Evaluation of Per- and Poly fluoroalkyl Substances (PFAS) *in vitro* toxicity testing for developmental neurotoxicity

Kelly E. Carstens[†*], Theresa Freudenrich[†], Kathleen Wallace[†], Seline Choo[‡], Amy Carpenter[‡], Marci Smeltz[§], Matthew S. Clifton[§], W. Matthew Henderson[§], Ann M. Richard [†], Grace Patlewicz [†], Barbara A. Wetmore[†], Katie Paul Friedman[†], Timothy Shafer[†]

[†]Center for Computational Toxicology and Exposure, ORD, US EPA, RTP, NC 27711
[‡]Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, TN 37830
[§]Center for Environmental Measurement and Modeling, ORD, US EPA, RTP, NC 27711

Corresponding author:
Kelly E. Carstens
109 T.W. Alexander Drive, Mail Drop D130-A
Research Triangle Park, NC 27711
Email: Carstens.kelly@epa.gov
Tel. 919-541-3834

Table of Contents

Figure S1: PFAS screening approach in the DNT NAMs
An original PFAS procurement set and previous DNT screening data (1) including 74 PFAS were screened in only mc screening. A subset of 136 PFAS were screened in a tiered screening approach (2) whereby PFAS were first screened in sc screening and subsequently screened in mc screening if the chemical demonstrated at least one positive response in the sc screening using a tiered screening analysis method described in the Supporting Information Methods. Note that the activity classifications ('Active', 'Inactive') from the tiered screening analysis differ from the ToxCast Analysis Pipeline (tcpl) due to differences in the methods (Supporting Information Methods).

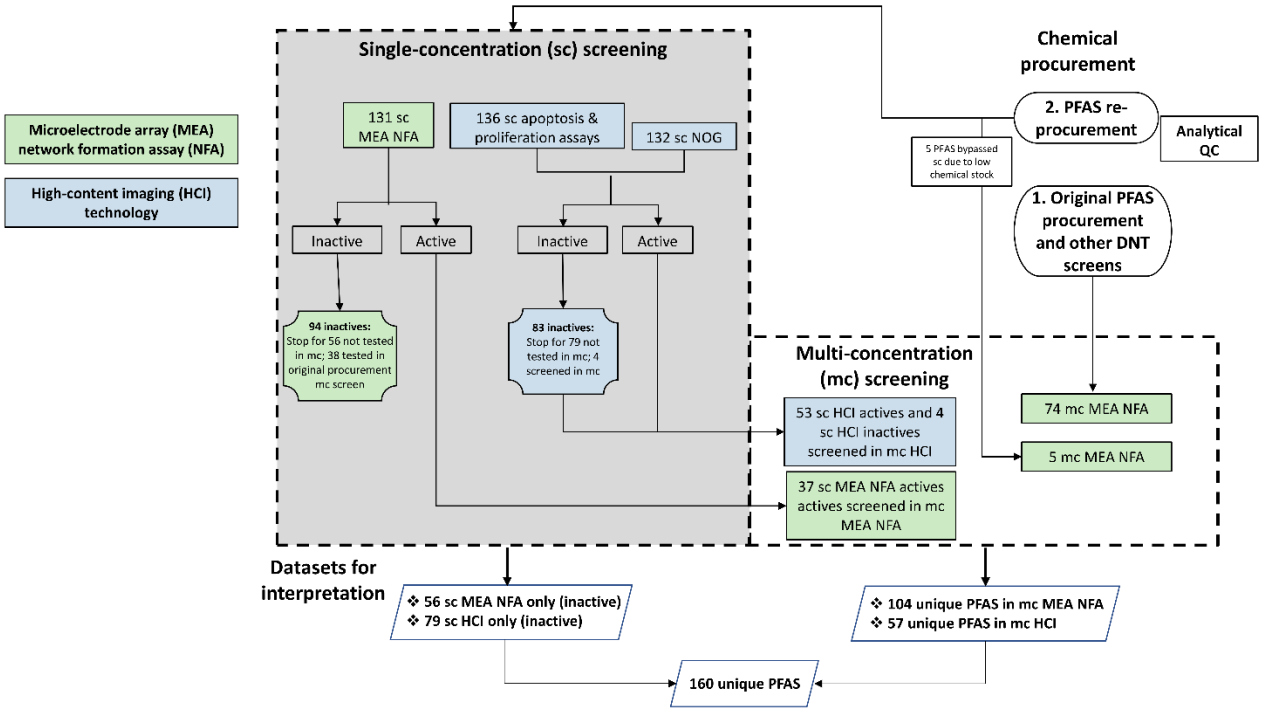


Figure S2: Frequency of PFAS representing six structural categories.
Of the 160 PFAS screened in the DNT NAM battery, 89 PFAS were captured by the six distinct structural groups based on classification guidance provided by the OECD/UNEP Global PFC group. The perfluoroalkyl acids (PFAAs) had the largest representation of the six structural groups with 29 PFAS. FASA: perfluoroalkane sulfonamide.

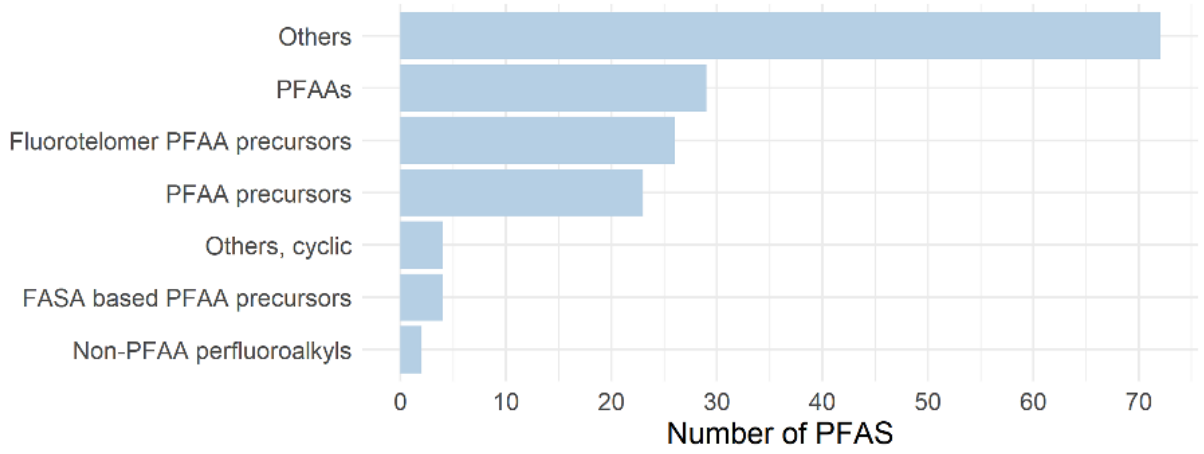


Figure S3. Analytical QC results and physicochemical properties
QC pass/ fail results were compared across four physicochemical properties: molecular weight (MW), logP, boiling point (BP), and log10 vapor pressure (log10.VP). The shapes indicate whether the PFAS was active or inactive in the DNT NAMs (active was defined as a chemical demonstrating at least one positive response; chemicals classified as equivocal were classified as inactive in this analysis). Horizontal lines indicate the mean, and the p-value indicates significance by a Student's t-test.

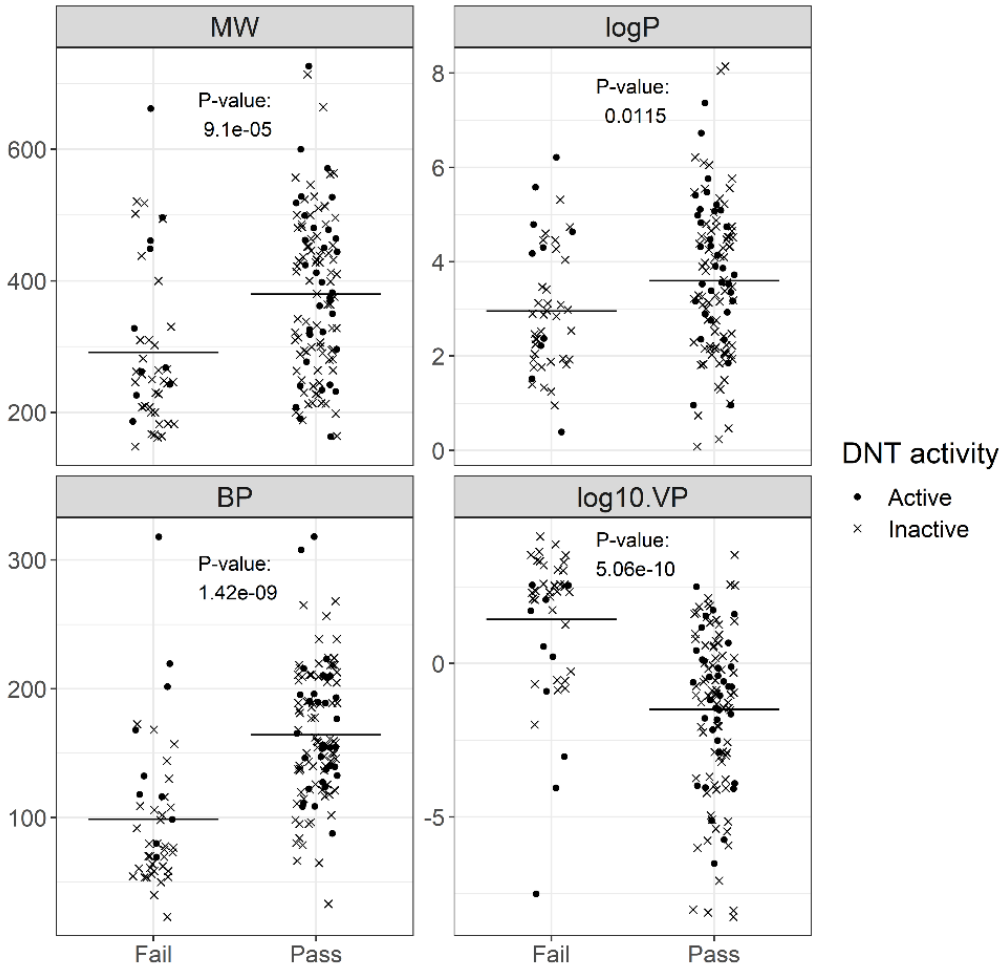
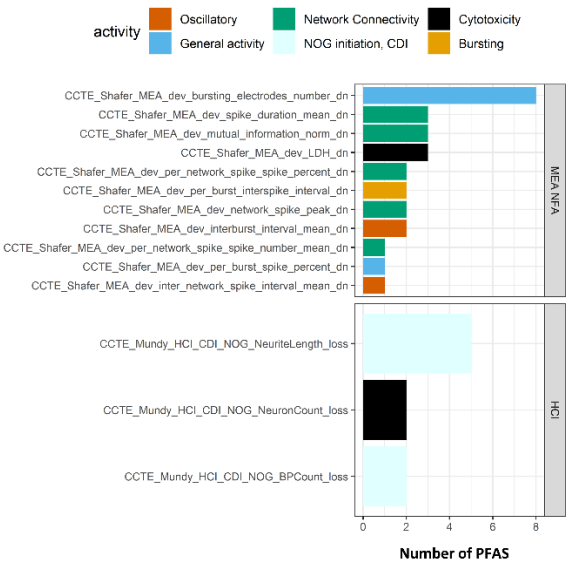


Figure S4: Assay endpoint sensitivity to PFAS
Ai: Bar plot indicates the number of chemicals for which each endpoint determined the minimum AC_{50} value per chemical. Bars are pseudo-colored by activity type. Note that not all PFAS were tested in both assay technologies therefore plots are faceted by assay technology. Aii: Table indicating the mean AC_{50} value by endpoint for all positive responses in the mc DNT NAM screening, excluding PFAS that failed QC. B: Concentration response curves (generated using the 'tcpl_v.2.1.0' R package) for the eight PFAS for which the endpoint measuring 'bursting_electrodes_number_dn' determined the minimum AC_{50} value. Red numbers indicate the curve ID shown in the table in C. C: Table with interpretation and conclusion for each concentration response curve shown in B. The 'hit sum' indicates the sum of positive responses for each chemical in the DNT NAM mc screening, the 'N mc endpoints tested' indicates the total number of endpoints measured for each chemical, 'Mean AC_{50}' and 'StDev AC_{50}' indicate the mean and standard deviation of the AC_{50} values for each compound in log10-uM and in uM, the 'QC Pass/ Fail' indicates the analysitcal QC testing results, and the curve ID corresponds to the curves shown in B.

Figure S4A

i) Endpoint that determines the minimum potency (AC_{50}) by chemical



ii) Mean AC_{50} by endpoint (excluding QC fail PFAS)

Endpoint	Mean AC_{50} (log10-uM)	StDev AC_{50} (log10-uM)
CCTE_Shafer_MEA_dev_bursting_electrodes_number_dn	0.315	0.529
CCTE_Shafer_MEA_dev_network_spike_number_dn	0.392	0.321
CCTE_Shafer_MEA_dev_per_burst_interspike_interval_dn	0.436	0.863
CCTE_Shafer_MEA_dev_burst_rate_dn	0.456	0.303
CCTE_Shafer_MEA_dev_mutual_information_norm_dn	0.474	0.383
CCTE_Shafer_MEA_dev_active_electrodes_number_dn	0.518	0.474
CCTE_Shafer_MEA_dev_per_network_spike_spike_percent_dn	0.548	0.359
CCTE_Shafer_MEA_dev_firing_rate_mean_dn	0.553	0.196
CCTE_Shafer_MEA_dev_network_spike_peak_dn	0.599	0.571
CCTE_Shafer_MEA_dev_network_spike_duration_std_dn	0.699	0.298
CCTE_Shafer_MEA_dev_per_burst_spike_percent_dn	0.758	0.298
CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean_dn	0.772	0.420
CCTE_Shafer_MEA_dev_burst_duration_mean_dn	0.783	0.368
CCTE_Shafer_MEA_dev_inter_network_spike_interval_mean_dn	0.794	0.136
CCTE_Shafer_MEA_dev_correlation_coefficient_mean_dn	0.832	0.404
CCTE_Shafer_MEA_dev_spike_duration_mean_dn	0.866	0.370
CCTE_Mundy_HCI_CDI_NOG_BPCount_loss	0.932	0.717
CCTE_Shafer_MEA_dev_interburst_interval_mean_dn	0.952	0.472
CCTE_Shafer_MEA_dev_LDH_dn	1.094	0.301
CCTE_Mundy_HCI_CDI_NOG_NeuronCount_loss	1.095	0.530
CCTE_Mundy_HCI_CDI_NOG_NeuriteLength_loss	1.104	0.384
CCTE_Shafer_MEA_dev_AB_dn	1.194	0.236
CCTE_Mundy_HCI_hNP1_Casp3_7_gain	NA	NA
CCTE_Mundy_HCI_hNP1_CellTiter_loss	NA	NA
CCTE_Mundy_HCI_hNP1_Pro_ObjectCount_loss	NA	NA
CCTE_Mundy_HCI_hNP1_Pro_ResponderAvgInten_loss	NA	NA
CCTE_Mundy_HCI_CDI_NOG_NeuriteCount_loss	NA	NA

Figure S4B

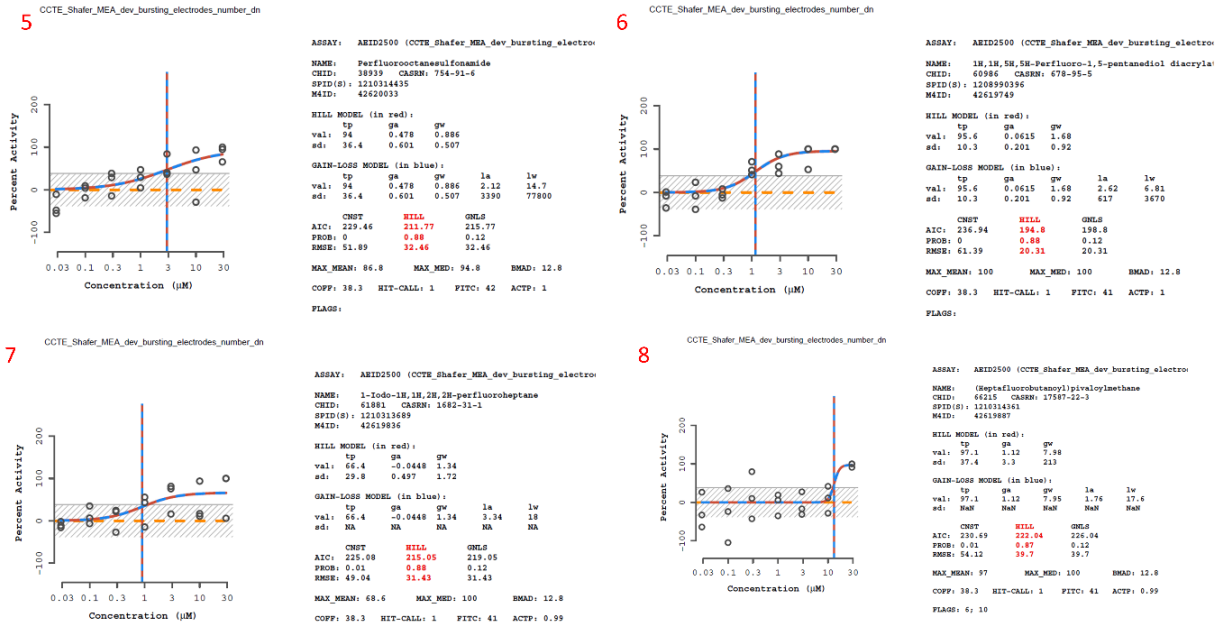
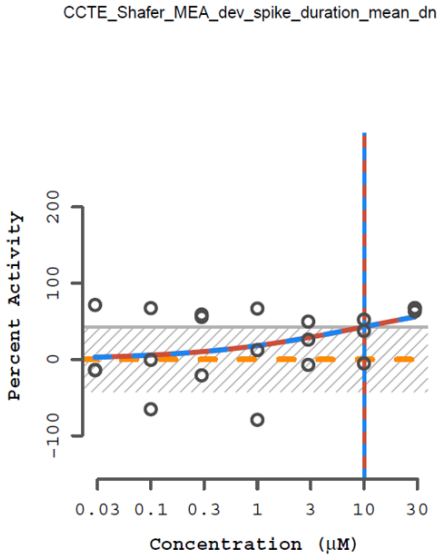


Figure S4C

DTXSID	Hit sum	N mc endpoints tested	Mean AC50 (log10-uM)	StDev AC50 (log10-uM)	Mean AC50 (µM)	StDev AC50 (µM)	QC Pass/ Fail	Concentration response curve ID (Supplemental Figure 4B)	Evaluation of the 'MEA_dev_bursting_electrodes_number_dn' curves (Supplemental Figure 4C)	Conclusion
DTXSID50218052	1	19	-1.001		0.100		F	1	This chemical demonstrates a low hit rate (1/19). There were no flags associated with the winning model. The low AC_{50} of -1.0 log10-µM appears to be associated with the gain-loss model as the winning model.	Although the model appears to be a good fit, based on the rule that any chemical that was only active in one mc MEA NFA endpoint (excluding cytotoxicity) is equivocal, this bioactivity was classified as **equivocal**. This compound also failed QC therefore bioactivity should be interpreted with caution.
DTXSID80379721	17	27	0.505	0.099	3.202	1.257	P	2	This chemical demonstrates a high hit rate (17/27 active endpoints) and the winning model fit was flagged for noise.	High confidence **active** based on the hit rate.
DTXSID90558000	7	27	0.375	0.816	2.374	6.549	P	3	This chemical demonstrates a high hit rate (7/27 active endpoints) and the winning model fit was flagged for borderline activity and efficacy values less than 50%.	High confidence **active** based on the hit rate.
DTXSID30627108	17	27	0.669	0.138	4.663	1.375	P	4	This chemical demonstrates a high hit rate (17/27 active endpoints) and the winning model fit looks good.	High confidence **active** based on the hit rate and curve.
DTXSID3038939	11	27	1.084	0.302	12.142	2.003	P	5	This chemical demonstrates a high hit rate (11/27 active endpoints) and the winning model fit looks good.	High confidence **active** based on the hit rate.
DTXSID5060986	13	27	0.474	0.237	2.979	1.725	P	6	This chemical demonstrates a high hit rate (13/27 active endpoints) and the winning model fit looks good.	High confidence **active** based on the hit rate and curve.
DTXSID9061881	8	19	0.981	0.424	9.565	2.653	P	7	This chemical demonstrates a high hit rate (8/19 active endpoints) and the winning model fit looks noisy but was not flagged for noise by tcpl.	High confidence **active** based on the hit rate.
DTXSID3066215	2	27	1.124	0.002	13.294	1.004	P	8	This chemical demonstrates a low hit rate (2/27 active endpoints) and the winning model fit was flagged for noise and activity only at the highest concentration. Given the low hit rate, the other positive response was inspected, which indicated cytotoxicity in the 'MEA_NFA_dev_LDH_dn' endpoint at a similar concentration (1.12 log10-µM versus 1.13 log10-µM, respectively.	High confidence **active**; the decrease in the number of bursting electrodes was likely associated with cytotoxic activity at the highest concentration tested.

Figure S5: Concentration response curve PFOA positive response
Concentration response curve (generated using the 'tcpl_v.2.1.0' R package) for the one positive response for PFOA in the mc MEA NFA.




```
ASSAY:   AEID2514 (CCTE_Shafer_MEA_dev_spike_duration_m

NAME:    Perfluorooctanoic acid
CHID:    31865   CASRN: 335-67-1
SPID(S): 1483052
M4ID:    42626756

HILL MODEL (in red):
      tp       ga       gw
val:  85.8     1        0.569
sd:   72.7     1.38     0.535

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  85.8     1        0.569    2.17     11.1
sd:   72.6     1.38     0.537    581      9280

      CNST       HILL       GNLS
AIC:  229.35     224.79     228.79
PROB: 0.08       0.81       0.11
RMSE: 50.02      41.28      41.28

MAX_MEAN: 66       MAX_MED: 67.3      BMAD: 14.2

COFF: 42.5   HIT-CALL: 1    FITC: 42   ACTP: 0.92

FLAGS:
```

Figure S6: Ranked AUC sum in the MEA NFA
The ranked AUC sum was calculated by taking the sum of all AUCs across the MEA NFA endpoint per PFAS (log2 transformed) and ranking the chemicals from highest to lowest. Tributyltin is included as a positive control for DNT NAM activity. Data points are pseudo-colored with the OECD structural category groupings.

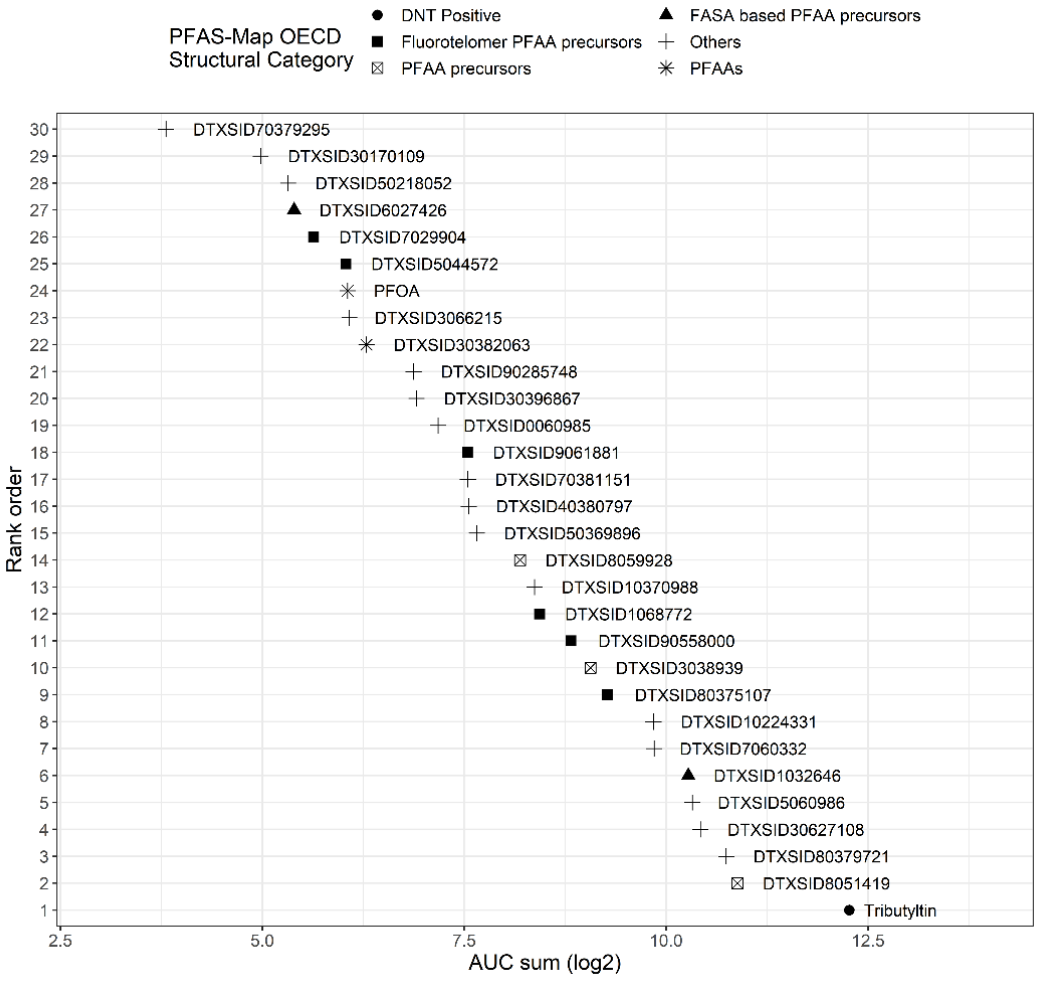


Figure S7: Heatmap of PFAS efficacy and/or potency in the HCI
Rows of the heatmap indicate chemical activity in each activity type. Column color legend indicates the efficacy and potency as measured by an area under the curve (AUC) metric. Yellow indicates inactivity (AUC of zero), whereas increasing pink to black colors indicates increasing AUC values (higher efficacy and/or potency). Tributyltin is included as a historically DNT NAM-active compound. Color of row text label (right) indicates whether the PFAS passed QC (black) or failed QC (red). An asterisk following the row text label indicates that the PFAS bioactivity was equivocal. Row color legend (left) indicates the number of C atoms in each chemical binned into three groups.

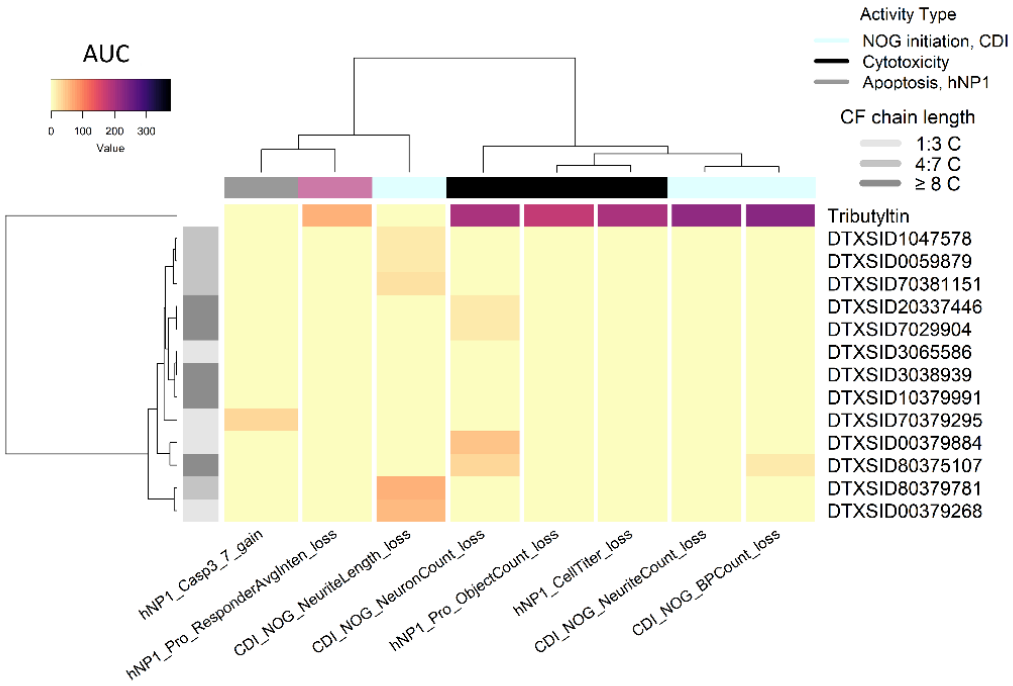


Figure S8: A, B: Concentration response curves for positive responses in HCI mc screening assays Concentration response curves (generated using the 'tcpl_v.2.1.0' R package) for the active PFAS in the HCI mc screening assays.

Figure S8A

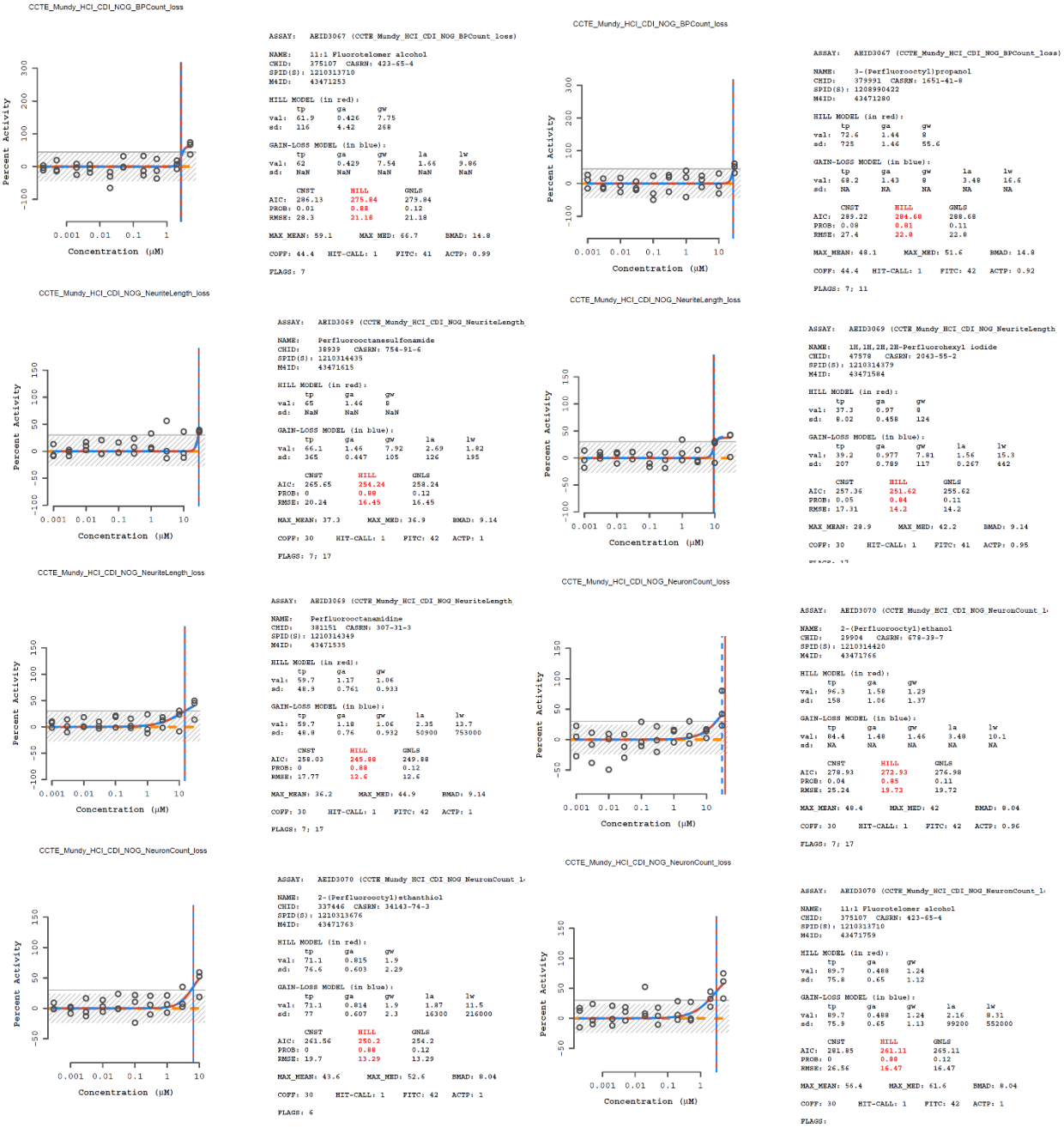
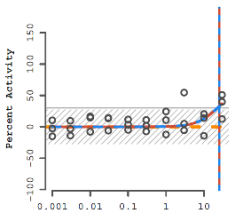


Figure S8B

CCTE_Mundy_HCI_CDI_NOG_NeuriteLength_loss

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ASSAY:   AEID3069 (CCTE_Mundy_HCI_CDI_NOG_NeuriteLength

NAME:    1H,1H,5H-Perfluoropentanol
CHID:    59879   CASRN: 355-80-6
SPID(S): 1210314395
M4ID:    43471561

HILL MODEL (in red):
       tp      ga      gw
val:  65.5     1.4    1.51
sd:   101    0.946    2.06

GAIN-LOSS MODEL (in blue):
       tp      ga      gw      la      lw
val:  65.5     1.4    1.51    2.37    8.09
sd:   99.7   0.938    2.04     883    8040

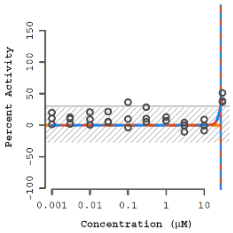
      CNST        HILL        GNLS
AIC:  261.27      255.38      259.38
PROB: 0.04        0.84        0.11
RMSE: 19.17       15.48       15.48

MAX_MEAN: 34.4      MAX_MED: 39.9      BMAD: 9.14

COFF: 30     HIT-CALL: 1    FITC: 42   ACTP: 0.96

FLAGS: 7; 17
```

CCTE_Mundy_HCI_CDI_NOG_NeuriteLength_loss

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ASSAY:   AEID3069 (CCTE_Mundy_HCI_CDI_NOG_NeuriteLength

NAME:    2,2,3,3,4,4,4-Heptafluorobutyl methacrylate
CHID:    65586   CASRN: 13695-31-3
SPID(S): 1210314373
M4ID:    43471582

HILL MODEL (in red):
       tp      ga      gw
val:  60.7     1.43    7.92
sd:   NaN      NaN     NaN

GAIN-LOSS MODEL (in blue):
       tp      ga      gw      la      lw
val:  59.6     1.43     8      3.02    17.7
sd:   NA       NA      NA      NA      NA

      CNST        HILL        GNLS
AIC:  259.72      244.84      248.84
PROB: 0           0.88        0.12
RMSE: 18.43       12.73       12.73

MAX_MEAN: 42.1      MAX_MED: 38.4      BMAD: 9.14

COFF: 30     HIT-CALL: 1    FITC: 42   ACTP: 1

FLAGS: 7; 17
```
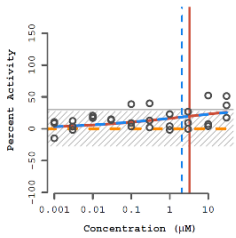

CCTE_Mundy_HCI_CDI_NOG_NeuriteLength_loss

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ASSAY:   AEID3069 (CCTE_Mundy_HCI_CDI_NOG_NeuriteLength

NAME:    3:3 Fluorotelomer carboxylic acid
CHID:    379268   CASRN: 356-02-5
SPID(S): 1210314388
M4ID:    43471625

HILL MODEL (in red):
       tp      ga      gw
val:  39.4    0.512    0.3
sd:   NaN      NaN     NaN

GAIN-LOSS MODEL (in blue):
       tp      ga      gw      la      lw
val:  36.9    0.32     0.3    1.97     14
sd:   NaN      NaN     NaN     NaN     NaN

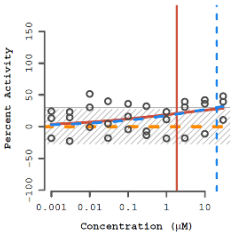
      CNST        HILL        GNLS
AIC:  271.11      255.01      259.66
PROB: 0           0.87        0.13
RMSE: 21.6        14.34       14.32

MAX_MEAN: 35.2      MAX_MED: 36.8      BMAD: 9.14

COFF: 30     HIT-CALL: 1    FITC: 42   ACTP: 1

FLAGS: 7; 17
```

CCTE_Mundy_HCI_CDI_NOG_NeuriteLength_loss

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ASSAY:   AEID3069 (CCTE_Mundy_HCI_CDI_NOG_NeuriteLength

NAME:    Perfluorocyclohexanecarbonyl fluoride
CHID:    379781   CASRN: 6588-63-2
SPID(S): 1210314391
M4ID:    43471512

HILL MODEL (in red):
       tp      ga      gw
val:  41.6    0.265    0.3
sd:   62.8     4.7     0.318

GAIN-LOSS MODEL (in blue):
       tp      ga      gw      la      lw
val:  60.5    1.31     0.3    2.31    8.38
sd:   206      9.6     0.66    691     6960

      CNST        HILL        GNLS
AIC:  288.88      283.53      287.45
PROB: 0.06        0.83        0.12
RMSE: 26.94       22.33       22.35

MAX_MEAN: 32.7      MAX_MED: 39.3      BMAD: 9.14

COFF: 30     HIT-CALL: 1    FITC: 42   ACTP: 0.94

FLAGS: 17
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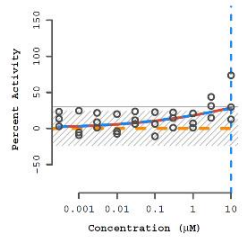
CCTE_Mundy_HCI_CDI_NOG_NeuronCount_loss

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ASSAY:   AEID3070 (CCTE_Mundy_HCI_CDI_NOG_NeuronCount_l

NAME:    3-(Perfluoro-3-methylbutyl)-1,2-propenoxide
CHID:    379884   CASRN: 54009-81-3
SPID(S): 1208990411
M4ID:    43471655

HILL MODEL (in red):
       tp      ga      gw
val:  68.9     1.5     0.3
sd:   256     9.46    0.423

GAIN-LOSS MODEL (in blue):
       tp      ga      gw      la      lw
val:  55.2     1      0.307   2.46    4.57
sd:   99      4.61    0.269   1210    3730

      CNST        HILL        GNLS
AIC:  270.31      254.09      258.95
PROB: 0           0.88        0.12
RMSE: 22.03       14.69       14.77

MAX_MEAN: 38.5      MAX_MED: 31.1      BMAD: 8.04

COFF: 30     HIT-CALL: 1    FITC: 42   ACTP: 1

FLAGS: 11; 17
```

Figure S9: Linear regression analysis comparing physicochemical properties and AC_{50} values
Scatter plots comparing AC_{50} values (log10-µM) in the DNT NAM battery and chemical properties: molecular weight (MW), C:F ratio, logP, boiling point (BP), and vapor pressure (VP (log10)), and CF chain length. A linear regression line equation and correlation coefficients (R) with p-values for significance ($p < 0.05$) are plotted.

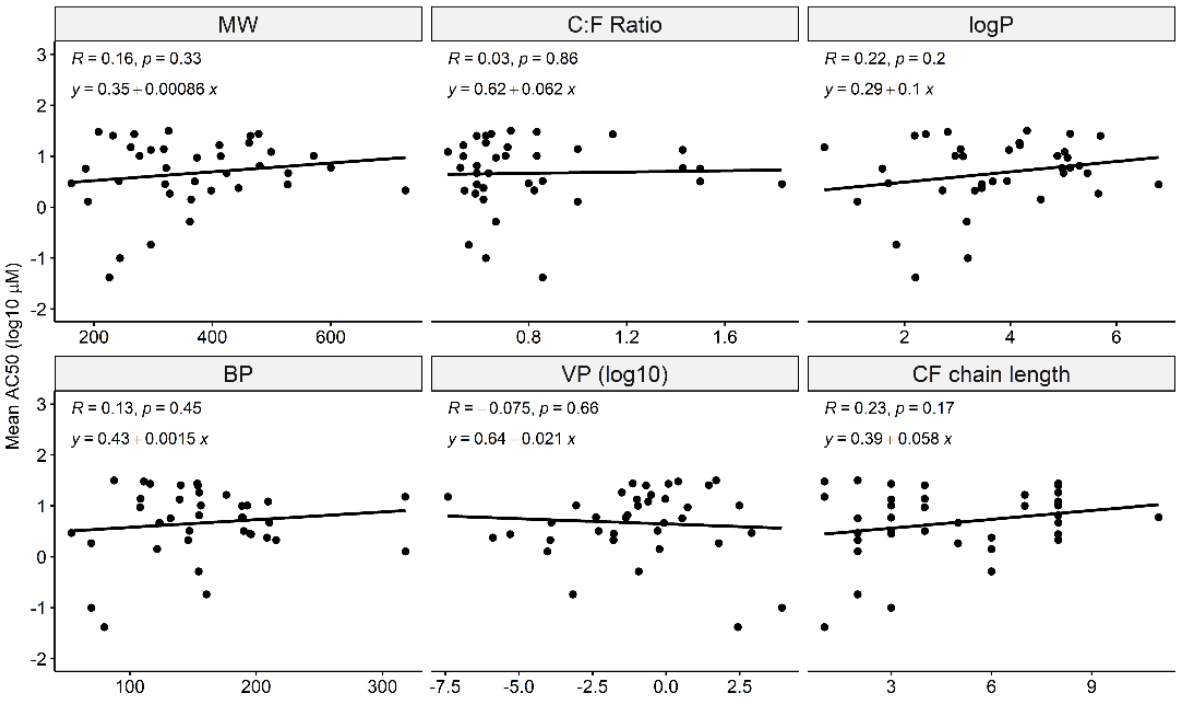


Figure S10: PFAS efficacy compared to all available chemicals tested in the DNT NAM battery. A: A cumulative density distribution plot of efficacy as measured by AUC sum (sum of all AUC values for each active endpoint by chemical) for all available chemicals screened in the DNT NAM data. B: A density plot comparing active PFAS efficacy (AUC sum) (gray) to all other active non-PFAS chemicals (blue). The x-axis indicates the log10 AUC sum values.

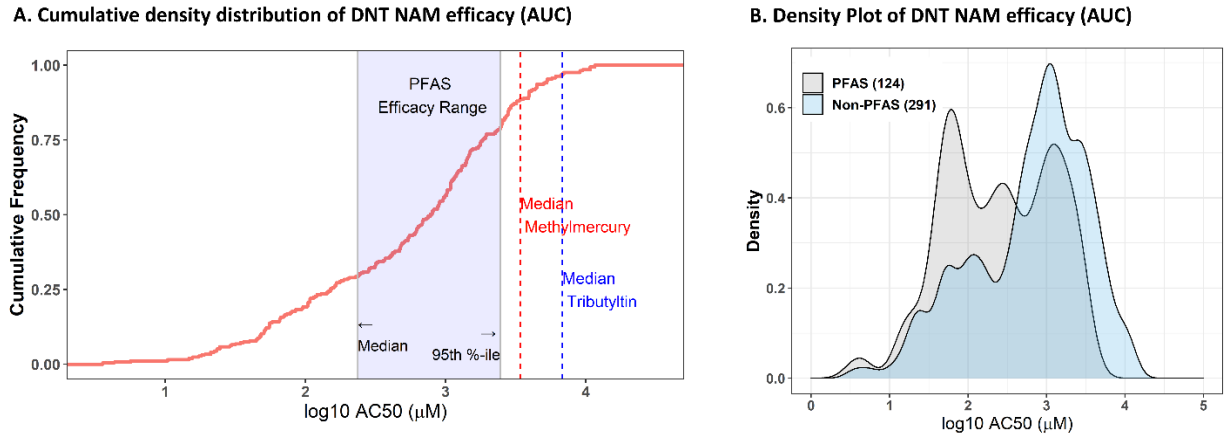
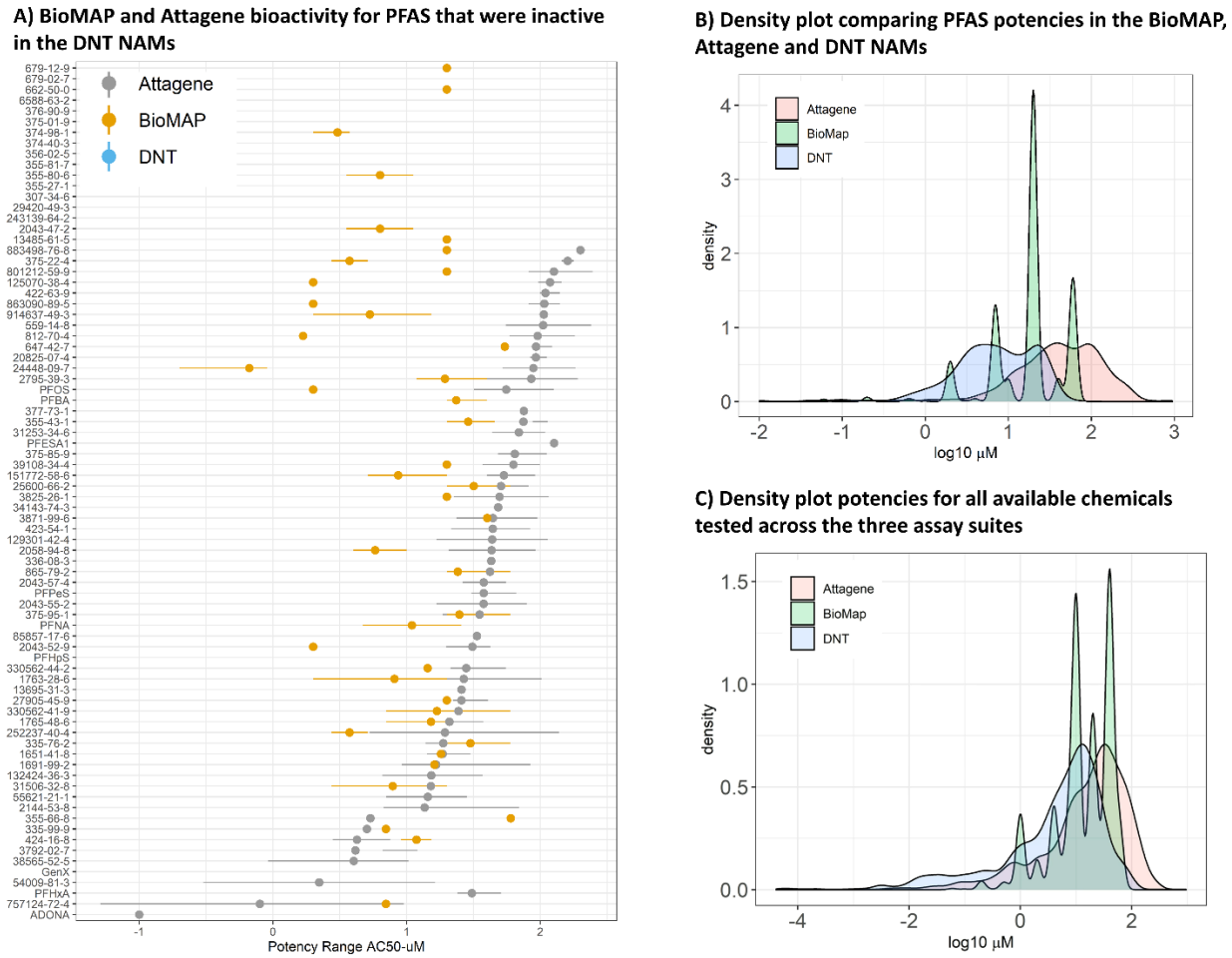


Figure S11: BioMAP and Attagene bioactivity comparison

A: Scatter plots comparing AC_{50} values (log10-uM) in the BioMAP (orange) and Attagene (gray) assays for PFAS that were inactive in the DNT NAMs. B: Density plot comparing the PFAS potencies in the BioMAP (green), Attagene (red), and DNT NAMs (green). C: Density plot comparing the potencies of all available chemical tested across BioMAP (green), Attagene (red), and DNT NAMs (green).



Supporting Information Methods

Single-concentration (sc) screening analysis

HCI assays (proliferation, apoptosis, NOG)

The goal of the single-concentration screen was to provide a health-protective estimate of which compounds would be a hit in the multiple-concentration screening, so in a first approach the hit-call determination was based on the same normalization methods used in the multiple-concentration screening in the ToxCast Pipeline (tcpl). For every HCI endpoint currently registered in tcpl (Table 1), the percent-of-control response values are calculated from the raw value (*rval*) and median DMSO *rval* on each plate (*bval)* with this formula:

$$resp.pc = \frac{rval - bval}{bval} * 100\%$$

Thus, the *resp.pc* represents a zero-centered, normalized response value. For all assay components except CCTE_Mundy_HCI_Cortical_NOG_Casp3_7, the *resp* value is multiplied by -1 so that a decrease in *resp* will lead to an increasing dose-response curve. The *bmad* is calculated as the median absolute deviation of the *resp.pc* in all control wells in the dataset. The current cutoff for HCI assay endpoints in multi-concentration screening in tcpl is defined as 3x*bmad* above the median control value or 30%, whichever is larger. (For 12 of the 17 assay endpoints currently in tcpl, 3x*bmad* is less than 30%).

Table 1: The 17 HCI endpoints in tcpl and the associated coff and 3xBMAD values for the multiple-concentration screening.

	aeid	aenm	bmad3	coff
1:	2777	CCTE_Mundy_HCI_Cortical_NOG_BPCount_loss	23.578699	30.00000
2:	2778	CCTE_Mundy_HCI_Cortical_NOG_NeuriteCount_loss	4.601172	30.00000
3:	2779	CCTE_Mundy_HCI_Cortical_NOG_NeuriteLength_loss	23.136021	30.00000
4:	2780	CCTE_Mundy_HCI_Cortical_NOG_NeuronCount_loss	22.087479	30.00000
5:	2781	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_BPCount_loss	17.737887	30.00000
6:	2782	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_CellBodySpotCount_loss	44.678480	44.67848
7:	2783	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteCount_loss	7.990187	30.00000
8:	2784	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteLength_loss	22.795974	30.00000
9:	2785	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength_loss	24.710000	30.00000
10:	2786	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuron_loss	36.575546	36.57555
11:	2787	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuronCount_loss	16.039668	30.00000
12:	2788	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_SynapseCount_loss	31.165243	31.16524
13:	2793	CCTE_Mundy_HCI_hNP1_Casp3_7_gain	9.269828	30.00000
14:	2794	CCTE_Mundy_HCI_hNP1_CellTiter_loss	5.874010	30.00000
15:	2795	CCTE_Mundy_HCI_hNP1_Pro_MeanAvgInten_loss	31.030868	31.03087
16:	2796	CCTE_Mundy_HCI_hNP1_Pro_ObjectCount_loss	23.183304	30.00000
17:	2797	CCTE_Mundy_HCI_hNP1_Pro_ResponderAvgInten_loss	34.292636	34.29264

Verifying the Proposed hit call method

The available multiple-concentration HCI data in tcpl were analyzed to verify if the median *resp.pc* at the highest concentration tested could provide a health-protective metric for the hit-call in a single-concentration screen against 2 or 3 x *bmad*. Particularly, the hits where the median *resp.pc* is less than the *max_med* were examined (e.g. if the dose-response curve is “biphasic” (aka ‘gain-loss’) or if the dose response curve is slightly noisy). The available HCI data in tcpl includes ~80+ compounds.

The 2x*bmad* threshold was found to detect all but 4 compounds that were labelled as “hits” in the multiple-concentration screening. The 4 compounds that were missed all fit a “gain-loss” dose-response curve.

	hitc	aeid	aenm	spid	bmad	med_resp_at_max_conc

1:	1	2780	CCTE_Mundy_HCI_Cortical_NOG_NeuronCount_loss	EX000389	7.362493	-3.0303030
2:	1	2785	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuriteSpotCountPerNeuriteLength_loss	TT0000177C03	8.236667	-11.1111111
3:	1	2787	CCTE_Mundy_HCI_Cortical_Synap&Neur_Matur_NeuronCount_loss	EX000402	5.346556	0.6858123
4:	1	2793	CCTE_Mundy_HCI_hNP1_Casp3_7_gain	TT0000177E03	3.089943	-1.6081565

Conclusion: 2x*bmad* will provide a good estimate for the single-concentration screen hit calls.

Applying Single-Concentration Hit Calls to PFAS data

- Pre-processing
    - Removed any data points where the Well quality is 0
    - Renamed any wells labelled as "Blank", "NA", or "Media" from each assay as "Media"
    - Defined the "wllt" (well type) as:
        - If Compund.Name is Media or DMSO, wllt = "n" to signify neutral controls. (In contrast, the tcpl single-concentration methods only utilize DMSO wells).
        - Otherwise, wllt = "t" (to signify test compounds)
- Found the median raw value of control wells on each plate (where wllt == "n") (*bval*)
- Calculated the normalized, zero-centered *resp.pc* from every raw value

$$resp.pc = \frac{rval - bval}{bval} * 100\%$$

- Calculated the rescaled Median Absolute Deviation of wells where wllt == "n" for each endpoint:

$$bmad = 1.4826 * median(|control_i - median(controls)|)$$

- Found the median *resp.pc* for each sample and endpoint
    - In order to be more health-protective, where there were only 2 replicates with Well Quality=1, set the *med_resp_up* as the maximum *resp.pc*, and the *med_resp_dn* as the minimum *resp.pc*
- Determined the hit calls for each sample for each endpoint:
    - If the median *resp.pc_dn* is less than or equal to -2x*bmad*, then the sample is a "down" hit
    - If the median *resp.pc_up* is greater than or equal to 2x*bmad*, then the sample is an "up" hit
    - Otherwise, the sample is a "no hit"

Final results

There are 53 samples with a hit in at least 1 assay (Supporting Information Methods Table 1). See 'hit_call_summary' sheet for summary of hit-call results for any HCI assay ('any_hit' column). For raw data values for this analysis, see sheets labeled 'all_values' or 'values_for_no_hit_compounds' for data on compounds with activity or no activity, respectively.

MEA NFA

This work aims to find an appropriate combination of endpoints and thresholds to determine which compounds tested in the Network Formation Assay (NFA) single-concentration screen are likely to be active in the NFA multi-concentration screen. The response values at the highest concentration tested and corresponding hit calls from the multi-concentration screen were used to select the most informative endpoints and to develop cutoffs. Then, the cutoffs for the selected set of endpoints were applied to the median percent-of-control response values of the PFAS NFA single-concentration screen to determine which compounds should be re-tested in the multi-concentration screen.

Multi-concentration Analysis

<u>Preparation of multi-concentration data</u>

The current multi-concentration NFA data set includes 422 samples, taken from several chemical sets, with the prefix "CCTE_Shafer_MEA_dev_." Data were processed as follows:

- Removed any hits from the multi-conc data with 3 or more flags.
- Removed any hits where the AC_{50} is less than the lowest concentration tested and the top modl parameter is less than 1.2*cutoff (fit category 36 or 45).

A sample was defined as "positive" in the multi-concentration screen if it has 3 or more unfiltered hits. This resulted in 236 "positives" and 186 "negatives."

Selection of Endpoints and Cutoffs

The goal of this analysis is to identify a combination of endpoints and cutoffs that will detect as many positives as possible while minimizing the number of false positives. There are 66 total endpoints to choose from: 2 cytotoxicity endpoints (LDH and AB), 17 parameters analyzed in the up and down direction, and 15 "DIV12" endpoints analyzed in the up and down direction (2 + 17*2 + 15*2 = 2+34+30 = 66 total).

Algorithm to compile a set of endpoints and cutoffs (based on the "greedy algorithm"):

1) Start with the 2 cytotoxicity endpoints (LDH and Alamar Blue) as the initial endpoints with a cutoff of 30% for each (corresponding to a 30% decrease in viability relative to controls). Any sample with a median response at the highest concentration tested above 30 for either endpoint is labelled as "sc_positive." This resulted in the detection of 178 true positives and 11 false negatives.
2) For each remaining endpoint, set the cutoff just above the highest response for the remaining undetected negatives. Specifically,
    a. Set the lower bound of the cutoff as the highest med_resp_max_conc of the undetected negatives for each endpoint
    b. Set the upper bound of the cutoff as the lowest med_resp_max_conc of the undetected positives that is above the lower bound of the cutoff
    c. Set the cutoff as the mid-point between the upper and lower bounds. In this way, the cutoff is high enough that no additional negatives will be a "hit."
3) Add the endpoint to the set that will add the most additional true positives. If there is a tie among endpoints, arbitrarily select one of them. Any sample with a median response at the highest concentration tested above the cutoff for the selected endpoint is labelled as "sc_positive."
4) Repeat from 2) until no additional true positives can be detected.

The algorithm was run at every juncture where there was a tie in the number of additional true positives at step 3. This resulted in 3 unique sets of endpoints and cutoffs (Supporting Information Methods Table 2). One of the 3 sets of endpoints with the corresponding cutoffs is shown below. Each row shows the accuracy of the detection of the multi-concentration positives after the addition of each endpoint.

aeid	aenm	coff	add_TP	add_FP	ttl_TP	ttl_FP	FP_rate	TP_rate	accuracy	rank
2529	CCTE_Shafer_MEA_dev_LDH_dn	30	170	9	170	9	4.83871	72.0339	82.22749	1
2530	CCTE_Shafer_MEA_dev_AB_dn	30	8	2	178	11	5.913978	75.42373	83.64929	2
2500	CCTE_Shafer_MEA_dev_bursting_electrodes_number_dn	47.0176	23	0	201	11	5.913978	85.16949	89.09953	3
3055	CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean_DIV12_up	63.308	7	0	208	11	5.913978	88.13559	90.75829	4
2527	CCTE_Shafer_MEA_dev_mutual_information_norm_up	118.434	4	0	212	11	5.913978	89.83051	91.70616	5
3042	CCTE_Shafer_MEA_dev_firing_rate_mean_DIV12_dn	55.5246	4	0	216	11	5.913978	91.52542	92.65403	6
3039	CCTE_Shafer_MEA_dev_per_burst_interspike_interval_DIV12_up	68.4416	3	0	219	11	5.913978	92.79661	93.36493	7
2512	CCTE_Shafer_MEA_dev_network_spike_peak_dn	53.0434	2	0	221	11	5.913978	93.64407	93.83886	8
2519	CCTE_Shafer_MEA_dev_per_network_spike_interspike_interval_mean_up	472.59	1	0	222	11	5.913978	94.0678	94.07583	9
3040	CCTE_Shafer_MEA_dev_per_burst_interspike_interval_DIV12_dn	52.8475	1	0	223	11	5.913978	94.49153	94.3128	10
3060	CCTE_Shafer_MEA_dev_network_spike_peak_DIV12_dn	26.6127	1	0	224	11	5.913978	94.91525	94.54976	11

The other 2 sets of endpoints varied by only 1 endpoint. They contained either "CCTE_Shafer_MEA_dev_mutual_information_norm_DIV12_up" or "CCTE_Shafer_MEA_dev_per_burst_spike_percent_DIV12_up" in place of "CCTE_Shafer_MEA_dev_mutual_information_norm_up."

Using any of the 3 sets of endpoints and the given cutoffs, 224 of the 236 positives could be detected with only 11 false positives out of the 186 negatives. (The true positives and false positives detected were identical with each of the 3 sets). This resulted in a final accuracy of accuracy of 94.55%. Interestingly, the majority of the true positives were detected by the LDH and Alamar Blue endpoints.

Below is a visual depiction of the detection of the multi-concentration positives and negatives (Figure 1). The endpoints are sorted by order of addition to the set.

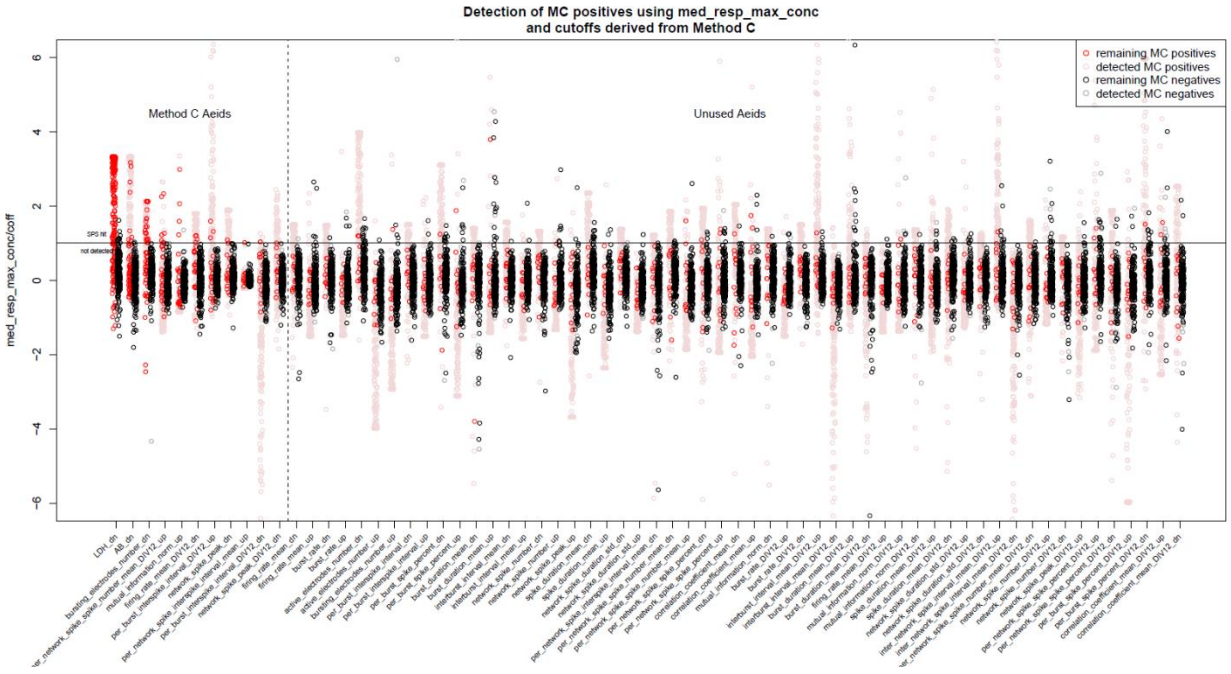


*Figure1. The plot shows that the responses in the 12 remaining undetected positives are largely not separable from the negatives. See Supporting Information Methods Table 2 for 3 unique sets of endpoints. The y-axis shows the median response at the highest conc tested for each sample, divided by the cutoff. Thus, any point above y=1 for the first 11 endpoints is a "hit" in the single-concentration screen. An arbitrary cutoff of 3*BMAD was used for the remaining 55 endpoints.*

Consideration of 3*BMAD for Cytotoxicity Cutoffs

The choice of 30% for the LDH and Alamar Blue cutoffs was somewhat arbitrary. In the multi-concentration screen in tcpl, the cutoff is set to 3 times the median absolute deviation of the percent-of-control values in DMSO wells (BMAD). This corresponds to a cutoff of 24% for LDH and 20% for Alamar Blue. The algorithm was run using 3*BMAD as the cutoffs for LDH and Alamar Blue. This resulted in 21 false positives and 227 true positives. The addition of 3 true positives at the cost of 10 additional false positives did not seem beneficial. Therefore, 30% as the cutoff for the 2 cytotoxicity endpoints.

Application of the Endpoints and Cutoffs to the PFAS data

Normalization and Hit Call Determination

Single-concentration screen data processing:

- Calculated the "bval" as the median endpoint value of the DMSO control wells from each MEA plate
- Calculated the normalized "response" values:
  - To measure the "up" response: $resp = \frac{rval - bval}{bval} * 100$
  - To measure the "down" response: $resp = -1 * \frac{rval - bval}{bval} * 100$
- Calculated the median response value of each endpoint for every sample
- Determined the hit calls using the endpoints selected in the multi-concentration analysis
  - If the median response value for a given sample was greater than or equal to the cutoff for any of the 11 endpoints in the set, then that sample was labelled as a "positive"
  - Otherwise, it was labelled as a "negative"

The hit-call determination was repeated with each of the 3 unique sets of endpoints found in the previous section. The first set resulted in 36 positives (including the positive control Bisphenol). The second set resulted in the same 36 positives plus 1 additional sample. The third set resulted in the same 37 positives as the previous sets, plus 1 additional sample. Since the third set resulted in the most hits, the third set was used to assign the final hit calls (Figure 2).

The number of hits per endpoint are summarized in the table and graph below:

	aenm	coff	num_hits
1:	CCTE_Shafer_MEA_dev_bursting_electrodes_number_dn	47.0176	26
2:	CCTE_Shafer_MEA_dev_LDH_dn	30.0000	18
3:	CCTE_Shafer_MEA_dev_firing_rate_mean_DIV12_dn	55.5246	13
4:	CCTE_Shafer_MEA_dev_AB_dn	30.0000	11
5:	CCTE_Shafer_MEA_dev_network_spike_peak_dn	53.0434	10
6:	CCTE_Shafer_MEA_dev_per_burst_interspike_interval_DIV12_up	68.4416	9
7:	CCTE_Shafer_MEA_dev_network_spike_peak_DIV12_dn	26.6127	3
8:	CCTE_Shafer_MEA_dev_per_network_spike_spike_number_mean_DIV12_up	63.3080	3
9:	CCTE_Shafer_MEA_dev_mutual_information_norm_up	118.4340	2
10:	CCTE_Shafer_MEA_dev_inter_network_spike_interval_mean_up	472.5900	0
11:	CCTE_Shafer_MEA_dev_per_burst_interspike_interval_DIV12_dn	52.8475	0

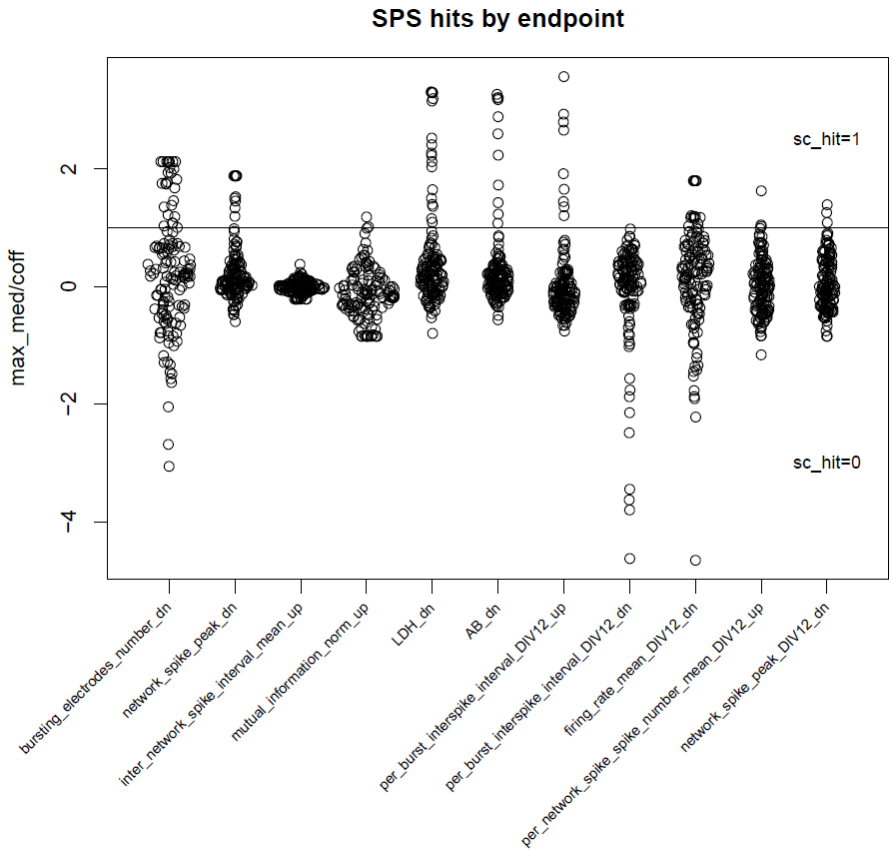


Figure 2: Each point corresponds to the median response value for a sample divided by the cutoff. Any point above the horizontal line where max_med/coff = 1 is a "positive."

Final results

Positive/negative determination for each sample and hit-calls for each sample for each of the 11 endpoints ("sc_hit") can be found in Supporting Information Methods Table 3. Cutoffs were set to the cutoffs determined in the multi-concentration analysis.