaling pathway. To identify ER antagonists, VM7-Luc-4E2 cell line (provided by Dr. Michael Denison from University of California) has been used to screen the Tox21 library of diverse environmental compounds. VM7Luc4E2 cell line endogenously expresses full-length ER-alpha and is stably transfected with a plasmid containing four estrogen responsive elements (ERE) upstream of a luciferase reporter gene.

The ERα_LUC_VM7 assays are qHTS format assays which measured the ability of a chemical to interfere with estrogen receptor alpha (ERα) by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human breast carcinoma (vMCF7) cell line which expresses endogenous full-length ERα and a luciferase reporter gene (ER-luc) to quantify xenoestrogenic activity**.** The cell line was previously thought to be an ovarian cancer line (BG-1). This change does not alter published validation studies. Additional documentation on this change can be found here: https://ntp.niehs.nih.gov/iccvam/methods/endocrine/VM7luc/VM7luc-vm7luc-june2016-508.pdf.

This assay is intended for use as a part of an integrated testing strategy, to screen a large structurally diverse chemical library for compounds with the potential to interact with estrogen receptor alpha mediated pathways and potentially affect endocrine systems in exposed populations. There is strong evidence that estrogen receptor agonism is the Molecular Initiating Event (MIE) in an Adverse Outcome Pathway (AOP) leading to reproductive dysfunction in oviparous vertebrates, and there is some evidence that estrogen receptor activation is the MIE for putative AOPs leading to reduced survival due to renal failure and leading to skewed sex ratios due to altered sexual differentiation in males (all AOPs currently under development). Chemical-activity profiles derived from this assay can inform prioritization decisions for compound selection in more resource intensive in vivo studies to further investigate the involvement of ER agonism in pathways leading to hazardous outcomes in biological systems.

Method Development Reference:

Rogers, J., & Denison, M. (2000). "Recombinant cell bioassays for endocrine disruptors: development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals". In Vitro Mol Toxicol 13(1), 67-82. (PMID: 10900408)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.216
Neutral control median absolute deviation, by plate:	7.325
Positive control well median response value, by plate:	-100.054
Positive control well median absolute deviation, by plate:	0.902
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.75
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-14
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-13.64
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	462.2
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-35.2

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Xenoestrogenic ligand-binding and ERα antagonism as monitored by measuring changes in luminescence resulting from activation of an estrogen-responsive luciferase reporter gene.

Analytical Elements:

The Tox21 VM7-luciferase ERα antagonist specificity assay was monitored for decreased activity (loss-of-signal) relative to DMSO (negative control) and 4-hydroxytamoxifen (positive control, 100% antagonist activity) measured in the presence of 0.5 nM estradiol agonist stimulation. Use of the 0.5 nM estradiol should result in a right-shifting of AC50s for competitive ER antagonists relative to the AEID 2053 assay results run with 0.1 nM estradiol. This is useful for distinguishing competitive antagonists from non-competitive or cytotoxic compounds. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Estrogen receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also

generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ACEA_T47D_80hr_Positive
ATG_ERE_CIS_up
ATG_ERa_TRANS_up
ATG_ERb_TRANS2_up
NVS_NR_bER
NVS_NR_hER
NVS_NR_mERa
OT_ER_ERaERa_0480
OT_ER_ERaERa_1440
OT_ER_ERaERb_0480
OT_ER_ERaERb_1440
OT_ER_ERbERb_0480
OT_ER_ERbERb_1440
OT_ERa_ERELUC_AG_1440
OT_ERa_ERELUC_ANT_1440
OT_ERa_EREGFP_0120
OT_ERa_EREGFP_0480
OT_ERb_ERELUC_ANT_1440
TOX21_ERa_BLA_Agonist_ratio
TOX21_ERa_BLA_Antagonist_ratio
TOX21_ERa_LUC_VM7_Agonist
TOX21_ERa_LUC_VM7_Agonist_10nM_ICI182780

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	4.083
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate

LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [5].

4. Assay Documentation

4.1. References

[1] Geisinger, K. R., et al. (1989). Cancer 63(2): 280-288. (PMID: 2910432)

[2] Baldwin, W. S., et al. (1998). In Vitro Cell Develop Biol -Animal 34(8): 649-654. (PMID: 9769151)

[3] Rogers, J. and M. Denison (2000). In Vitro Mol Toxicol 13(1): 67-82. (PMID: 10900408)

[4] OECD (2012). Test No. 457: VM7luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Publishing.

[5] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion

AOP, Adverse Outcome Pathway

E2, Estradiol

EDC, Endocrine Disrupting Compounds

ER, Estrogen Receptor

ERE, Estrogen Response Element

FBS, Fetal Bovine Serum

FRD, Flying Reagent Dispenser

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

MIE, Molecular Initiating Event

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors

qHTS, quantitative high-throughput screening

TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)

109 T.W. Alexander Drive (MD-B-205-01)

Research Triangle Park, NC 27711

919-541-4219

Date of Assay Document Creation:

3 June 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (see existing annotations)

Tox21_FXR_BLA_agonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Peroxisome Farnesoid X (FXR) Agonist qHTS Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The farnesoid-X-receptor (FXR), a ligand-activated nuclear hormone receptor, is highly expressed in liver, intestine, kidney and adrenal cortex. Natural ligands of FXR are the bile acids (e.g., cholic acid, chenodeoxy cholic acid). FXR is an important regulator of diverse metabolic pathways, including bile acid homeostasis, lipid and glucose metabolism. To identify compounds that activate FXR signaling, GeneBLAzer FXR-UAS-bla HEK 293T cell line (Invitrogen, Carlsbad, CA) containing a beta-lactamase reporter gene under the control of a UAS response element was used to screen the Tox21 compound library. This experimental system expresses a fusion protein of a human farnesoid X receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds stimulates β-lactamase reporter gene expression. FXR activation by test compounds was assessed following 24-hour incubation in 1536-well microtiter plates. The cytotoxicity of the Tox21 compound library against the FXR-bla cell line was tested in parallel by measuring the cell viability using CellTiter-Glo assay in the same wells.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from induced FXR gene expression.

Experimental System:

GeneBLAzer® FXR-UAS-bla HEK293T cells contain the ligand-binding domain (LBD) of the human farnesoid X receptor fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer® UAS-bla HEK293T cell line. GeneBLAzer® UAS-bla HEK 293T cells (Invitrogen catalog #K1104) stably express a beta-lactamase reporter gene under the transcriptional control of an Upstream Activator Sequence (UAS). When an agonist binds to the LBD of the GAL4(DBD)-FXR(LBD) fusion protein, it translocates to the nucleus where it binds to the UAS inducing transcription of beta-lactamase. When an agonist binds to the LBD of the GAL4 (DBD)-FXR (LBD) fusion protein, the protein binds to the UAS, resulting in increased expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM, with Glutamax	Invitrogen	10569
Phenol Red free DMEM	Invitrogen	21063
Dialyzed FBS	Invitrogen	26400
Nonessential amino acids (NEAA)	Invitrogen	11140
DPBS	Invitrogen	14190
Sodium Pyruvate	Invitrogen	11360
Penicillin/Streptomycin	Invitrogen	15140
HEPES (1M pH 7.3)	Invitrogen	15630
0.05% Trypsin/EDTA	Invitrogen	25300
Hygromycin	Invitrogen	10687
Zeocin	Invitrogen	R25001
LiveBLAzer FRET B/G Loading Kit: Solution A, B, and C	Invitrogen	K1030
Recovery Cell Culture Freezing Medium	Invitrogen	12648
Fetal bovine serum (FBS), charcoal stripped	Invitrogen	12676
Chenodeoxycholic acid (CDCA)	Sigma	C9377
Black, clear-bottom, 1536-well assay plates	Greiner BioOne	789092-F
PinTool	Kalypsys	
BioRAPTR, Microfluidic Workstation	Beckmen	
EnVision plate reader	Perkin Elmer	

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	-	90%	-
Phenol Red free DMEM	-	98%	-	-
Dialyzed FBS	10%	-	10%	-
Charcoal-Stripped FBS	-	2%	-	-
Penicillin/Streptomycin	100U/mL;100 µg/mL	100U/mL;100 µg/mL	100U/mL;100 µg/mL	-
Sodium Pyruvate	-	1 mM	-	-
NEAA	0.1 mM	0.1 mM	0.1 mM	-
HEPES (pH 7.3)	25 mM	-	25 mM	-

Hygromycin	100 µg/ml	-	-	-
Zeocin	100 µg/ml	-	-	-

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a 15 ml conical tube

1.2.2 Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.

1.2.3 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.

1.2.4 Transfer the vial contents drop-wise into 14 mL of thaw medium in a sterile 15-mL conical tub.

1.2.5 Centrifuge cells at 900 rpm for 4 minutes and resuspend in thaw medium.

1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37 °C/5% CO2 incubator.

1.2.7 Switch to growth medium at first passage.

1.3. Propagation method

1.3.1 Aspirate medium, rinse once in DPBS, add 0.05% Trypsin/EDTA and swirl to coat the cell evenly.

1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.

1.3.3 Centrifuge cells at 900 rpm for 4 minutes and resuspend in growth medium.

1.3.4 Cell should be passage at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture and resuspend in assay medium.

2.2 Dispense 5000 cells/5µL/well into 1536-well tissue treated black, clear-bottom plates using a Multi-drop dispenser.

2.3 After the cells were incubated at 37°C for 5 hours, 23 nL of control or compounds dissolved in DMSO were transferred to the assay plate by a PinTool resulting in a 217-fold dilution.

2.4 Incubate the plates for 16 hours at 37°C.

2.5 Add 1 µL of 6X LiveBLAzer-FRET B/G (CCF4-AM) Substrate Mixture to each well using a BioRAPTR dispenser.

2.6 After two hours incubation at room temperature, measure fluorescence intensity at 460 and 530 nm emission and 405 nm excitation by an Envision detector. Data is expressed as the ratio of 460nm/530nm emissions.

Protocol Summary

FXR-bla cells were dispensed at 5000 cells/5uL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 C and 5% CO2 for 5 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The assay plates were incubated at 37C for 16 h. After 1 uL of LiveBLAzer B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN), the plates were incubated at room temperature for 2 h, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 4uL/well of CellTiter-Glo reagent was added into the assay

plates using a Bioraptr FRD. After 30 min incubation at room temperature, the luminescence intensity in the plates was measured using a ViewLux plate reader (PerkinElmer)

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [3]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote FXR mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 farnesoid-X-receptor β-lactamase agonist assay screened a library of diverse environmental compounds to probe for xenobiotic ligand-binding and potential to induce FXR-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 16-hour incubation of cells with test compounds a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for an additional hour, in the dark. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Farnesoid-X-receptor (FXR) is a ligand-activated nuclear receptor which regulates the expression of genes involved in bile acid homeostasis and has a role in the regulation of glucose and lipid metabolic pathways. FXR is primarily expressed in the liver, kidney, intestine and adrenal cortex, and regulates the expression of target genes by binding either as a monomer or as a heterodimer with the retinoid X receptor (RXR). Numerous studies have reported that FXR exerts protective function during cholestasis, diabetes, liver regeneration, and cancer. The FXR-RXR heterodimer, when bound to DNA, can act as transcriptional activators or inhibitors. FXR is activated by bile acids and the main endogenous ligand for FXR is chenodeoxycholic acid (CDCA). FXR reduces bile acid concentration in the liver by repressing genes involved in bile acid synthesis and regulates lipid metabolism.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.0890
Neutral control median absolute deviation, by plate:	2.638
Positive control well median response value, by plate:	99.490
Positive control well median absolute deviation, by plate:	16.56
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.43
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	6

SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	37.49
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-1026.38
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-27.69

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

FXR ligand-binding and agonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the FXR_BLA_Agonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (Chenodeoxycholic acid, CDCA; 100% agonist activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of CDCA activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up
ATG_FXR_TRANS_up
NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3

Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	1.720
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)

[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)

[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)

[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

ATCC, American Tissue Culture Collection

AIC, Akaike Information Criterion

CDCA, Chenodeoxycholic acid

FBS, Fetal Bovine Serum

FRD, Flying Reagent Dispenser

FXR, Farnesoid X receptor

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

LBD, Ligand-Binding Domain

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors

qHTS, Quantitative High-Throughput Screening

UAS, Upstream Activator Sequence

TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 October 2016

Date of Revisions:

2016

Author of Revisions:

EPA NCCT

5. Supporting Information (existing annotations):

Tox21_FXR_BLA_antagonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Farnesoid X (FXR) Antagonist qHTS Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The farnesoid-X-receptor (FXR), a ligand-activated nuclear hormone receptor, is highly expressed in liver, intestine, kidney and adrenal cortex. Natural ligands of FXR are the bile acids (e.g., cholic acid, chenodeoxy cholic acid). FXR is an important regulator of diverse metabolic pathways, including bile acid homeostasis, lipid and glucose metabolism. To identify compounds that inhibit FXR signaling, GeneBLAzer FXR-UAS-bla HEK 293T cell line (Invitrogen, Carlsbad, CA) containing a beta-lactamase reporter gene under the control of a UAS response element was used to screen the Tox21 compound library. This experimental system expresses a fusion protein of a human farnesoid X receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds stimulates β-lactamase reporter gene expression. FXR interference by test compounds was assessed following 24-hour incubation in 1536-well microtiter plates. The cytotoxicity of the Tox21 compound library against the FXR-bla cell line was tested in parallel by measuring the cell viability using CellTiter-Glo assay in the same wells.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from induced FXR gene expression.

Experimental System:

GeneBLAzer® FXR-UAS-bla HEK293T cells contain the ligand-binding domain (LBD) of the human farnesoid X receptor fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer® UAS-bla HEK293T cell line. GeneBLAzer® UAS-bla HEK 293T cells (Invitrogen catalog #K1104) stably express a beta-lactamase reporter gene under the transcriptional control of an Upstream Activator Sequence (UAS). When an agonist binds to the LBD of the GAL4(DBD)-FXR(LBD) fusion protein, it translocates to the nucleus where it binds to the UAS inducing transcription of beta-lactamase. When an agonist binds to the LBD of the GAL4 (DBD)-FXR (LBD) fusion protein, the protein binds to the UAS, resulting in increased expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number

DMEM, with Glutamax	Invitrogen	10569
Phenol Red free DMEM	Invitrogen	21063
Dialyzed FBS	Invitrogen	26400
Nonessential amino acids (NEAA)	Invitrogen	11140
DPBS	Invitrogen	14190
Sodium Pyruvate	Invitrogen	11360
Penicillin/Streptomycin	Invitrogen	15140
HEPES (1M pH 7.3)	Invitrogen	15630
0.05% Trypsin/EDTA	Invitrogen	25300
Hygromycin	Invitrogen	10687
Zeocin	Invitrogen	R25001
LiveBLAzer FRET B/G Loading Kit: Solution A, B, and C	Invitrogen	K1030
Recovery Cell Culture Freezing Medium	Invitrogen	12648
Fetal bovine serum (FBS), charcoal stripped	Invitrogen	12676-011
Chenodeoxycholic acid (CDCA)	Sigma	C9377
Black, clear-bottom, 1536-well assay plates	Greiner BioOne	789092-F
PinTool	Kalypsys	
BioRAPTR, Microfluidic Workstation	Beckmen	
EnVision plate reader	Perkin Elmer	

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	-	90%	-
Phenol Red free DMEM	-	98%	-	-
Dialyzed FBS	10%	-	10%	-
Charcoal-Stripped FBS	-	2%	-	-
Penicillin/Streptomycin	100U/mL;100 µg/mL	100U/mL;100 µg/mL	100U/mL;100 µg/mL	-
Sodium Pyruvate	-	1 mM	-	-
NEAA	0.1 mM	0.1 mM	0.1 mM	-
HEPES (pH 7.3)	25 mM	-	25 mM	-
Hygromycin	100 µg/ml	-	-	-
Zeocin	100 µg/ml	-	-	-

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a 15 ml conical tube

1.2.2 Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes.

1.2.3 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.

1.2.4 Transfer the vial contents drop-wise into 14 mL of thaw medium in a sterile 15-mL conical tub.

1.2.5 Centrifuge cells at 900 rpm for 4 minutes and resuspend in thaw medium.

1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37 °C/5% CO2 incubator.

1.2.7 Switch to growth medium at first passage.

1.3. Propagation method

1.3.1 Aspirate medium, rinse once in DPBS, add 0.05% Trypsin/EDTA and swirl to coat the cell evenly.

1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.

1.3.3 Centrifuge cells at 900 rpm for 4 minutes and resuspend in growth medium.

1.3.4 Cell should be passage at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture and resuspend in assay medium.

2.2 Dispense 5000 cells/5µL/well into 1536-well tissue treated black, clear-bottom plates using a Multi-drop dispenser.

2.3 After the cells were incubated at 37°C for 5 hours, 23 nL of control or compounds dissolved in DMSO were transferred to the assay plate by a PinTool resulting in a 217-fold dilution.

2.4 Add 1 µL of agonist (CDCA) at 300 µM in assay medium to the column 1-2 and column 5-48. Add 1 µL of assay medium to the column 3-4.

2.5 Incubate the plates for 16 hours at 37°C.

2.6 Add 1 µL of 6X LiveBLAzer-FRET B/G (CCF4-AM) Substrate Mixture to each well using a BioRAPTR dispenser.

2.7 After two hours incubation at room temperature, measure fluorescence intensity at 460 and 530 nm emission and 405 nm excitation by an Envision detector. Data is expressed as the ratio of 460nm/530nm emissions.

Protocol Summary

FXR-bla cells were dispensed at 5000 cells/5uL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 C and 5% CO2 for 5 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1 µL Chenodeoxycholic acid (50 uM, final concentration in the wells). The assay plates were incubated at 37C for 16 h. After 1 µL of LiveBLAzer B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN), the plates were incubated at room temperature for 2 h, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 4µL/well of CellTiter-Glo reagent was added into the assay plates using a Bioraptr FRD. After 30 min incubation at room temperature, the luminescence intensity in the plates was measured using a ViewLux plate reader (PerkinElmer).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [3]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote FXR mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 farnesoid-X-receptor β-lactamase antagonist assay screened a library of diverse environmental compounds to probe for xenobiotic ligand-binding and potential to inhibit FXR-dependent transcription, monitored through *bla* reporter gene signal activation using a

mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 16 hour incubation of cells with test compounds and a small amount of agonist (50 µM chenodeoxycholic acid) a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for an additional hour, in the dark. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Farnesoid-X-receptor (FXR) is a ligand-activated nuclear receptor which regulates the expression of genes involved in bile acid homeostasis and has a role in the regulation of glucose and lipid metabolic pathways. FXR is primarily expressed in the liver, kidney, intestine and adrenal cortex, and regulates the expression of target genes by binding either as a monomer or as a heterodimer with the retinoid X receptor (RXR). Numerous studies have reported that FXR exerts protective function during cholestasis, diabetes, liver regeneration, and cancer. The FXR-RXR heterodimer, when bound to DNA, can act as transcriptional activators or inhibitors. FXR is activated by bile acids and the main endogenous ligand for FXR is chenodeoxycholic acid (CDCA). FXR reduces bile acid concentration in the liver by repressing genes involved in bile acid synthesis and regulates lipid metabolism.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.127
Neutral control median absolute deviation, by plate:	4.829
Positive control well median response value, by plate:	-100.38
Positive control well median absolute deviation, by plate:	6.094
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.67
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-13
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-20.67

Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	778.69
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-37.07

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

FXR ligand-binding and antagonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the FXR_BLA_Antagonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (Guggulsterone; 100% antagonist activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of Guggulsterone activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. FXR activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_IR1_CIS_up
ATG_FXR_TRANS_up
NVS_NR_hFXR_Agonist
NVS_NR_hFXR_Antagonist
OT_FXR_FXRSRC1_0480
OT_FXR_FXRSRC1_1440
Tox21_FXR_BLA_agonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM

Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	2.790
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [4].

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)

[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)

[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)

[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

ATCC, American Tissue Culture Collection

AIC, Akaike Information Criterion

FBS, Fetal Bovine Serum

FRD, Flying Reagent Dispenser

FXR, Farnesoid X receptor

ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

LBD, Ligand-Binding Domain

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods

NR, Nuclear Receptors

qHTS, Quantitative High-Throughput Screening

RXR, Retinoid X Receptor

UAS, Upstream Activator Sequence

TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 October 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information (existing annotations):

Tox21_PPARd_BLA_agonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Peroxisome Proliferator-activated Receptor Delta (PPARd) Agonist qHTS Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors of the nuclear receptor superfamily with three distinct subtypes namely PPAR alpha, PPAR delta (PPARδ) (also called PPAR beta) and PPAR gamma. All these subtypes form heterodimers with Retinoid X receptor (RXR) to regulate transcription of various genes and have different physiological functions. Although the function of PPARδ is less well known, the recent identification of subtype specific synthetic ligands and the creation of animal models revealed its role in the regulation of cholesterol and lipid metabolism. To identify the compounds that activate PPARδ signaling, GeneBLAzer PPAR delta UAS-bla HEK293H cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS) was used to screen the Tox21 compound library. This experimental system expresses a fusion protein of a peroxisome proliferator-activated receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds stimulates β-lactamase reporter gene expression. PPARδ activation by test compounds was assessed following 24-hour incubation in 1536-well microtiter plates. The cytotoxicity of the Tox21 compound library against the PPARδ-bla cell line was tested in parallel by measuring the cell viability using CellTiter-Glo assay (Promega, Madison, WI) in the same wells.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from xenobiotic PPAR delta gene expression.

Experimental System:

GeneBLAzer® PPAR delta 293T DA (Division-arrested) cells and PPAR delta-UAS-bla 293T cells contain a peroxisome proliferator-activated receptor delta (PPARδ) ligand-binding domain/Gal4 DNA-binding domain chimera, stably integrated into the CellSensor® UAS-bla 293T cell line. CellSensor® UAS-bla 293T contains a beta-lactamase reporter gene under control of a UAS response element stably integrated into 293T cells. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4 (DBD)-PPAR delta (LBD) fusion protein, the protein binds to the UAS, resulting in expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM+Glutamax	Invitrogen	10569
Phenol Red free DMEM	Invitrogen	21063
Dialyzed FBS	Invitrogen	26400
Charcoal-Stripped FBS	Invitrogen	12676
Sodium Pyruvate	Invitrogen	11360
Penn-strep	Invitrogen	15140
NEAA	Invitrogen	11140
HEPES	Invitrogen	15630
HygromycinB	Invitrogen	10687
Zeocin	Invitrogen	R25001
Multidrop	Thermofisher	-
BiorapTR dispenser	Beckman Coulter	-
Envision plate reader	Perkin Elmer	-
LiveBLAzer B/G FRET substrate	Invitrogen	K1030

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	-	90%	-
Phenol Red free DMEM	-	98%	-	-
Dialyzed FBS	10%	-	10%	-
Charcoal-Stripped FBS	-	2%	-	-
Penn-strep	1%	1%	1%	-
Sodium Pyruvate	-	-	1 mM	-
NEAA	0.1 mM	-	0.1 mM	-
HEPES	25 mM	-	25 mM	-
Hygromycin	80 µg/ml	-	-	-
Zeocin	100 µg/ml	-	-	-

1.2. Thawing method

1.2.1 -Place 14 mL of pre-warmed thaw medium into a 15 ml conical tube

1.2.2 -Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.

1.2.3 -Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO

1.2.4 -Discard the supernatant and transfer the precipitated cells to T175 flask using 30 ml thawing medium

1.3. Propagation method

1.3.1 - Detach the cells from the flask using TrypLExpress

1.3.2 -The cells are re-seeded in T-175 flask at 2.5-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing

2.2 -Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust the required cell density

2.3 -Plate the cells in black-clear bottom 1536 well plate at 3000/well/6µL through 8 tip Multidrop plate dispenser

2.4 -Incubate for 5hrs at 37°C / 99% Humidity / 5% CO2

2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool

2.6 -Incubate for 17hrs at 37°C / 99% Humidity / 5% CO2

2.7 -Add 1 µL of CCF4 (FRET Substrate) dye using a single tip plate dispenser (Bioraptr)

2.8 -Incubate at room temperature for 1hrs in the dark

2.9 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamaze protocol optimized for this cell type

2.10 -Add 3 µL of Cell Titer Glo and Incubate at room temperature for 0.5 hrs in dark for both agonist and antagonist mode

2.11 -Read on ViewLux protocol optimized for this cell type for agonist mode

PPARd-bla cells were dispensed at 3000 cells/6µL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 °C and 5% CO2 for 5 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The assay plates were incubated at 37C for 17 h. After 1 µL of LiveBLAzer™ B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA), the plates were incubated at room temperature for 2 h, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 3 µL/well of CellTiter-Glo reagent was added into the assay plates using a Bioraptr FRD. After 30 min incubation at room temperature, the luminescence intensity in the plates was measured using a ViewLux plate reader (PerkinElmer).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [3]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with

extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 peroxisome proliferator-activated receptor delta β-lactamase agonist assay screened a library of diverse environmental compounds to probe for xenobiotic ligand-binding and potential to induce PPAR delta-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 17-hour incubation of cells with test compounds a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for an additional 2 hours in the dark. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. Following CCF4 incubation and detection, 3µL of CellTiter-Glo reagent is added to each well, and incubated for 30 minutes before cytotoxicity readout is measured on a ViewLux microtiter plate reader. The test compounds were selected

based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical). 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Peroxisome proliferator-activated receptor delta is a ligand-activated nuclear receptor which is expressed ubiquitously and may have a role in regulating the differentiation of adipocytes, in keratinocyte differentiation and in the regulation of cholesterol and lipid metabolism [4, 5]. The PPARd_BLA_Agonist assay used Fluorescence Resonance Energy Transfer (FRET) substrate to generate a ratiometric reporter response to receptor ligand-binding to allow monitoring of PPARδ activity relative to a known receptor agonist. This assay is designed to help identify environmental compounds with a capacity for PPAR delta ligand-binding activity. The Tox21 PPARδ bla assays are qHTS format assays which measured the ability of a chemical to interact with PPARδ by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses PPARδ and a one-hybrid GAL4 system to quantify xenobiotic PPARδ agonism.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.144
Neutral control median absolute deviation, by plate:	4.064
Positive control well median response value, by plate:	99.74
Positive control well median absolute deviation, by plate:	7.63
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.64
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	24.57
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-693.78
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-26.69

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPAR delta ligand-binding and agonism as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the PPARd_BLA_Agonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (L-165,041; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of L-165,041 activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
ATG_PPARd_TRANS_up
Tox21_PPARd_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	2.916
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives,

food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [6].

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archives Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Schmuth, M., et al. (2004). J Invest Derm 122(4): 971-983. (PMID: 15102088)
[5] Seimandi, M., et al. (2005). Anal Biochem 344(1): 8-15. (PMID: 16038868)
[6] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

ATCC, American Tissue Culture Collection
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
PPAR, Peroxisome Proliferator-Activated Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

15 July 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information

Tox21_PPARd_BLA_antagonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Peroxisome Proliferator-activated Receptor Delta (PPARd) Antagonist qHTS Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors of the nuclear receptor superfamily with three distinct subtypes namely PPAR alpha, PPAR delta (PPARδ) (also called PPAR beta) and PPAR gamma. All these subtypes form heterodimers with Retinoid X receptor (RXR) to regulate transcription of various genes and have different physiological functions. Although the function of PPARδ is less well known, the recent identification of subtype specific synthetic ligands and the creation of animal models revealed its role in the regulation of cholesterol and lipid metabolism. To identify the compounds that inhibit PPAR-delta signaling, GeneBLAzer PPAR delta UAS-bla HEK293H cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an Upstream Activator Sequence (UAS) was used to screen the Tox21 compound library. This experimental system expresses a fusion protein of a peroxisome proliferator-activated receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds stimulates β-lactamase reporter gene expression. PPARδ inhibition by test compounds was assessed following 24 hour incubation in 1536-well microtiter plates. The cytotoxicity of the Tox21 compound library against the PPARδ-bla cell line was tested in parallel by measuring the cell viability using CellTiter-Glo assay (Promega, Madison, WI) in the same wells.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from xenobiotic PPAR delta gene expression.

Experimental System:

GeneBLAzer® PPAR delta 293T DA (Division-arrested) cells and PPAR delta-UAS-bla 293T cells contain a peroxisome proliferator-activated receptor delta (PPARδ) ligand-binding domain/Gal4 DNA-binding domain chimera, stably integrated into the CellSensor® UAS-bla 293T cell line. CellSensor® UAS-bla 293T contains a beta-lactamase reporter gene under control of a UAS response element stably integrated into 293T cells. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an antagonist binds to the LBD of the GAL4 (DBD)-PPAR delta (LBD) fusion protein, binding to the UAS is impeded, resulting in interfered expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 (Dohr et al. 1995). The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid (Bylund et al. 2004). HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM+Glutamax	Invitrogen	10569
Phenol Red free DMEM	Invitrogen	21063
Dialyzed FBS	Invitrogen	26400
Charcoal-Stripped FBS	Invitrogen	12676
Sodium Pyruvate	Invitrogen	11360
Penn-strep	Invitrogen	15140
NEAA	Invitrogen	11140
HEPES	Invitrogen	15630
HygromycinB	Invitrogen	10687
Zeocin	Invitrogen	R25001
Multidrop	Thermofisher	-
BiorapTR dispenser	Beckman Coulter	-
Envision plate reader	Perkin Elmer	-
LiveBLAzer B/G FRET substrate	Invitrogen	K1030

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	-	90%	undefined
Phenol Red free DMEM	-	98%	-	-
Dialyzed FBS	10%	-	10%	undefined
Charcoal-Stripped FBS	-	2%	-	-
Penn-strep	1%	1%	1%	-
Sodium Pyruvate	-	-	1 mM	-
NEAA	0.1 mM	-	0.1 mM	-
HEPES	25 mM	-	25 mM	-
Hygromycin	80 µg/ml	-	-	-
Zeocin	100 µg/ml	-	-	-

1.2. Thawing method

1.2.1 -Place 14 mL of pre-warmed thaw medium into a 15 ml conical tube

1.2.2 -Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.

1.2.3 -Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO

1.2.4 -Discard the supernatant and transfer the precipitated cells to T175 flask using 30 ml thawing medium

1.3. Propagation method

1.3.1 - Detach the cells from the flask using TrypLExpress

1.3.2 -The cells are re-seeded in T-175 flask at 2.5-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing

2.2 -Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust the required cell density

2.3 -Plate the cells in black-clear bottom 1536 well plate at 3000/well/5µL through 8 tip Multidrop plate dispenser

2.4 -Incubate for 5hrs at 37°C / 99% Humidity / 5% CO2

2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool

2.6 -Add 1 µL of buffer and 1µL of Agonist concentration to respective columns as per plate map for antagonist mode

2.7 -Incubate for 17hrs at 37°C / 99% Humidity / 5% CO2

2.8 -Add 1 µL of CCF4 (FRET Substrate) dye using a single tip plate dispenser (Bioraptr)

2.9 -Incubate at room temperature for 2hrs in the dark

2.10 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamase protocol optimized for this cell type

2.11 -Add 3 µL of Cell Titer Glo and Incubate at room temperature for 0.5 hrs in dark for both agonist and antagonist mode

2.12 -Read on ViewLux protocol optimized for this cell type for antagonist mode

PPARd-bla cells were dispensed at 3000 cells/5uL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 C and 5% CO2 for 5 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1ul L-165,041 (300 nM, final concentration in the wells). The assay plates were incubated at 37 C for 17 h, and then 1 uL of LiveBLAzerTM B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The assay plates were incubated at room temperature for 2 h and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 3 ul/well of CellTiter-Glo reagent was added into the assay plates using a Bioraptr FRD. After 30 min incubation at room temperature, the luminescence intensity in the plates was measured using a ViewLux plate reader (PerkinElmer).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) (Michael et al. 2008). GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

2. Assay Component Descriptions

Assay Objectives:

The Tox21 peroxisome proliferator-activated receptor delta β-lactamase antagonist assay screened a library of diverse environmental compounds to probe for xenobiotic ligand-binding and potential to suppress PPARδ-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. Each well contained 0.3 µM L-165,041 to stimulate receptor activity and MK886 (a leukotriene inhibitor) served as a positive control. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 17 hour incubation of cells with test compounds a membrane-permeable FRET-based substrate

CCF4-AM is introduced and incubated for an additional hour. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader and CellTiter-Glo assay reagent (Promega) is also incubated with test system for 30 minutes before readout to detect cell viability.

The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical). 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Peroxisome proliferator-activated receptor delta is a ligand-activated nuclear receptor which is expressed ubiquitously and may have a role in regulating the differentiation of adipocytes, in keratinocyte differentiation and in the regulation of cholesterol and lipid metabolism (Schmuth et al. 2004, Seimandi et al. 2005). The PPARδ_BLA_Antagonist assay used Fluorescence Resonance Energy Transfer (FRET) substrate to generate a ratiometric reporter response to receptor ligand-binding to allow monitoring of PPARδ activity relative to a known receptor antagonist. This assay is designed to help identify environmental compounds with a capacity for PPAR delta interfering activity. The Tox21 PPARδ bla assays are qHTS format assays which measured the ability of a chemical to interact with PPARδ by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses PPARδ and a one-hybrid GAL4 system to quantify xenobiotic PPARδ agonism.

Method Development Reference:

Inglese, J., Auld, D. S., Jadhav, A., Johnson, R. L., Simeonov, A., Yasgar, A., Zheng, W., & Austin, C. P. (2006). "Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries". Proc Natl Acad Sci 103(31), 11473-11478. (PMID: 16864780)

Xia, M., Huang, R., Sun, Y., Semenza, G. L., Aldred, S. F., Witt, K. L., Inglese, J., Tice, R. R., & Austin, C. P. (2009). "Identification of chemical compounds that induce HIF-1alpha activity". Toxicol Sci 112(1), 153-163. (PMID: 19502547)

Assay Quality Statistics:

Neutral control well median response value, by plate:	10.1035
Neutral control median absolute deviation, by plate:	1.4826
Positive control well median response value, by plate:	133.452
Positive control well median absolute deviation, by plate:	11.6836
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.68
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	11
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	90.3
Signal-to-noise (median across all plates, using negative control wells):	NA

Signal-to-background (median across all plates, using positive control wells):	14.2
Signal-to-background (median across all plates, using positive control wells):	NA
CV (median across all plates):	0.15

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPAR delta antagonism and ligand interference and as monitored by FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the PPARd_BLA_antagonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (MK886; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of MK886 activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR delta interence was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	6
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.3 µM
Standard maximum concentration tested:	100 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	1.302
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals (Richard et al. 2016).

4. Assay Documentation

4.1. References

4.2. Abbreviations and Definitions

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;

Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation

Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency;

Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. Supporting Information (existing annotations):

Tox21_PPARg_BLA_Agonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Peroxisome Proliferator-activated Receptor Gamma (PPARg) Agonist qHTS Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors of the nuclear receptor superfamily with three distinct subtypes namely PPAR alpha, PPAR delta (also called PPAR beta) and PPAR gamma. All these subtypes heterodimerize with Retinoid X receptor (RXR) and these heterodimers regulate transcription of various genes. The PPARγ receptor is involved in the regulation of glucose and lipid metabolism. To identify the compounds that activate PPARγ signaling, GeneBLAzer® PPAR gamma UAS-bla HEK293H cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under control of an upstream activator sequence (UAS) stably integrated into HEK293H cells was used to screen the Tox21 compound library. This experimental system expresses a fusion protein of a peroxisome proliferator-activated receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds stimulates β-lactamase reporter gene expression. PPARγ activation by test compounds was assessed following 24 hour incubation in 1536-well microtiter plates.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from xenobiotic PPAR delta gene expression.

Experimental System:

GeneBLAzer® PPARγ 293H DA (Division-arrested) cells and PPAR gamma-UAS-bla 293H cells contain a peroxisome proliferator-activated receptor gamma (PPARγ) ligand-binding domain/Gal4 DNA-binding domain chimera, stably integrated into the CellSensor® UAS-bla 293H cell line. CellSensor® UAS-bla 293H contains a beta-lactamase reporter gene under control of a UAS response element stably integrated into 293H cells. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4 (DBD)-PPARγ (LBD) fusion protein, the protein binds to the UAS, resulting in expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 [1]. The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid [2]. HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM+Glutamax	Invitrogen	11965
Phenol Red free DMEM	Invitrogen	21063
Dialyzed FBS	Invitrogen	26400
Charcoal-Stripped FBS	Invitrogen	12676-029
Sodium Pyruvate	Invitrogen	11360
Penn-strep	Invitrogen	15140
NEAA	Invitrogen	11140
HEPES	Invitrogen	15630
Hygromycin	Invitrogen	10687-010
Geneticin	Invitrogen	10131-027
Multidrop	Thermofisher	-
BiorapTR dispenser	Beckman Coulter	-
Envision plate reader	Perkin Elmer	-
LiveBLAzer B/G FRET substrate	Invitrogen	K1030

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	-	90%	-
Phenol Red free DMEM	-	99%	-	-
Dialyzed FBS	10%	-	10%	-
Charcoal-Stripped FBS	-	1%	-	-
Penn-strep	1%	1%	1%	-
Sodium Pyruvate	1 mM	-	1 mM	-
NEAA	0.1 mM	-	0.1 mM	-
HEPES	25 mM	-	25 mM	-
Hygromycin	100 ug/ml	-	-	-
Geneticin	500 ug/ml	-	-	-

1.2. Thawing method

1.2.1 -Place 14 mL of pre-warmed thaw medium into a 15 ml conical tube

1.2.2 -Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.

1.2.3 -Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO

1.2.4 -Discard the supernatant and transfer the precipitated cells to T175 flask using 30 ml thawing medium

1.3. Propagation method

1.3.1 - Detach the cells from the flask using TrypLExpress

1.3.2 -The cells are re-seeded in T-175 flask at 3-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing

2.2 -Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust the required cell density

2.3 -Plate the cells in black-clear bottom 1536 well plate at 3000/well/6µL through 8 tip Multidrop plate dispenser

2.4 -Incubate for 5hrs at 37°C / 99% Humidity / 5% CO2

2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool

2.6 -Incubate for 17hrs at 37°C / 99% Humidity / 5% CO2

2.7 -Add 1 µL of CCF4 (FRET Substrate) dye using a single tip plate dispenser (Bioraptr)

2.8 -Incubate at room temperature for 1hrs in the dark

2.9 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamaze protocol optimized for this cell type

PPARγ-bla cells were dispensed at 3000 cells/6uL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37°C and 5% CO2 for 5 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA). The assay plates were incubated at 37C for 17 h. After 1 uL of LiveBLAzerTM B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA), the plates were incubated at room temperature for 1 h, and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [3]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program

National Center for Advancing Translational Sciences [NCATS]

NIH Chemical Genomics Center [NCGC]

U.S. Environmental Protection Agency [EPA]

National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 peroxisome proliferator-activated receptor gamma β-lactamase agonist assay screened a library of diverse environmental compounds to probe for xenobiotic ligand-binding and potential to induce PPARγ-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 17 hour incubation of cells with test compounds a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for an additional hour, in the dark. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. The test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known ER agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 μM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor which regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of

energy homeostasis. PPARγ is primarily expressed in adipose tissue, macrophages and in the colon where it controls adipocyte differentiation, lipid storage and inflammatory responses. PPARγ agonists, the thiazolidinediones (TZDs), improve insulin sensitivity, lower glucose levels, and lower plasma triglycerides and free fatty acid (FFA) levels by enhancing their uptake into adipocytes. The PPARg_BLA_Agonist assay used Fluorescence Resonance Energy Transfer (FRET) substrate to generate a ratiometric reporter response to receptor ligand-binding to allow monitoring of PPARγ activity relative to a known receptor agonist. This assay is designed to help identify environmental compounds with a capacity for PPARγ ligand-binding activity. The Tox21 PPARγ bla assays are qHTS format assays which measured the ability of a chemical to interact with PPARγ by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses PPARγ and a one-hybrid GAL4 system to quantify xenobiotic PPARγ agonism**.**

Method Development Reference:

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.093
Neutral control median absolute deviation, by plate:	3.487
Positive control well median response value, by plate:	99.805
Positive control well median absolute deviation, by plate:	6.461
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.7
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	14
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	28.66
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-953.1
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-34.37

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPAR gamma ligand-binding and agonism; measured by monitoring FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the PPARg_BLA_Agonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (rosiglitazone; 100% activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of rosiglitazone activity, and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model

which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR gamma activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
ATG_PPARg_TRANS_up
NVS_NR_hPPARg
OT_PPARg_PPARgSRC1_0480
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_antagonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	2.084
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds

recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [11].

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

ATCC, American Tissue Culture Collection
AIC, Akaike Information Criterion
AOP, Adverse Outcome Pathway
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
MIE, Molecular Initiating Event
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
PPAR, Peroxisome Proliferator-Activated Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

12 September 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information

Tox21_PPARg_BLA_antagonist_ratio

Assay Name: Tox21 Beta-lactamase HEK293 Peroxisome Proliferator-activated Receptor Gamma (PPARg) Antagonist qHTS Assay

1. Assay Descriptions

1.1. Overview

Assay Summary:

The peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors of the nuclear receptor superfamily with three distinct subtypes namely PPAR alpha, PPAR delta (also called PPAR beta) and PPAR gamma (PPARγ). All these subtypes heterodimerize with Retinoid X receptor (RXR) and these heterodimers regulate transcription of various genes. PPARγ receptor (glitazone receptor) is involved in the regulation of glucose and lipid metabolism. To identify the compounds that inhibit PPAR-gamma signaling, GeneBLAzer PPAR gamma UAS-bla HEK293H cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under control of an Upstream Activator Sequence (UAS) was used to screen the Tox21 compound library. This experimental system expresses a fusion protein of a peroxisome proliferator-activated receptor ligand-binding domain coupled to GAL4 DNA-binding domain which when activated by xenobiotic compounds modulates β-lactamase reporter gene expression. PPARγ interactions with tested compounds was assessed following 24 hour incubation in 1536-well microtiter plates. To detect loss of signal due to compound cytotoxicity, a CellTiter-Glo fluorescence assay to measure ATP production was run concurrently in all wells using tetraoctylammonium bromide as a positive control for cell death.

1.2. Assay Definition

Assay Throughput:

Stably transfected HEK293T cells are aliquoted into 1536-well microtiter plates and incubated with test compounds for 24 hours prior to monitoring fluorescence emission resulting from PPAR delta gene expression.

Experimental System:

GeneBLAzer® PPARγ 293H DA (Division-arrested) cells and PPAR gamma-UAS-bla 293H cells contain a peroxisome proliferator-activated receptor gamma (PPARγ) ligand-binding domain/Gal4 DNA-binding domain chimera, stably integrated into the CellSensor® UAS-bla 293H cell line. CellSensor® UAS-bla 293H contains a beta-lactamase reporter gene under control of a UAS response element stably integrated into 293H cells. These cells stably express a β-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an antagonist binds to the LBD of the GAL4 (DBD)-PPARγ (LBD) fusion protein, binding to the UAS is impeded, resulting in interfered expression of β-lactamase.

The HEK-293 cell line is a human embryonic kidney cell line (of unknown parentage) transformed with sheared adenovirus 5 DNA by Frank Graham in 1973 (Dohr et al. 1995). The transformation incorporated approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells, and subsequent cytogenetic characterization established that the 293 line is pseudotriploid (Bylund et al. 2004). HEK293 cells are popular for their ease of growth and transfection cells, and are frequently used to produce exogenous proteins or viruses for pharmaceutical and biomedical research purposes.

Xenobiotic Biotransformation Potential:

The intrinsic production of Phase I/II enzymes has not been well characterized in this cell line and metabolic activation/detoxification of test compounds is potentially limited.

Basic Procedure:

MATERIALS and INSTRUMENTS:

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM+Glutamax	Invitrogen	11965
Phenol Red free DMEM	Invitrogen	21063
Dialyzed FBS	Invitrogen	26400
Charcoal-Stripped FBS	Invitrogen	12676-029
Sodium Pyruvate	Invitrogen	11360
Penn-strep	Invitrogen	15140
NEAA	Invitrogen	11140
HEPES	Invitrogen	15630
Hygromycin	Invitrogen	10687-010
Geneticin	Invitrogen	10131-027
Multidrop	Thermofisher	-
BiorapTR dispenser	Beckman Coulter	-
Envision plate reader	Perkin Elmer	-
LiveBLAzer B/G FRET substrate	Invitrogen	K1030

PROCEDURE:

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Freezing Medium	-	-	-	100%
DMEM+Glutamax	90%	-	90%	-
Phenol Red free DMEM	-	99%	-	-
Dialyzed FBS	10%	-	10%	-
Charcoal-Stripped FBS	-	1%	-	-
Penn-strep	1%	1%	1%	-
Sodium Pyruvate	1 mM	-	1 mM	-
NEAA	0.1 mM	-	0.1 mM	-
HEPES	25 mM	-	25 mM	-
Hygromycin	100 ug/ml	-	-	-
Geneticin	500 ug/ml	-	-	-

1.2. Thawing method

1.2.1 -Place 14 mL of pre-warmed thaw medium into a 15 ml conical tube

1.2.2 -Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.

1.2.3 -Mix the entire content of the vial to 14 ml of pre-warmed medium and centrifuge to remove DMSO

1.2.4 -Discard the supernatant and transfer the precipitated cells to T175 flask using 30 ml thawing medium

1.3. Propagation method

1.3.1 - Detach the cells from the flask using TrypLExpress

1.3.2 -The cells are re-seeded in T-175 flask at 3-4 million

2. Assay Protocol

2.1 -Spin down the cells after rinsing the cells with DPBS and trypsinizing

2.2 -Resuspend the pellet with assay medium followed by filtering through cell strainer and adjust the required cell density

2.3 -Plate the cells in black-clear bottom 1536 well plate at 3000/well/5µL through 8 tip Multidrop plate dispenser

2.4 -Incubate for 5hrs at 37°C / 99% Humidity / 5% CO2

2.5 -Transfer 23nL of compounds from the library collection and positive control to the assay plates through Pintool

2.6 -Add 1 uL of buffer and 1uL of Agonist concentration to respective columns as per plate map for antagonist mode

2.7 -Incubate for 17hrs at 37°C / 99% Humidity / 5% CO2

2.8 -Add 1 µL of CCF4 (FRET Substrate) dye using a single tip plate dispenser (Bioraptr)

2.9 -Incubate at room temperature for 1hrs in the dark

2.10 -Read the fluorescence intensity through Envision plate reader using Beta-Lactamaze protocol optimized for this cell type

2.11 -Add 3 uL of Cell Titer Glo and Incubate at room temperature for 0.5 hrs in dark

2.12 -Read on ViewLux protocol optimized for this cell type for antagonist mode

PPARg-bla cells were dispensed at 3000 cells/5uL/well in 1536-well black wall/clear bottom plates using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37 C and 5% CO2 for 5 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station (Kalypsys, San Diego, CA), followed by addition of 1ul Rosiglitazone (50 nM, final concentration in the wells). The assay plates were incubated at 37°C for 17 h, and then 1 uL of LiveBLAzerTM B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The assay plates were incubated at room temperature for 1 h and fluorescence intensity was measured by an Envision plate reader (PerkinElmer, Shelton, CT). For cell viability readout that measures cytotoxicity, 4 ul/well of CellTiter-Glo reagent was added into the assay plates using a Flying Reagent Dispenser. After 30 min incubation at room temperature, the luminescence intensity in the plates was measured using a ViewLux plate reader (PerkinElmer).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) (Michael et al. 2008). GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote peroxisome proliferator receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with

extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Huang, R., Xia, M., Cho, M. H., Sakamuru, S., Shinn, P., Houck, K. A., Dix, D. J., Judson, R. S., Witt, K. L., Kavlock, R. J., Tice, R. R., & Austin, C. P. (2011). "Chemical genomics profiling of environmental chemical modulation of human nuclear receptors". Environ Health Perspect 119(8), 1142-1148. (PMID: 21543282)

Huang, R., Xia, M., Sakamuru, S., Zhao, J., Shahane, S. A., Attene-Ramos, M., Zhao, T., Austin, C. P., & Simeonov, A. (2016). "Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization". Nature Communications 7, 10425. (PMID: 26811972)

Teng, C., Goodwin, B., Shockley, K., Xia, M., Huang, R., Norris, J., Merrick, B. A., Jetten, A. M., Austin, C. P., & Tice, R. R. (2013). "Bisphenol A affects androgen receptor function via multiple mechanisms". Chem-Biol Interact 203(3), 556-564. (PMID: 23562765)

Method Updates / Confirmatory Studies:

None reported

2. Assay Component Descriptions

Assay Objectives:

The Tox21 peroxisome proliferator-activated receptor gamma β-lactamase antagonist assay screened a library of diverse environmental compounds to probe for xenobiotic ligand-interference and potential to suppress PPARγ-dependent transcription, monitored through *bla* reporter gene signal activation using a mammalian one-hybrid GAL4 system. The assay is run in triplicate on 1536-well microplates. HEK293T cells are plated the day of the assay and following 17 hour incubation of cells with test compounds a membrane-permeable FRET-based substrate CCF4-AM is introduced and incubated for an additional hour. Once in the cell, cytoplasmic esterases trap the negatively charged CCF4 substrate in the cytosol and *bla* expression is quantified by measuring the ratio of blue (460nm, product) to green (530nm, substrate) fluorescence. Fluorescence signals are monitored using an Envision plate reader. The test compounds were selected based on various

criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical). 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at potentially interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated nuclear receptor which regulates the expression of genes involved in fatty acid-oxidation and is a major regulator of energy homeostasis. PPARγ is primarily expressed in adipose tissue, macrophages and in the colon where it controls adipocyte differentiation, lipid storage and inflammatory responses. PPARγ agonists, the thiazolidinediones (TZDs), improve insulin sensitivity, lower glucose levels, and lower plasma triglycerides and free fatty acid (FFA) levels by enhancing their uptake into adipocytes. The PPARg_BLA_Antagonist assay used Fluorescence Resonance Energy Transfer (FRET) substrate to generate a ratiometric reporter response to receptor ligand-binding to allow monitoring of PPARγ activity relative to a known receptor antagonist. This assay is designed to help identify environmental compounds with a capacity for PPARγ ligand-binding interference. The Tox21 PPARγ bla assays are qHTS format assays which measured the ability of a chemical to inhibit PPARγ by monitoring modulation of fluorescence reporter gene signals. This assay utilized a human embryonic kidney cell line (HEK293T) which expresses PPARγ and a one-hybrid GAL4 system to quantify xenobiotic PPARγ antagonism.

Method Development Reference:

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.154
Neutral control median absolute deviation, by plate:	10.059
Positive control well median response value, by plate:	-100.23
Positive control well median absolute deviation, by plate:	5.506
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.53
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	-9
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	-9.95
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	583.55
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-61.24

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

PPAR gamma ligand-binding and antagonism; measured by monitoring FRET emission resulting from GAL4/β-lactamase gene expression.

Analytical Elements:

BLA expression in the PPARg_BLA_Antagonist assay is quantified by measuring the ratio of product (blue; 460 nm) to substrate (green; 530 nm) fluorescence. Raw plate readouts were normalized relative to positive controls (GW9662; 100% PPARγ antagonist activity) and negative controls (DMSO; baseline activity) and then subjected to a NCGC in-house pattern correction algorithm (developed using DMSO-only plates run at the beginning and end of the compound plate stack). Data was analyzed as percentage of GW9662 activity and concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. PPAR gamma activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_PPRE_CIS_up
ATG_PPARg_TRANS_up
NVS_NR_hPPARg
OT_PPARg_PPARgSRC1_0480
OT_PPARg_PPARgSRC1_1440
Tox21_PPARg_BLA_agonist_ratio

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	80 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	6.000
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	35.98

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives,

food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals (Richard et al. 2016).

4. Assay Documentation

4.1. References

[1] Dohr, O., et al. (1995). Archiv Biochem Biophys 321(2): 405-412. (PMID: 7646066)
[2] Bylund, L., et al. (2004). Cytogenet Genome Res 106(1): 28-32. (PMID: 15218237)
[3] Michael, S., et al. (2008). Assay Drug Dev Technol 6(5): 637-657. (PMID: 19035846)
[4] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

ATCC, American Tissue Culture Collection
AIC, Akaike Information Criterion
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
LBD, Ligand-Binding Domain
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
PPAR, Peroxisome Proliferator-Activated Receptors
qHTS, Quantitative High-Throughput Screening
UAS, Upstream Activator Sequence
TF, Transcription Factor

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

2017

Date of Revisions:

Author of Revisions:

5. Potential Regulatory Applications

Context of use:

Examples of end use scenarios could include, but are not limited to:

Support Category Formation and Read-Across: The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across;
Priority Setting: The assay might help prioritize substances within an inventory for more detailed evaluation
Screening Level Assessment of a Biomarker or Mechanistic Activity or Response: The screening level assessment may be sufficient to identify a hazard provide a gauge of potency;
Integrated approaches to testing and assessment (IATA): The assay may form one component of an IATA;

6. **Supporting Information (existing annotations):**

Tox21_TR_LUC_GH3_Agonist

Assay Name: Tox21 GH3 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Agonists of the Thyroid Receptor (TR) Signaling Pathway by Monitoring Thyroid Response Element Activation

1. Assay Descriptions

1.1. Overview

Assay Summary:

Thyroid receptor (TR), a nuclear hormone receptor, plays an important role in development, proliferation, differentiation, metabolism, brain function, and cardiovascular system. TR-interacting compounds have been shown to disrupt thyroid homeostasis [1]. To profile the Tox21 compound library's potential to mimic TH and disrupt signaling pathways, a cell-based GH3-TRE-Luc assay was used to measure the activation of TR following xenobiotic exposures. Activity was measured in GH3 (rat pituitary tumor) cells stably expressing a TR activity sensor consisting of two TR response elements and a luciferase reporter gene in 1536-well plates following 24-hour incubation with test chemicals. Increased luciferase activity indicates elevated levels of TR transactivation.

1.2. Assay Definition

Assay Throughput:

GH3.TRE-Luc cells are aliquoted into 1536-well microtiter plates (1500 cells/5 µL/well) and incubated for 4 hours prior to 24-hour exposure to test compounds and monitoring of increased luminescence resulting from xenobiotic-induced TR gene expression.

Experimental System:

GH3 cell line was derived from rat pituitary tumor cells and has been routinely employed for studying effects of TH disruption using the T-screen assay [2, 3] and is reported to retain unique characteristics of the original differentiated tissue such as production of growth hormone and prolactin [3-5]. Moreover, this cell line endogenously expresses both TH receptor isoforms (α and β) in very high amounts and they respond to physiological concentrations of TH by proliferating [6]. The GH3.TRE-Luc cell line, developed in the laboratory of Dr. Albertinka J. Murk (Wageningen University) is derived from GH3 cells and stably expresses a modified firefly luciferase reporter gene under the regulation of a TR activity sensor consisting of a pair of thyroid hormone response elements (TREs). [7].

Xenobiotic Biotransformation Potential:

GH3 cells display an increased level of cell proliferation and growth hormone secretion in response to physiologic levels of thyroid hormones. TRE-LUC cells are activated by the thyroid hormone triiodothyronine (T3) and all trans retinoic acid but not RAR or LXR specific ligands. [7] CYP1A1 and 1B1 (but not 1A2) have been shown to be inducible by PCBs in GH3 cells [8].

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM:F12	Invitrogen	Gibco, 10565
Fetal Bovine Serum	Hyclone	Hyclone, Sh30071.03
Pen/Strep	Invitrogen	Invitrogen, 15140
Insulin	Sigma	Sigma, I6634

Ethanolamine	Sigma	Sigma, E0135
Sodium Selenite	Sigma	Sigma, S5261
Human Apotranferin	Sigma	Sigma, T2036
Bovine Serum Albumin	Sigma	Sigma, A9647
TrypLE Express	Invitrogen	Invitrogen, 12605
PBS w/o Calcium and Magnesium	Invitrogen	Invitrogen, 14190
Recovery Cell Culture Medium	Invitrogen	Invitrogen, 12648
Centrifuge	Sorvall Legend XTR	Thermo Fisher Science 75004520
Bioraptr Microfluidic Workstation	Beckmen	-
Pintool	Kalypsys	-
White, TC, Sterile 1536-Well Assay Plates	Greiner Bio-One	Greiner, 789173-F
Viewlux Plate Reader	Perkinelmer	-
T3 (Agonist control compound)	Calbiochem	Calbiochem, 642511
DMSO	AMRESCO	Kd Medical, RGE-3070
One-Glo	Promega	Promega, E6120

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Culture Medium	-	-	-	100%
DMEM: F12	90%	100%	90%	-
Fetal Bovine Serum	10%	-	10%	-
Pen/Strep	100U/mL-100µg/mL	-	100U/mL-100µg/mL	-
Insulin	-	10µg/mL	-	-
Ethanolamine	-	10µM	-	-
Sodium Selenite		10ng/mL	-	-
Human apo-Transferrin	-	10µg/mL	-	-
Bovine Serum Albumin	-	500µg/mL	-	-

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a T75 flask

1.2.2 Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.

1.2.3 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.

1.2.4 Transfer the vial contents drop-wise into 10 mL of Thaw Medium in a sterile 15-mL conical tube

1.2.5 Centrifuge cells at 1000 rpm for 4 minutes

1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37°C/5% CO2 incubator

1.2.7 Switch to growth medium at first passage.

1.3. Propagation method

1.3.1 Aspirate medium, rinse once in DPBS, add TrypLE Express (3 mL for a T75 flask and 5 mL for a T175 flask and 7.5 mL for T225 flask) and swirl to coat the cell evenly.

1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.

1.3.3 Centrifuge cells at 1000 rpm for 4 minutes and resuspend in Growth Medium.

1.3.4 Cell should be passage or fed at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture in Growth Medium and resuspend in assay medium

2.2 Dispense 1500 cells/5 µL/well into 1536-well tissue treated white solid plates using a BioRAPTR dispenser.

2.3 After the cells were incubated at 37°C for 4 hours, 23 nL of compounds dissolved in DMSO, positive controls or DMSO were transferred to the assay plate by a PinTool

2.4 Incubate the plates for 24 hours at 37°C.

2.5 Add 5 µL of One-Glo to each well using a BioRAPTR dispenser and incubate the plate at room temperature for 30 mins.

2.6 Measure luminescence using Viewlux

GH3.TRE-Luc cells were dispensed at 1500 cells/5 µL/well into 1536-well white solid bottom plates using a Multidrop Combi dispenser (Thermo Fisher Scientific Inc., Waltham, MA). After the assay plates were incubated at 37°C for 4 h, 23 nL of library compound or controls (T3 or DMSO) was transferred to the assay plates by a pintool station (Kalypsys, San Diego, CA). The assay plates were incubated at 37°C for 24 hours. 5 µL of ONE-Glo Luciferase Assay reagent (Promega, Madison, WI) was added to each well using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA), followed by 30 min incubation at room temperature. The luminescence intensity of plates was recorded by a ViewLux plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [3].

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote thyroid receptor mediated gene expression, and is intended to provide information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program

National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2011

Assay Publication:

Freitas, J., Cano, P., Craig-Veit, C., Goodson, M. L., Furlow, J. D., & Murk, A. J. (2011). "Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay". Toxicol In Vitro 25(1), 257-266.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 thyroid receptor luciferase GH3 agonist assay screened a large library of diverse environmental compounds to probe for xenobiotic activity and potential to induce thyroid-dependent transcription, monitored through luciferase reporter gene signal activation using a TR-luciferase reporter gene construct. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 24-hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in agonist mode using luciferase detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

Thyroid hormone signaling is essential for normal brain development both before and after birth and has profound effects on cellular metabolism in almost all organs. One potential mechanism by which endocrine disrupting chemicals may produce toxic effects is by interfering with the ability of thyroid hormones (T3, triiodothyronine and T4, thyroxine) to direct normal development and metabolism. Thyroid hormone interfering compounds can result in neurological disorders by interfering with normal developmental processes. Thyroid hormones also have important roles in the initiation and proliferation of central nervous system and cardiovascular tissues. An important component of an endocrine disruptor screening program should be the inclusion of assays designed to screen TH disrupting compounds. The GH3_TRE_LUC cell line is a TR responsive system which is a sensitive and reliable method to evaluate the potential for xenobiotic compounds to act

as ligands to the thyroid hormone receptor. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of TR binding and interference with endogenous thyroid signaling by monitoring the increase in luminescent signals relative to a thyroid hormone (20mM T3) as a positive control, and indicator of receptor activation.

Method Development Reference:

Gutleb, A. C., Meerts, I. A., Bergsma, J. H., Schriks, M., & Murk, A. J. (2005). "T-Screen as a tool to identify thyroid hormone receptor active compounds". Environ Toxicol Pharmacol 19(2), 231-238. (PMID: 21783481)

Assay Quality Statistics:

Neutral control well median response value, by plate:	-0.040
Neutral control median absolute deviation, by plate:	1.602
Positive control well median response value, by plate:	99.59
Positive control well median absolute deviation, by plate:	12.64
Negative control well median, by plate:	NA
Negative control well median absolute deviation value, by plate:	NA
Z' (median across all plates, using positive control wells):	0.57
Z' (median across all plates, using negative control wells):	NA
SSMD (median across all plates, using positive control wells):	8
SSMD (median across all plates, using negative control wells):	NA
Signal-to-noise (median across all plates, using positive control wells):	62.07
Signal-to-noise (median across all plates, using negative control wells):	NA
Signal-to-background (median across all plates, using positive control wells):	-2484.75
Signal-to-background (median across all plates, using negative control wells):	NA
CV (median across all plates):	-39.06

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Thyroid receptor ligand-binding and agonism, measured by monitoring increased luminescence resulting from thyroid response element-driven expression of luciferase.

Analytical Elements:

The Tox21_TR_LUC_GH3_Agonist assay was monitored for increased luminescence (gain-of-signal) relative to 20mM T3 (positive control) signal, using DMSO (negative control) as a baseline for chemical-TR activity, and response was reported as a percent of positive control activity. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Thyroid receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were

determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up
ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_up
LTEA_HepaRG_THRSP_dn
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Antagonist

3.2. Assay Performance

Assay Performance Measures:

Nominal number of tested concentrations:	15
Target (nominal) number of replicates:	3
Standard minimum concentration tested:	0.001 µM
Standard maximum concentration tested:	90 µM
Baseline median absolute deviation for the assay – based on the response values at the 2 lowest tested concentrations (bmad):	0.643
Response cutoff used to derive hit calls (e.g., 5*bmad, 10*bmad):	20

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [9].

4. Assay Documentation

4.1. References

[1] Crofton, K. M. (2008). Int J Androl 31(2): 209-223.
[2] Gutleb, A. C., et al. (2005). Environ Toxicol Pharmacol 19(2): 231-238. (PMID: 21783481)
[3] Hohenwarter, O., et al. (1996). Anal Biochem 234(1): 56-59.

[4] Ghisari, M. and E. C. Bonefeld-Jorgensen (2005). Mol Cell Endocrinol 244(1): 31-41. (PMID: 16221524)
[5] Samuels, H., et al. (1988). J Clin Invest 81(4): 957.
[6] Freitas, J., et al. (2011). Toxicol In Vitro 25(1): 257-266.
[7] Freitas, J., et al. (2014). Current Chem Genom Transl Med 8: 36.
[8] Gauger, K. J., et al. (2007). Environ Health Perspect: 1623-1630.
[9] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods
NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
T3, Triiodothyronine
T4, Thyroxine
TF, Transcription Factor
TH, Thyroid Hormone
TR, Thyroid Receptor
TRE, Thyroid Hormone Response Elements

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

3 August 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information

Tox21_TR_LUC_GH3_Antagonist

Assay Name: Tox21 GH3 Cell-Based qHTS Luciferase Assay to Identify Small Molecule Antagonists of the Thyroid Receptor (TR) Signaling Pathway by Monitoring Thyroid Response Element Inhibition

1. Assay Descriptions

1.1. Overview

Assay Summary:

Thyroid receptor (TR), a nuclear hormone receptor, plays an important role in development, proliferation, differentiation, metabolism, brain function, and cardiovascular system. TR-interacting compounds have been shown to disrupt thyroid homeostasis [1]. To profile the Tox21 compound library's potential to interfere in TR signaling pathways, a cell-based GH3-TRE-Luc assay was used to measure the inhibition of TR following xenobiotic exposures. Activity was measured in GH3 (rat pituitary tumor) cells stably expressing a TR activity sensor consisting of two TR response elements and a luciferase reporter gene in 1536-well plates following 24-hour incubation with test chemicals. Increased luciferase activity indicates elevated levels of TR transactivation, and to detect TR antagonism, this assay is designed to monitor for loss-of-signal against thyroid hormone (T3) agonists. The cytotoxicity of the Tox21 compound library against the GH3.TRE-Luc cell line was tested in parallel by measuring the cell viability using CellTiter-Fluor assay (Promega, Madison, WI) in the same wells, using tetraoctylammonium bromide as a positive control for cytotoxicity.

1.2. Assay Definition

Assay Throughput:

GH3.TRE-Luc cells are aliquoted into 1536-well microtiter plates (1500 cells/5 µL/well) and incubated for 4 hours prior to 24-hour exposure to test compounds in the presence of T3 (agonist). Antagonistic activity is monitoring by measuring decreased luminescence resulting from xenobiotic-repression of TR gene expression.

Experimental System:

GH3 cell line was derived from rat pituitary tumor cells and has been routinely employed for studying effects of TH disruption using the T-screen assay [2, 3] and is reported to retain unique characteristics of the original differentiated tissue such as production of growth hormone and prolactin [3-5]. Moreover, this cell line endogenously expresses both TH receptor isoforms (α and β) in very high amounts and they respond to physiological concentrations of TH by proliferating [6]. The GH3.TRE-Luc cell line, developed in the laboratory of Dr. Albertinka J. Murk (Wageningen University) is derived from GH3 cells and stably expresses a modified firefly luciferase reporter gene under the regulation of a TR activity sensor consisting of a pair of thyroid hormone response elements (TREs). [7].

Xenobiotic Biotransformation Potential:

GH3 cells display an increased level of cell proliferation and growth hormone secretion in response to physiologic levels of thyroid hormones. TRE-LUC cells are activated by the thyroid hormone triiodothyronine (T3) and all trans retinoic acid but not RAR or LXR specific ligands. [2] CYP1A1 and 1B1 (but not 1A2) have been shown to be inducible by PCBs in GH3 cells [3].

Basic Procedure:

Materials

Supplies/Medium/Reagent	Manufacturer	Vender/Catalog Number
DMEM:F12	Invitrogen	Gibco, 10565

Fetal Bovine Serum	Hyclone	Hyclone, Sh30071.03
Pen/Strep	Invitrogen	Invitrogen, 15140
Insulin	Sigma	Sigma, I6634
Ethanolamine	Sigma	Sigma, E0135
Sodium Selenite	Sigma	Sigma, S5261
Human Apotranferin	Sigma	Sigma, T2036
Bovine Serum Albumin	Sigma	Sigma, A9647
TrypLE Express	Invitrogen	Invitrogen, 12605
PBS w/o Calcium And Magnesium	Invitrogen	Invitrogen, 14190
Recovery Cell Culture Medium	Invitrogen	Invitrogen, 12648
Centrifuge	Sorvall Legend Xtr	Thermo Fisher Science 75004520
Bioraptr Microfluidic Workstation	Beckmen	-
Pintool	Kalypsys	-
White, TC, Sterile 1536-Well Assay Plates	Greiner Bio-One	Greiner, 789173-F
Viewlux Plate Reader	Perkinelmer	-
T3 (Agonist control compound)	Calbiochem	Calbiochem, 642511
DMSO	Amresco	Kd Medical, Rge-3070
Cell Titer Glo	Promega	Promega, G7572
Tetraoctylammonium bromide	Sigma	Sigma, 294136

1. Cell handling:

1.1. Media Required:

Component	Growth Medium	Assay Medium	Thaw Medium	Freezing Medium
Recovery Cell Culture Medium	-	-	-	100%
DMEM: F12	90%	100%	90%	-
Fetal Bovine Serum	10%	-	10%	-
Pen/Strep	100U/mL-100µg/mL	-	100U/mL-100µg/mL	-
Insulin	-	10µg/mL	-	-
Ethanolamine	-	10µM	-	-
Sodium Selenite		10ng/mL	-	-
Human Apo-transferrin	-	10µg/mL	-	-
Bovine Serum Albumin	-	500µg/mL	-	-

1.2. Thawing method

1.2.1 Place 14 mL of pre-warmed thaw medium into a T75 flask

1.2.2 Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
1.2.3 Decontaminate the vial by wiping with 70% ethanol before opening in a biological safety cabinet.
1.2.4 Transfer the vial contents drop-wise into 10 mL of Thaw Medium in a sterile 15-mL conical tube
1.2.5 Centrifuge cells at 1000 rpm for 4 minutes
1.2.6 Transfer contents to the T75 tissue culture flask containing Thaw Medium and place flask in a humidified 37°C/5% CO2 incubator
1.2.7 Switch to growth medium at first passage.
1.3. Propagation method
1.3.1 Aspirate medium, rinse once in DPBS, add TrypLE Express (3 mL for a T75 flask and 5 mL for a T175 flask and 7.5 mL for T225 flask) and swirl to coat the cell evenly.
1.3.2 Add an equal volume of Growth Medium to inactivate Trypsin after 2-3 minutes incubation at 37°C.
1.3.3 Centrifuge cells at 1000 rpm for 4 minutes and resuspend in Growth Medium.
1.3.4 Cell should be passage or fed at least twice a week.

2. Assay Protocol

2.1 Harvest cells from culture in Growth Medium and resuspend in assay medium
2.2 Dispense 1500 cells/5 µL/well into 1536-well tissue treated white solid plates using a BioRAPTR dispenser.
2.3 After the cells were incubated at 37°C for 4 hours, 23 nL of compounds dissolved in DMSO, positive controls or DMSO were transferred to the assay plate by a PinTool
2.4 Dispense 1µL of T3 or buffer control using BioRaptr
2.5 Incubate the plates for 23.5 hours at 37°C.
2.6 Add 5µL of Cell Titer Glo to each well using a BioRAPTR dispenser and incubate the plate at room temperature for 30min.
2.7 Measure luminescence using Viewlux

GH3.TRE-Luc cells were dispensed at 1500 cells/4 uL/well into 1536-well white solid bottom plates using a Multidrop Combi dispenser (Thermo Fisher Scientific Inc., Waltham, MA). After the assay plates were incubated at 37 °C for 4 hours, 23 nL of library compound or DMSO controls was transferred to the assay plates by a pintool station (Kalypsys, San Diego, CA), followed by addition of 1 ul of T3 (1 nM, final concentration in the wells) to stimulate TR transactivation. The assay plates were incubated at 37 °C for 23.5 h, and then 1 uL of CellTiter-Fluor reagent (Promega, Madision, WI) of measuring cytotoxicity was added to each well using a Bioraptr Flying Reagent Dispenser (FRD) workstation (Beckman Coulter, Indianapolis, IN, USA). The plates were incubated at 37 °C and 5% CO2 for additional 30 min, and fluorescence intensity was measured by a ViewLux plate reader (PerkinElmer, Shelton, CT).

Proprietary Elements:

This assay is not proprietary; The Tox21 qHTS robotic platform has a 1536-well per run capacity and is capable of fully-automated (hands-free) assay execution (liquid dispensing and aspiration, plate centrifugation and incubation, et cetera) and signal recording (plate readout) [4]. GeneBLAzer® System is publicly available through Invitrogen.

Caveats:

The assay described here is intended to provide initial (screening) information about the capacity for a chemical to promote thyroid receptor mediated gene expression, and is intended to provide

information on a large number of diverse compounds; caution is advised with extrapolation of these results to prediction of organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems, but can aid in the prioritization of compound selection for more resource intensive toxicity studies.

1.3. Assay References

Assay Source Contact Information:

U.S. Tox21 Program
National Center for Advancing Translational Sciences [NCATS]
NIH Chemical Genomics Center [NCGC]
U.S. Environmental Protection Agency [EPA]
National Institutes of Environmental Health Sciences [NIEHS]
National Toxicology Program [NTP]
U.S. Food and Drug Administration [FDA]

Assay Contact: Ruili Huang
NIH Chemical Genomics Center, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA

Assay Publication Year:

2014

Assay Publication:

Freitas, J., Cano, P., Craig-Veit, C., Goodson, M. L., Furlow, J. D., & Murk, A. J. (2011). "Detection of thyroid hormone receptor disruptors by a novel stable in vitro reporter gene assay". Toxicol In Vitro 25(1), 257-266.

Method Updates / Confirmatory Studies:

None reported.

2. Assay Component Descriptions

Assay Objectives:

The Tox21 thyroid receptor luciferase GH3 antagonist assay screened a large library of diverse environmental compounds to probe for xenobiotic activity and potential to suppress thyroid-dependent transcription, monitored through decreased luciferase reporter gene signal activation using a TR-luciferase reporter gene construct stimulated by 20µM of the thyroid hormone T3. The assay is run in triplicate on a 1536-well microplate and bioluminescence was measured following 24-hour incubation of cells with test compounds and 30 min incubation of test system with ONE-Glo™ luciferase assay reagent to detect TR inhibition. The bioluminescent signal was monitored using a Promega ViewLux plate reader. Test compounds were selected based on various criteria, e.g., exposure hazard, physicochemical properties, availability and affordability and each assay incorporated 88 chemical duplications (each derived from the same primary stock solution as a sample chemical) and 39 different reference chemicals with known agonistic/antagonistic activities. 10% of the chemicals tested were duplicated chemical structures sourced from separate venders or from different production lots to assess sample variability. Following the incubation period, the cell culture was screened for bioluminescent signals in antagonist mode using luciferase-coupled ATP detection technology. Each compound was tested in a concentration-response format, using 15 concentrations ranging from 1.1 nM to 92 µM. Loss-of-signal due to antagonism was distinguished from cytotoxicity by Compound auto-fluorescence was monitored using auto-fluorescence assays run at interfering wavelengths to allow for filtering of artifacts before analytical endpoint evaluation.

Scientific Principles:

TH is essential for normal brain development both before and after birth and has profound effects on cellular metabolism in almost all organs. One potential mechanism by which endocrine disrupting chemicals may produce toxic effects is by interfering with the ability of thyroid hormones (T3, triiodothyronine and T4, thyroxine). Thyroid hormone interfering compounds can result in neurological disorders by interfering with normal developmental and metabolic processes. Thyroid hormones also have important roles in the initiation and proliferation of central nervous system and cardiovascular tissues. An important component of an endocrine disruptor screening program should be the inclusion of assays designed to screen TH disrupting compounds. The GH3_TRE_LUC cell line is a TR responsive system which is a sensitive and reliable method to evaluate the potential for xenobiotic compounds to act as interfering ligands to the thyroid hormone receptor. This assay is designed to screen a large, structurally diverse chemical library to identify compounds capable of interference with endogenous thyroid signaling by monitoring the increase in luminescent signals relative to a thyroid hormone (T3), positive control and indicator of receptor activation.

Method Development Reference:

Gutleb, A. C., Meerts, I. A., Bergsma, J. H., Schriks, M., & Murk, A. J. (2005). "T-Screen as a tool to identify thyroid hormone receptor active compounds". Environ Toxicol Pharmacol 19(2), 231-238. (PMID: 21783481)

Assay Quality Statistics:

3. Assay Endpoint Descriptions

3.1. Data Interpretation

Biological Response:

Thyroid receptor ligand-binding and antagonism, measured by monitoring decreased luminescence resulting from repressed thyroid response element-driven expression of luciferase

Analytical Elements:

The Tox21_TR_LUC_GH3_Antagonist assay was monitored for decreased luminescence (loss-of-signal) relative to 0.001 mM T3 (positive control, 100% activity) signal, using DMSO (negative control) as a signal baseline, and response was reported as a percent of positive control (T3) activity. Concentration-response relationships were determined based on a range of 15 chemical concentrations. All statistical analyses were conducted using R programming language, employing *tcpl* package to generate model parameters and confidence intervals. Each chemical concentration series is fitted to three predictive models; a constant function (no activity), a 4-parameter Hill function and a gain-loss function (two sequential Hill functions, which allows for curves with both increasing and decreasing trajectories). The model which produces the lowest Akaike Information Criterion (AIC) value is considered the winning model and used in further analysis as the most appropriate predictor of xenobiotic effects. Thyroid receptor activation was determined based on a chemical fulfilling the following criteria; either the median of normalized response values at a single concentration falls above the signal noise band (in this assay, any response over 5 times the baseline median absolute deviation); if the modeled top of the curve was above the established response cutoff; and if the Hill or Gain-Loss model had a lower AIC value than the Constant model. An AC_{50} (concentration in µM at 50% of maximum activity; modl_ga), Hill-slope (modl_gw for Hill, modl_gw (gain) and modl_lw (loss) for Gain-Loss functions), and maximum activity (modl_tp) were determined for each active test chemical. Winning model probability (modl_prob) and RMSE (modl_rmse) are also generated for each active chemical response series and all data are publicly available on the ToxCast data download page (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

Related ToxCast Assays:

ATG_THRa1_TRANS_up
ATG_THRb_TRANS2_up
LTEA_HepaRG_THRSP_up
LTEA_HepaRG_THRSP_dn
NVS_NR_hTRa_Antagonist
Tox21_TR_LUC_GH3_Agonist

3.2. Assay Performance

Assay Performance Measures:

Reference Chemicals / Predictive Capacity:

Chemical Library Scope and Limitations:

The ToxCast chemical library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. EPA's ToxCast inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also considered practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Operating within these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary in vivo and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. The chemical inventory used in this assay includes the "e1k" chemical inventory which includes compounds recognized as known estrogen receptor (ER) and androgen receptor (AR) active reference chemicals [9].

4. Assay Documentation

4.1. References

[1] Crofton, K. M. (2008). Int J Androl 31(2): 209-223.
[2] Gutleb, A. C., et al. (2005). Environ Toxicol Pharmacol 19(2): 231-238. (PMID: 21783481)
[3] Hohenwarter, O., et al. (1996). Anal Biochem 234(1): 56-59.
[4] Ghisari, M. and E. C. Bonefeld-Jorgensen (2005). Mol Cell Endocrinol 244(1): 31-41. (PMID: 16221524)
[5] Samuels, H., et al. (1988). J Clin Invest 81(4): 957.
[6] Freitas, J., et al. (2011). Toxicol In Vitro 25(1): 257-266.
[7] Freitas, J., et al. (2014). Current Chem Genom Transl Med 8: 36.
[8] Gauger, K. J., et al. (2007). Environ Health Perspect: 1623-1630.
[9] Richard, A. M., et al. (2016). Chem Res Toxicol Article ASAP. (PMID: 27367298)

4.2. Abbreviations and Definitions

AIC, Akaike Information Criterion
EDC, Endocrine Disrupting Compounds
FBS, Fetal Bovine Serum
FRD, Flying Reagent Dispenser
ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM, National Toxicology Program Interagency Center for Evaluation of Alternative Toxicological Methods
NR, Nuclear Receptors
qHTS, Quantitative High-Throughput Screening
T3, Triiodothyronine
T4, Thyroxine
TF, Transcription Factor
TH, Thyroid Hormone
TR, Thyroid Receptor
TRE, Thyroid Hormone Response Elements

4.3. Assay Documentation Source

Contact Information:

U.S. EPA National Center for Computational Toxicology (NCCT)
109 T.W. Alexander Drive (MD-B-205-01)
Research Triangle Park, NC 27711
919-541-4219

Date of Assay Document Creation:

3 August 2016

Date of Revisions:

Author of Revisions:

5. Supporting Information