EPA
United States
Environmental Protection
Agency

High-Throughput Transcriptomics

Joshua A. Harrill

USEPA Center for Computational Toxicology and Exposure (CCTE)

German Federal Institute for Risk Assessment
Challenges in Public Health Protection in the 21st Century: New Methods, Omics and Novel Concepts in Toxicology
November 16th, 2021

EPA United States Environmental Protection Agency

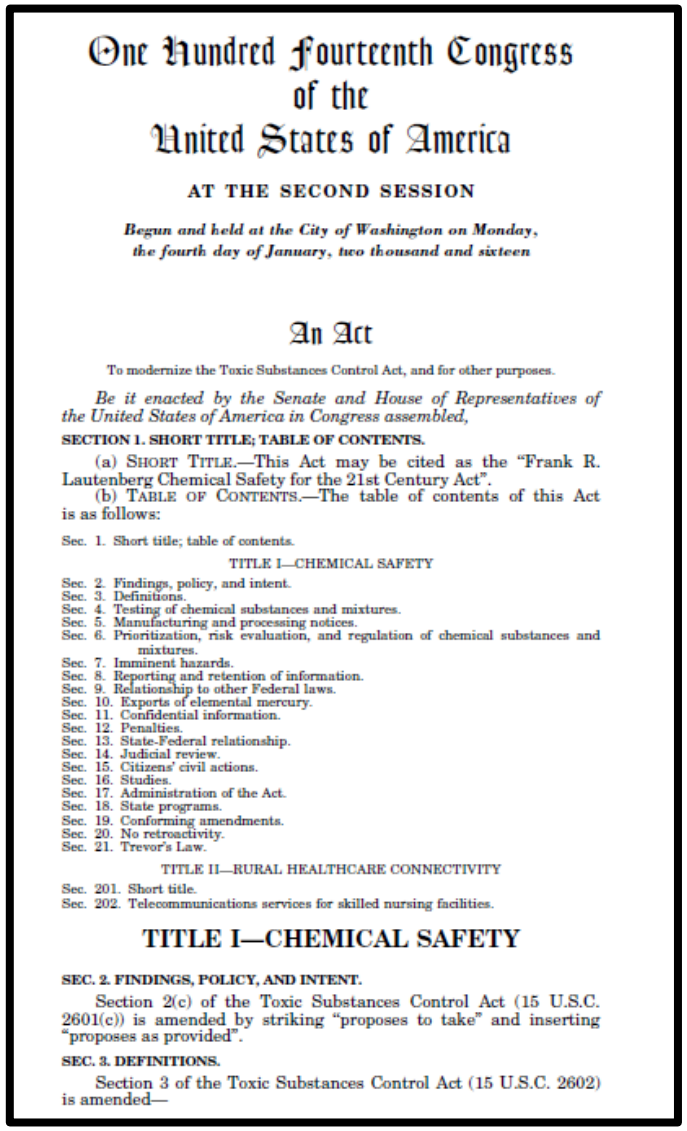
Disclaimer

The views expressed in this presentation are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency, nor does mention of trade names or products represent endorsement for use.

Outline

- **Background**
  - New Approach Methods (NAMs)
  - Center Computational Toxicology and Exposure (CCTE)
  - Blueprint for Computational Toxicology at EPA
- **High-Throughput Transcriptomics (HTTr)**
  - TempO-Seq Technology
  - Pilot Study in MCF7 Cells
  - Screening Results in MCF7 Cells
- **Potential Applications of HTTr-Derived PODs**
  - Bioactivity to Exposure Ratio (BER) Analysis

Regulatory Driver for Development & Use of NAMs by US EPA



One Hundred Fourteenth Congress
of the
United States of America

AT THE SECOND SESSION

Begun and held at the City of Washington on Monday, the fourth day of January, two thousand and sixteen

An Act

To modernize the Toxic Substances Control Act, and for other purposes.

Be it enacted by the Senate and House of Representatives of the United States of America in Congress assembled,

SECTION 1. SHORT TITLE; TABLE OF CONTENTS.

(a) SHORT TITLE.—This Act may be cited as the "Frank R. Lautenberg Chemical Safety for the 21st Century Act".

(b) TABLE OF CONTENTS.—The table of contents of this Act is as follows:

Sec. 1. Short title; table of contents.

TITLE I—CHEMICAL SAFETY

Sec. 2. Findings, policy, and intent.
Sec. 3. Definitions.
Sec. 4. Testing of chemical substances and mixtures.
Sec. 5. Manufacturing and processing notices.
Sec. 6. Prioritization, risk evaluation, and regulation of chemical substances and mixtures.
Sec. 7. Imminent hazards.
Sec. 8. Reporting and retention of information.
Sec. 9. Relationship to other Federal laws.
Sec. 10. Exports of elemental mercury.
Sec. 11. Confidential information.
Sec. 12. Penalties.
Sec. 13. State-Federal relationship.
Sec. 14. Judicial review.
Sec. 15. Citizens' civil actions.
Sec. 16. Studies.
Sec. 17. Administration of the Act.
Sec. 18. State programs.
Sec. 19. Conforming amendments.
Sec. 20. No retroactivity.
Sec. 21. Trevor's Law.

TITLE II—RURAL HEALTHCARE CONNECTIVITY

Sec. 201. Short title.
Sec. 202. Telecommunications services for skilled nursing facilities.

TITLE I—CHEMICAL SAFETY

SEC. 2. FINDINGS, POLICY, AND INTENT.

Section 2(c) of the Toxic Substances Control Act (15 U.S.C. 2601(c)) is amended by striking "proposes to take" and inserting "proposes as provided".

SEC. 3. DEFINITIONS.

Section 3 of the Toxic Substances Control Act (15 U.S.C. 2602) is amended—

The Toxic Substances Control Act (TSCA), as amended by the Frank R. Lautenberg Chemical Safety for the 21st Century Act, directs EPA to:

1. **Reduce and replace**, to the extent practicable and scientifically justified, the use of vertebrate animals in the testing of chemical substances or mixtures;
2. Promote the development and timely incorporation of **alternative test methods or strategies** that do not require new vertebrate animal testing

"Alternative test methods" – Tools of the Trade

1. ***Computational toxicology and bioinformatics.***
2. ***High-throughput screening methods.***
3. Testing of categories of chemical substances.
4. ***Tiered testing methods.***
5. ***In vitro studies.***
6. Systems Biology.
7. ICCVAM or OECD validated assays.
8. Industry consortia that develop information submitted under this title.

"Alternative test methods" → "New Approach Methods (NAMs)"

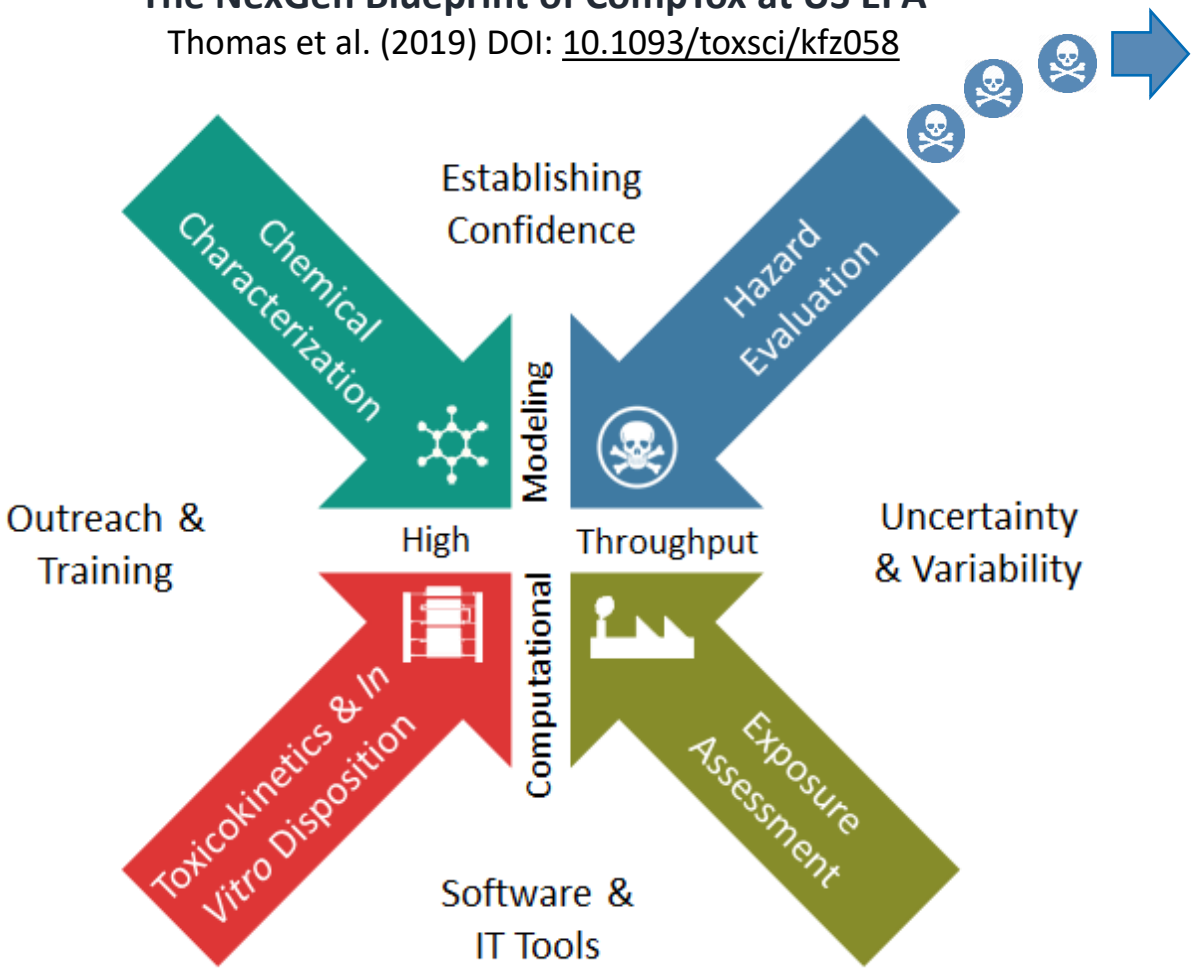
Any technology, methodology, approach or combination thereof that can be used to provide information on chemical hazard and risk that avoids the use of intact animals.

2016

https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/alternative-test-methods-and-strategies-reduce

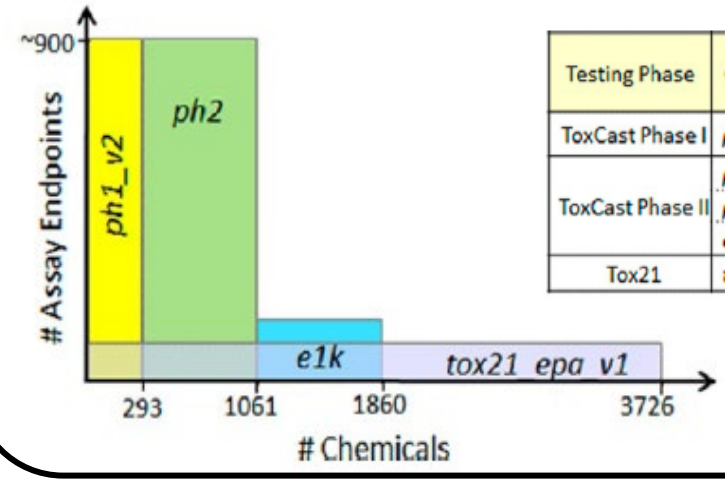
Computational Toxicology Research Areas

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



ToxCast: Uses targeted high-throughput screening (HTS) assays to expose living cells or isolated proteins to chemicals and assess bioactivity and potential toxic effects.

Richard et al. (2016) DOI: 10.1021/acs.chemrestox.6b00135



Testing Phase	Chemical Set	Unique Chemicals	Assay Endpoints
ToxCast Phase I	*ph1_v1*	310	~700
ToxCast Phase II	*ph1_v2*	293	~200
	ph2	768	~900
	e1k	799	~50
Tox21	*tox21_epa_v1*	3726	~80

Mostly targeted assays (*chemical X → target Y*).
Incomplete coverage of human biological space.

New Strategy for Hazard Evaluation: Improve efficiency and increase biological coverage by using broad-based (i.e. non-targeted) assays that cast the broadest net possible for capturing the potential molecular and phenotypic responses of human cells in response to chemical exposures.

NAMs-Based Tiered Hazard Evaluation Approach (1)

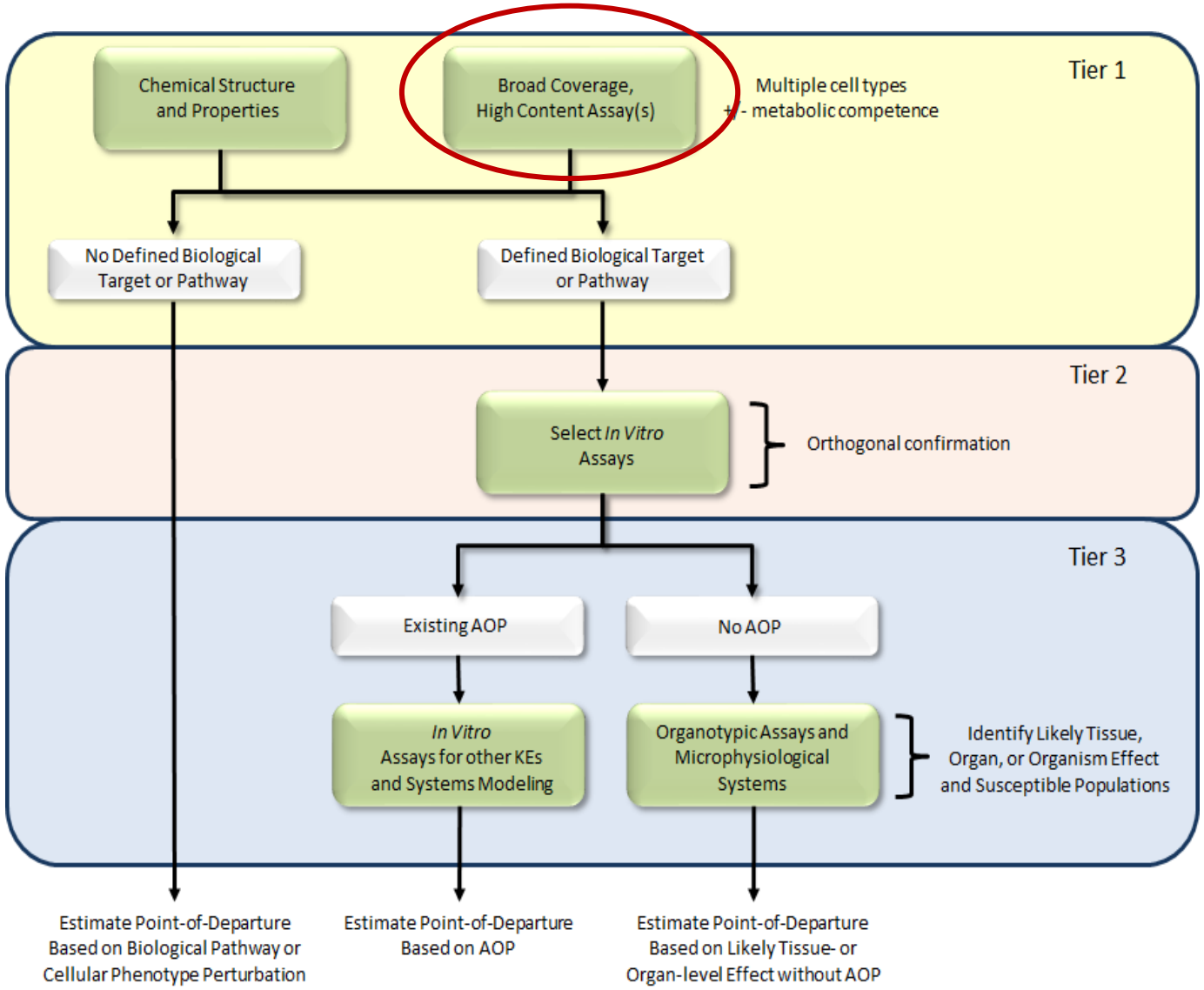
High throughput profiling (HTP) assays are proposed as the first tier in a NAMs-based hazard evaluation approach.

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]**



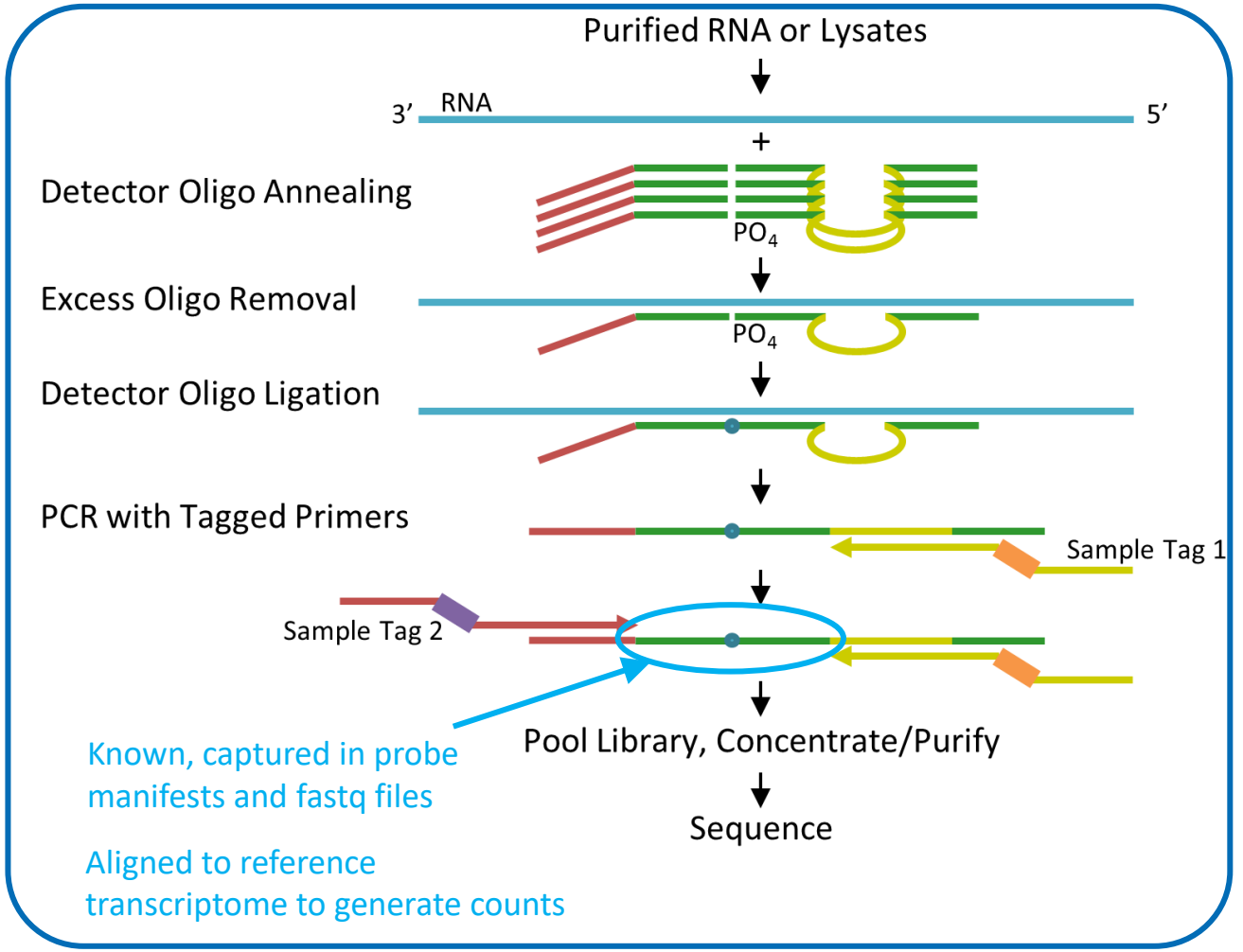
2016 — 2018 — 2019 — 2020

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

MCF7 Pilot Experimental Design

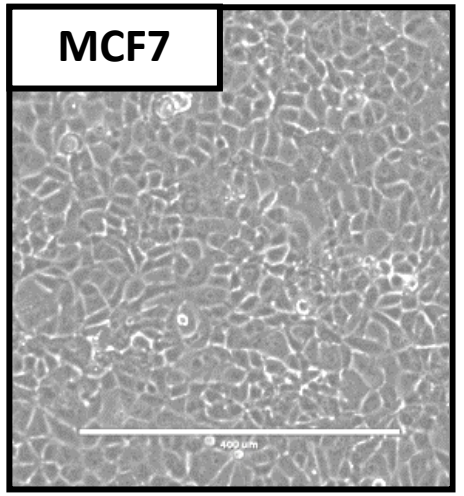
High-Throughput Transcriptomics Platform for Screening Environmental Chemicals

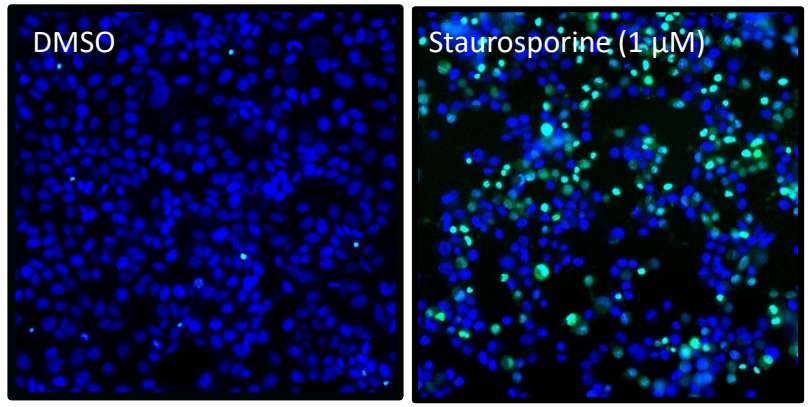
Joshua A. Harrill,*,1 Logan J. Everett,* Derik E. Haggard,*,† Thomas Sheffield,*,† Joseph L. Bundy,* Clinton M. Willis,*,‡ Russell S. Thomas,* Imran Shah,* and Richard S. Judson*

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

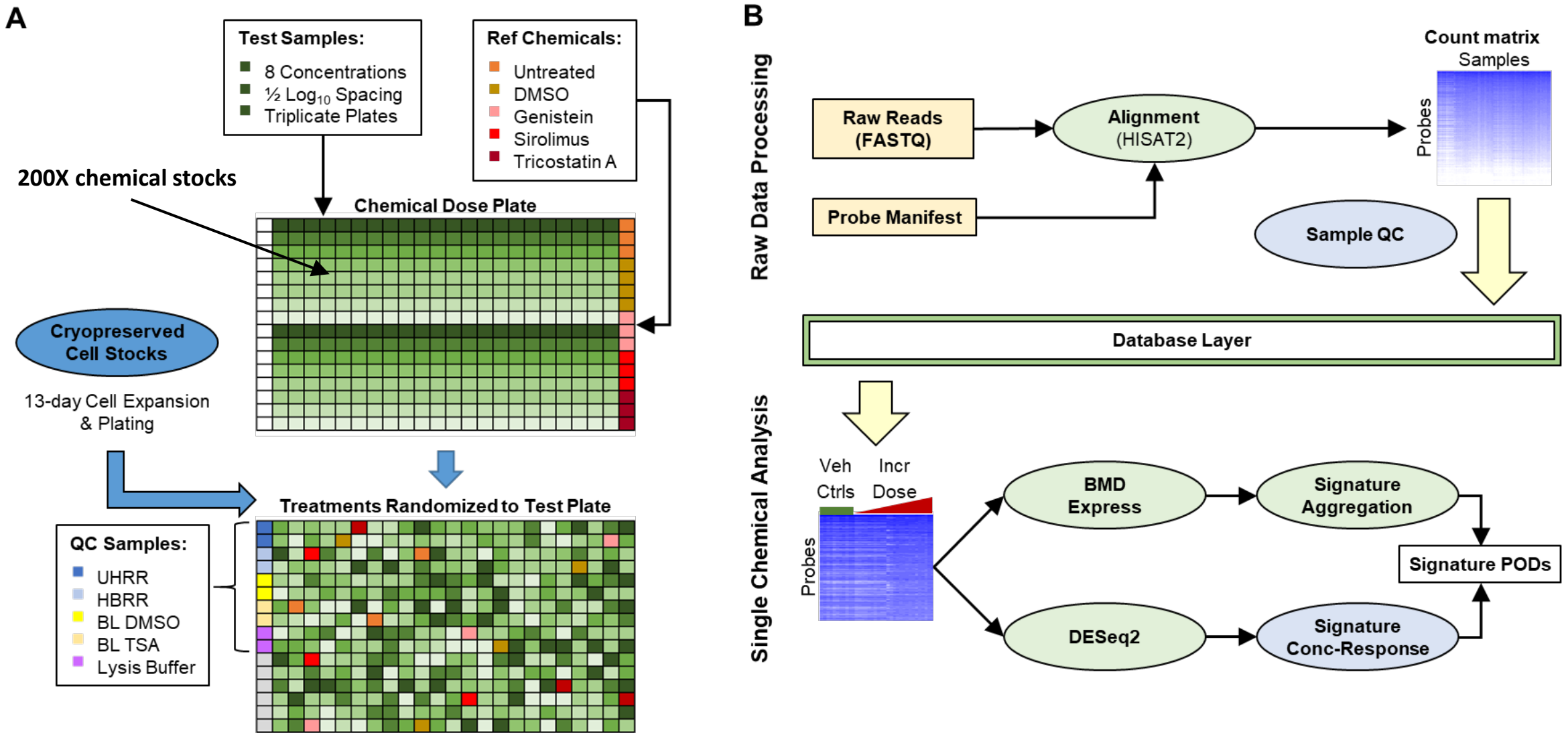
Table 1. Chemicals Used in the Study

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

HTTr Experimental Design and Bioinformatics Workflow



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

Gene Signatures

- Understanding the biological meaning of changes in gene expression for **10,000 – 20,000 genes** is difficult.
- Analyzing responses at the level of the gene signature aids in **data interpretation**.
- Takes into account coordinated changes in gene expression that may not be identified using gene level fitting approaches.
- **Examples of signature types**:
  - Genes that are perturbed in diseased tissue vs. healthy tissue.
  - Genes perturbed by gene knockdowns / knockouts.
  - Genes perturbed by drugs or other chemicals with known (or unknown) mechanisms.
- **Example use:**
  - If an unknown *chemical X* perturbs genes that are also perturbed by a well-characterized chemical with a specific mechanism of action, then one can infer the *chemical X* may affect the same molecular target(s).
- **CCTE signature collection:**
  - Compiled from many public sources (MSigDB[1], BioPlanet[2], DisGeNET[3], Connectivity Map[4]) → ~10,000 signatures.
- **Signature Scoring Method:**
  - Single Sample Gene Set Enrichment Analysis (ssGSEA)[5]

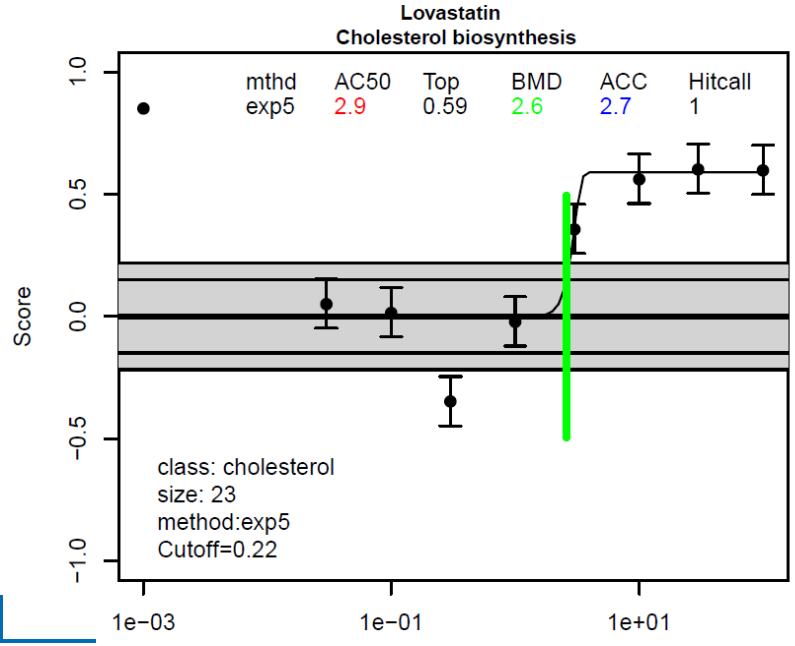
[1] *Liberzon et al., Bioinformatics. 2011 Jun 15;27(12):1739-40*
[2] *Huang et al., Front Pharmacol. 2019 Apr 26;10:445*
[3] *Pinero et al., Database (Oxford). 2015 Apr 15;2015:bav028*
[4] *Subramanian et al., Science. 2006 Sep 29;313(5795):1929-35.*
5 Barbie et al ., Nature. Nov 5;462(7269):108-12.

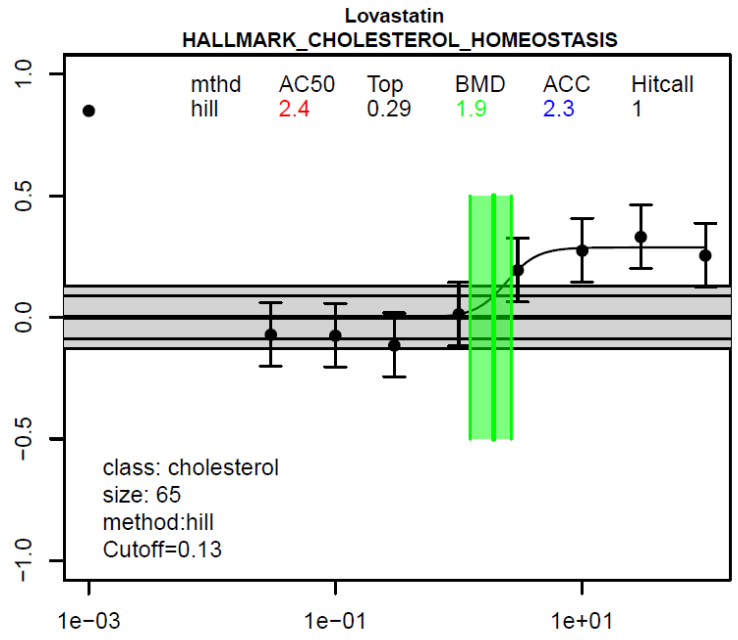
Concentration-Response Modeling of Signature Scores (1)

Concentration response modeling of signature scores using ***tcplfit2*** (https://rdrr.io/github/USEPA/CompTox-ToxCast-tcplFit2/)

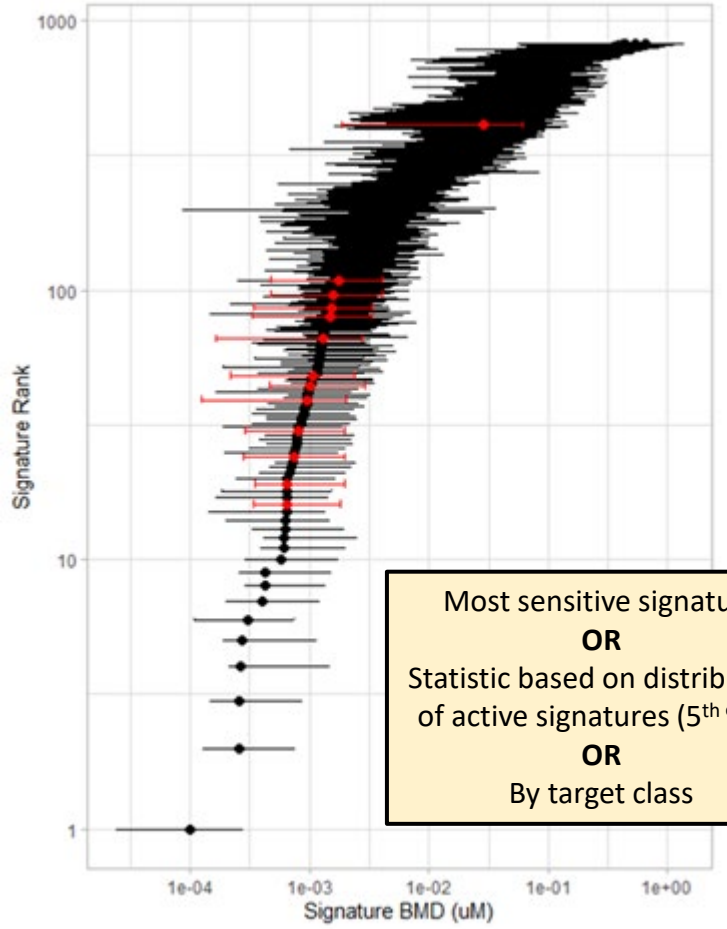
New and/or improved functionality of *tcplfit2* (versus *tcpl*):

- All curve forms from *tcpl* and BMDExpress are included.
- Calculates benchmark concentrations (BMCs) in addition to AC50s.
- Models in the "up" and "down" direction.
- Provides continuous hit calls for identifying high confidence and low confidence hits.





Concentration-Response Modeling of Signature Scores

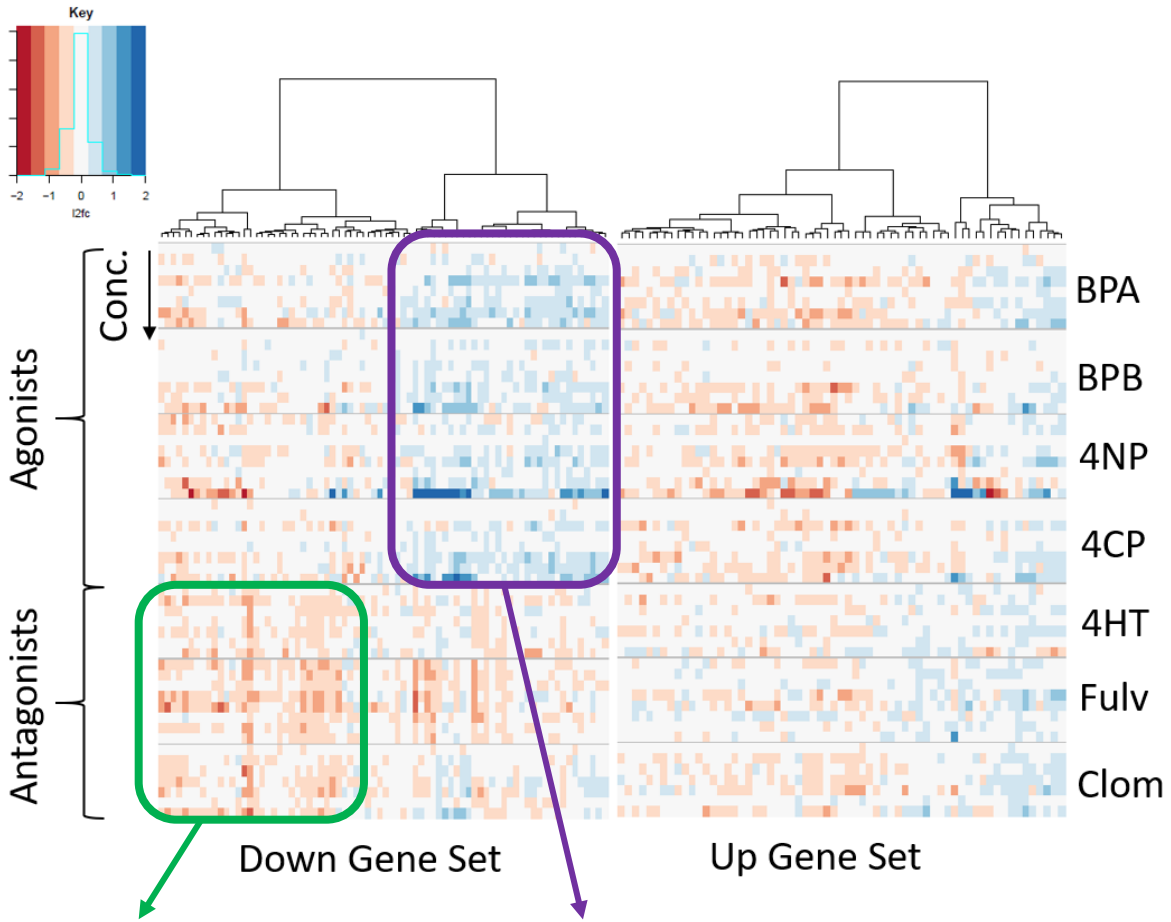


Most sensitive signature
OR
Statistic based on distribution of active signatures (5th %ile)
OR
By target class

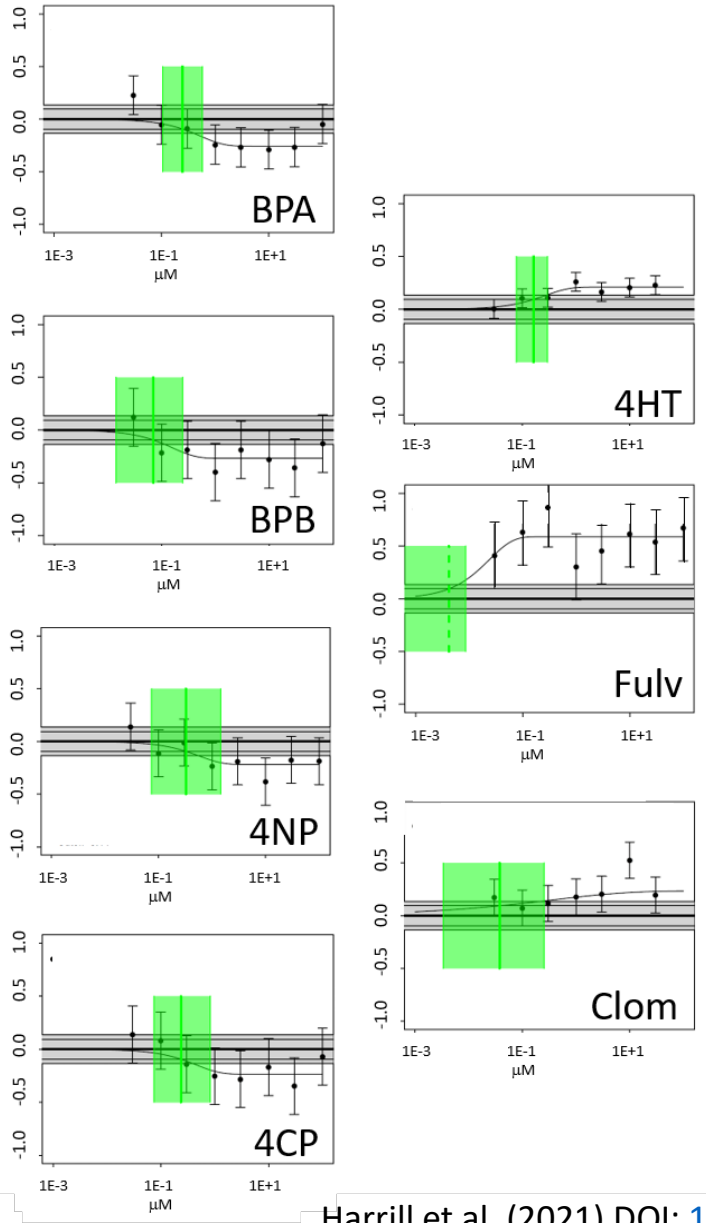
Concentration-Response Modeling of Signature Scores (2)

A

Fulvestrant Signature
(Top 100 Up & Down Genes)



B



These gene level data are noisy!

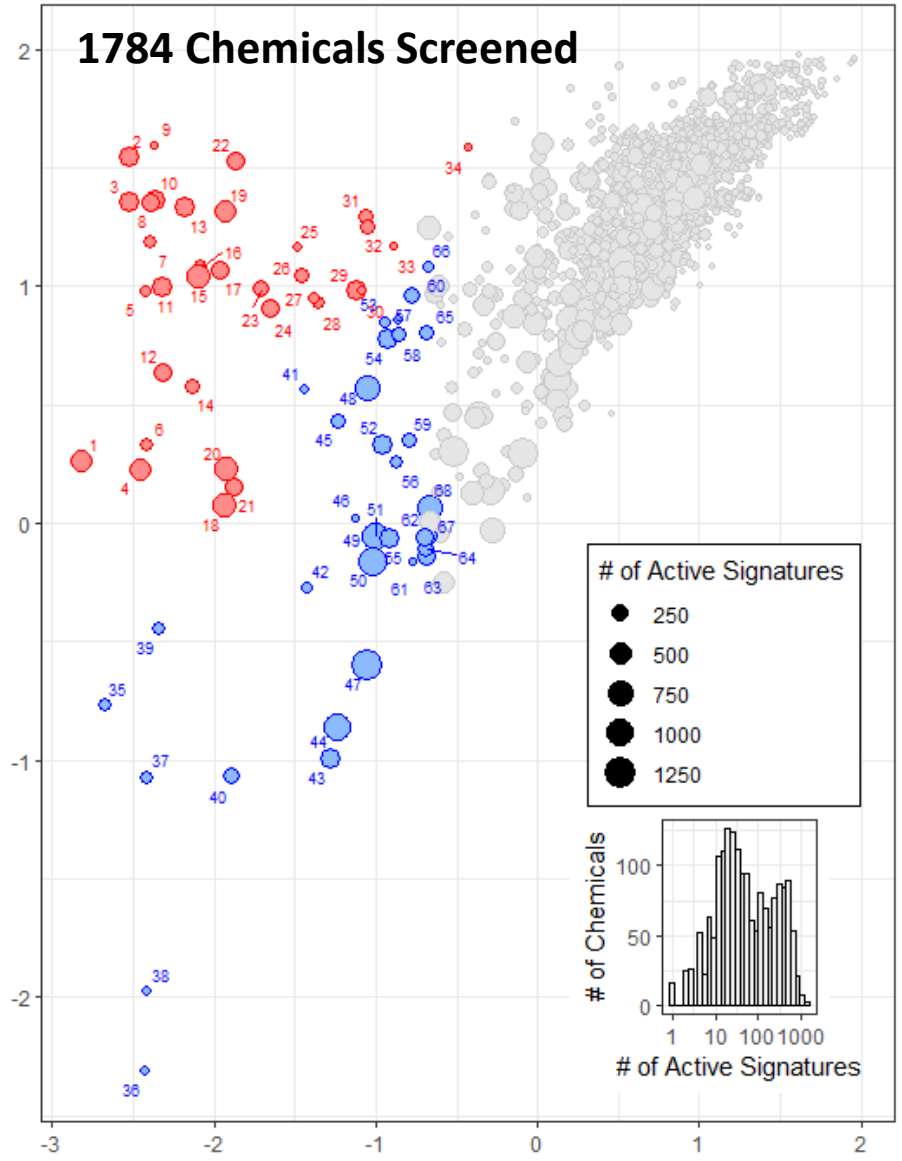
Signature level results display correct directionality!

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

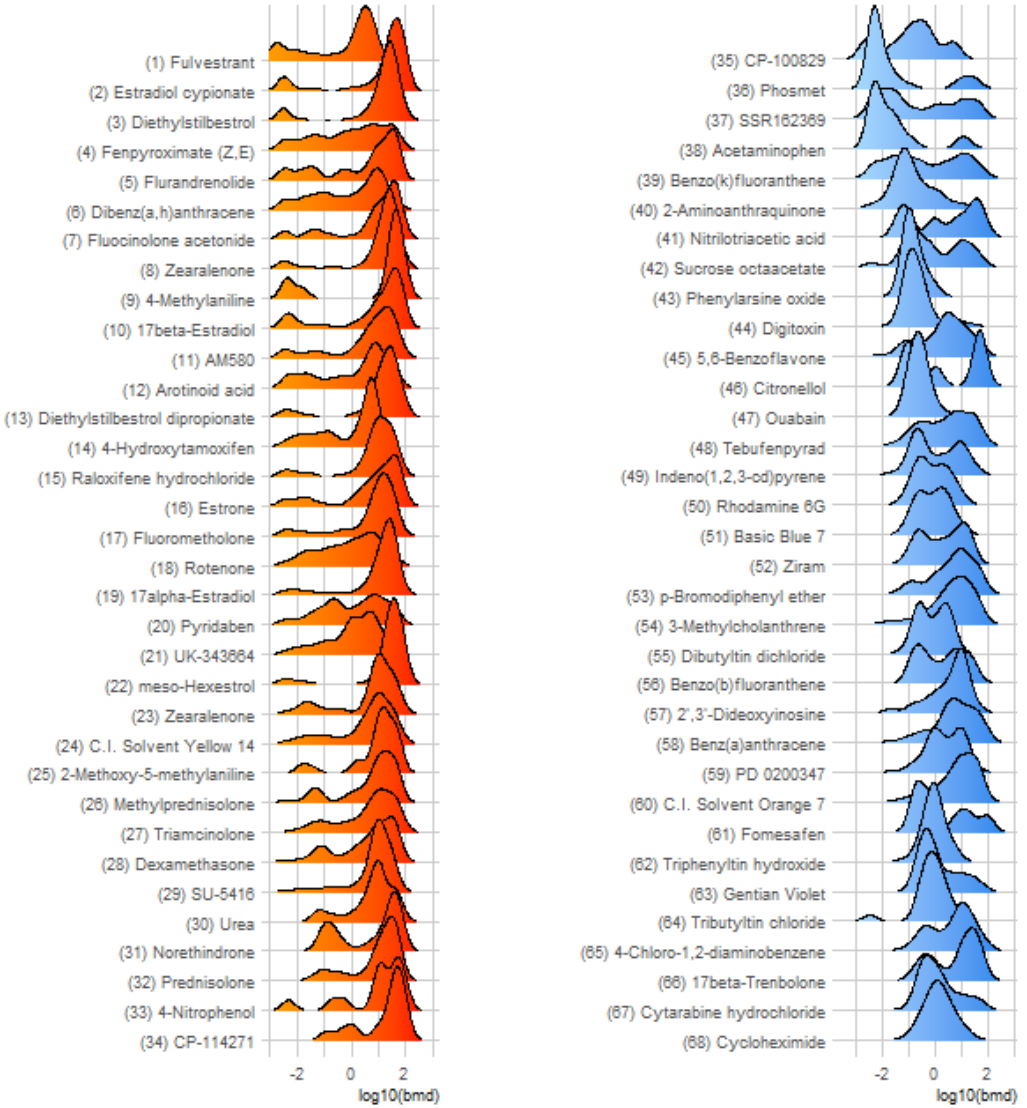
The expression of fulvestrant signature "down" genes goes up following ER agonist treatment

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

MCF7 HTTr Screening Results (1)



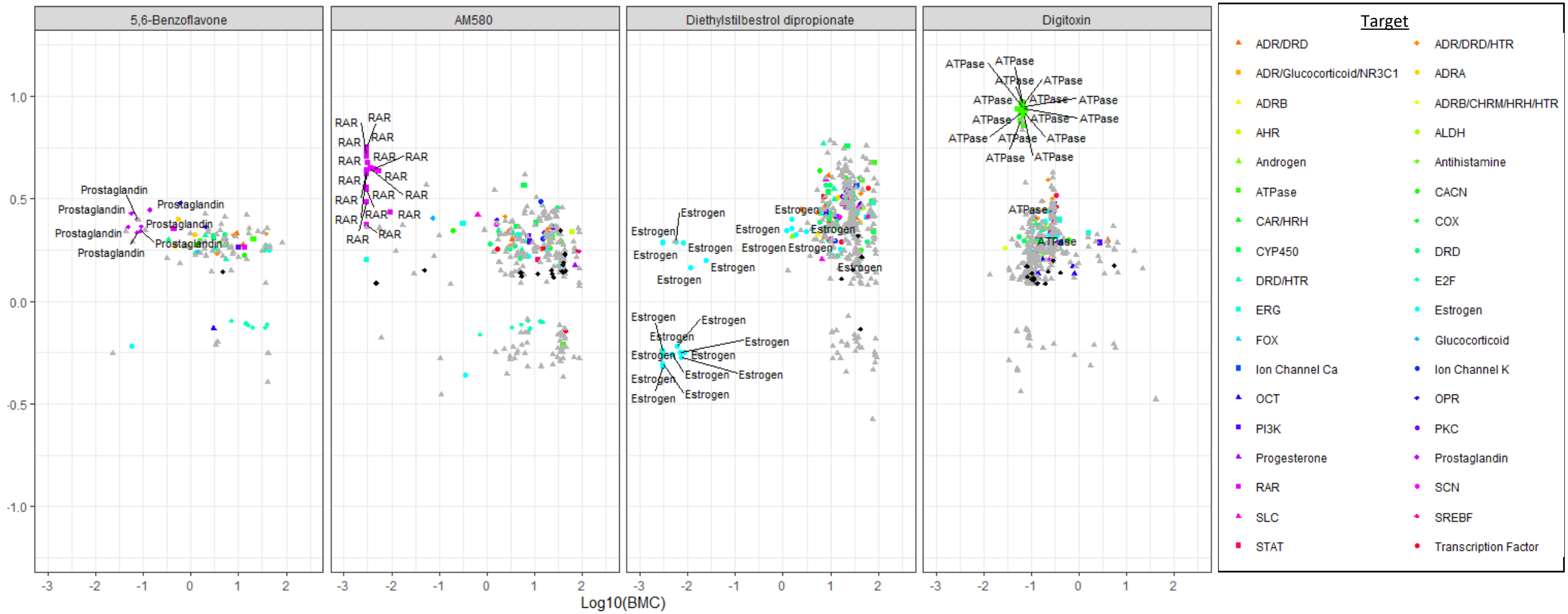
Distribution of BMCs of Active Signatures



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

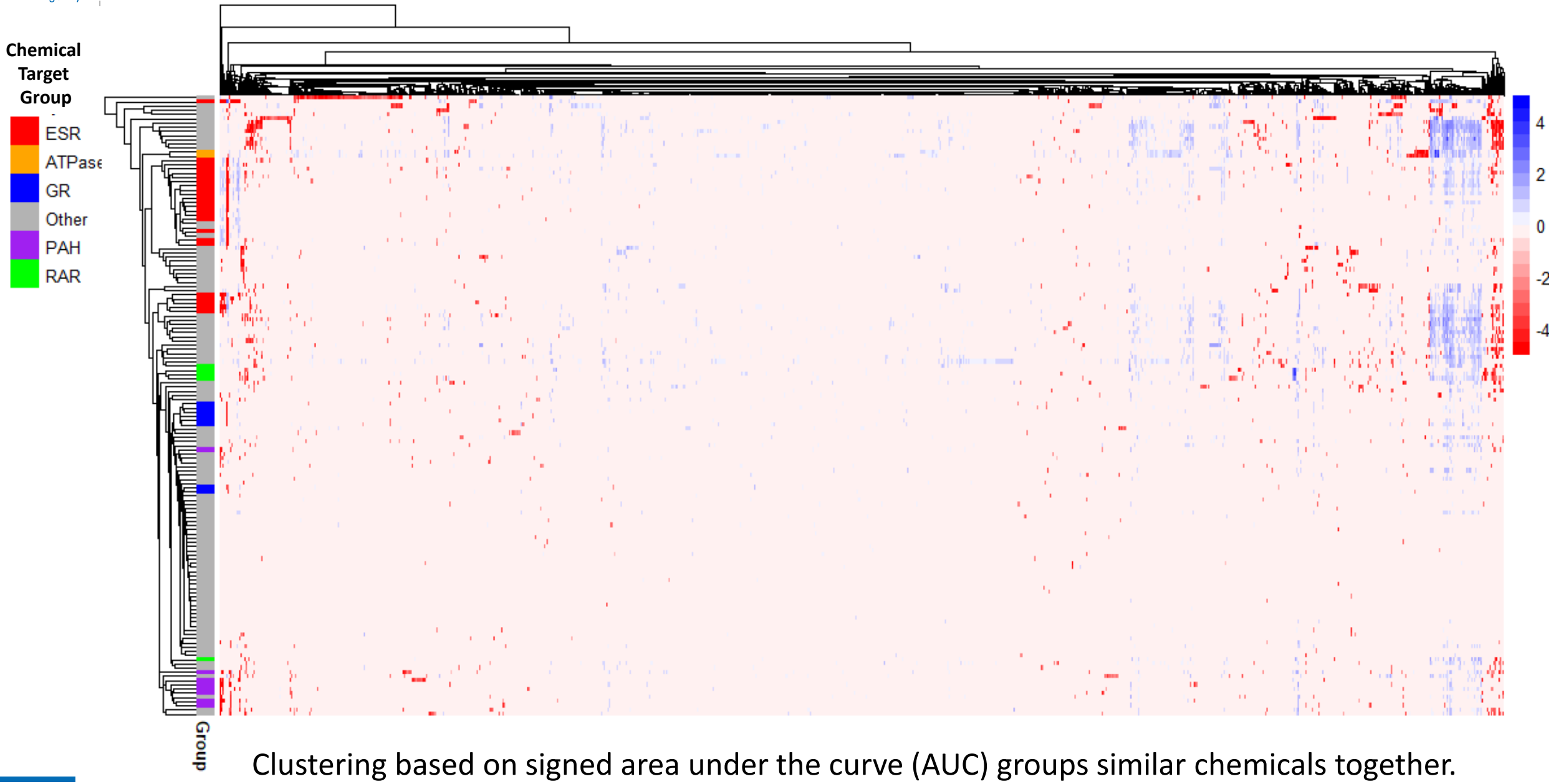
Chemicals with known pharmacological targets show an “early wave” of biological activity.

MCF7 HTTr Screening Results (2)



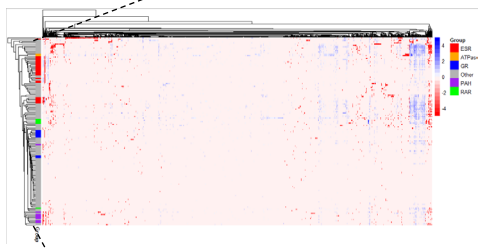
The most potent and efficacious signature hits correspond to known mechanisms for these chemicals.

MCF7 HTTr Screening Results (3)



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

MCF7 HTTr Screening Results (4)



1 Polycyclic Aromatic Hydrocarbons (PAH)

TP0001719N15_C.I. Solvent Yellow 14
EPAPLT0027E14_1-Naphthol
TP0001969O02_Indeno(1,2,3-cd)pyrene
TP0001720M20_Dibenz(a,h)anthracene
TP0001967A02_C.I. Solvent Orange 7
TP0001719J03_Benzo(b)fluoranthene
EPAPLT0027I03_5,6-Benzoflavone
TP0001969L02_Benzo(k)fluoranthene
EPAPLT0027G09_1,4-Dihydroxyanthracene-9,10-dione
EPAPLT0027C20_SU-5416
TP0001967G01_3-Methylcholanthrene

2 Glucocorticoid Receptor (GR)

EPAPLT0027K14_Fluorometholone
EPAPLT0027M18_Fluocinolone acetonide
EPAPLT0027L16_Flurandrenolide
TP0001967P03_Prednisolone
TP0001967N05_Methylprednisolone
TP0001967B09_Dexamethasone

3 Retinoic Acid Receptor (RAR)

EPAPLT0027C17_Bexarotene
EPAPLT0027L01_AM580
EPAPLT0027J15_Arotinoid acid
TP0001720E04_Triphenyltin hydroxide
EPAPLT0027J09_Thimerosal
TP0001718L05_Tributyltin methacrylate
TP0001718I16_Ziram
TP0001720P03_Thiram

4 Selective Estrogen Receptor Modulator (SERM)

TP0001719N17_Tamoxifen
TP0001718E16_Raloxifene hydrochloride
TP0001719H18_Tamoxifen citrate
TP0001718D16_Clomiphene citrate (1:1)
TP0001718F22_4-Hydroxytamoxifen

5 Estrogens

TP0001720H24_Zearalenone
EPAPLT0027G08_Zearalenone
TP0001719I06_Perfluorodecanoic acid
TP0001719J11_Norethindrone
TP0001969J07_Bromofos
TP0001719M14_Tetrac
EPAPLT0027F15_Ethisterone
TP0001719I02_Daidzein
TP0001718I04_5alpha-Dihydrotestosterone
TP0001718G13_17beta-Trenbolone
TP0001719B08_meso-Hexestrol
TP0001719E11_Estrone
TP0001718E09_Diethylstilbestrol
EPAPLT0027J06_Dehydroepiandrosterone
TP0001967N07_Diethylstilbestrol dipropionate
TP0001718D07_17alpha-Estradiol
TP0001719G02_Levonorgestrel
TP0001967M08_dl-Norgestrel
TP0001718K19_Genistein
TP0001718A17_17beta-Estradiol
EPAPLT0027D13_Estradiol cypionate
TP0001967D06_Ouabain
TP0001969O01_Digitoxin

6 ATPase

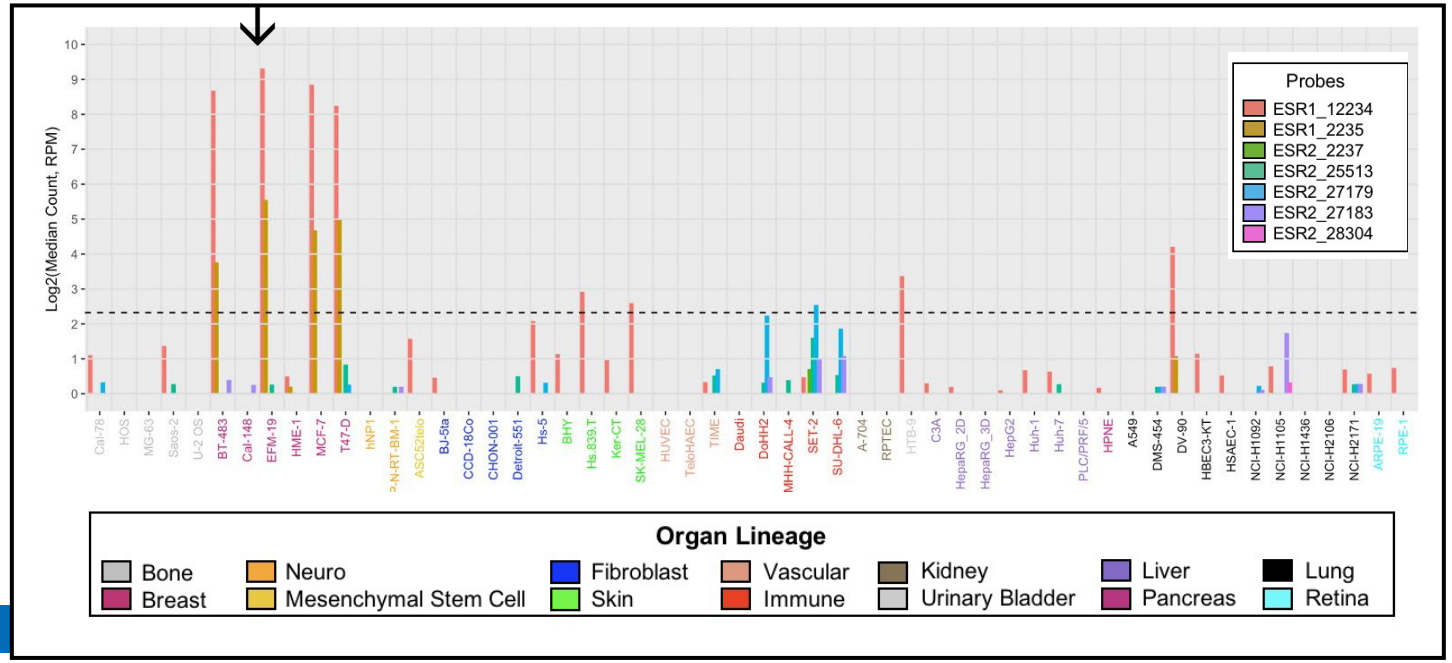
Comparison of Transcriptional BPACs to ER Model

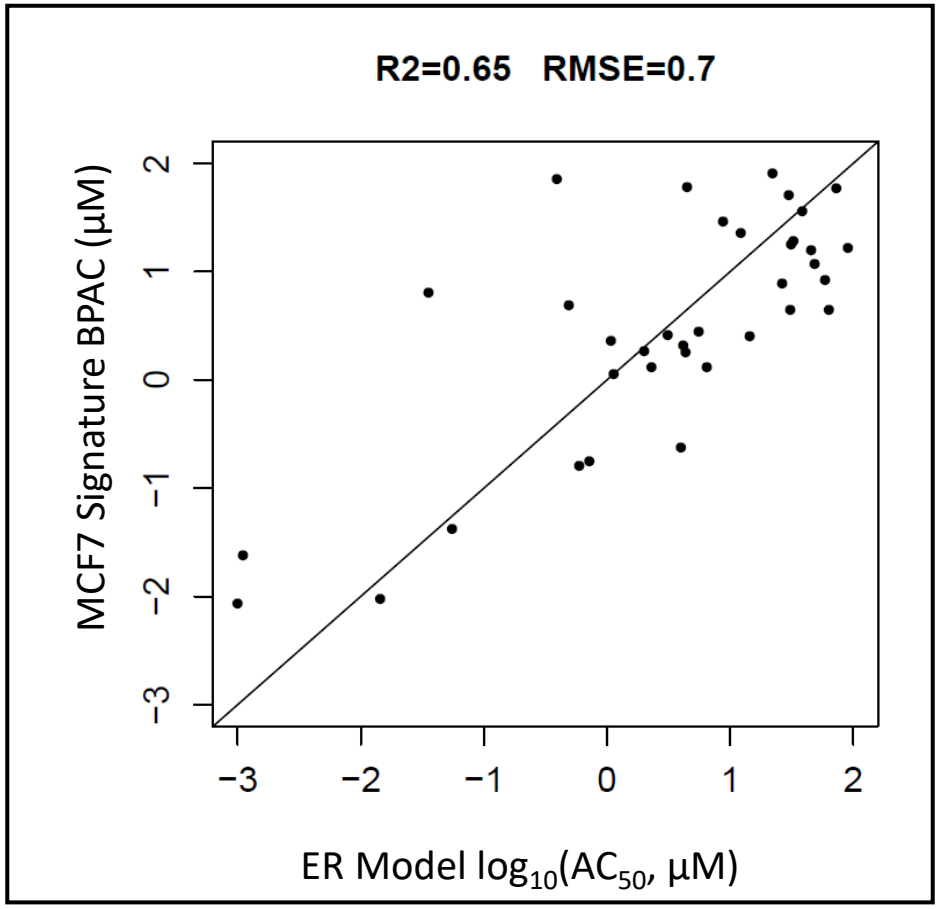
- US EPA has developed a battery of 18 ToxCast assays to predict activity at the estrogen receptor (Brown et al. (2015) DOI: 10.1021/acs.est.5b02641)

- Log_{10} AC_{50} values from the ToxCast ER model assays were compared to transcriptomic signature BPACs in MCF7 cells for a collection of 37 estrogenic chemicals.

- Signature-based BPACs are concordant with ER model predictions. →

- Estrogen receptor is also abundantly expressed in MCF7 cells (and other breast-derived cell lines).





Harrill et al., (unpublished). DO NOT CITE OR QUOTE

EPA
United States
Environmental Protection
Agency

Potential Applications for HTTr-Derived Molecular PODs

HTP Screening Experimental Designs

Parameter	Multiplier	Notes			
Chemicals	462	APCRA retrospective case study chemicals			
Cell Types	4	U-2 OS		HepaRG-2D	MCF7
Assay Formats	2	HTPP	HTTr	HTTr	HTTr
Exposure Durations	Variable	24 HR	24 HR	24 HR	6 HR
Concentrations:	8	3.5 log_{10} units; ~half-log_{10} spacing			
Biological Replicates:	Variable	4	3	3	3

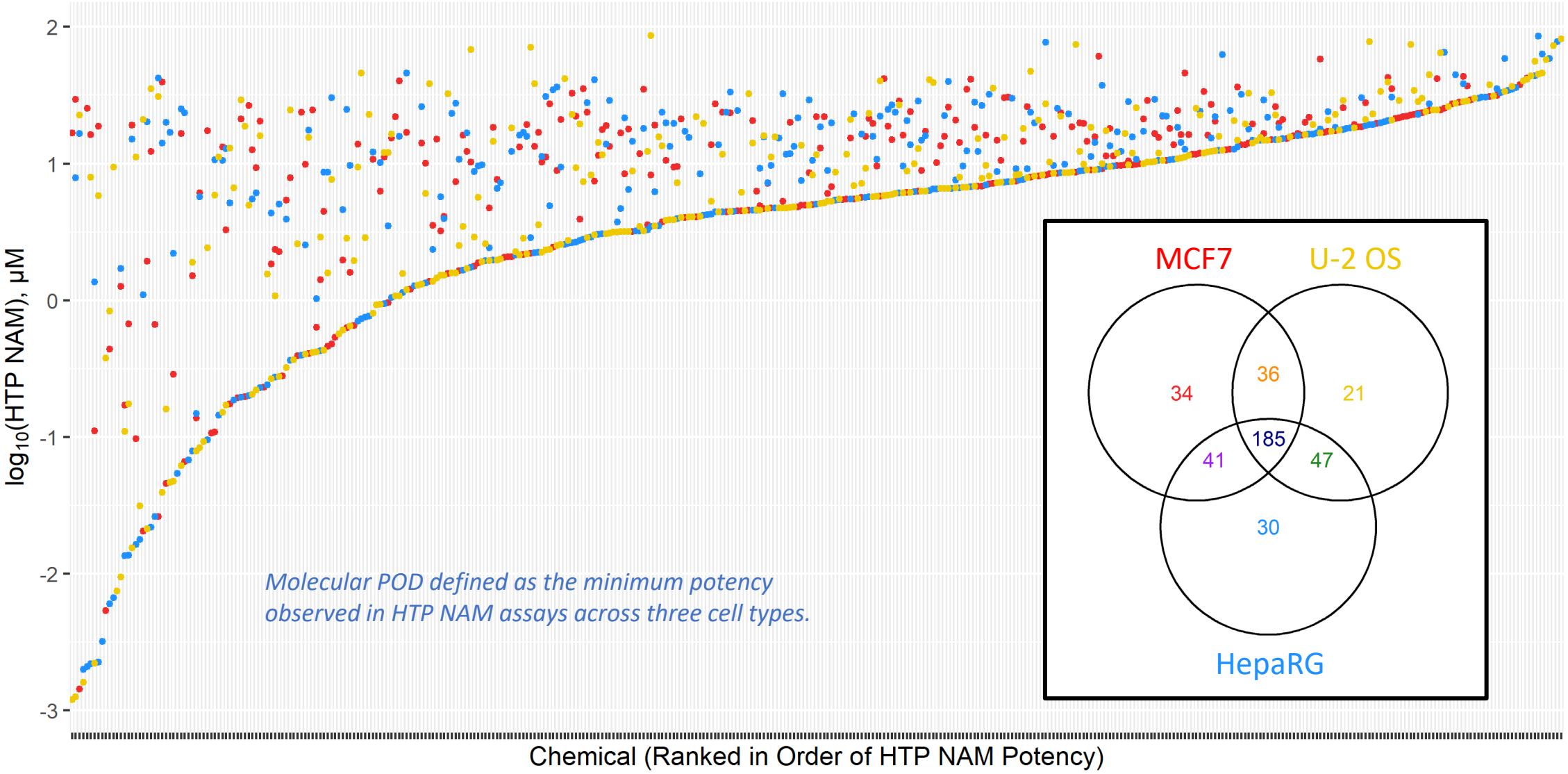
APCRA
ACCELERATING THE PACE OF CHEMICAL RISK ASSESSMENT

"Advancing Methodology" case study: **deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates.**

APCRA Chemicals → PK parameters necessary for *in vitro* to *in vivo* extrapolation (IVIVE)
in vivo toxicity data

Kavlock et al. (2018)
Chem. Res. Tox; 31(5): 287-290

Comparison of Screening Results Across Cell Lines



There are chemicals that have more potent bioactivity in one cell line as opposed to another.

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)

HTP Potency Estimate (µM)

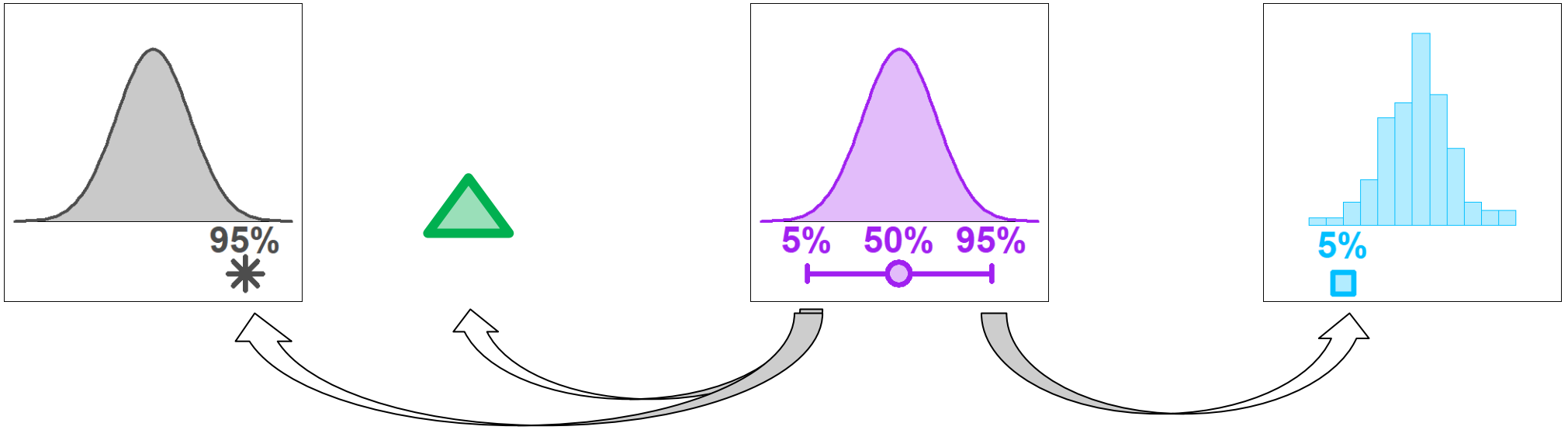
***In vitro*-to-*in vivo* extrapolation (IVIVE)**
high-throughput toxicokinetics (httk)

HTP AED (mg/kg bw/day)

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

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

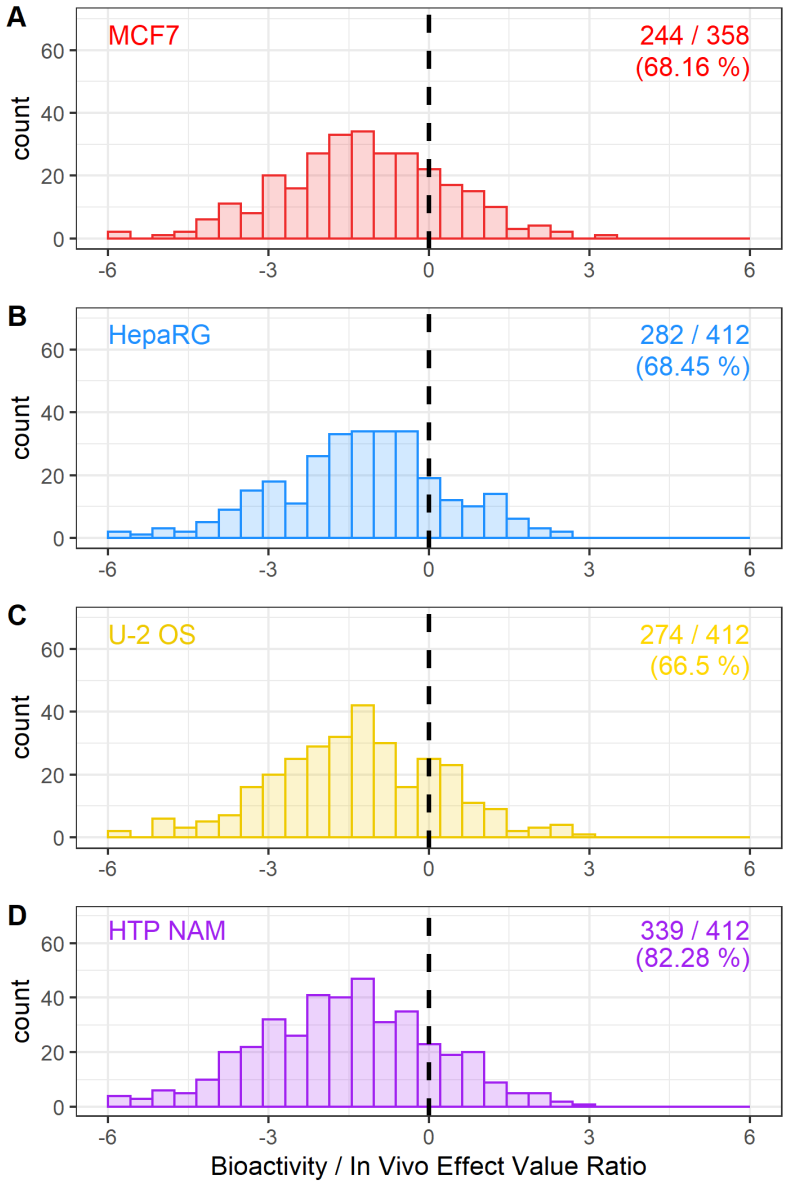
- Mammalian species
- oral exposures
- Various study types
- NOEL, LOEL, NOAEL, LOAEL
- mg/kg/day

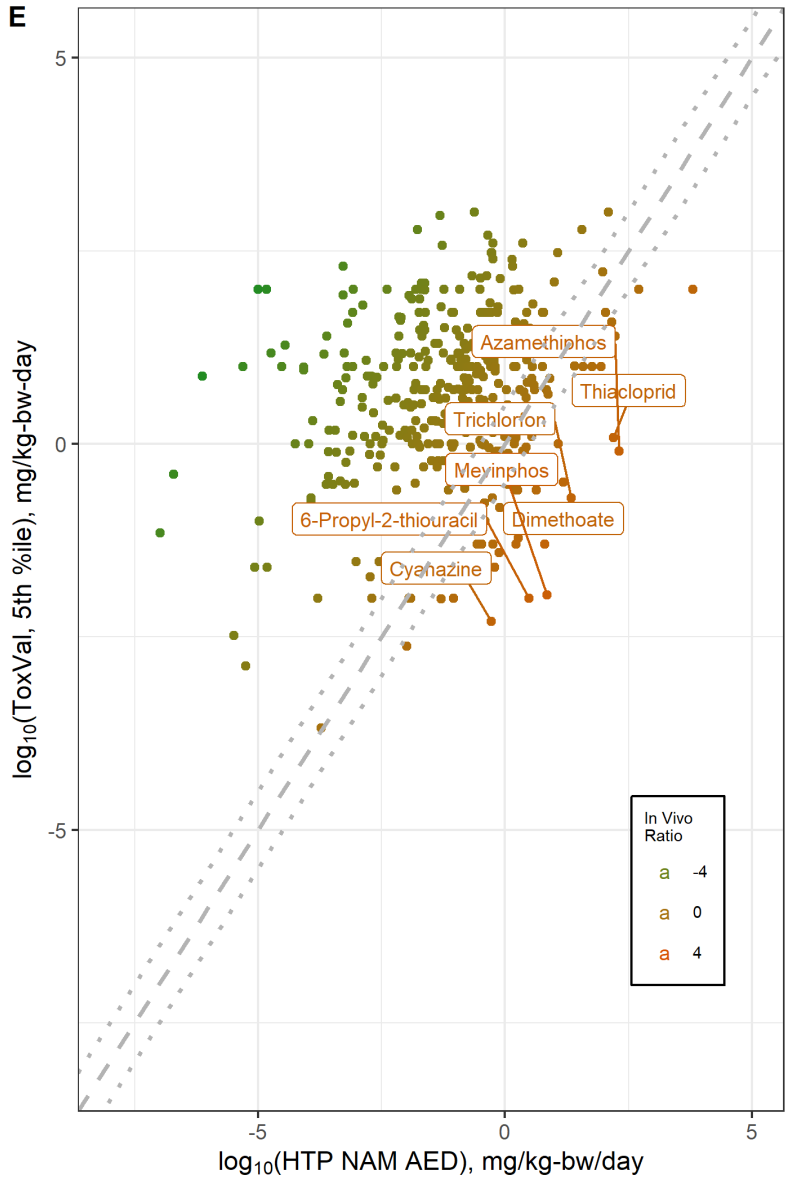


POD: point-of-departure
AED: administered equivalent dose

Bioactivity / *In Vivo* Effect Value Ratio Analysis

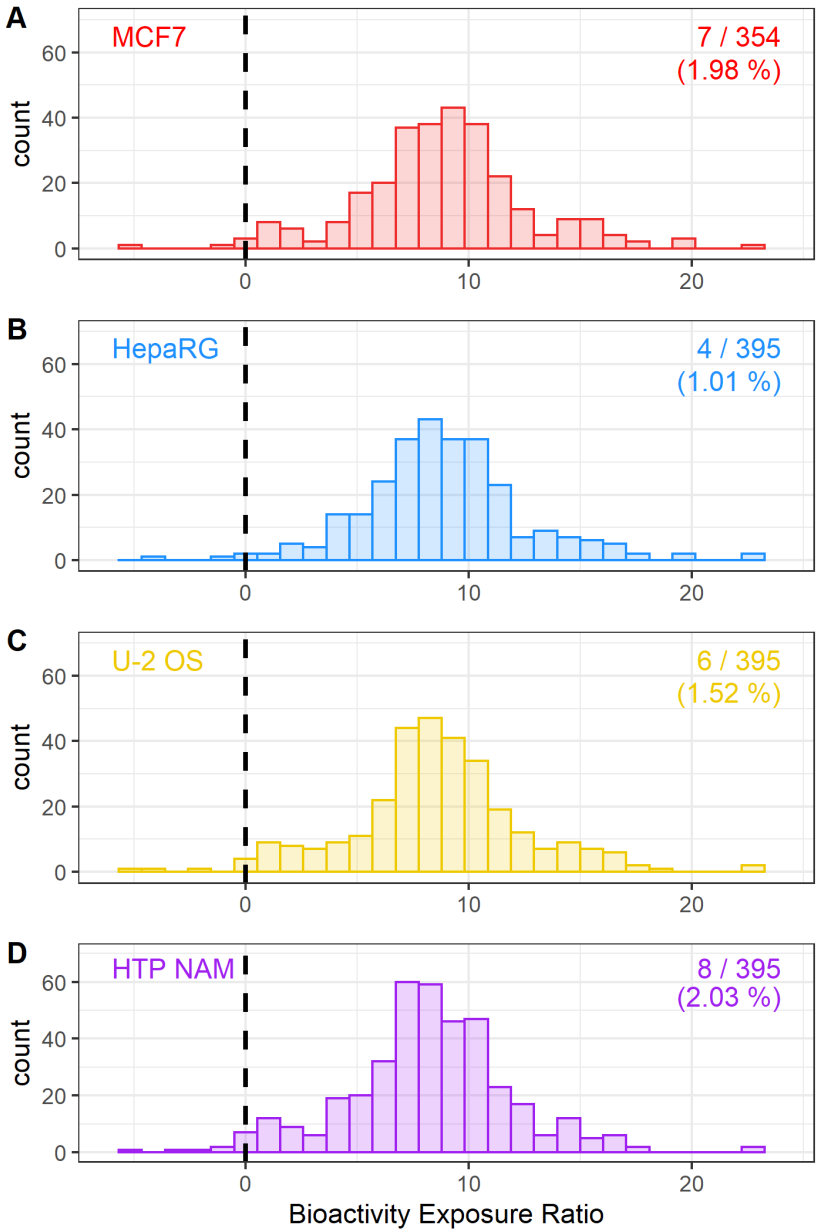
- **Negative ratios** indicate that AEDs derived from HTP NAMs molecular PODs are **conservative** surrogates for traditional *in vivo* PODs.
- When cell lines are considered individually, **~66-68%** of chemicals had negative ratios.
- When considered in combination, the number and percentage of chemicals with negative ratios **increased (82.3 %)**.
- Paul-Friedman et al. (2020) (PMID: 31532525)
  - Using ToxCast, **89 %** of APCRA chemicals had negative ratios.
- Positive ratios observed for several organophosphate and carbamate pesticides.

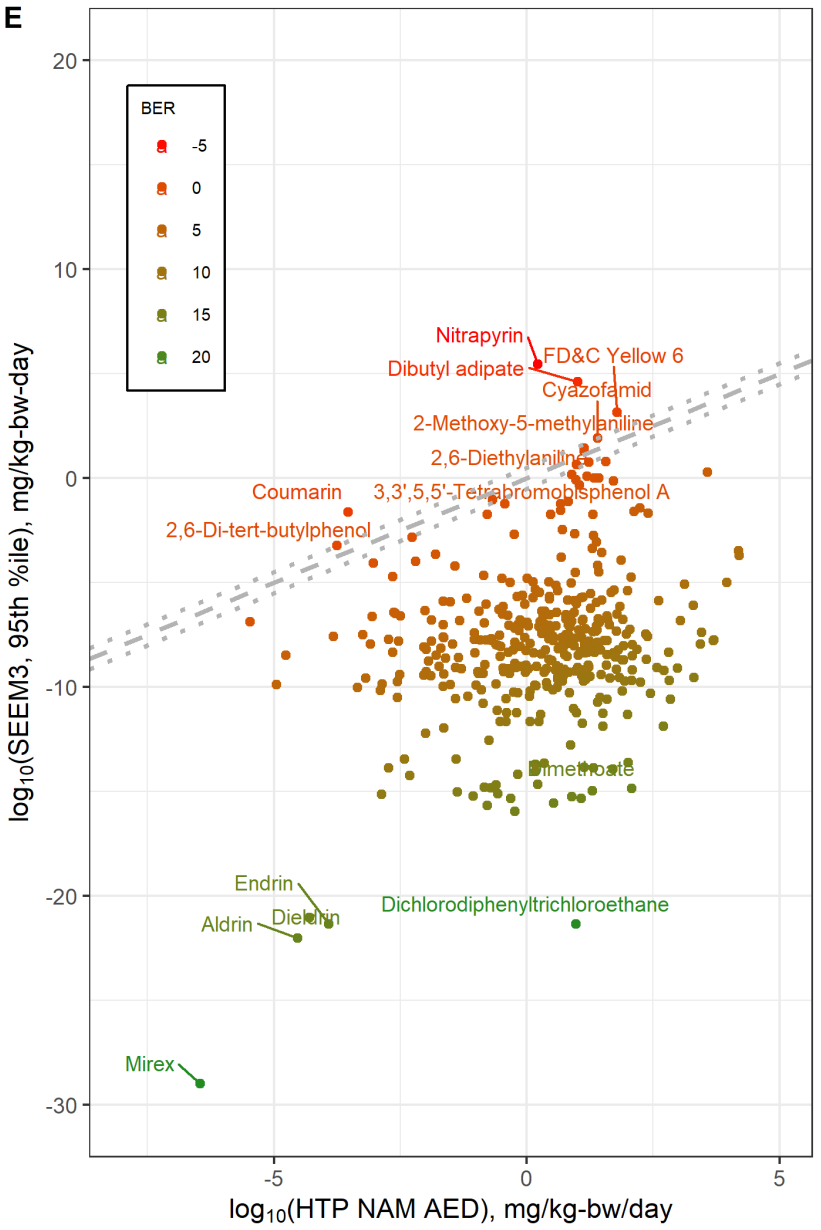




Bioactivity Exposure Ratio (BER) Analysis

- **Negative ratios** indicate a potential for human exposure to chemicals in a range that is bioactive in vitro.
- When cell lines are considered individually, **~1-2%** of chemicals had negative ratios.
- When considered in combination, the percentage of chemicals with negative ratios **did not appreciably change**.
- Positive ratios observed for several chemicals found in consumer products.
- Most extreme negative ratios associated with banned or limited use organochlorine pesticides.





Summary and Conclusions

- **High-Throughput Profiling:** Developed experimental designs and scalable laboratory workflows for high-throughput transcriptomics screening of environmental chemicals that can be used in multiple human-derived cell types.
- **Potency Estimation:** Developed high-throughput concentration-response modeling workflows to identify thresholds for perturbation of gene expression (e.g. BPACs).
- **IVIVE:** Potency estimates can be converted to administered equivalent doses (AEDs) using high-throughput toxicokinetic modeling.
- **Bioactivity to *In Vivo* Effect Value Ratio Analysis:** AEDs derived from HTP assays were conservative compared to traditional PODs a majority of the time. Performance improved to ~80% when results from multiple cell types were considered in combination.
- **Bioactivity to Exposure Ratio (BER) Analysis:** AEDs derived from HTP assays were compared to high-throughput exposure predictions. There were very few chemicals where AEDs were within the range of exposure predictions.
- **Comparison to ToxCast:** Applications using HTP NAMs potencies as input yielded comparable results compared to the use of ToxCast NAMs potencies.

Acknowledgements

Office of Research and Development (ORD)
Center for Computational Toxicology and Exposure (CCTE)

- Johanna Nyffeler
- Clinton Willis
- Rick Brockway
- Megan Culbreth
- Dan Hallinger
- Terri Fairley

- Ann Richard
- Kathy Coutros
- Maureen Gwinn
- Sandy Roberts
- Russell Thomas

- Logan Everett
- Imran Shah
- Richard Judson
- Derik Haggard
- Thomas Sheffield
- Joseph Bundy
- Woody Setzer

- Katie Paul Friedman
- John Wambaugh

National Toxicology Program
U.S. Department of Health and Human Services

- Scott Auerbach

BioSpyder™

- Jo Yeakley
- Bruce Seligmann
- Joel McComb
- Pete Shepherd
- Milos Babic
- Dalia Gonzalez
- Kyle LeBlanc
- Garrett McComb