Supporting Information

Li et al. 10.1073/pnas.1714109114

SI Materials and Methods

Cell Culture and Treatment. TK6 cells were grown in RPMI 1640 medium supplemented with 10% FBS as described previously (8). The cell culture density was maintained at a concentration of 1–10 × 10^5 cells/mL. On the day before treatment, 5 mL of cells at a concentration of 2 × 10^5 cells/mL were seeded in six-well plates. All chemicals were purchased from Sigma-Aldrich. Stocks of chemicals were prepared according to the manufacturer's instructions. Stock solutions were added to the culture medium, followed by incubation at 37 °C in 5% CO_2 for 4 h. Simultaneously, the control for each treatment was treated with the vehicle, H_2O, PBS, or DMSO. Each treatment was performed in triplicate. At the end of the 4-h treatment period, cells were pelleted and subjected to RNA isolation. Dose–response studies were performed using six concentrations with fivefold dilution for all testing chemicals to determine the concentration for the microarray experiments. For IR exposure, cells were irradiated at a dose of 4 Gy using a gamma ray (^{137}Cs) irradiator.

To assess chemicals requiring metabolic activation, cells were treated with the chemicals in the presence of 1% 5,6 benzoflavone/phenobarbital-induced rat liver S9 with NADPH generating system cofactors (Moltox) at two concentrations for 8 h. The concentration and treatment time were based on the earlier study (11).

Cell Viability Assay. The cytotoxicity of each compound was measured using the MTT assay. TK6 cells were treated with a range of doses from 3 nM to 100 μM using 10 different concentrations with threefold dilution for 4 h in triplicate. At the end of the 4-h treatment, the medium was removed and cells were washed twice with PBS. Fresh medium was added to the cells, and cells recovered at 37 °C in 5% CO_2 for 20 h. Cell viability was measured at the end of recovery period using the MTT Assay Kit (Cayman Chemical) following the manufacturer's instructions. If the IC_{50} was not clearly observed, then a second concentration range (up to 5 mM) was used.

RNA Isolation and qRT-PCR. RNA was extracted using the TRIzol protocol (Invitrogen) according to the manufacturer's instructions and then purified using the RNeasy Mini Kit (Qiagen). Purified RNA was subjected to spectrometry and Bioanalyzer analysis (Agilent) to determine the quantity and quality, respectively. qRT-PCR was performed with an iCycler (Bio-Rad) to measure the expression of *ATF3*, *CDKN1A*, and *GADD45A*. Brilliant III Ultra-Fast QRT-PCR Master Mix (Agilent) was used, and primer/probe sets for the TaqMan gene expression assays were purchased from Applied Biosystems. Each assay was done in triplicate. Expression levels of genes were normalized with *GAPDH*. The relative mRNA induction fold change was calculated (8). When more than one concentration met the criteria and behaved similarly, the lower concentration was selected.

Microarray Procedures. RNAs from the dose setting experiment of each compound at their selected concentrations were pooled together, and 5 μg of pooled RNA from treated and vehicle control was labeled with the Fairplay III Microarray Labeling Kit (Agilent). The resultant cDNA was labeled with either Cy3 or Cy5. The labeled cDNAs were then purified, and cDNA yield and dye incorporation were measured with a NanoDrop ND-1000 spectrophotometer. Hybridization to the Agilent SurePrint G3 human whole genome 8 × 60k microarray kit was conducted following the manufacturer's protocol, and dye swaps were performed. Arrays were scanned with an Agilent DNA microarray scanner. Feature Extraction version 10.7 (Agilent) was used to filter, normalize, and calculate the signal intensity and ratios. Processed data were subjected to analysis using GeneSpring version 12.0 (Agilent).

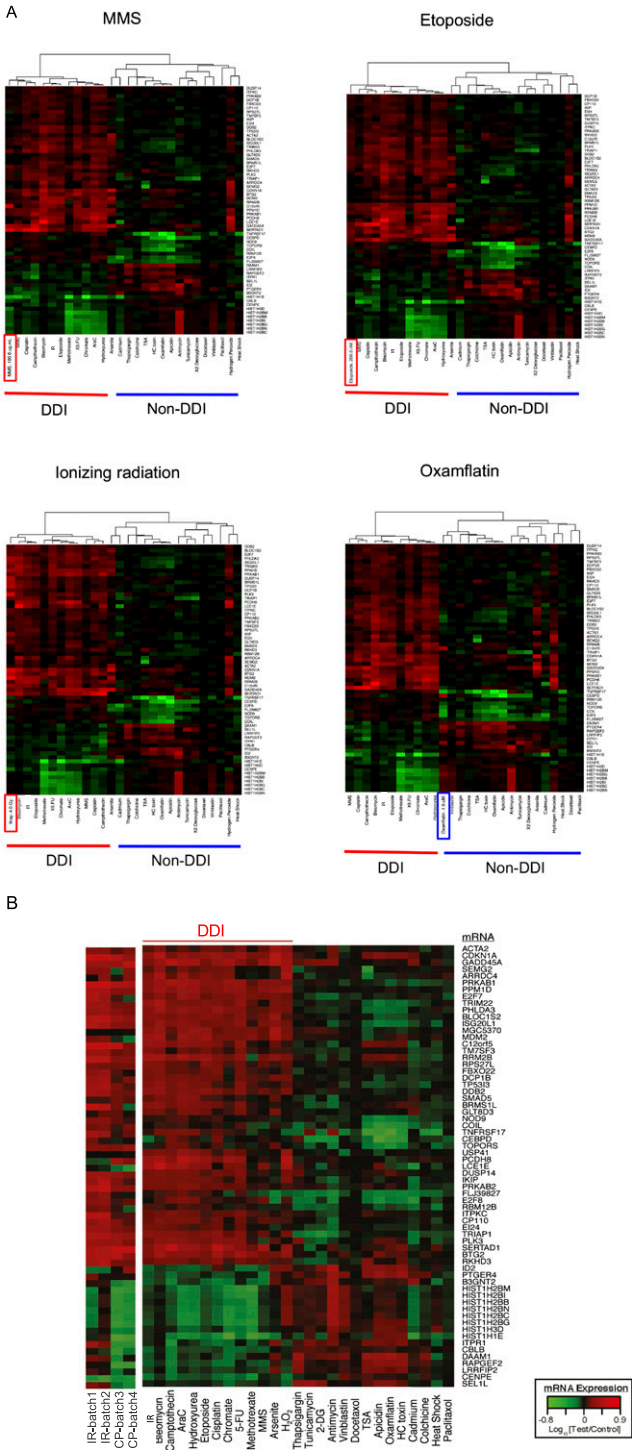


Fig. S1. Demonstration of technical robustness, reproducibility and batch variation of this toxicogenomic system. (*A*) Four agents (MMS, etoposide, oxamflatin, and IR) were selected from the original training set to confirm the reproducibility of genotoxicity prediction using the TGx-DDI biomarker. The microarray profiles for each agent were compared with the previously published dataset (8). As anticipated, the treatments clustered with their expected categories by 2DC using the TGx-DDI biomarker. For the three DNA-damaging agents (red box), the compounds clustered with the genotoxic agents. For oxamflatin (blue box), a nongenotoxicant, this agent clustered with the non–DNA-damaging agents. (*B*) Comparison of IR and cisplatin (CP) treatments that served as positive controls for different batches of cells used.

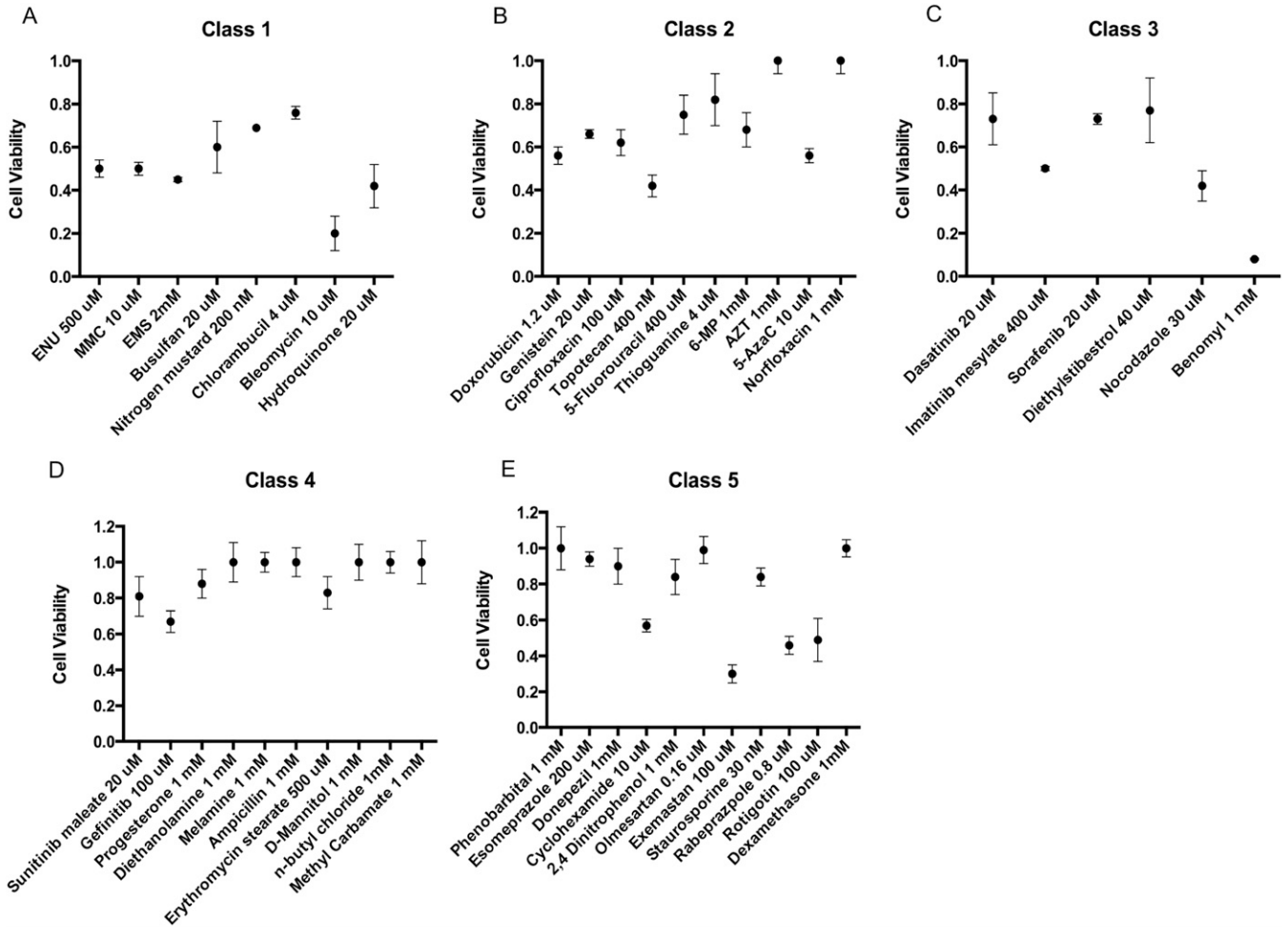


Fig. S2. Cell viability at selected doses for each compound was measured with the MTT assay. After the 4-h treatment, cells were incubated in fresh medium for 20 h. Cell viability was measured at the end of this 20-h recovery period using the MTT assay. *A–E* show the results of class 1–5, respectively. All but three compounds—bleomycin (class 1), benomyl (class 3), and exemastane (class 5)—all compounds yielded >40% viability.

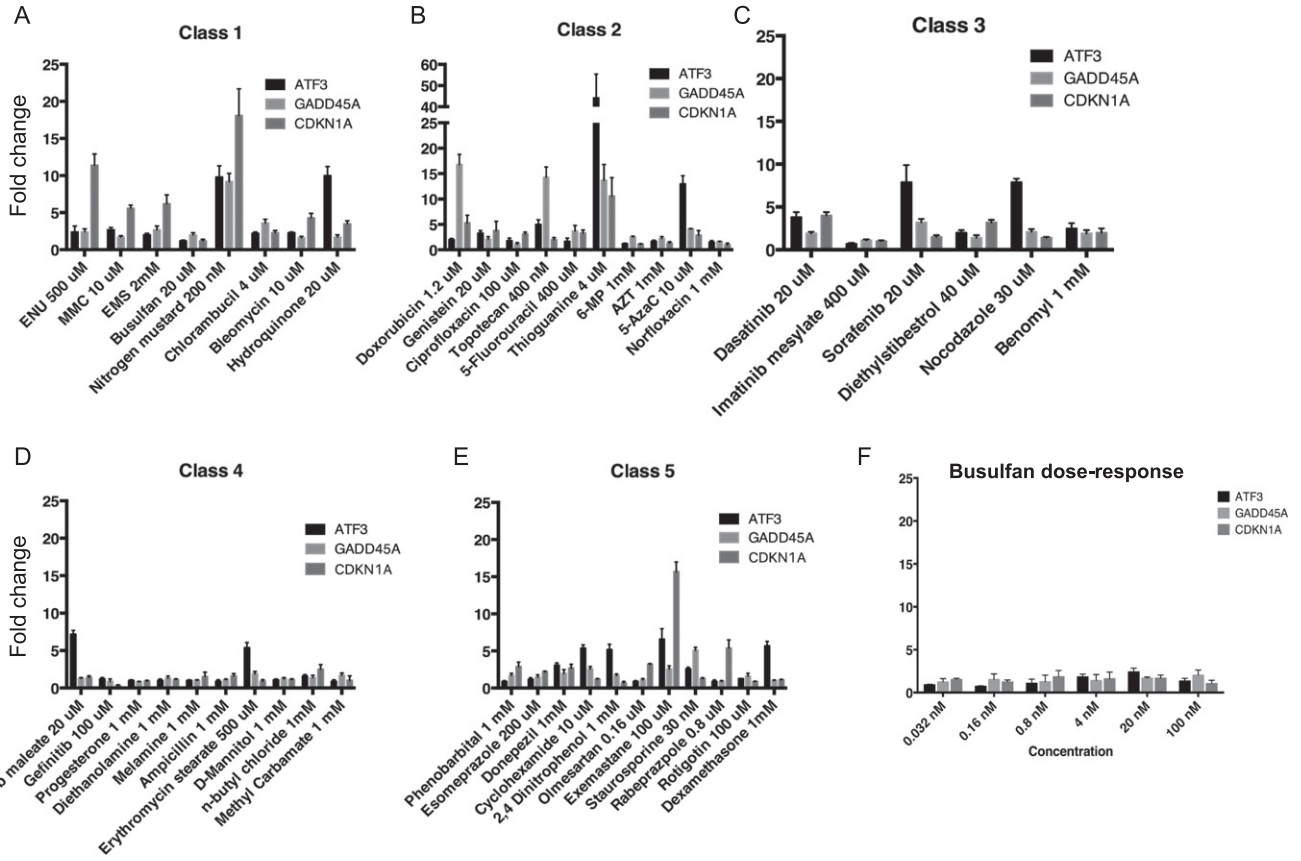
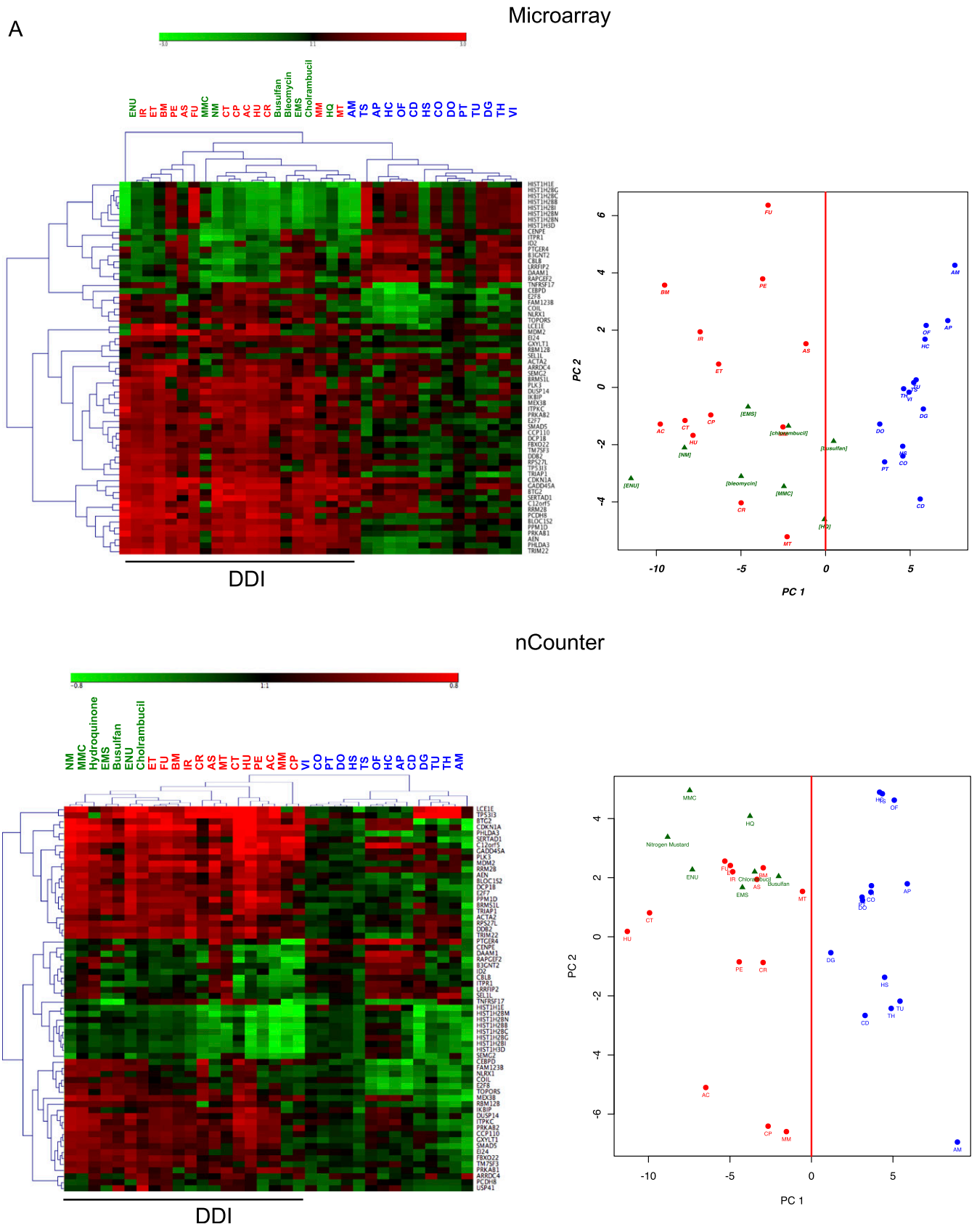


Fig. S3. Concentration range-finding studies were conducted by assessing the expression of three stress responsive genes—*CDKN1A*, *GADD45A*, and *ATF3*—using qRT-PCR. To enable comparison of transcriptome profiles across the whole set of agents at a single concentration per chemical, and to establish a strategy for setting concentrations for new test compounds, we followed the qRT-PCR stress gene panel expression protocol established in our previous study (8). The ratio designates the relative change in gene expression compared with vehicle-treated control cells. *A*–*E* are expression-level changes of these three genes for class 1–5, respectively, at the dose selected for transcriptomics. *F* shows the dose response results of busulfan. In brief, cells were treated over a broad concentration range, and results are shown for the concentration for each agent selected for subsequent microarray experiments.



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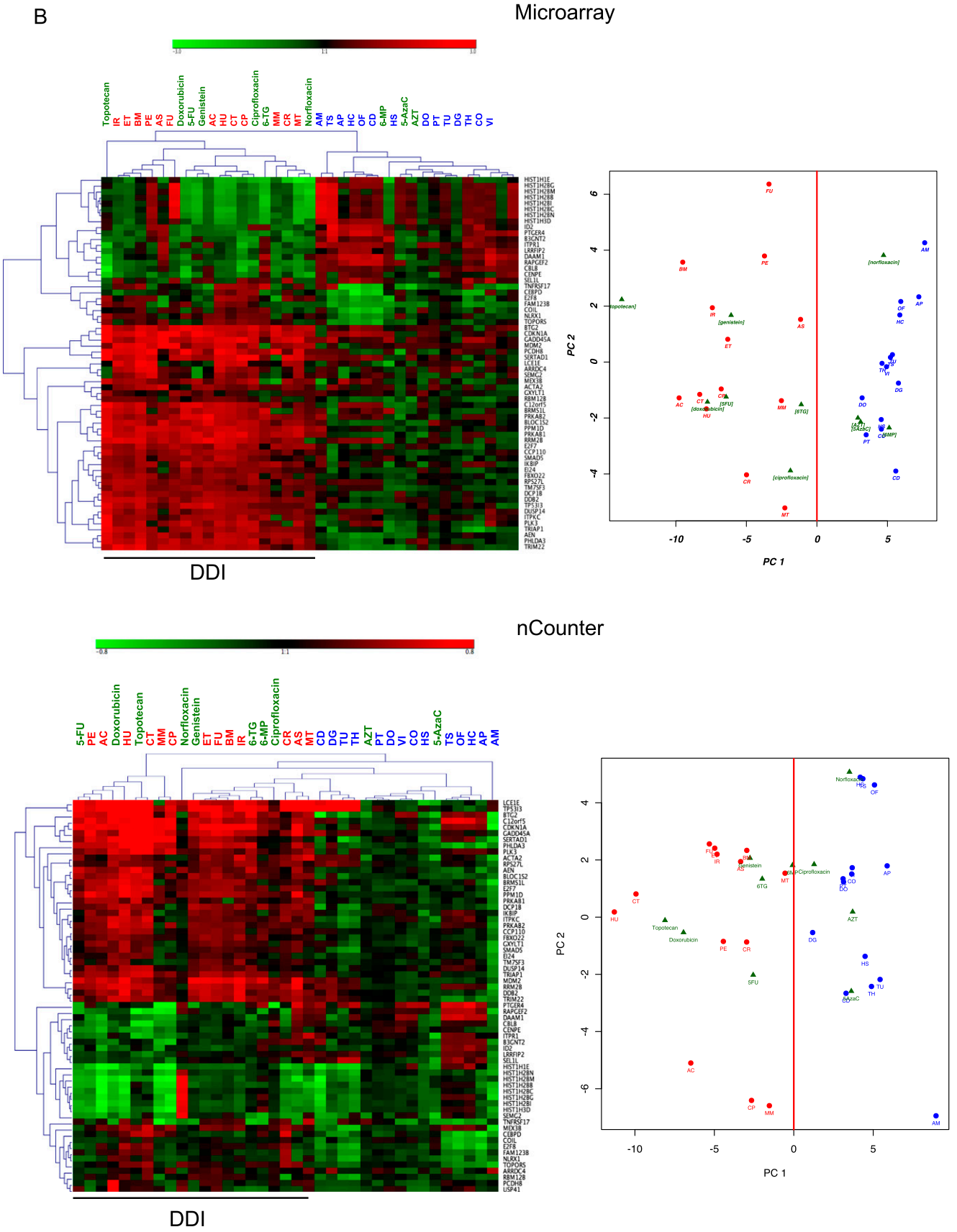

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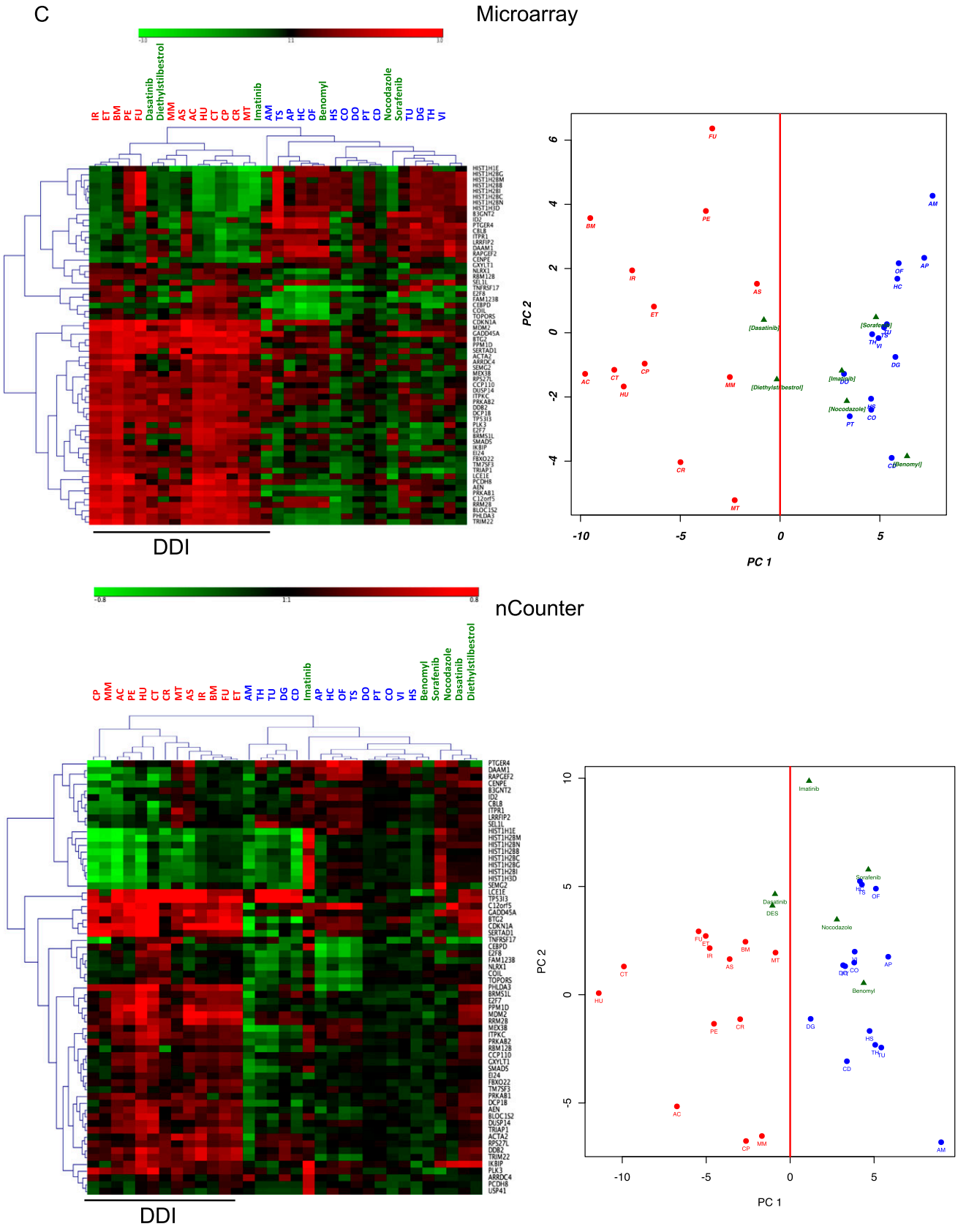

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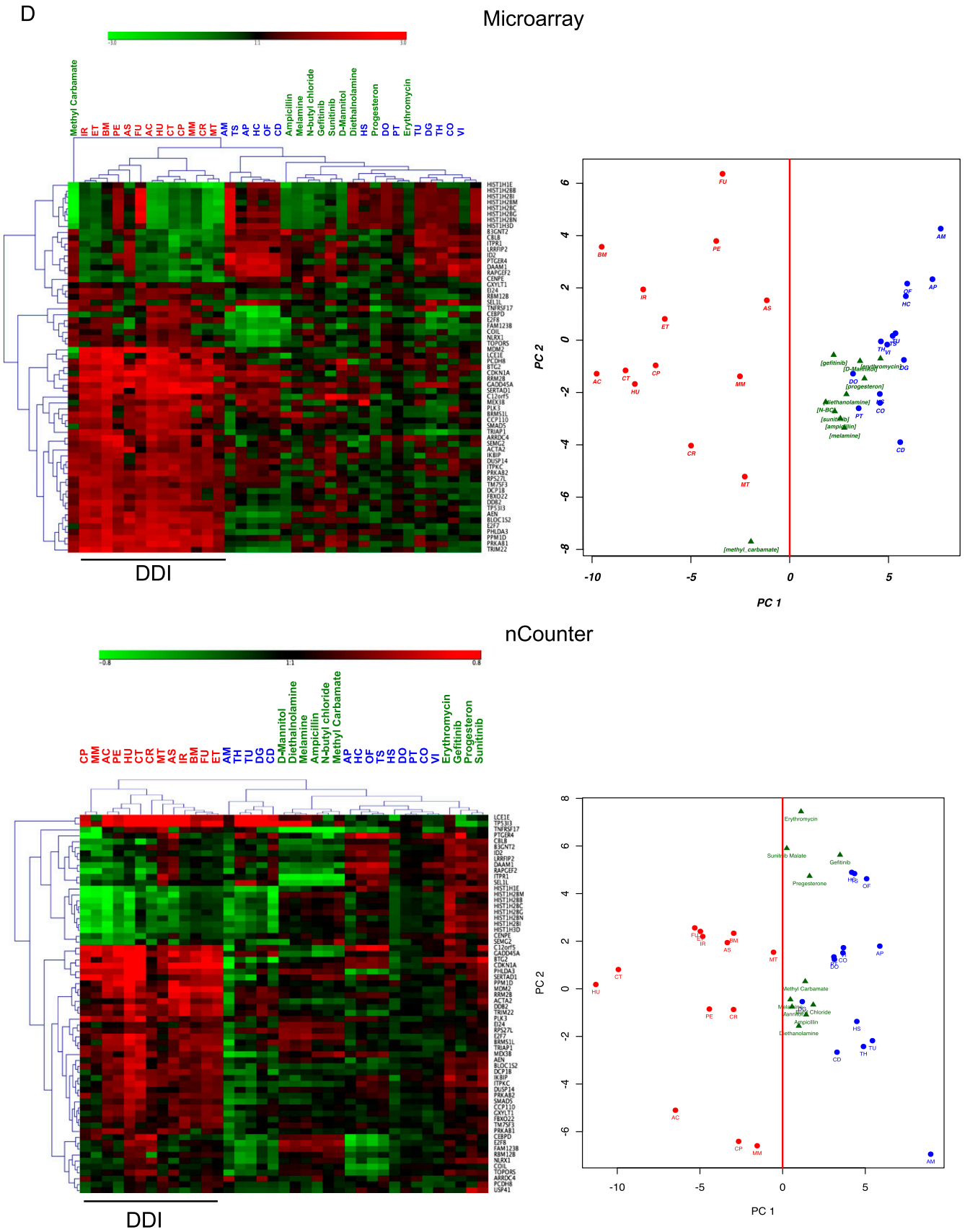

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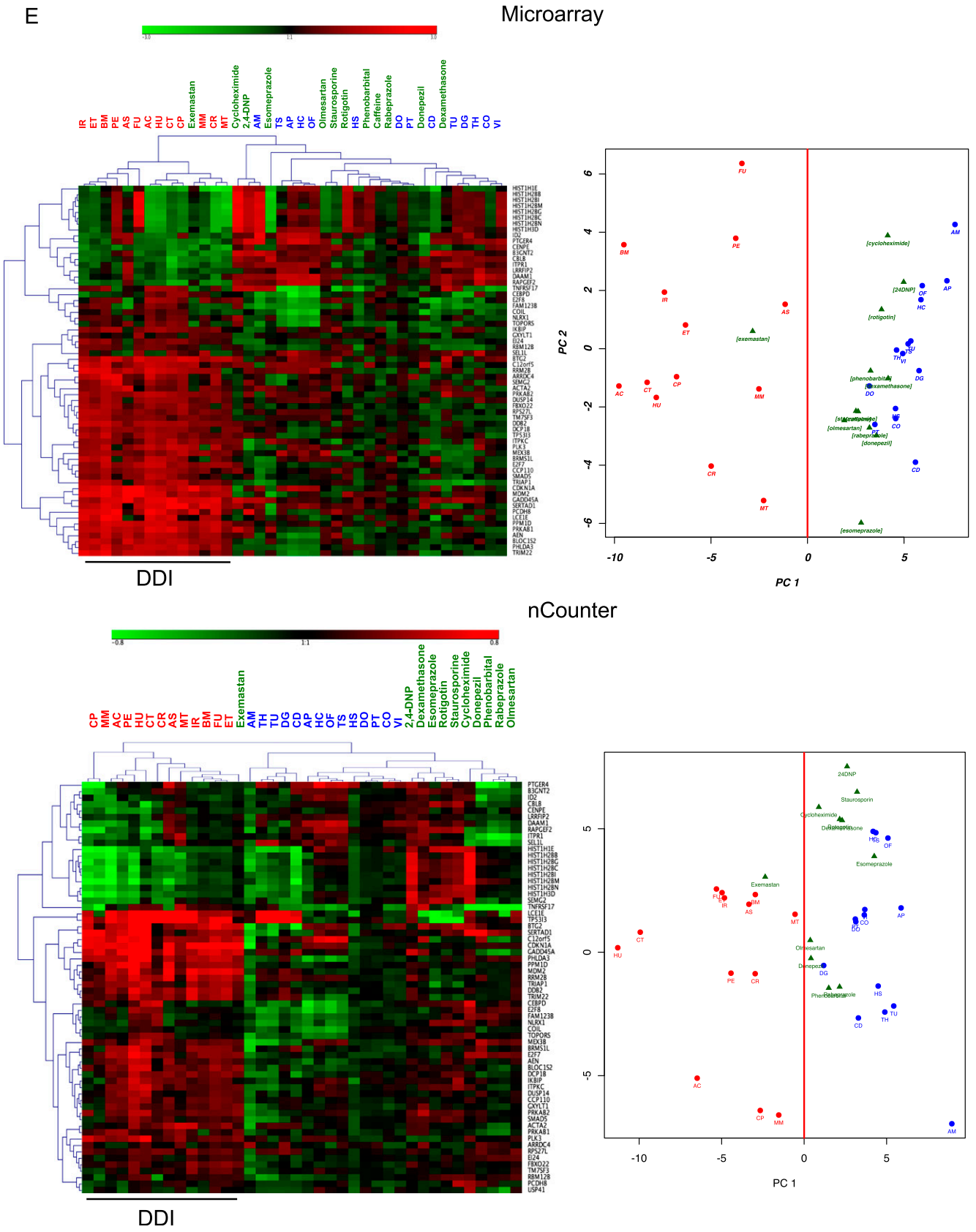
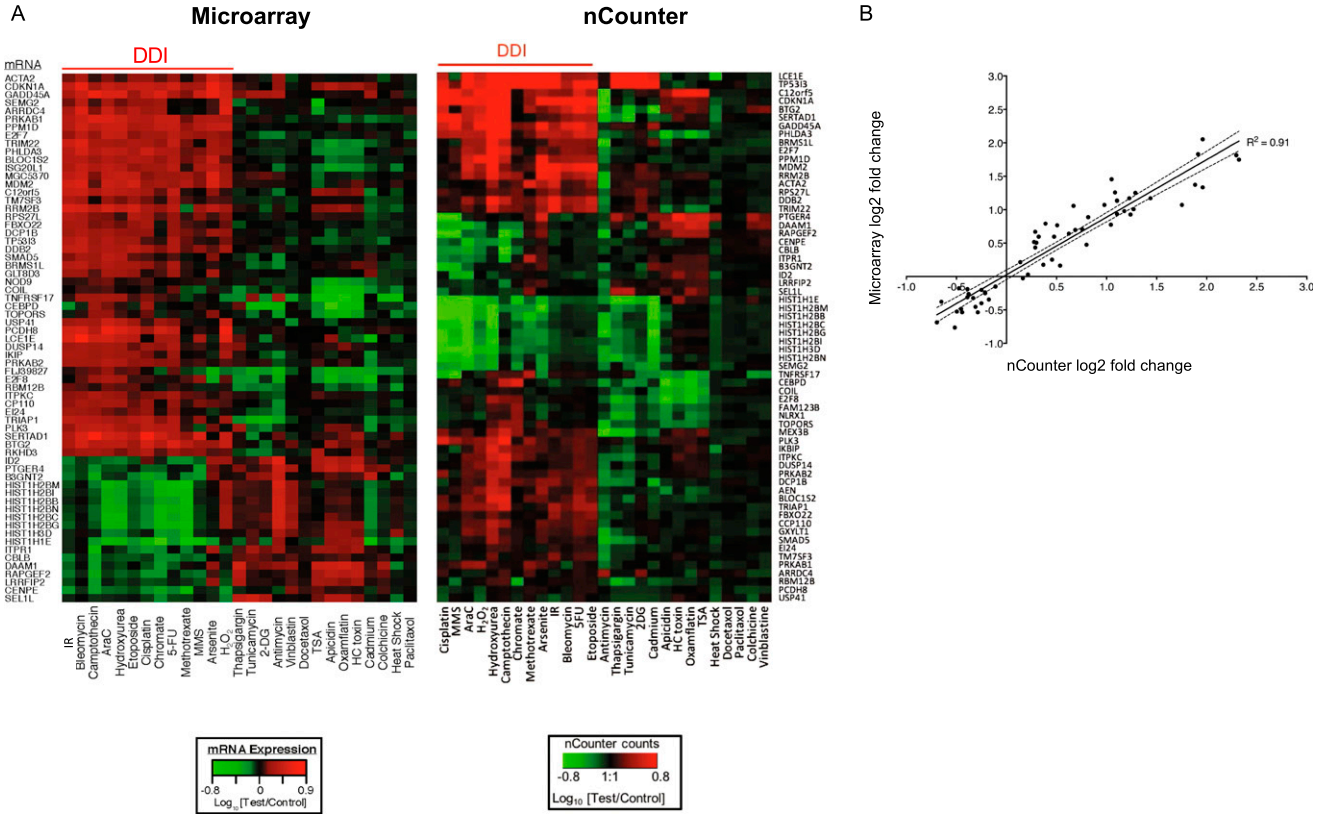


Fig. S4. Results of two DDI prediction methods using TGx-DDI, 2DC and PCA, for microarray data and nCounter data of the validation chemicals from five general classes. These two methods are described detail in *Materials and Methods*. (*Left*) 2DC results. (*Right*) PCA results. (*Top*) Microarray data. (*Bottom*) nCounter data. In both plots, the compounds in the learning set were labeled in two-letter short form, with red indicating DDI and blue indicating non-DDI.

Legend continued on following page

The short-forms for the corresponding compounds are as follows: AC, AraC; AM, antimycin; AP, apicidin; AS, arsenite; BM, bleomycin; CD, cadmium; CO, colchicine; CP, cisplatin; CR, chromate; CT, camptothecin; DG, 2-DG; DO, docetaxel; ET, etoposide; FU, 5-FU; HC, HC-toxin; HS, heat shock; HU, hydroxyurea; IR, ionizing radiation; MM, MMS; MT, methotrexate; OF, oxamflatin; PE, peroxide; PT, paclitaxel; TH, thapsigargin; TS, Trichostatin A; TU, tunicamycin; VI, vinblastin. Compounds in dark green are the test compounds in each class. NM (in class 1) stands for nitrogen mustard. In brief, a chemical clustering with the DDI branch in the 2DC plot is called DDI, and vice versa for non-DDI agents. In the PCA plot, chemicals with a negative first principal component (PC1) are classified as DDI, and those with a positive PC1 are classified as non-DDI. The plots of classes 1–5 are displayed in *A*–*E*, respectively. The microarray results show that all but one validation chemicals in class 5 are classified as non-DDI. These chemicals are known to have irrelevant positive results in a chromosomal aberration assay. Consistent with the results from microarray profiling, the nCounter results show that all but one validation chemicals in class 5 are classified as non-DDI.



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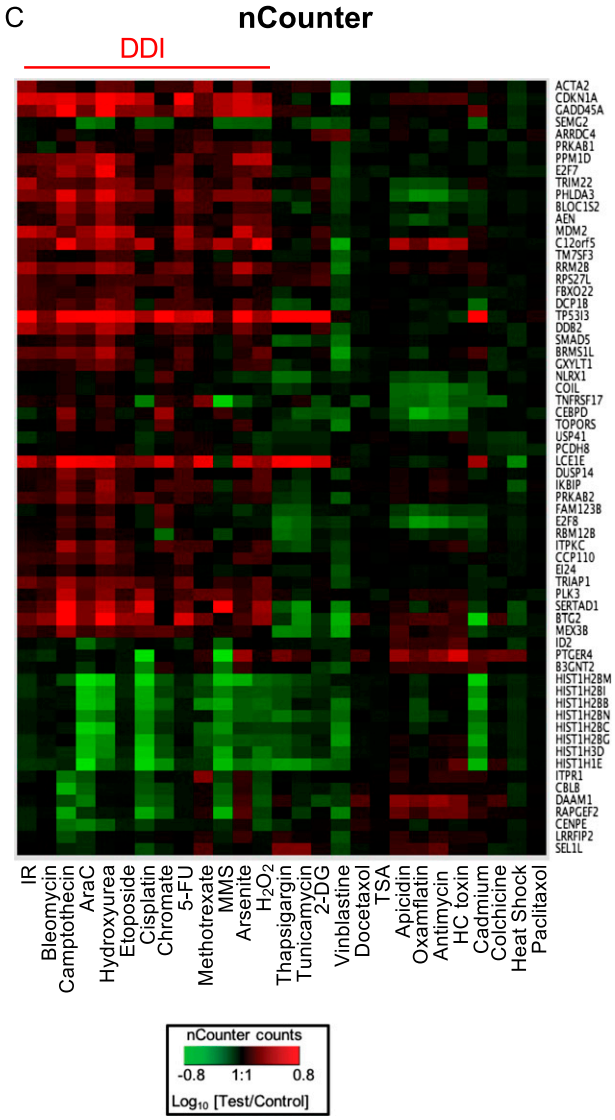

Fig. S5. Performance of TGx-DDI on different platforms. (*A*) Comparison of performance of TGx-DDI by microarray and NanoString nCounter. The DDI prediction was based on nCounter results using the 2DC method as shown in the heatmaps. (*B*) Log2 fold change correlation of genes in TGx-DDI as measured by NanoString nCounter and microarray analysis. A linear fit yields a correlation coefficient of 0.91. (*C*) nCounter results (as shown in *A*) using the orders for both genes (vertical order) and chemicals (horizontal order) identical to those of the microarray data.

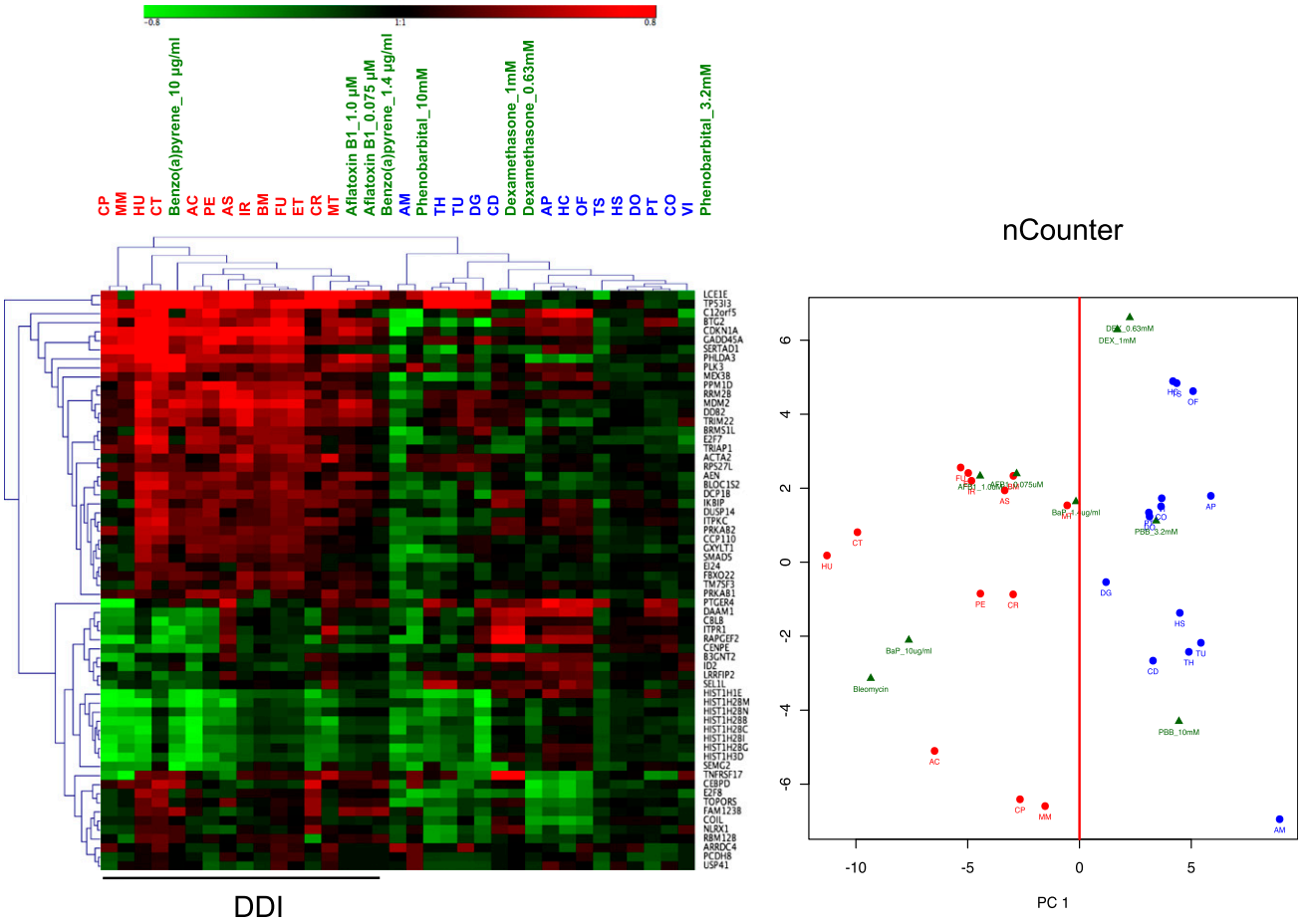

Fig. S6. 2DC (*Left*) and PCA (*Right*) analyses of nCounter data for TGx-DDI for chemicals requiring metabolic activation. As in Fig. S4, in both plots, agents in red and blue are the DDI and non-DDI agents, respectively, from the original training set. Agents in green are the validation chemicals for the five classes. In brief, chemicals clustering with the DDI branch in the 2DC plot are classified as DDI, and vice versa for non-DDI agents. In the PCA plot, chemicals with a negative first principal component (PC1) are classified as DDI, and those with a positive PC1 are classified as non-DDI.

Table S1. Representative recent references addressing issues with current in vitro genotoxic assays

Issue discussed	Summary of selected references
Assessment of specificity issue of in vitro genotoxic assays	A battery of three of the most commonly used in vitro genotoxicity tests has been evaluated for its ability to discriminate rodent carcinogens and noncarcinogens, from a large database of more than 700 chemicals. Very low specificity. When all three tests were performed, 75–95% of noncarcinogens gave positive (i.e., false positive) results in at least one test in the battery (5).
	Results of the MLA and the chromosomal aberration test obtained for rodent carcinogens and noncarcinogens were analyzed in more detail. Correlation between tumor profile and pattern of genotoxicity results was investigated (27).
Discussion of causative factors in false-positive results	Genotoxicity tests in mammalian cells in vitro produce a remarkably high and unacceptable occurrence of irrelevant positive results (e.g., compared with rodent carcinogenicity).
	Cell lines commonly used for genotoxicity testing have various deficiencies that may contribute to the high false- positive rate (28).
	High concentrations of test compounds and high levels of cytotoxicity are potential sources of false-positive results (28).
	"Promiscuous activation," the decisive in vivo enzyme, is missing in vitro (28).
	Underestimation of cytotoxicity may lead to selection of inappropriately toxic concentrations for analysis, with the potential for generation of irrelevant positive results (29).
Guideline for follow-up actions from positive results and considerations of new assays	A flowchart provides guidance on the decision process for follow-up actions in case of clear positive results in vitro (30).
	Consideration of a hypothesized mode of action is a powerful approach to direct possible follow-up testing for positive results in vitro (30).
	Lists of three groups of chemicals are recommended for assessment of the performance of new genotoxicity tests. One group is for irrelevant positive results in vitro (31).
	Cell systems, preferably of human origin, that are p53 and DNA-repair proficient, have defined phase 1 and phase 2 metabolism covering a broad set of enzyme forms, and are used within the context of appropriately set limits of concentration and cytotoxicity offer the best hope for reduced false-positives in the future (28).

While the rate of false (or irrelevant) positives often varies with the particular study, it remains substantial.

Table S2. Correlation coefficient for cisplatin treatments

Sample ID	Cisplatin 1	Cisplatin 2	Cisplatin 3	Cisplatin 4
Cisplatin 1	–	0.97	0.95	0.96
Cisplatin 2	0.97	–	0.95	0.95
Cisplatin 3	0.95	0.95	–	0.96
Cisplatin 4	0.96	0.95	0.96	–

Correlation coefficiency is calculated across the four replicate treatments with 80 μM cisplatin on 432 significantly perturbed genes relative to solvent controls ($P < 0.01$, Bonferroni correction).

Table S3. Validated compounds listed by class, with the assessed concentration and summarized results of toxicogenomic assay and the genotoxicity battery findings

Class	Agents	Concentration	Determination
Class 1	*N*-ethyl-*N*-nitrosourea (ENU)	500 μM	RT-PCR
	Mitomycin (MMC)	10 μM	RT-PCR
	Ethyl methanesulfonate (EMS)	2 mM	RT-PCR
	Bleomycin	20 μM	RT-PCR
	Nitrogen mustard	200 nM	RT-PCR
	Chlorambucil	4 μM	RT-PCR
	Busulfan	10 μM	RT-PCR
	Hydroquinone	20 μM	RT-PCR
Class 2	Topoisomerase inhibitors		
	Doxorubicin	1.2 μM	RT-PCR
	Genistein	20 μM	RT-PCR
	Topotecan	400 nM	RT-PCR
	Norfloxacin	1 mM	Specified
	Ciprofloxacin	100 μM	RT-PCR
	Antimetabolites		
	5-Fluorouracil (5-FU)	400 μM	RT-PCR
	Thioguanine (6-TG)	4 μM	RT-PCR
	Thiopurine (6-MP)	1 mM	RT-PCR
	Azidothymidine (AZT)	1 mM	RT-PCR
	5-Azacytidine (5-AzaC)	10 μM	RT-PCR
Class 3	Antimitotic agents		
	Diethylstilbestrol	40 μM	RT-PCR
	Nocodazole	30 μM	RT-PCR
	Benomyl	1 mM	RT-PCR
	Kinase inhibitors		
	Dasatinib	20 μM	RT-PCR
	Imatinib mesylate	400 μM	Cytotoxicity
	Sorafenib	20 μM	RT-PCR
Class 4	Kinase inhibitors		
	Sunitinib maleate	20 μM	RT-PCR
	Gefinitib	100 μM	Cytotoxicity
	Nongenotoxic carcinogens		
	Progesterone	1 mM	Specified
	Diethanolamine	1 mM	Specified
	Melamine	1 mM	
	Antibiotics		
	Ampicillin	1 mM	Specified
	Erythromycin stearate	500 μM	RT-PCR
	Others		
	D-Mannitol	1 mM	Specified
	n-Butyl chloride	1 mM	Specified
	Methyl carbamate	1 mM	Specified
Class 5	Phenobarbital	1 mM	RT-PCR
	Esomeprazole	200 μM	RT-PCR
	Donepezil	1 mM	RT-PCR
	Cyclohexamide	10 μM	RT-PCR
	2,4-Dinitrophenol (2,4-DNP)	1 mM	RT-PCR
	Olmesartan	0.16 μM	RT-PCR
	Exemastan	100 μM	RT-PCR
	Rabeprazole-NA	0.8 μM	RT-PCR
	Rotigotin	100 μM	Cytotoxicity
	Dexamethasone	1 mM	RT-PCR
	Staurosporine	30 nM	RT-PCR

The concentration used in microarray experiments is shown in the second column. The dose determination in the third column was based either on the induction of stress genes (qRT-PCR) or on cytotoxicity. In the cases where neither stress gene induction nor cytotoxicity was seen, 1 mM was used ("specified").

Table S4. Comparison of gene expression measurement technologies: global profiling (microarray and RNA-Seq) and targeted methods (multiplex qRT-PCR and nCounter)

	Profiling		Targeted	
Feature	Microarray	RNA-Seq	qRT-PCR	nCounter
Sensitivity	Medium	Medium/High	High	High
Need amplification and/or library	Yes	Yes	Yes	No (thus great precision)
High-throughput	No	No	No	Yes
Automated	No	No	Yes	Yes
Cost of reagents	High	High	Medium	Low
Cost of labor	High	High	Medium	Low
Readout	Analog	Digital	Analog	Digital