EPA
United States
Environmental Protection
Agency

Molecular Point of Departure (mPOD) Determination From *In Vitro* High-Throughput Transcriptomics Data.

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ECETOC
Workshop On Omics Threshold On Non-Adversity
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EPA United States Environmental Protection Agency

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NAMs-Based Tiered Hazard Evaluation Approach

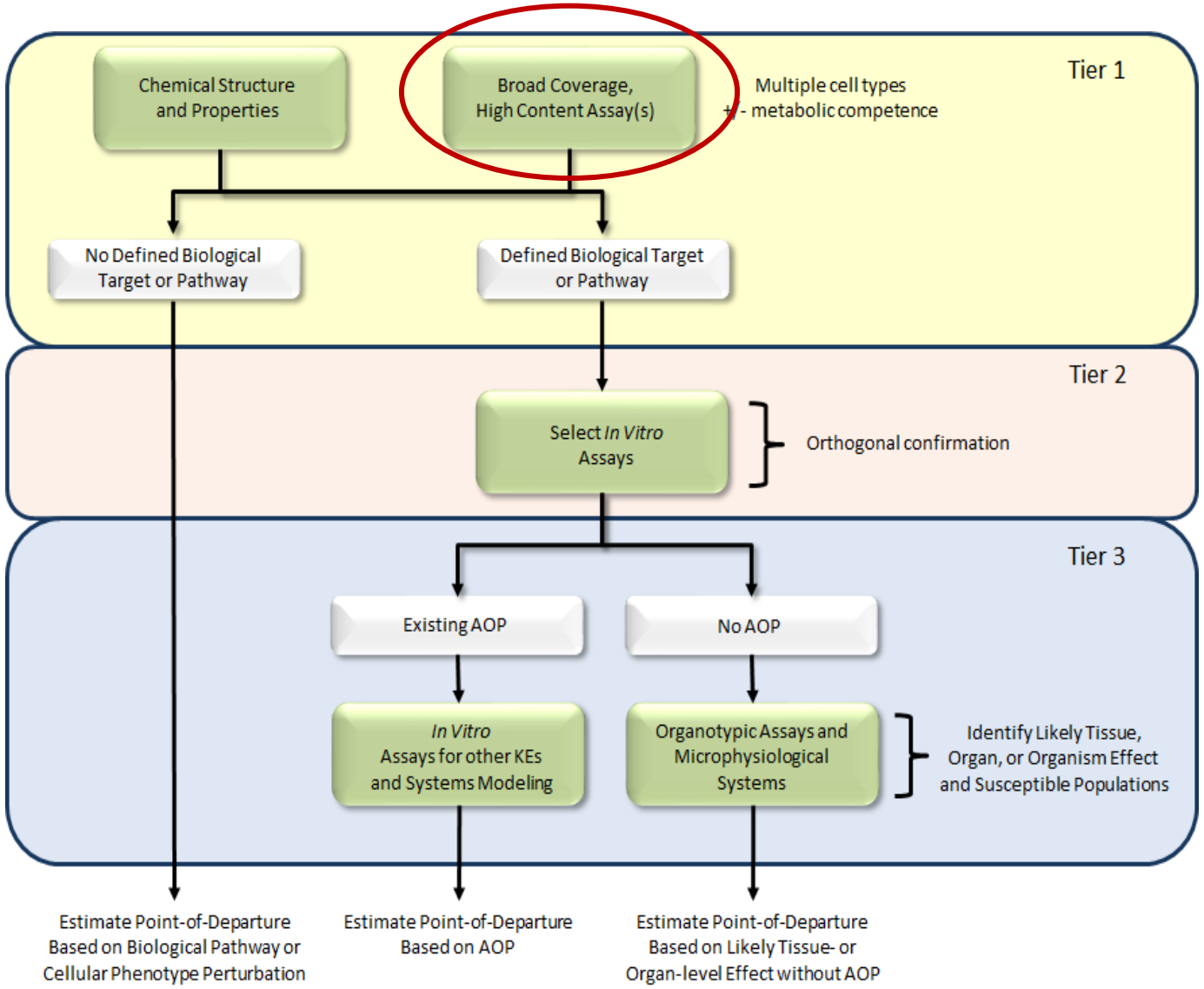
High throughput profiling (HTP) assays are proposed as the first tier in a NAMs-based hazard evaluation approach.

HTP Assay Criteria:

1. Yield bioactivity profiles that can be used for **potency estimation**, **mechanistic prediction** and evaluation of **chemical similarity**.
2. Compatible with multiple human-derived culture models.
3. Concentration-response screening mode.
4. Cost-effective.

To date, EPA has identified and implemented two HTP assays that meet this criteria.

- **High-Throughput Transcriptomics [HTTr]**
- **High-Throughput Phenotypic Profiling [HTPP]**

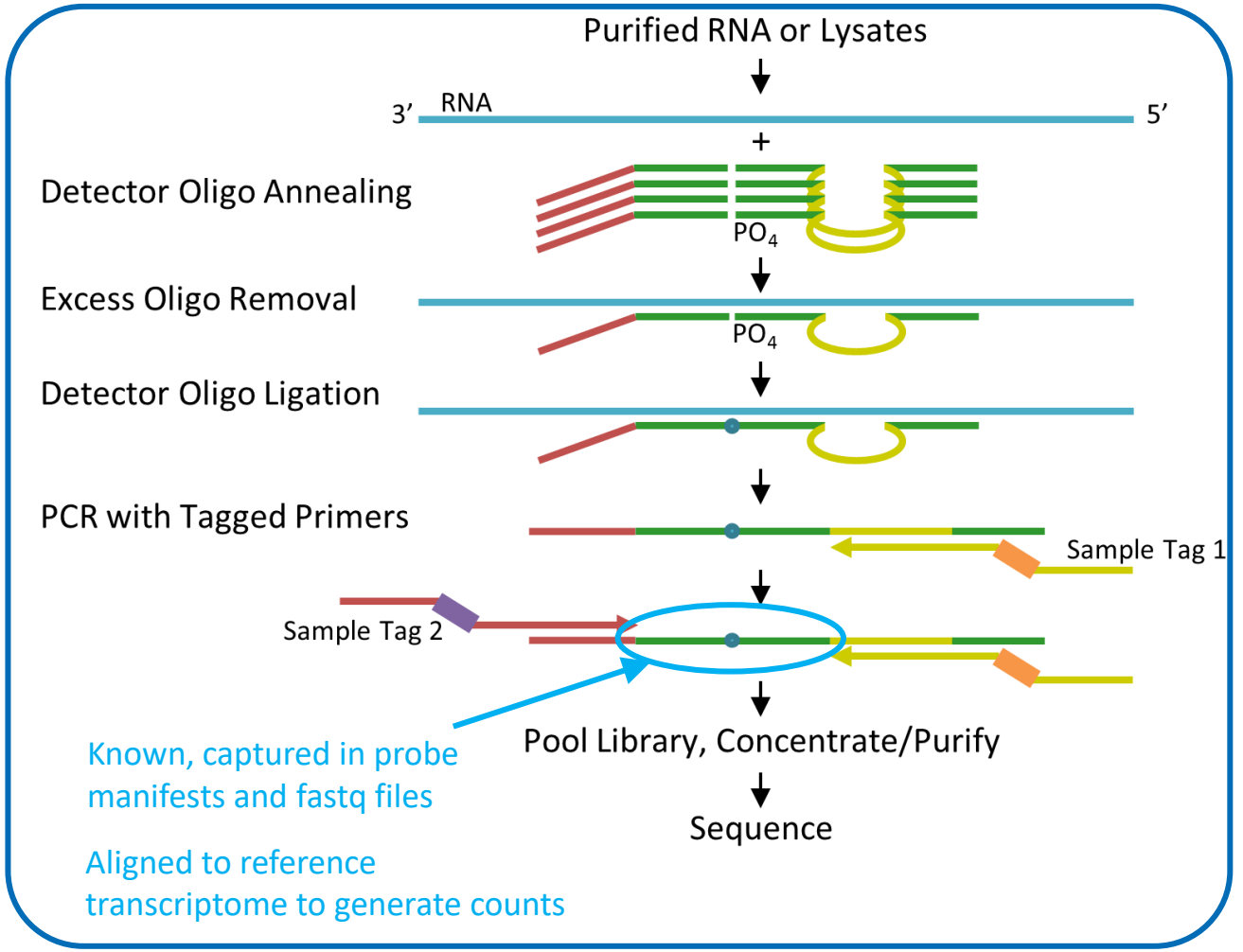


The NexGen Blueprint of CompTox at US EPA
Thomas et al. (2019) DOI: 10.1093/toxsci/kfz058

Templated Oligo with Sequencing Readout (TempO-Seq)

- The **TempO-Seq** human whole transcriptome assay measures the expression of greater than 20,000 transcripts.
- Requires only picogram amounts of total RNA per sample.
- Compatible with purified RNA samples or **cell lysates**.
- Lysates are barcoded according to sample identity and combined in a single library for sequencing using industry standard instruments.
- Scalable, targeted assay:
  - 1) specifically measures transcripts of interest
  - 2) ~50-bp reads for all targeted genes
  - 3) requires less flow cell capacity than RNA-Seq

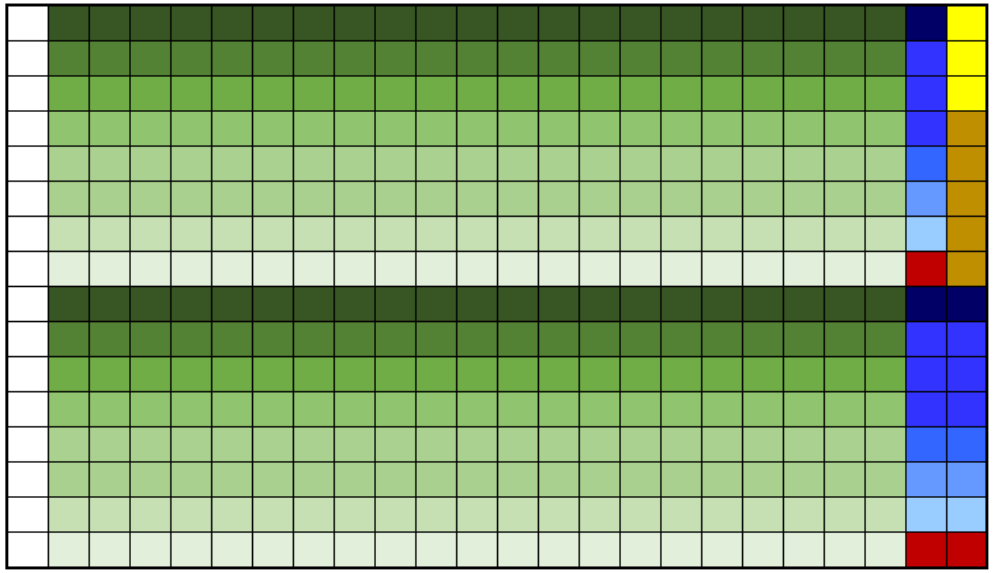
TempO-Seq Assay Illustration



Yeakley et al. (2017) DOI: 10.1371/journal.pone.0178302

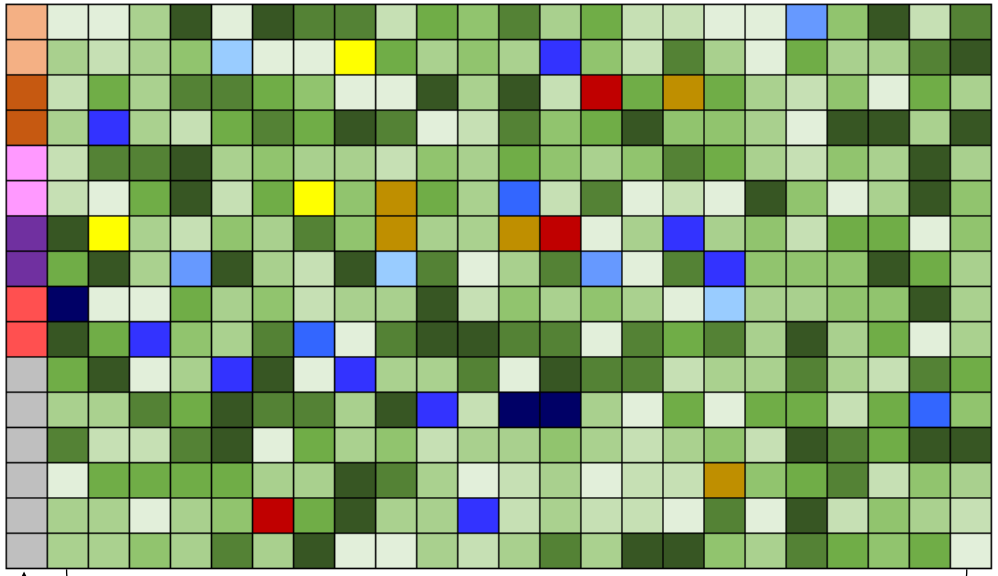
Generic Experimental Design for HTTr

Dose Plate



LabCyte® Echo 550 Acoustic Dispenser

Assay Plate



= Test chemicals in 8-point dilution series

= Vehicle controls (DMSO)

= Reference chemicals in 7-point dilution series

= Trichostatin A (cell type agonist reference chem)

= Staurosporine (cell type agnostic cell viability control)

Used to track assay performance.

= Reference RNAs

= Reference Lysates

= Bulk Lysates

Used to track assay performance independent of Chemical treatments and responsivity of culture.

= Reserved for sequencing vendor

MCF7 Pilot Experimental Design

High-Throughput Transcriptomics Platform for Screening Environmental Chemicals

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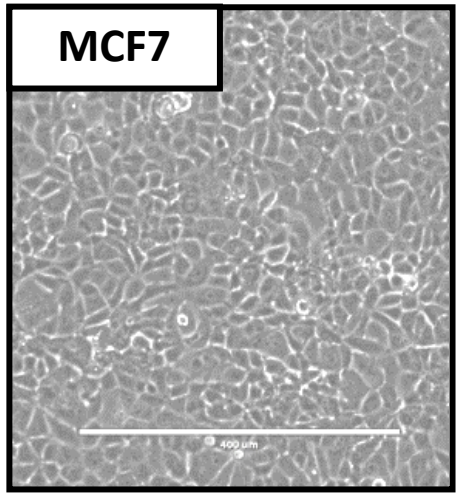
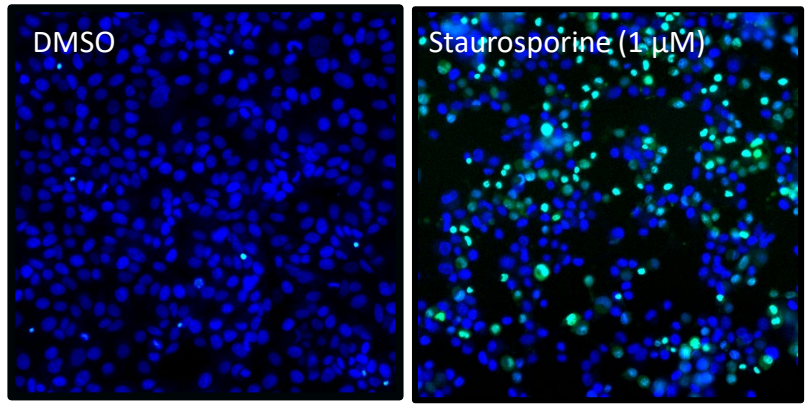
TOXICOLOGICAL SCIENCES, 2021, 1–22

doi: 10.1093/toxsci/kfab009
Advance Access Publication Date: 4 February 2021
Research Article

Parameter	Multiplier	Notes
Cell Type(s)	1	MCF7
Assay Formats:	2	High-Throughput Transcriptomics Cell Viability
Culture Condition	1	DMEM + 10% HI-FBS
Chemicals	44	ToxCast chemicals
Time Points:	1	6 hours
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing
Biological Replicates:	3	Independent cultures





CellEvent Caspase 3/7

Harrill et al. (2021) DOI: 10.1093/toxsci/kfab009

MCF7 Pilot Chemical List

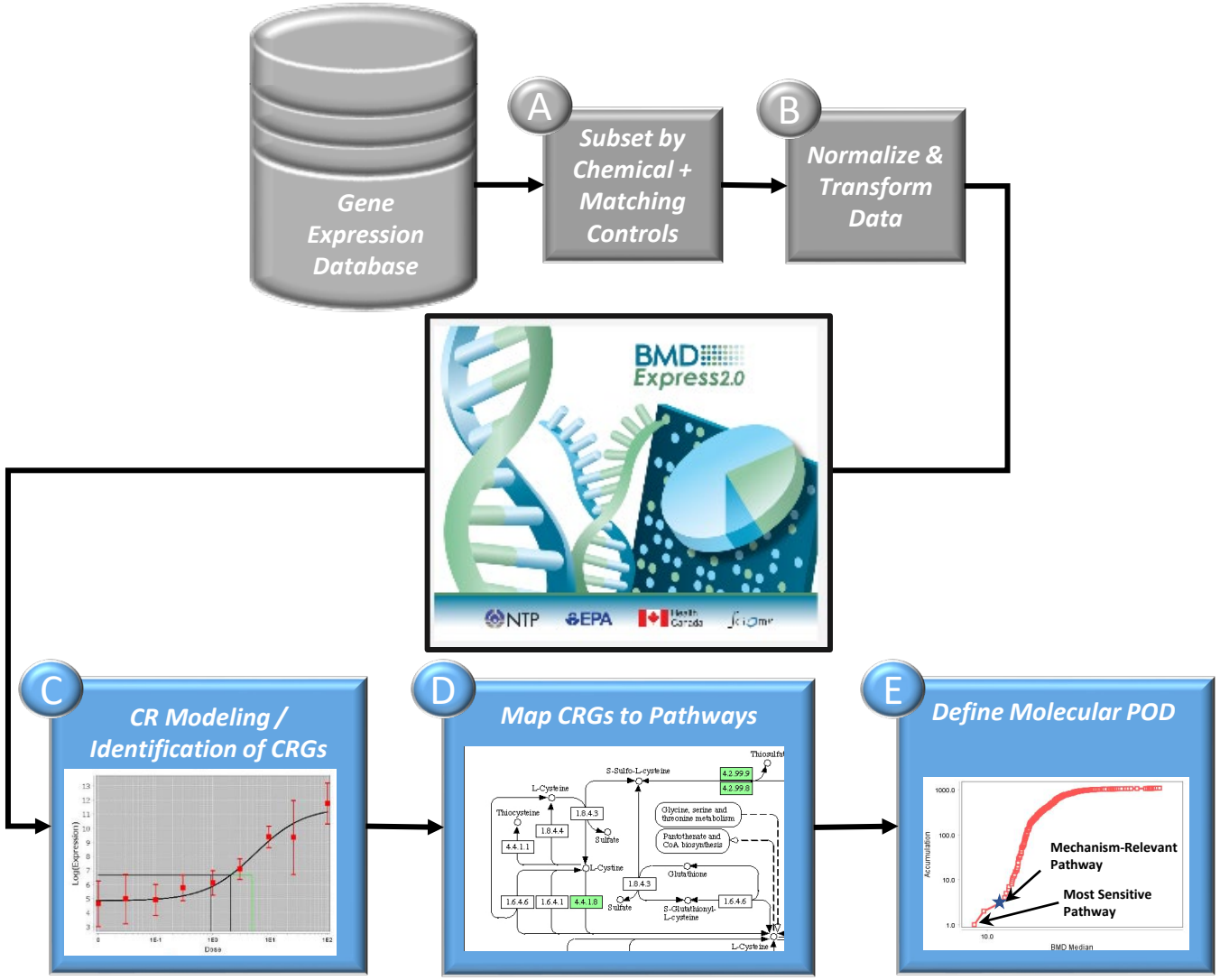
Table 1. Chemicals Used in the Study

Name	Target Annotation
Cyproterone acetate	AR antagonist
Flutamide	AR antagonist
Nilutamide	AR antagonist
Vinclozolin	AR antagonist
Amiodarone hydrochlorid	Blocks myocardial calcium, potassium and sodium channels
Cladribine	DNA synthesis inhibitor
4-Cumylphenol	ER agonist
4-Nonylphenol, branched	ER agonist
Bisphenol A	ER agonist
Bisphenol B	ER agonist
4-Hydroxytamoxifen	ER antagonist
Clomiphene citrate (1:1)	ER antagonist
Fulvestrant	ER antagonist
Cyproconazole	Ergosterol-biosynthesis inhibitor. Pan-cyp inhibitor
Imazalil	Ergosterol-biosynthesis inhibitor. Pan-cyp inhibitor
Prochloraz	Ergosterol-biosynthesis inhibitor. Pan-cyp inhibitor
Propiconazole	Ergosterol-biosynthesis inhibitor. Pan-cyp inhibitor
Atrazine	Herbicide, photosystem II inhibitor
Cyanazine	Herbicide, photosystem II inhibitor
Simazine	Herbicide, photosystem II inhibitor
Butafenacil	Herbicide, PPO inhibition
Fomesafen	Herbicide, PPO inhibition
Lactofen	Herbicide, PPO inhibition
Lovastatin	HMGCR inhibitor
Simvastatin	HMGCR inhibitor
Maneb	Inhibition of metal-dependent and sulfhydryl enzyme systems
Thiram	Inhibition of metal-dependent and sulfhydryl enzyme systems
Ziram	Inhibition of metal-dependent and sulfhydryl enzyme systems
Reserpine	Inhibition of the ATP/Mg2+ pump
Rotenone	Mitochondria (complex I inhibitor)
Pyraclostrobin	Mitochondria (complex III inhibitor)
Trifloxystrobin	Mitochondria (complex III inhibitor)
Fenpyroximate (Z, E)	Mitochondrial electron transport inhibitor
Clofibrate	PPARα agonist, upregulates extrahepatic lipoprotein lipase
Fenofibrate	PPARα agonist, upregulates extrahepatic lipoprotein lipase
Farglitazar	PPARγ agonist
Perfluorooctanoic acid (PFOA)	PPARγ, PPARα agonist
Perfluorooctanesulfonic acid (PFOS)	PPARγ, PPARα agonist
Troglitazone	PPARγ, PPARα agonist
Cycloheximide	Protein synthesis inhibitor
Bifenthrin	Sodium channel modulator
Cypermethrin	Sodium channel modulator
Tetrac	T4 synthesis inhibitor
3,5,3′-triiodothyronine	THR agonist

- Chemicals were selected that cover a broad range of molecular targets with some redundancy within target class.
- Intentionally selected some chemicals whose molecular targets are not expressed in MCF7 cells (or in mammalian tissues).

Harrill et al. (2021) DOI: 10.1093/toxsci/kfab009

BMDExpress for mPOD Determination



Based on National Toxicology Program Approach to Genomic Dose-Response Modeling (NTP RR 5)

BMDExpress Parameter	Criteria
Pre-filter:	|FC| > 2 at any test concentration
Models	Hill, Power, Linear, Poly2, Exponential 2|3|4|5
BMR Factor:	1.349*SD of controls (10%)
Best Model Selection:	Lowest AIC
Hill Model Flagging:	'k' < 1/3 Lowest Positive Dose Exclude Flagged Hill from Best Model Selection
Conc-Response Hit Criteria	(0.1*lowest conc. < BMC < highest conc.) BMC fit p-value > 0.1 BMCL / BMCU < 40
Gene Set Analysis:	≥ 3 Concentration-responsive genes ≥ 5% Gene Set Coverage
Gene Set Collections:	MSigDB (Liberzon et al. 2015) BioPlanet (Huang et al. 2019) CMAP (Subramanian et al. 2005)
Molecular Point of Departure	Most Sensitive Gene Set

Harrill et al. (2021) DOI: 10.1016/j.cotox.2019.05.004

Modeling of Signature Scores for mPOD Determination (1)

Step 1: Inputs

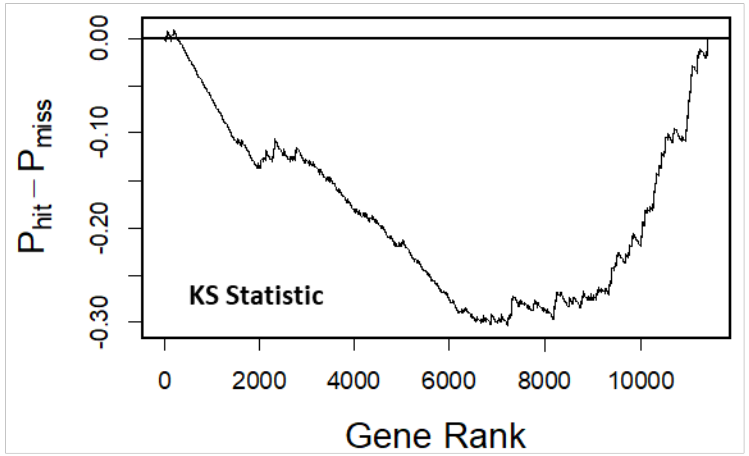
Experimental Data: Chemical_Conc × Gene matrix of $\log_2$ (fold-change) (l2fc) values.

Signature Collections: MSigDB (*Liberzon et al. 2015*), BioPlanet (*Huang et al. 2019*), CMAP (*Subramanian et al. 2005*)

Step 2: Pathway Scoring

Scores based on single sample GSEA method (Barbie et al. 2009)

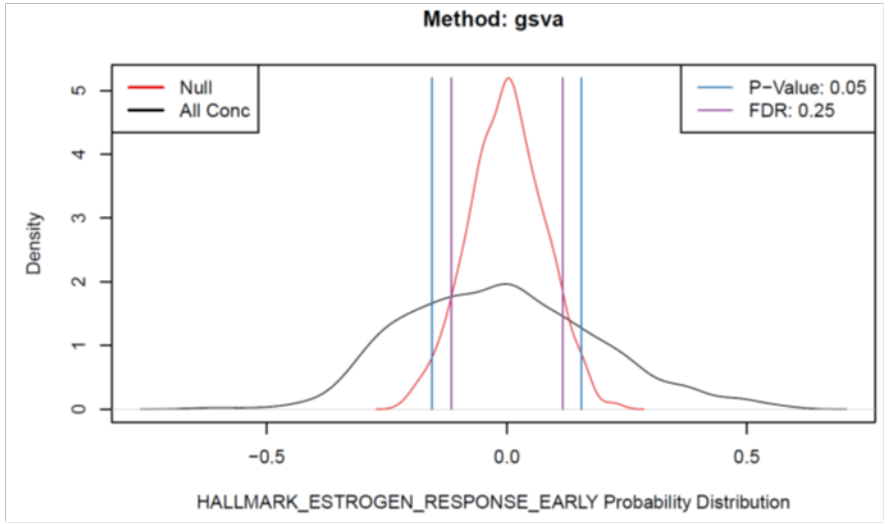
K-S statistic



Chemical_Conc × Pathway matrix of scores.

Step 3: Cut-off Estimation via NULL Modeling

- For each gene, **resample** l2fc based on the cross-sample gene distribution
- Calculate **pathway scores for "null" data**
  - One null distribution (n = 1000 scores) / pathway

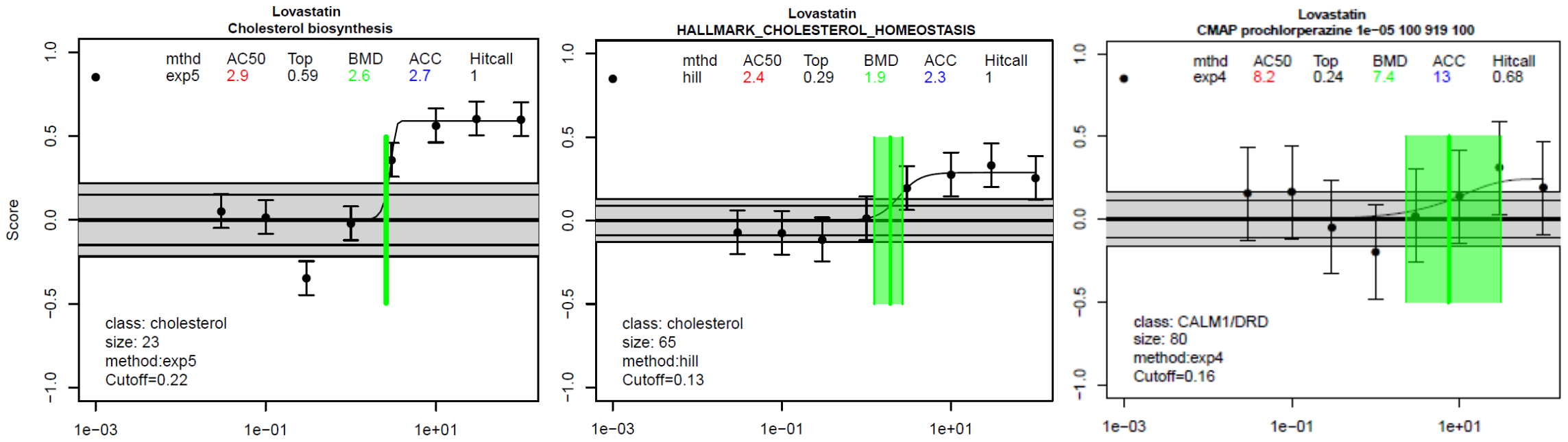


Analysis by Thomas Sheffield and Richard Judson

Modeling of Signature Scores for mPOD Determination (2)

Step 4: CR Modeling

Concentration response modeling of signature scores using *tcplfit2* (Sheffield et al. (2021) *10.1093/bioinformatics/btab779*)



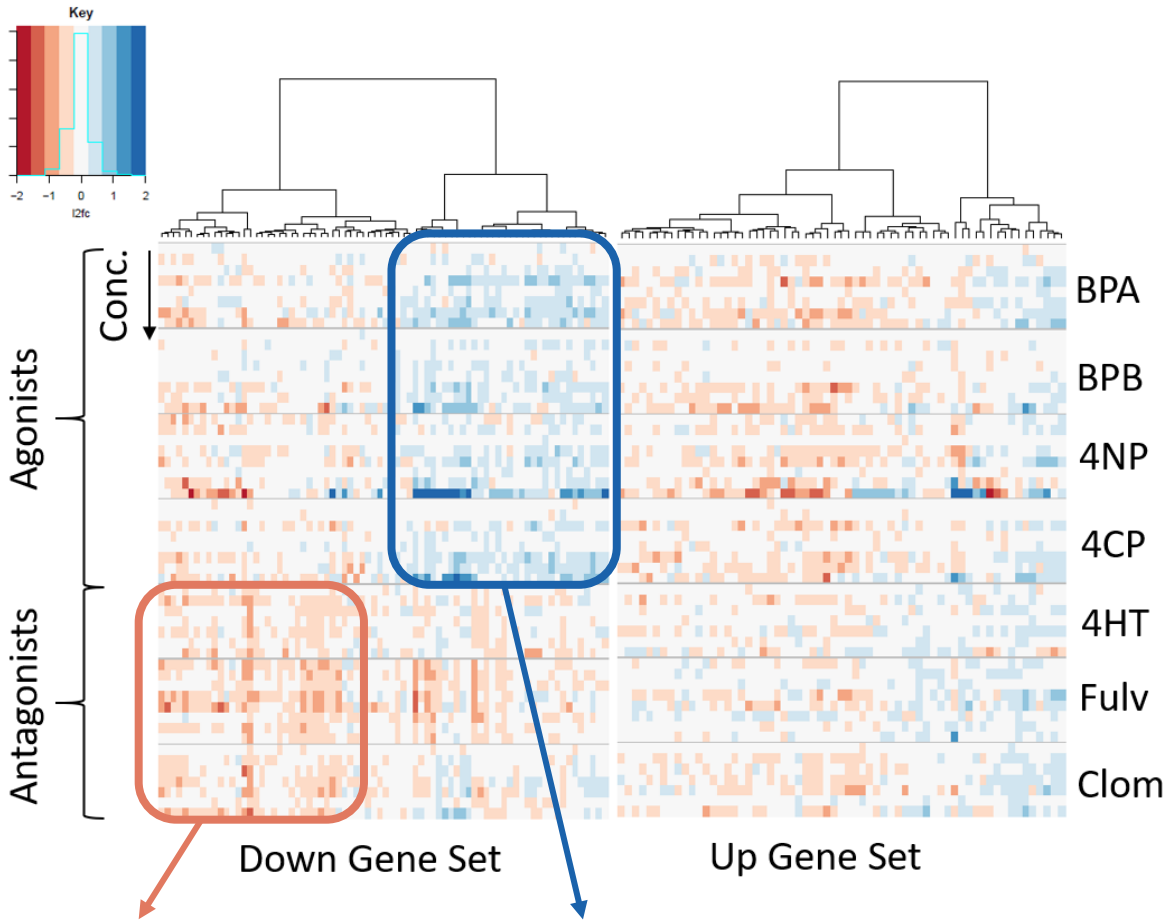
- Takes into account coordinated changes in gene expression that may not be identified using gene level fitting approaches.
- All curve forms from BMDExpress, plus constant model.
- Provides continuous hit calls for identifying high confidence and low confidence hits.

MCF7 Pilot Results: Directionality of Signature Scores

These gene level data are noisy!

A

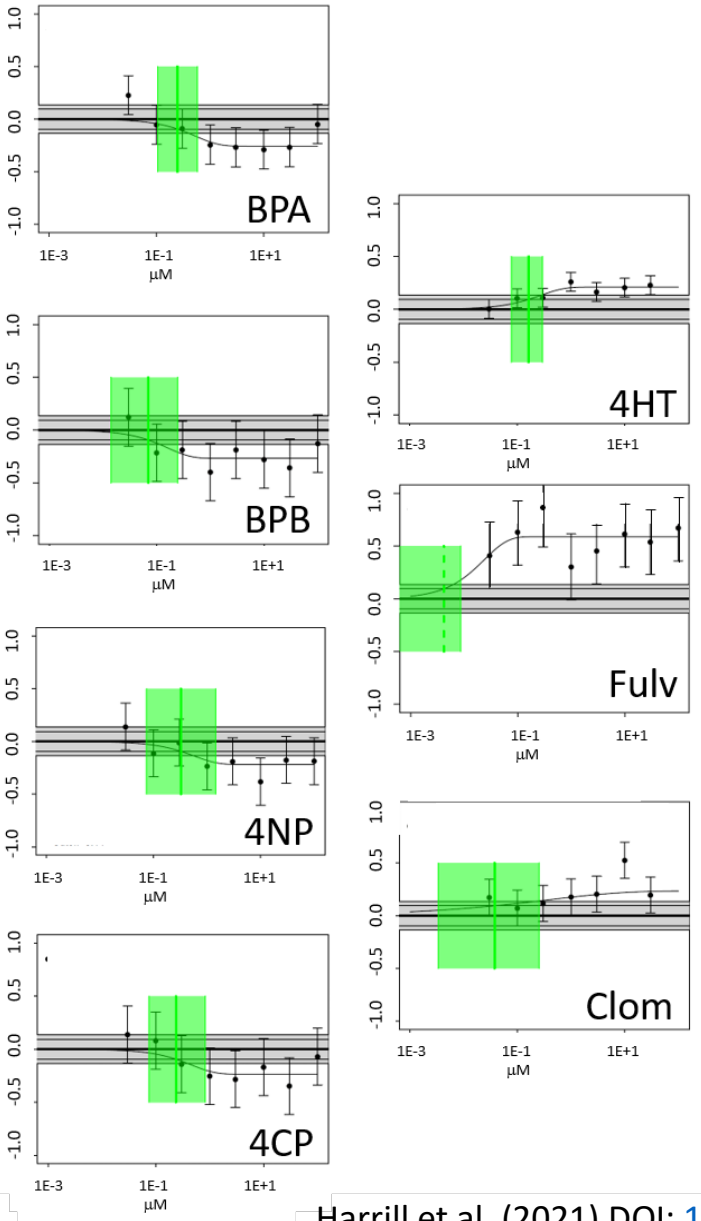
Fulvestrant Signature (Top 100 Up & Down Genes)



The expression of fulvestrant signature "down" genes goes down following ER antagonist treatment

The expression of fulvestrant signature "down" genes goes up following ER agonist treatment

B



Signature level results display correct directionality!

Harrill et al. (2021) DOI: 10.1093/toxsci/kfab009

MCF7 Pilot Results: Comparison of mPOD Approaches

BPAC = Biological Pathway Altering Concentration

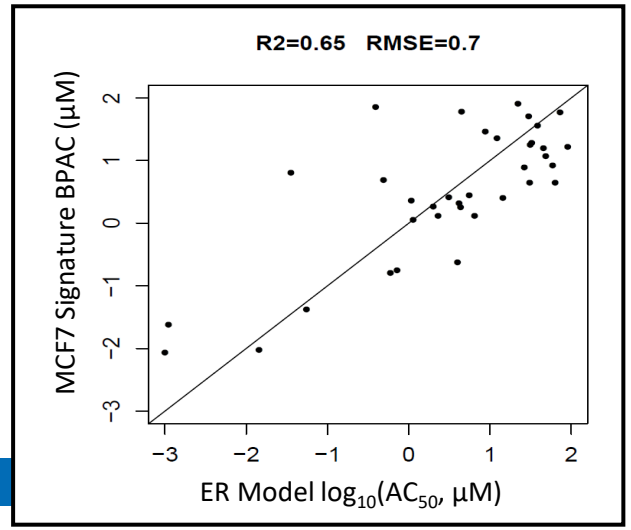
$BPAC_{Sig}$ → 5th lowest BPAC of active signatures

$BPAC_{BMDX}$ → Most sensitive signature / pathway

$BPAC_{HTS}$ → Lower 5th percentile of active AC50 values for ToxCast assays that pass a series of quality filters.

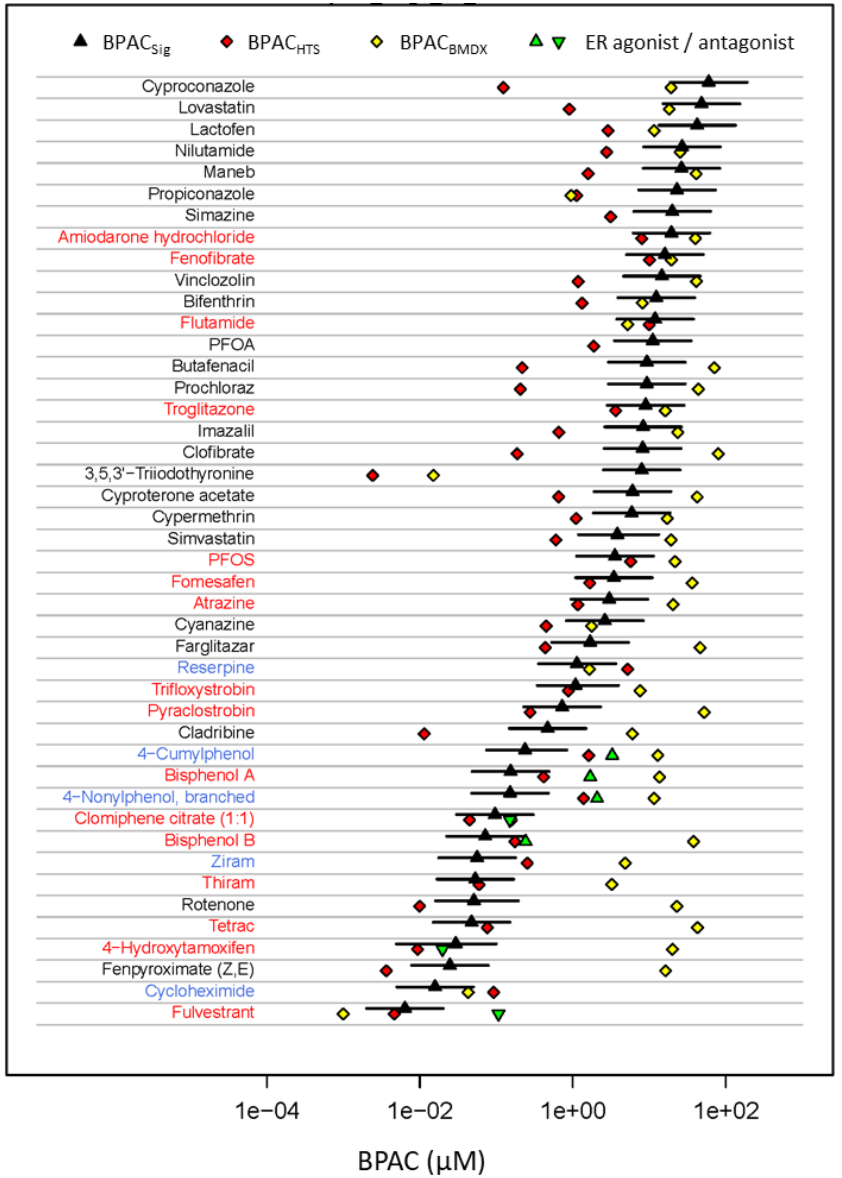
$BPAC_{HTS}$ and $BPAC_{Sig}$ are in better agreement than $BPAC_{HTS}$ and $BPAC_{BMDX}$

In most of these cases, $BPAC_{HTS}$ is also more potent than $BPAC_{BMDX}$.



Signature-based BPACs in MCF7 are concordant with ToxCast estrogen receptor (ER) model predictions.

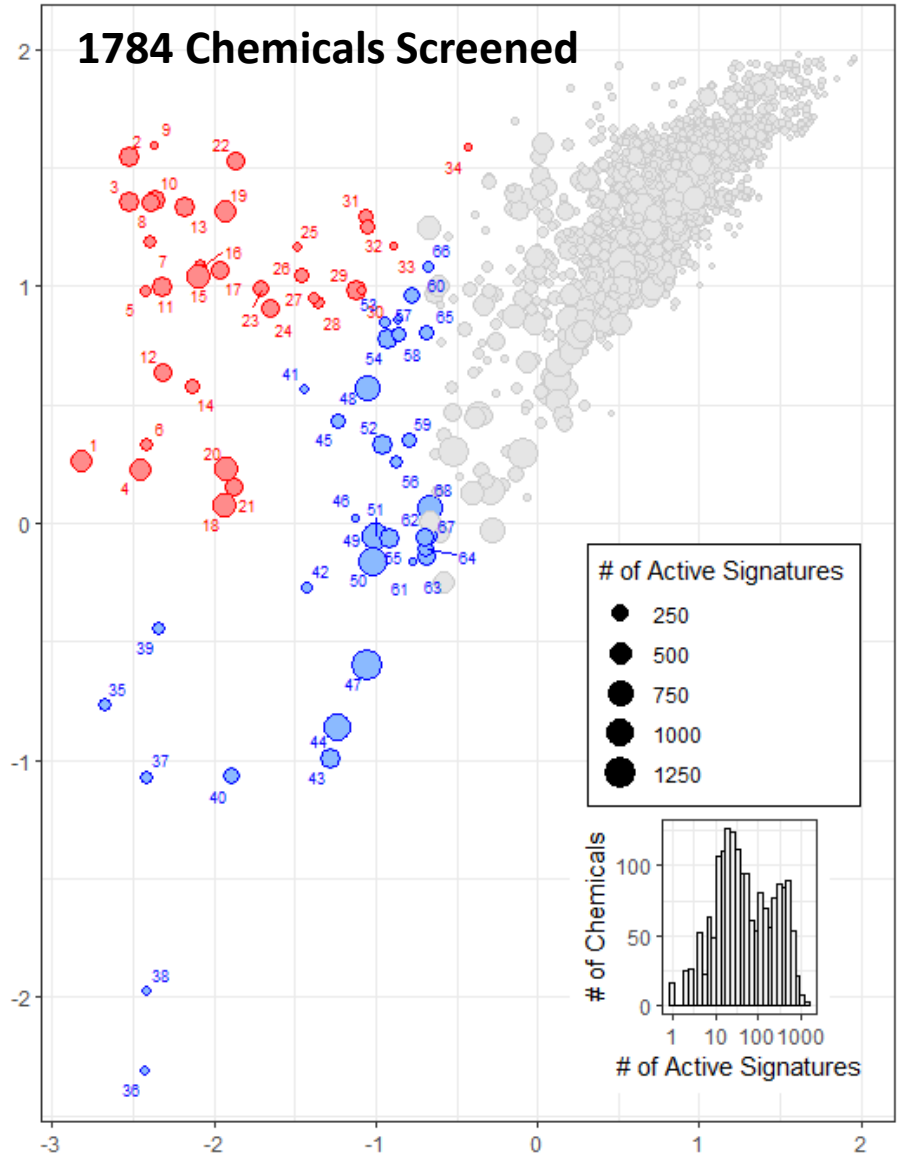
- Brown et al. (2015) DOI: 10.1021/acs.est.5b02641



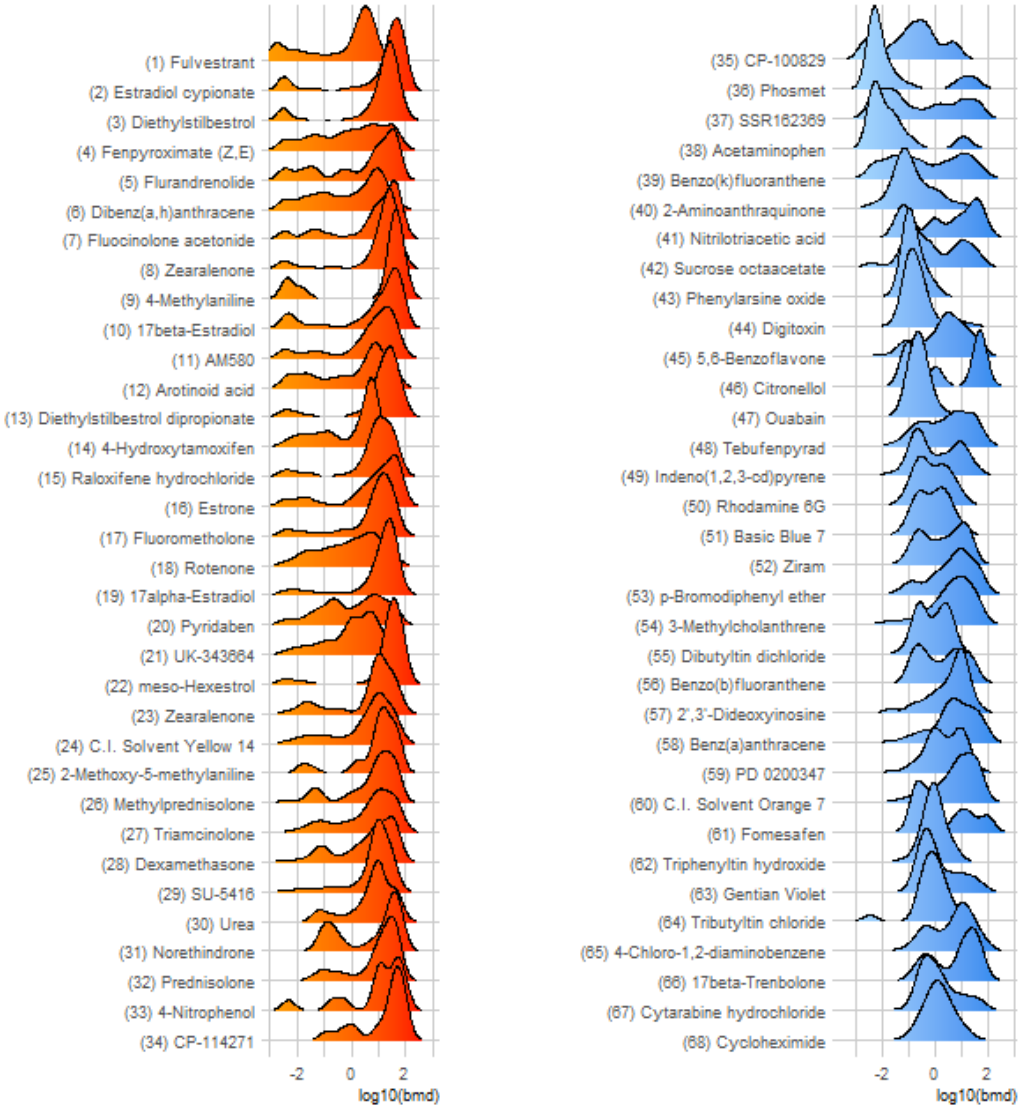
Harrill et al. (2021) DOI: 10.1093/toxsci/kfab009

MCF7 HTTr Screening Results (1)

United States Environmental Protection Agency



Distribution of BMCs of Active Signatures

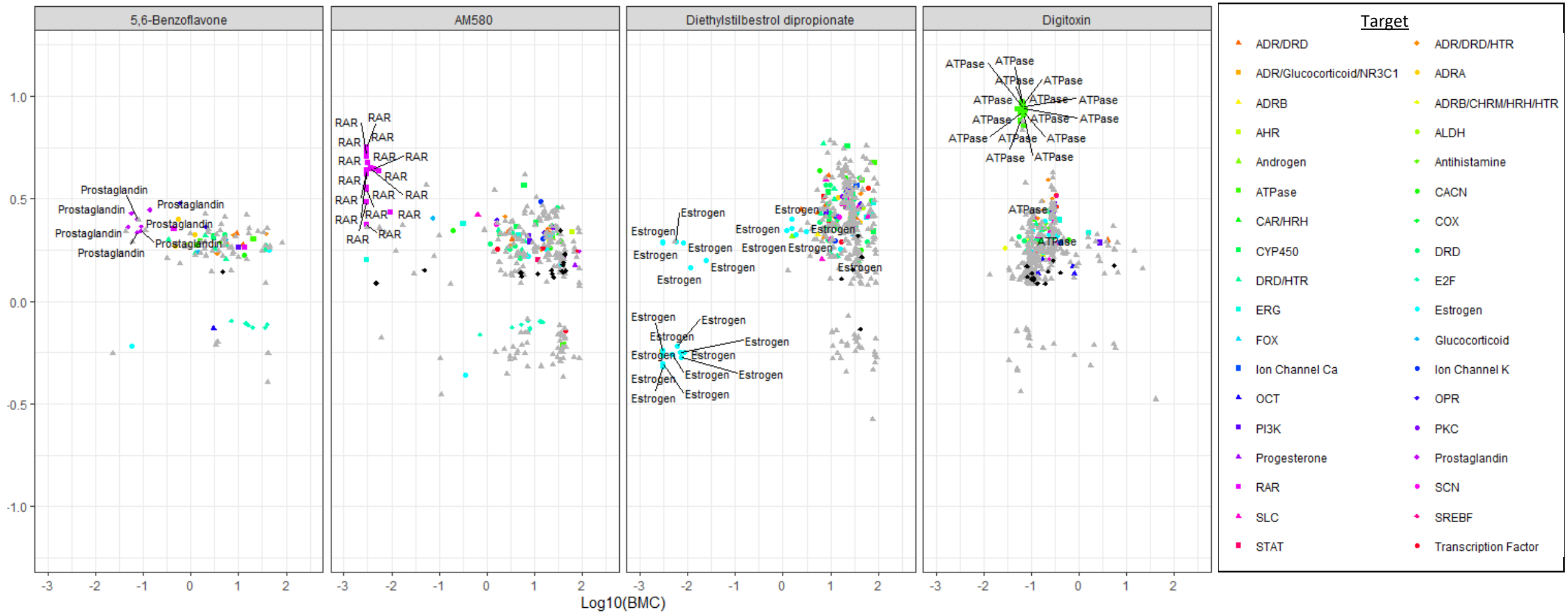


Other potent toxicants (organometallics, dyes, etc) cause many signatures to be affected near the onset of biological activity.

Chemicals with known pharmacological targets show an “early wave” of biological activity.

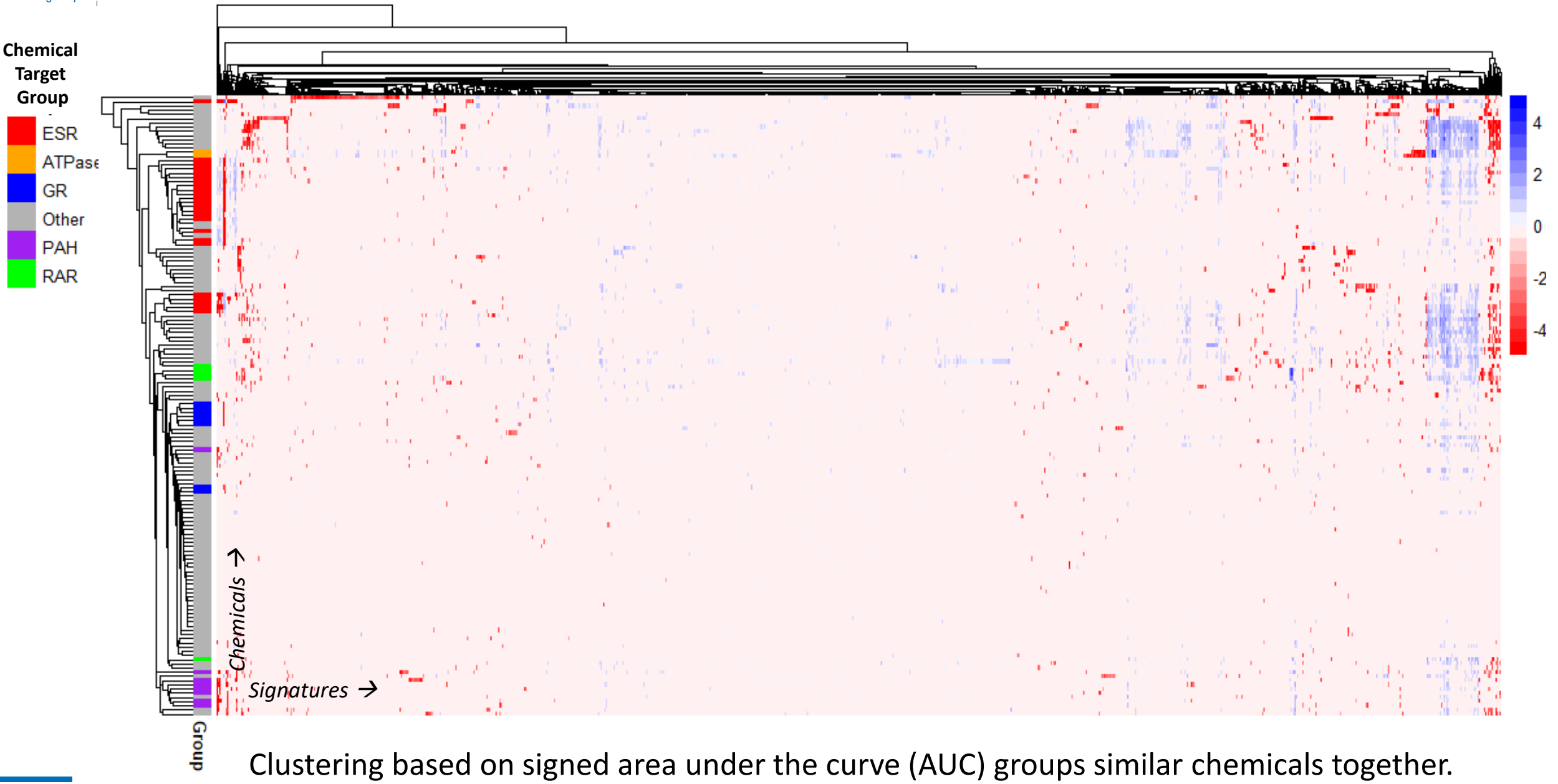
Unpublished Data
Do Not Cite or Quote

MCF7 HTTr Screening Results (2)



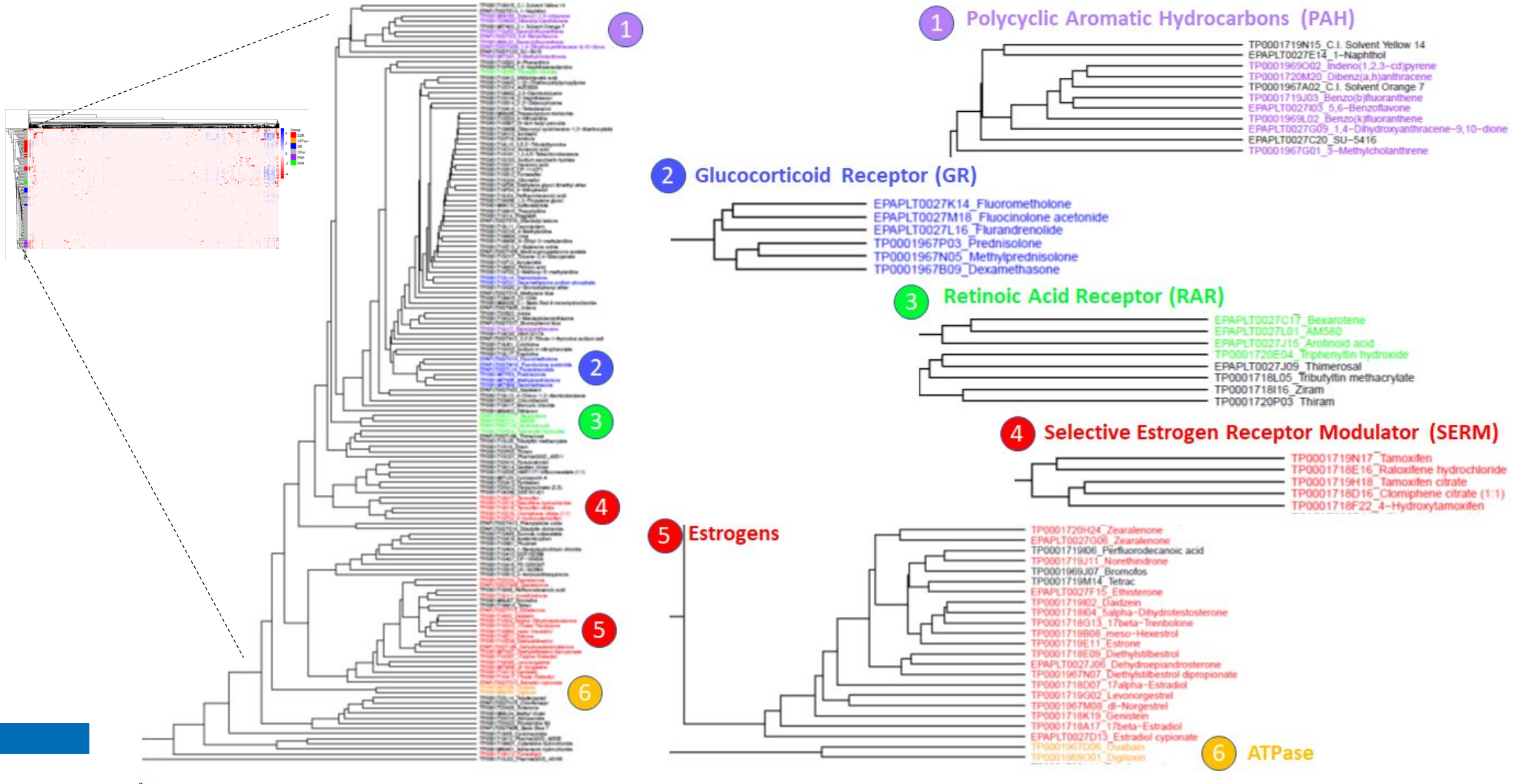
The most potent and efficacious signature hits correspond to known mechanisms for these chemicals.

MCF7 HTTr Screening Results (3)



Clustering based on signed area under the curve (AUC) groups similar chemicals together.

MCF7 HTTr Screening Results (4)



In Vitro to *In Vivo* Extrapolation (IVIVE) Using High-Throughput Toxicokinetic (httk) Modeling

Predicted exposure

Exposure predictions (EPA ExpoCast)

- Systematic Empirical Evaluation of Models (SEEM) version 3
- Inferred from human biomonitoring data, production volume and use categories (industrial / consumer use)

New approach methodologies (NAMs)

Toxicological threshold of concern (TTC)

HTTr Potency Estimate (µM)

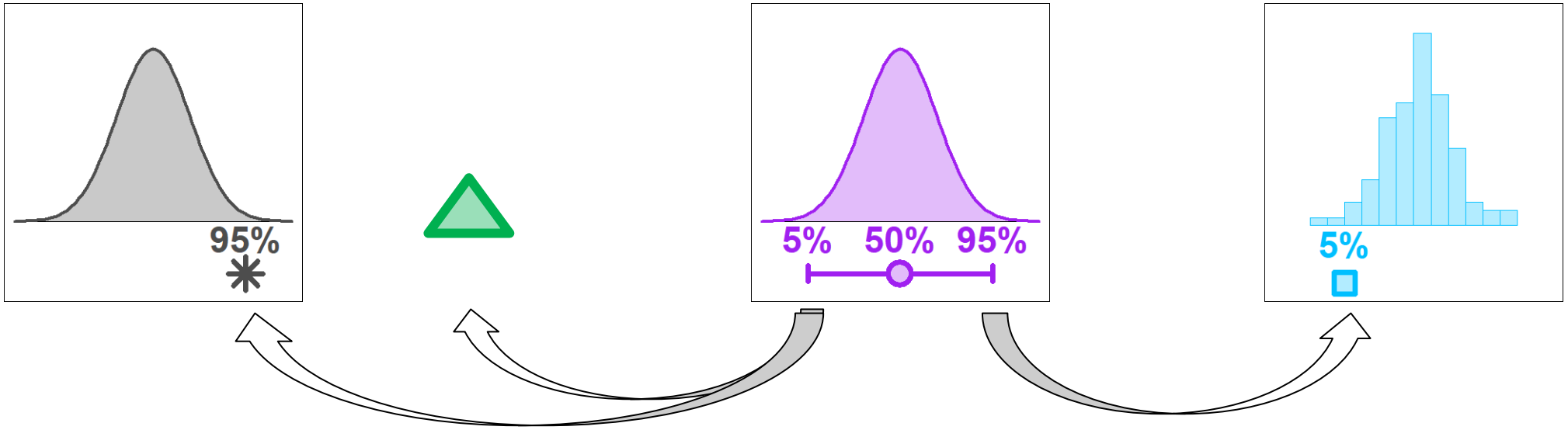
***In vitro*-to-*in vivo* extrapolation (IVIVE)**
high-throughput toxicokinetics (httk)

HTTr AED (mg/kg bw/day)

***in vivo* point-of-departure**

Database of *in vivo* effect values (EPA – ToxValDB)

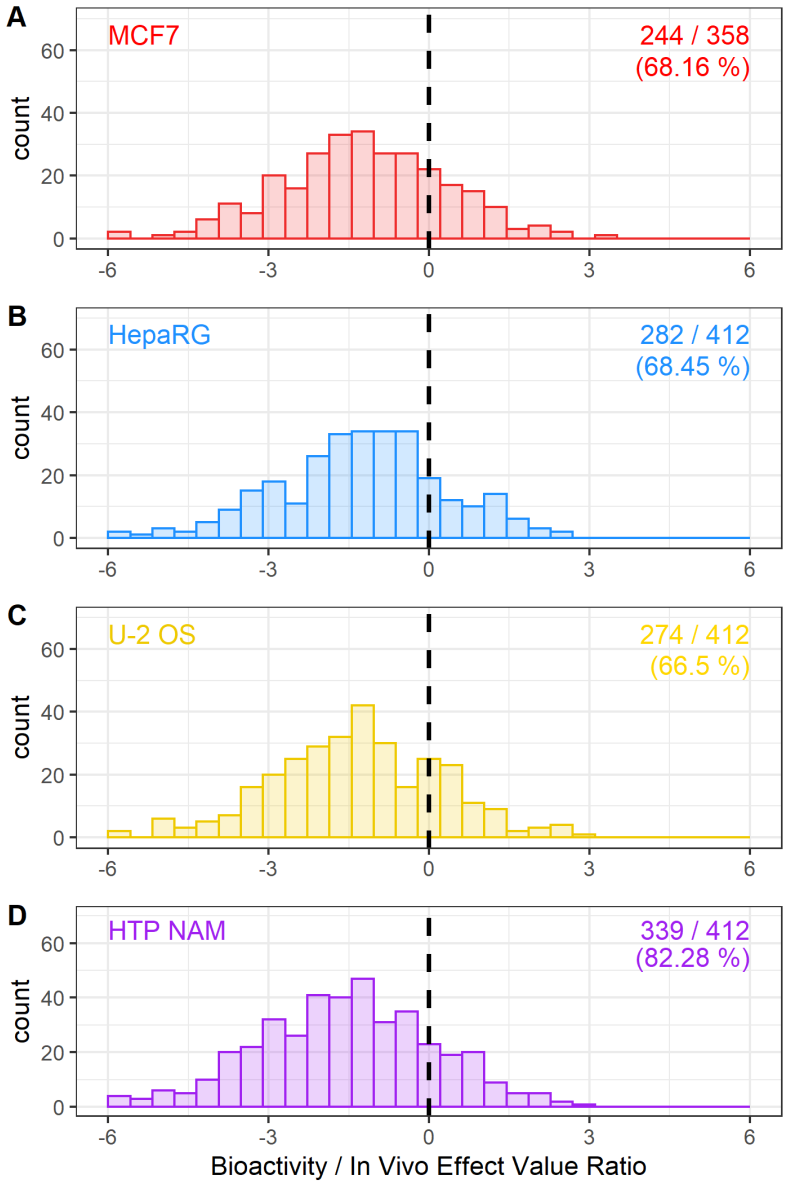
- Mammalian species
- oral exposures
- Various study types
- NOEL, LOEL, NOAEL, LOAEL
- mg/kg/day

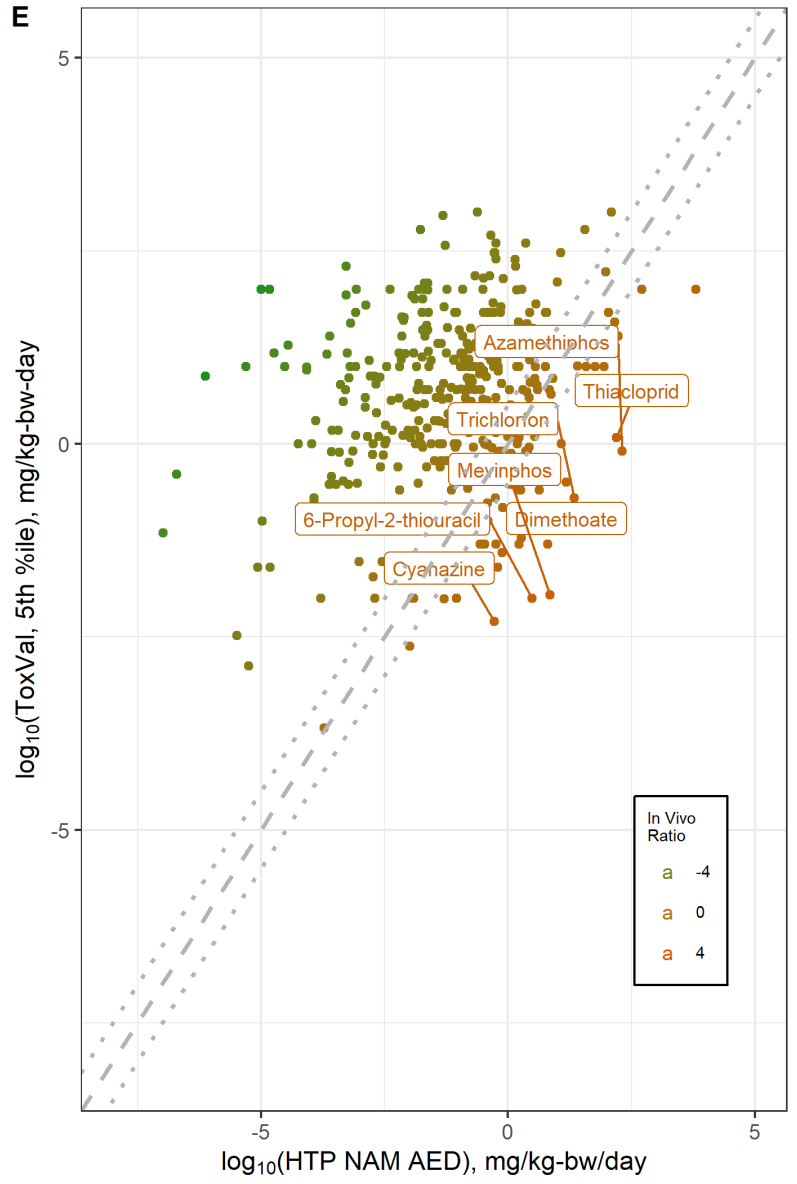


POD: point-of-departure
AED: administered equivalent dose

Bioactivity / *In Vivo* Effect Value Ratio Analysis

- **Negative ratios** indicate that AEDs derived from HTP NAMs molecular PODs are **conservative** surrogates for traditional *in vivo* PODs.
- When cell lines are considered individually, **~66-68%** of chemicals had negative ratios.
- When considered in combination, the number and percentage of chemicals with negative ratios **increased (82.3 %)**.
- Paul Friedman et al. (2020) (PMID: 31532525)
  - Using ToxCast, **89 %** of APCRA chemicals had negative ratios.
- When multiple cell types are considered, mPODs from HTTr screening appear to be conservative surrogates for *in vivo* PODs.
- Correlation of *in vitro* and *in vivo* is low.





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