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US EPA CSS-HERA Board of Scientific Counselors Chemical Safety Subcommittee Meeting

US EPA CSS-HERA BOSC Meeting – February 2-5, 2021

CSS Session 1A Slides

The work presented within represents US EPA Office of Research and Development research activities. Material includes both peer reviewed, published results and work-in-progress research. Please do not cite or quote slides.

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High-Throughput Phenotypic Profiling

Joshua A. Harrill, Ph.D.

Rapid Assay Development Branch

Biomolecular and Computational Toxicology Division

Center for Computational Toxicology and Exposure

Office of Research and Development, U.S. EPA

Disclaimer

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Research Drivers

- There are many chemicals in U.S. commerce with the potential to enter the environment that are poorly characterized in terms of human health hazards.

- Traditional toxicity testing approaches in laboratory animals are expensive and time-consuming and therefore cannot be used to efficiently address this large data gap.

- Animal-free New Approach Methods (NAMs) provide a means for accelerating the pace of chemical hazard assessment using models anchored in human biology.

- EPA has been tasked with and is committed to reducing the use of animals in toxicity testing and expanding the use of NAMs in chemical risk assessment

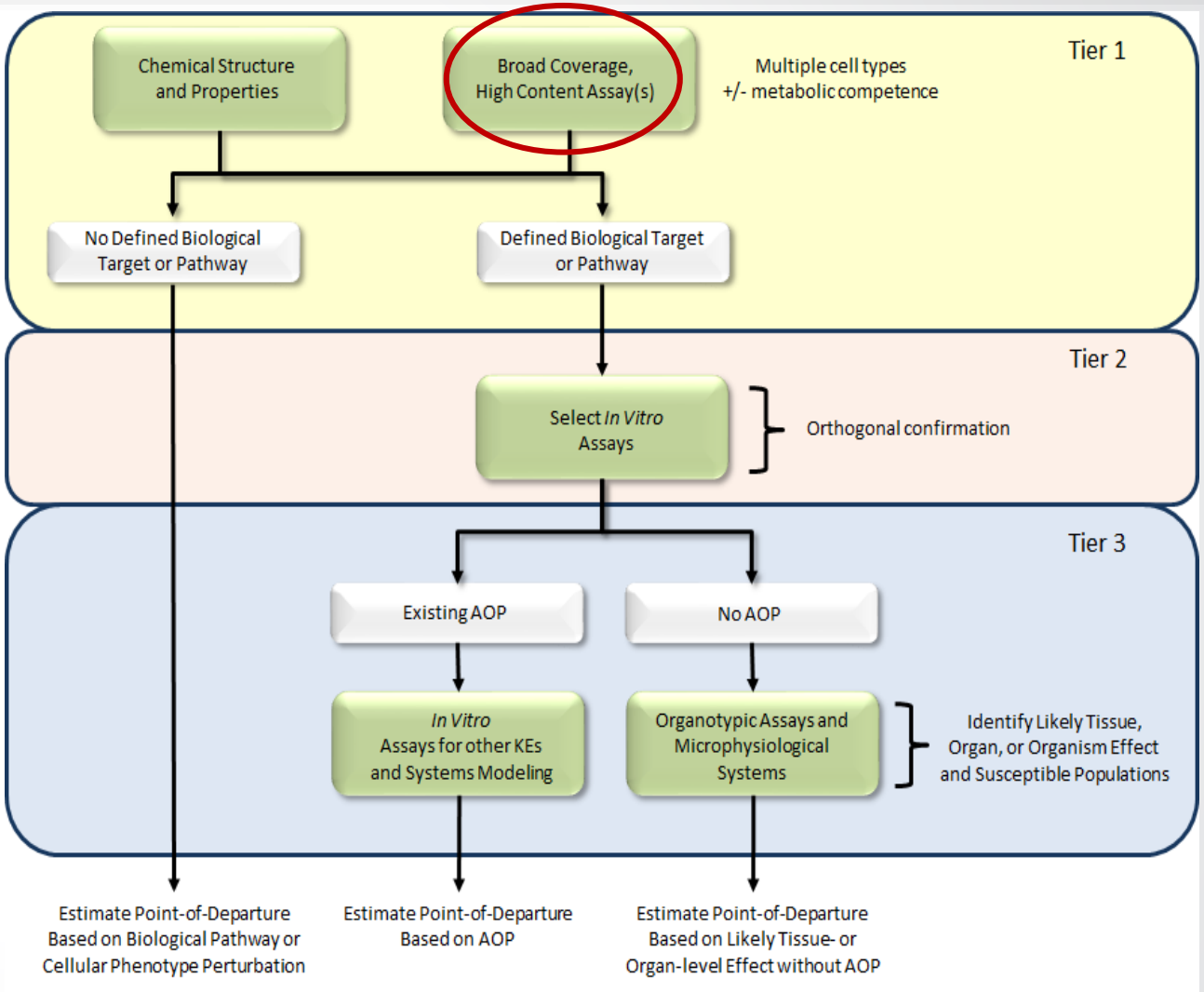
    - (**June '16**) Frank R. Lautenberg Chemical Safety for the 21st Century Act (*15 U.S.C. §2601*)
    - (**June '18**) US EPA Strategic Plan to Promote the Development and Implementation of Alternative Test Methods within the TSCA Program (*EPA-740-R1-8004*).
    - (**Sept '19**) Administrator's Directive to Prioritize Efforts to Reduce Animal Testing (*Wheeler 2019*)
    - (**June '20**) US EPA New Approach Methods Work Plan (*EPA 615B2000*)

NAMs-Based, Tiered Hazard Evaluation Strategy

- **New Approach Methodologies (NAMs)** are 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.

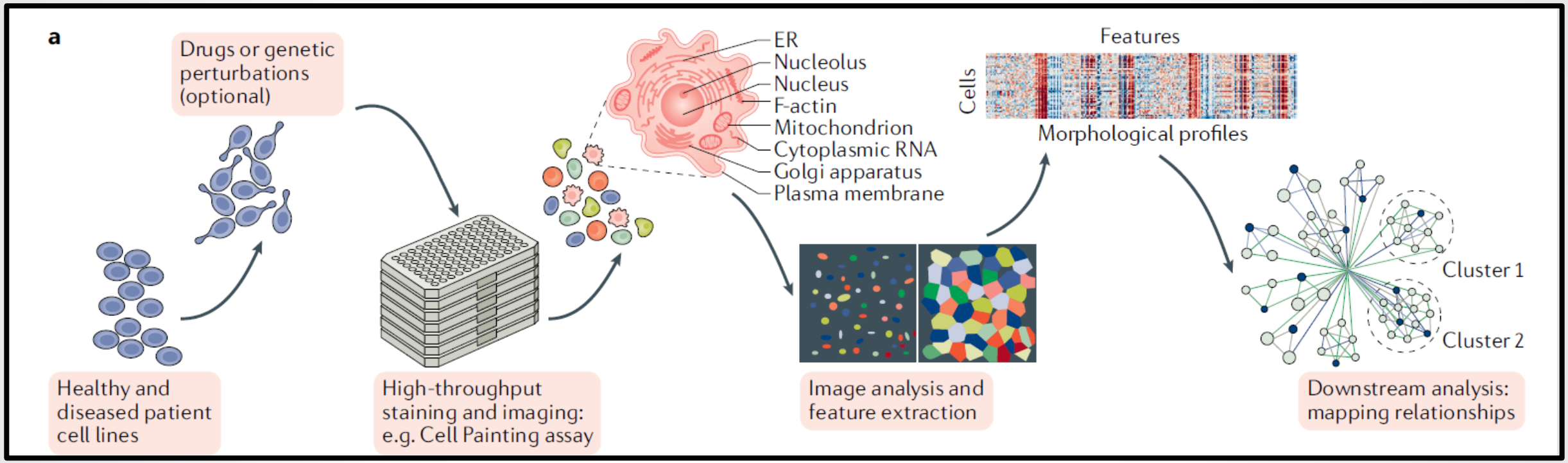
- US EPA CompTox Blueprint advocates the use of **high throughput profiling (HTP) assays** as the first tier in a NAMs-based hazard evaluation strategy.

- **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.



The NexGen Blueprint of CompTox at USEPA, Tox. Sci. 2019; 169(2):317-322

Imaging-Based High-Throughput Phenotypic Profiling (HTPP)



Chandrasekaran et al. Nat Rev Drug Discov. 2020 Dec 22:1–15

- A high-throughput testing strategy where rich information present in biological images is reduced to multidimensional numeric profiles and mined for information characteristic to a chemical's biological activity.

- Originated in the pharmaceutical sector and has been used in drug development to understand disease mechanisms and predict chemical activity, toxicity and/or mechanism-of-action

HTPP with the Cell Painting Assay

Cell Painting is a profiling method that measures a large variety of phenotypic features in fluoroprobe labeled cells *in vitro*.

- High-throughput
- Cost-effective (¢ / well)
- Scalable
- Reproducible
- Amenable to lab automation
- Deployable across multiple human-derived cell types.
- Infrastructure investment
- High volume data management

Laboratory & bioinformatics workflows for conduct of this assay have been established at CCTE.

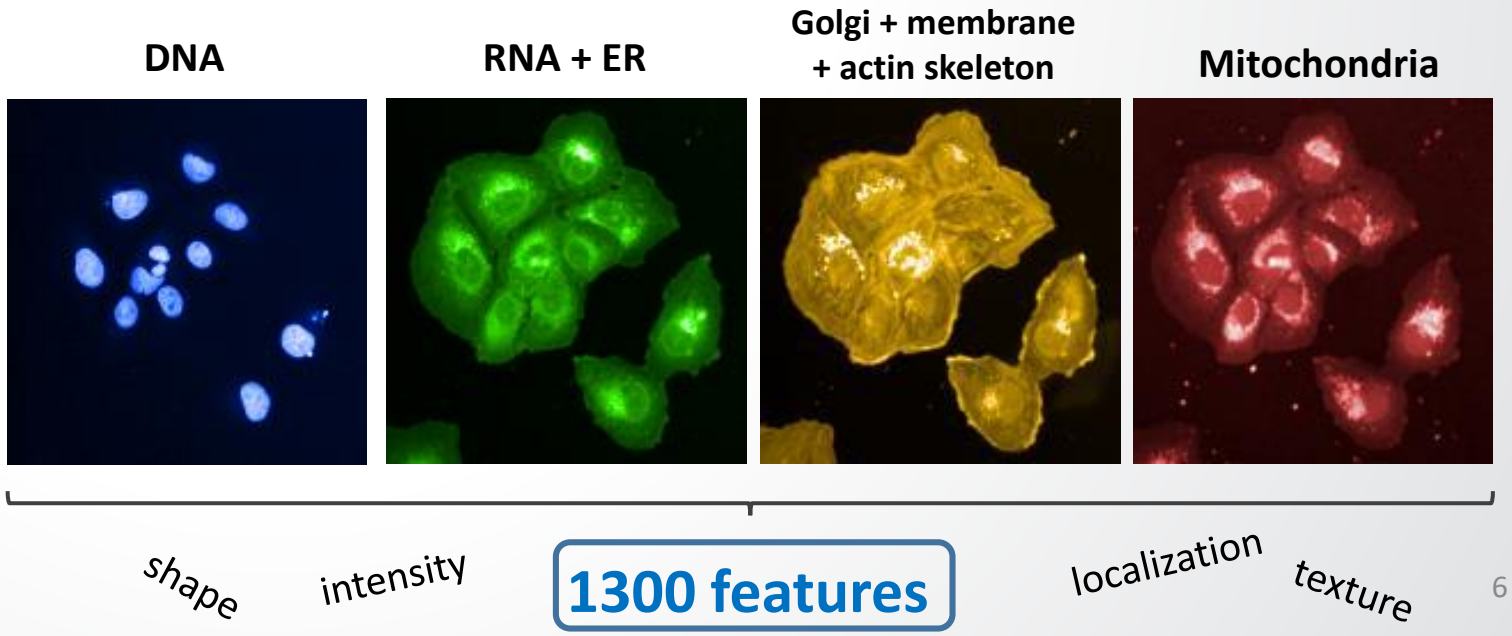
OPEN ACCESS Freely available online

PLOS ONE

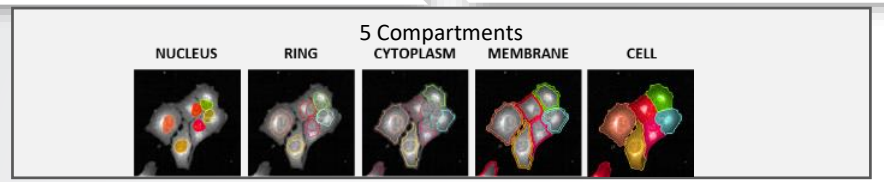
Multiplex Cytological Profiling Assay to Measure Diverse Cellular States

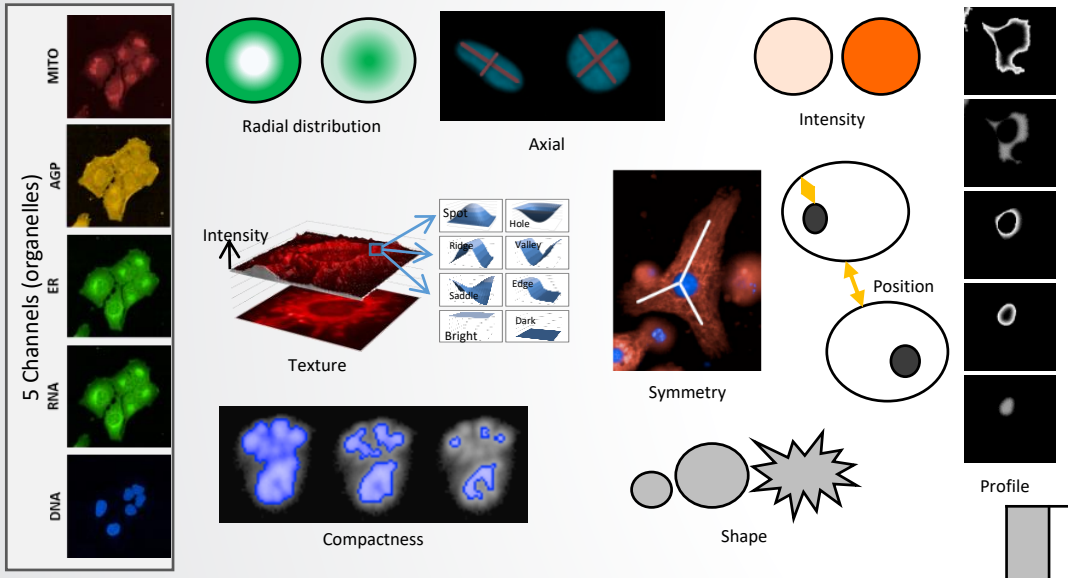
Sigrun M. Gustafsdottir, Vebjorn Ljosa, Katherine L. Sokolnicki, J. Anthony Wilson, Deepika Walpita, Melissa M. Kemp, Kathleen Petri Seiler, Hyman A. Carrel, Todd R. Golub, Stuart L. Schreiber, Paul A. Clemons, Anne E. Carpenter, Alykhan F. Shamji

Broad Institute of Harvard and MIT, Cambridge, Massachusetts, United States of America



Imaging & Phenotypic Feature Extraction





49 Feature Categories
(ex. MITO_Texture_Cytoplasm)

1300 features / cell

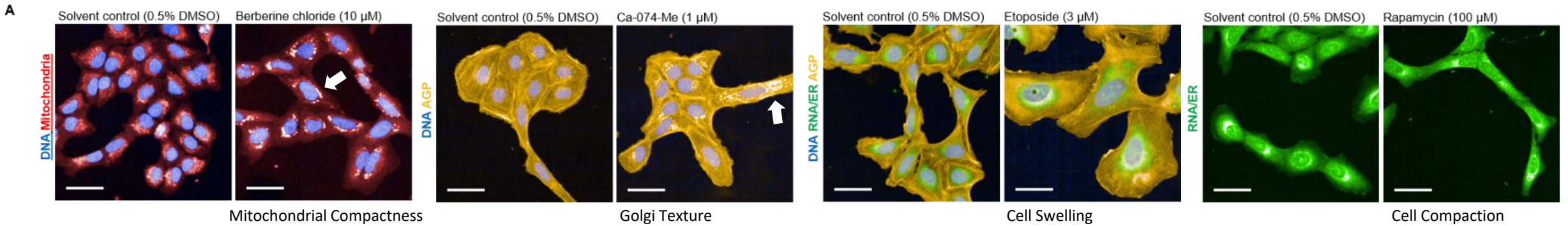
Channel	Module: Position [7]	Module: Basic morph-ology [5]	Module: SCARP morphology: Symmetry [80]	Module: SCARP morphology: Compactness [40]	Module: SCARP morphology: Axial [20]	Module: SCARP morphology: Radial [28]	Module: SCARP morphology: Profile [20-30]	Module: Intensity [9]	Module: Texture [14]
DNA			Nuclei	Nuclei	Nuclei	Nuclei Cell	Nuclei Cytoplasm	Nuclei	Nuclei
RNA			Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei	Nuclei
ER			Cell	Cell	Cell	Cell	Cytoplasm	Ring Cytoplasm	Ring Cytoplasm
AGP			Cell	Cell	Cell	Cell	Nuclei Cytoplasm	Ring Cytoplasm Membrane	Ring Cytoplasm Membrane
Mito			Cell	Cell	Cell	Cell	Nuclei Cytoplasm	Ring Cytoplasm	Ring Cytoplasm
Not associated with a channel	Nuclei Cell	Nuclei Cell							

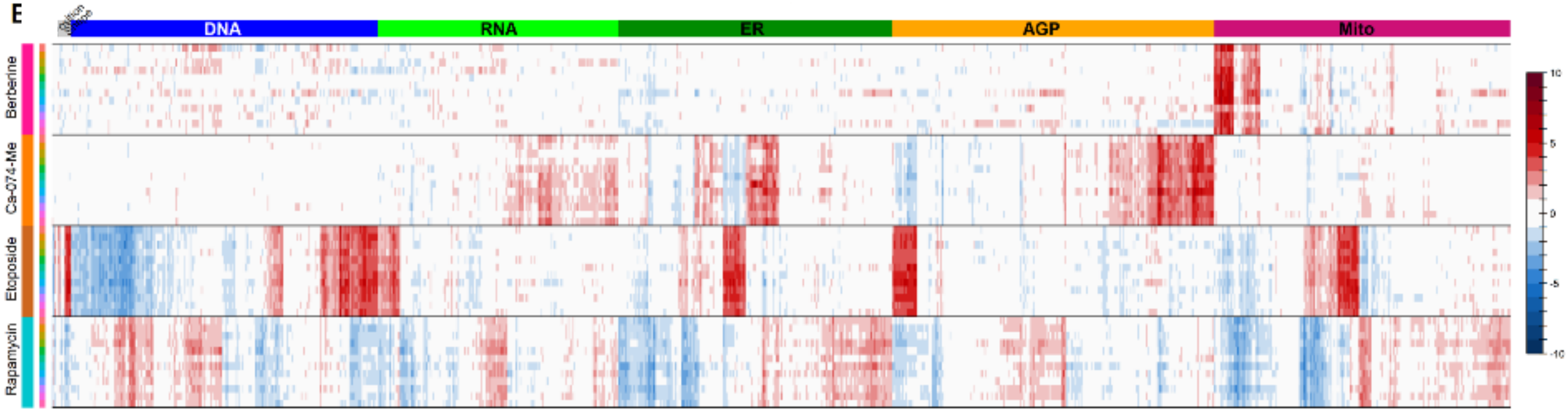
PerkinElmer Opera Phenix

Modality: Confocal (single *z*)
Objective: 20X Water
Plate: CellCarrier-384 Ultra
Fields: 5 or 9

With illustrations from Perkin Elmer

Examples of Chemical Induced Phenotypes





- Strong phenotypes are observed qualitatively and produce distinct profiles when measured quantitatively.

Adapted from Nyffeler et al. Toxicol Appl Pharmacol. 2020 Jan 15;389:114876

HTPP Data Analysis Pipeline

Data reduction

cell-level data

Normalization
MAD normalization

$$\frac{\text{cell value} - \text{median}_{DMSO}}{1.4826\ \text{MAD}_{DMSO}}$$

normalized cell-level data

Aggregation
median

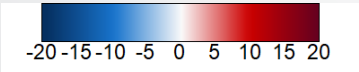
well-level data

Standardization
Z transformation

Cell Count Info
Conc. > 50% cell loss

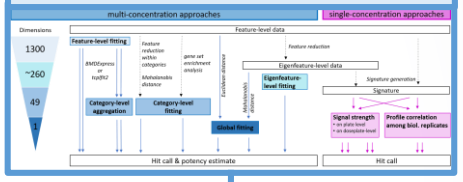
scaled well-level data

clipped well-level data



Concentration Response Modeling

Calculate Response Metrics



See Nyffeler et al. SLAS Discov. 2020 Aug 29: doi: 10.1177/2472555220950245

Fit Multiple Curve Shapes

Best Model Selection

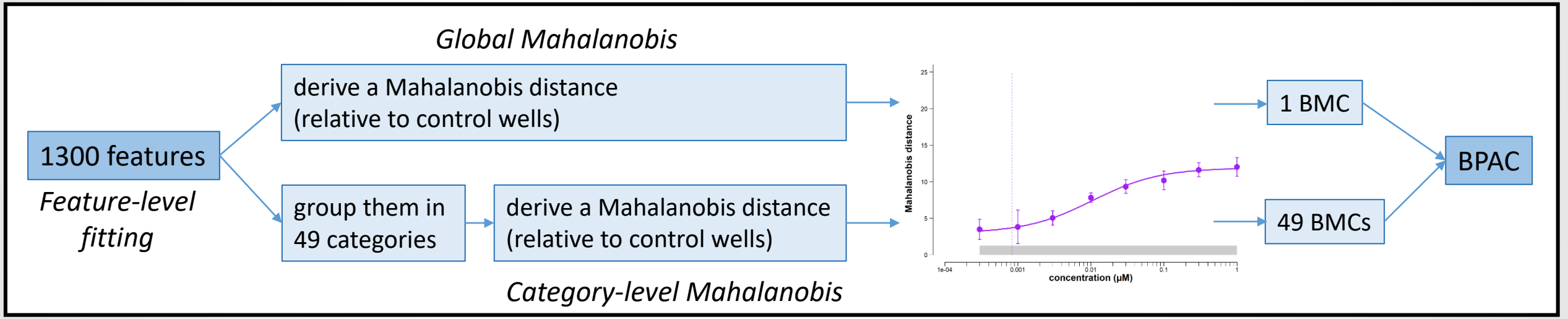
BMC

Berberine chloride
Mito_Cells_Morph_STAR

Phenotype Altering Concentration (PACs)

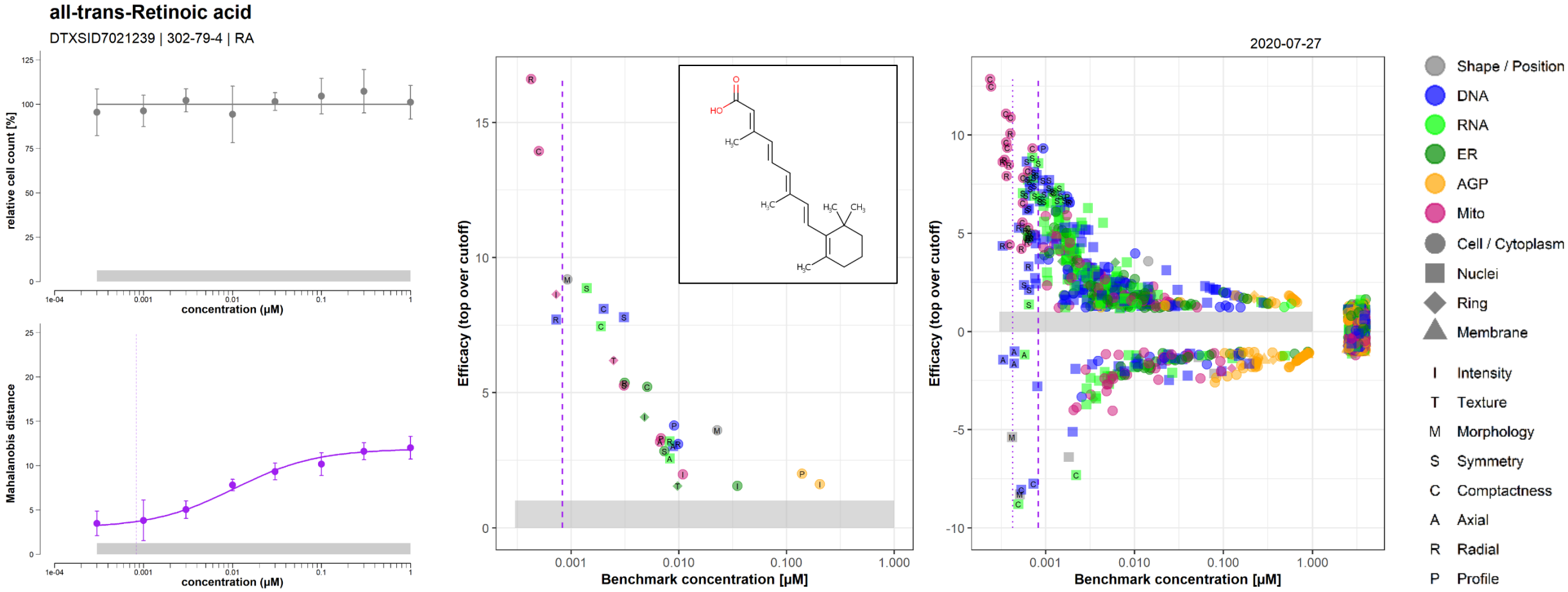
Mahalanobis Distance (D_M):

- A multivariate distance metric that measures the distance between a point (vector) and a distribution.
- Accounts for unpredictable changes in cell states across test concentrations and inherent correlations in profiling data.



- Chemicals where a BMC can be determined using either the global or category D_M approach are considered active.
- The minimum of the global or most sensitive category BMC is the **Phenotype Altering Concentration (PAC)**

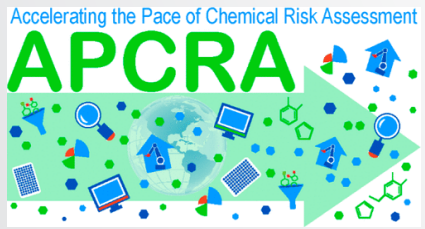
Concentration-Response Modeling Example



- Phenotypic effects can be observed below the threshold for cytotoxicity and in the absence of cytostatic effects.
- Category and feature-level modeling can reveal which organelles exhibit treatment-related changes in morphology.

ToxCast Chemical Screen – Experimental Design (1)

Parameter	Multiplier	Notes
Cell Type(s)	1	U-2 OS
Culture Condition	1	DMEM + 10% HI-FBS
Chemicals	1,202	Selected from US EPA ToxCast chemical collection **Includes 179 chemicals with annotated molecular targets** **Includes 462 APCRA case study chemicals**
Time Points:	1	24 hours
Assay Formats:	2	High Throughput Phenotypic Profiling (Cell Painting) *High Throughput Transcriptomics (TempO-Seq)*
Concentrations:	8	3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing
Biological Replicates:	4	--

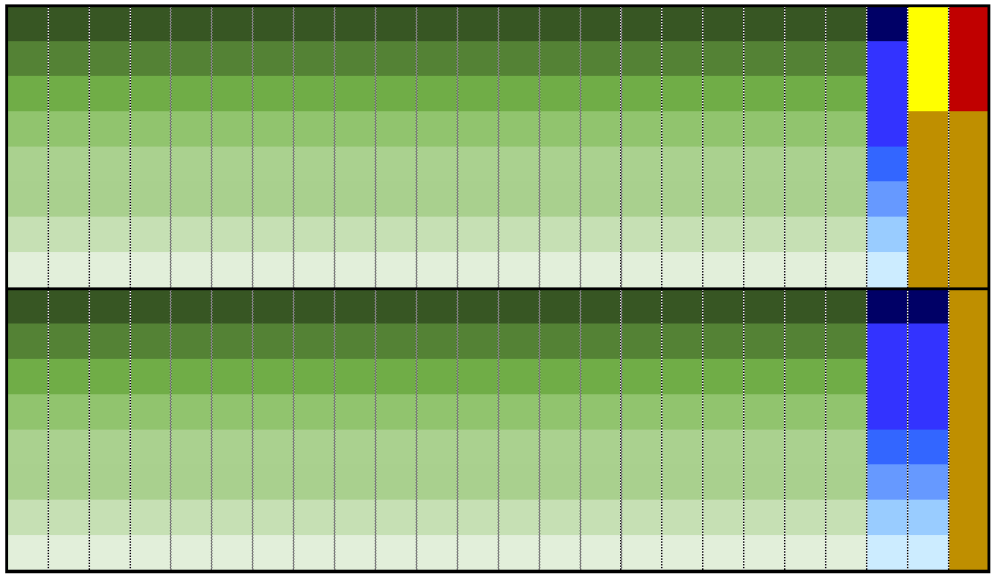


Kavlock et al. (2018)
Chem. Res. Tox; 31(5): 287-290

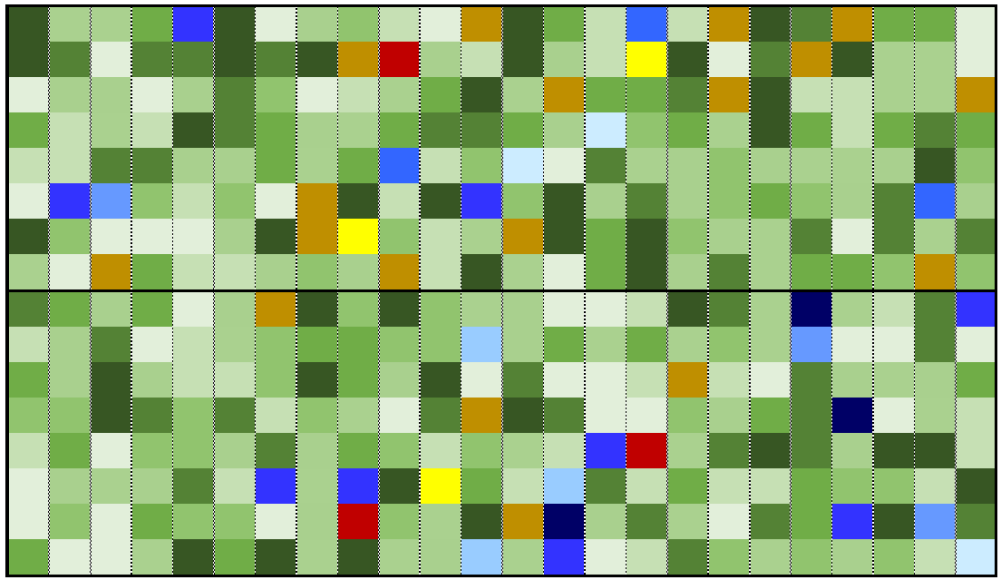
International collaboration of regulatory scientists focused on next generation chemical risk assessment including **deriving quantitative estimates of risk based on NAM-derived potency information and computational exposure estimates.**

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

ToxCast Chemical Screen – Experimental Design (2)

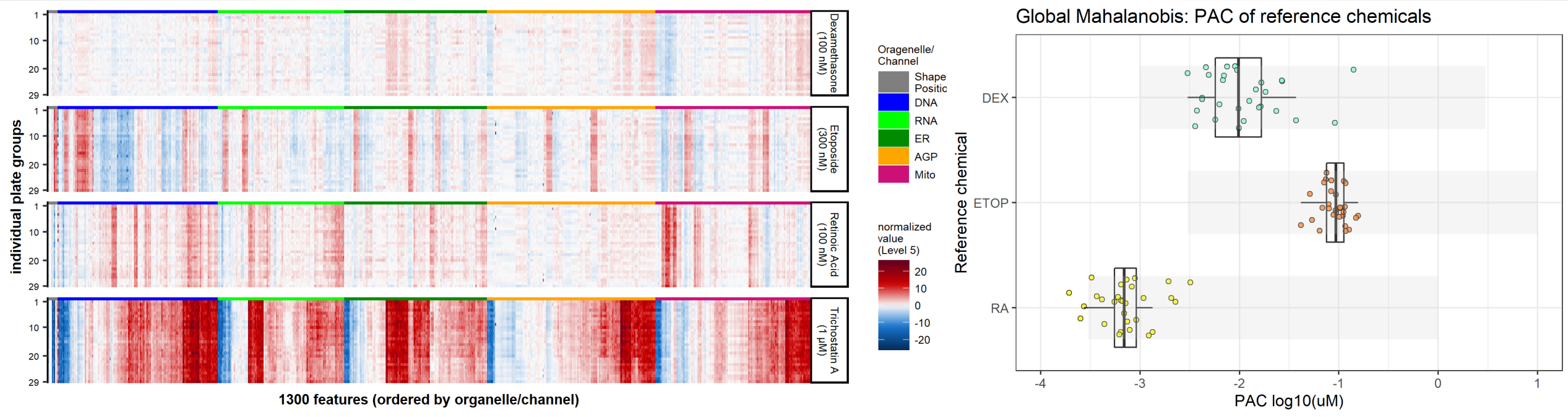


Treatment
Randomization



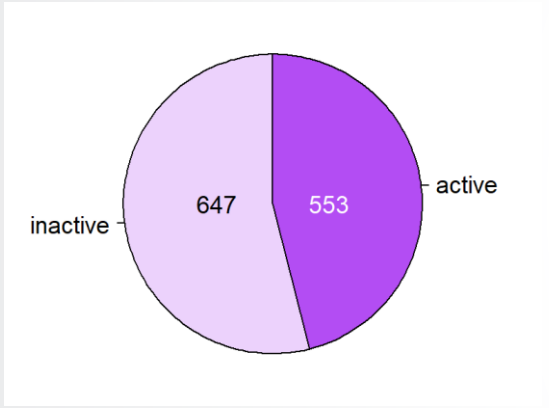
Label	Reference Chemicals:	Molecular Mechanism-of-Action	Test Concentrations
A	Etoposide	DNA topoisomerase inhibitor	0.03 - 10 µM
B	all-trans-Retinoic Acid	Retinoic acid receptor agonist	0.0003 – 1 µM
C	Dexamethasone	Glucocorticoid receptor agonist	0.001 – 3 µM
D	Trichostatin A	Histone deacetylase inhibitor	1 µM
E	Staurosporine	Cytotoxicity control	1 µM
F	DMSO	Vehicle control	0.5 %

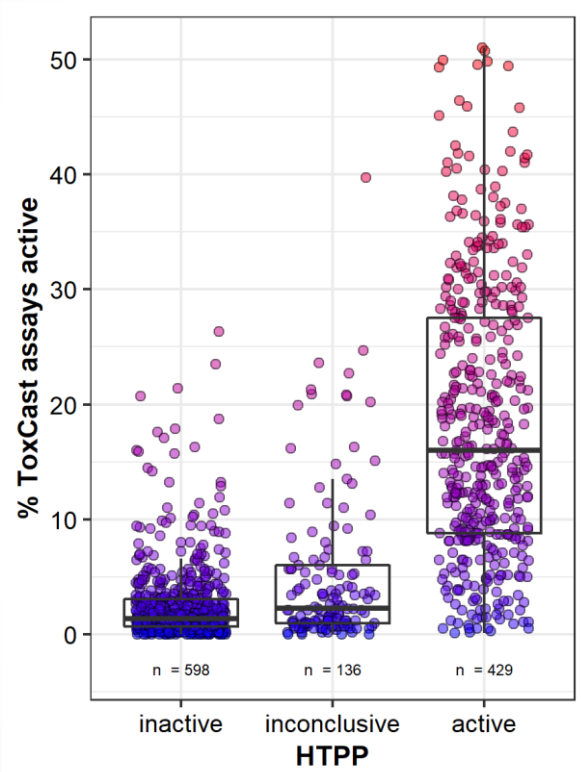
Assay Performance / Reproducibility

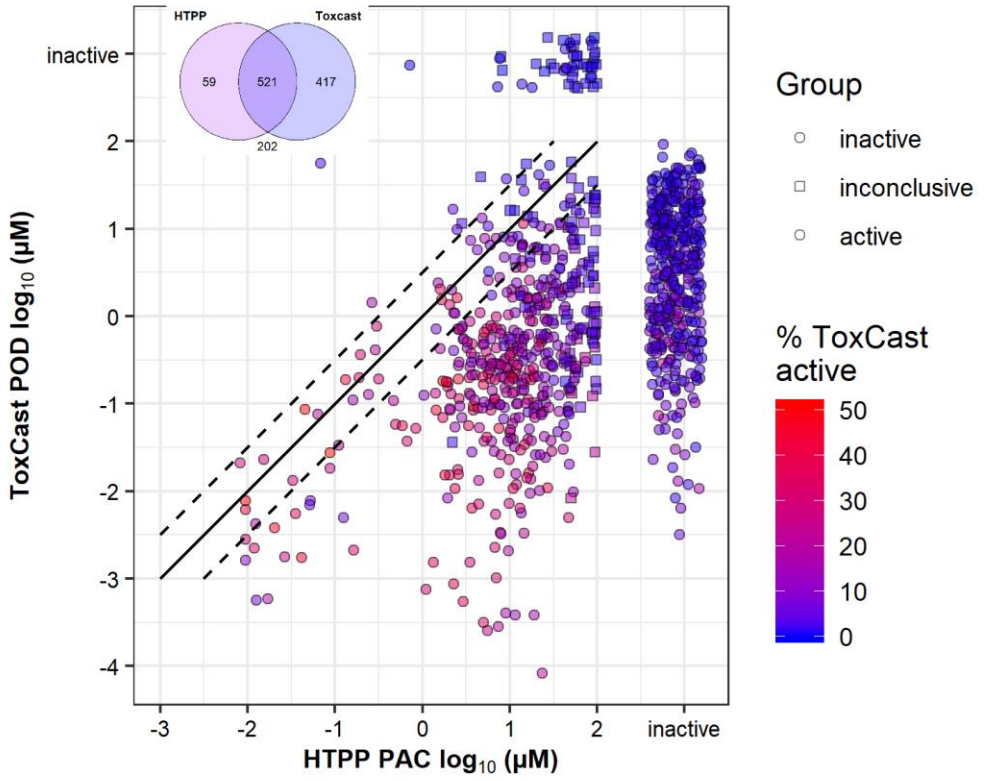


- Reference chemicals produce **reproducible** and **distinct** profiles.
- Reference chemicals produce reproducible **potency** estimates (PACs).

ToxCast Chemical Screening Results

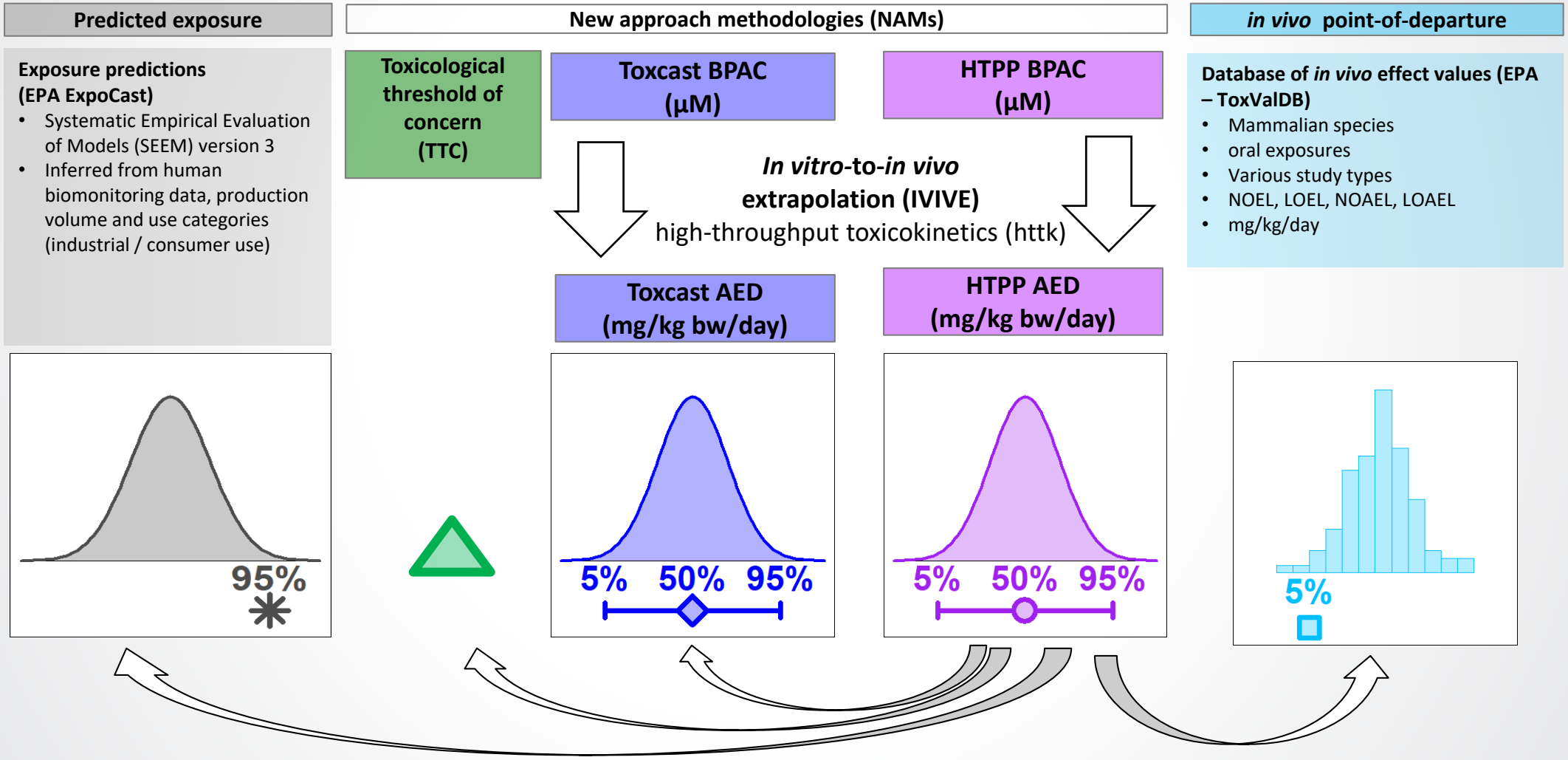






- Chemicals active in HTPP are more often ‘promiscuous’ in ToxCast.
- Chemicals active in HTPP produce less potency PACs compared to ToxCast.

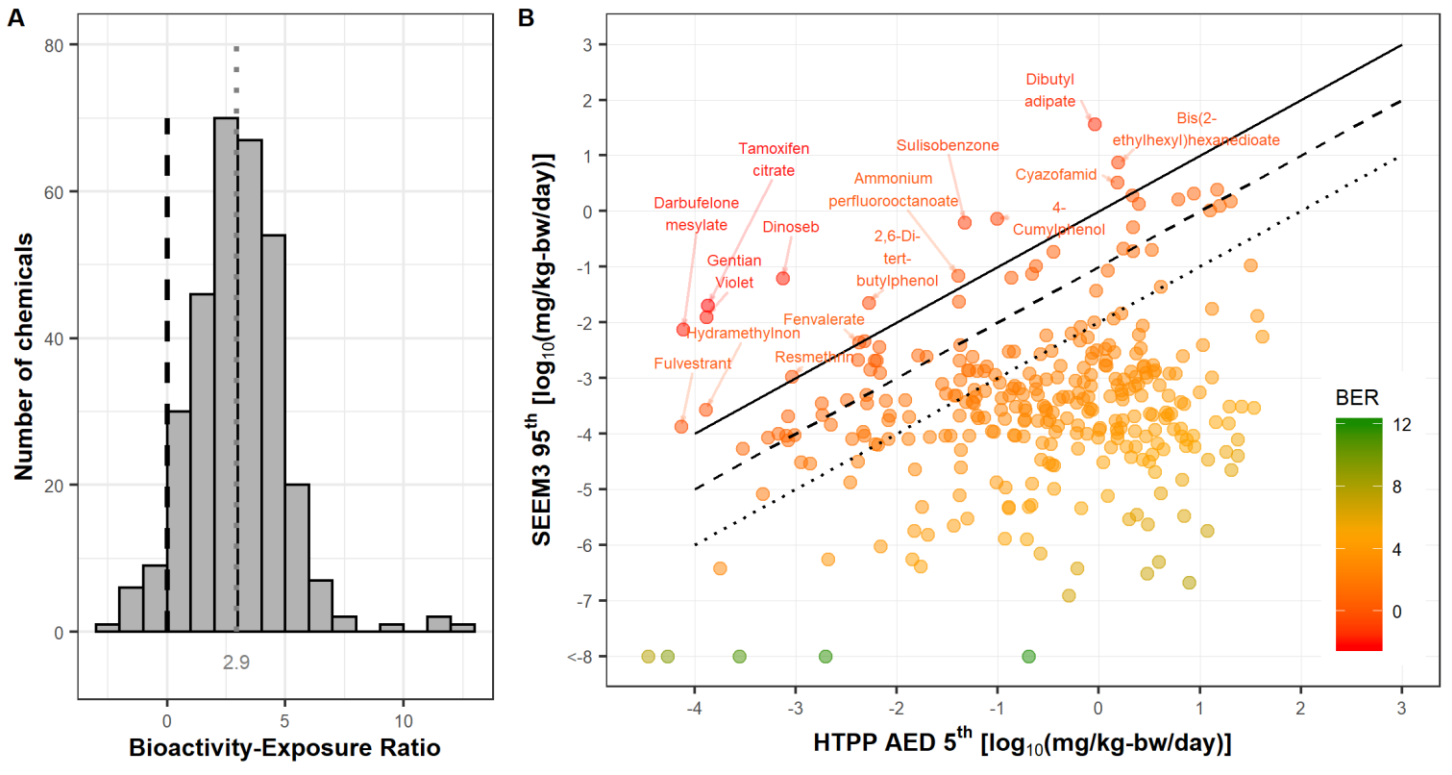
In Vitro to *In Vivo* Extrapolation (IVIVE)



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

Bioactivity to Exposure Ratio (BER) Analysis

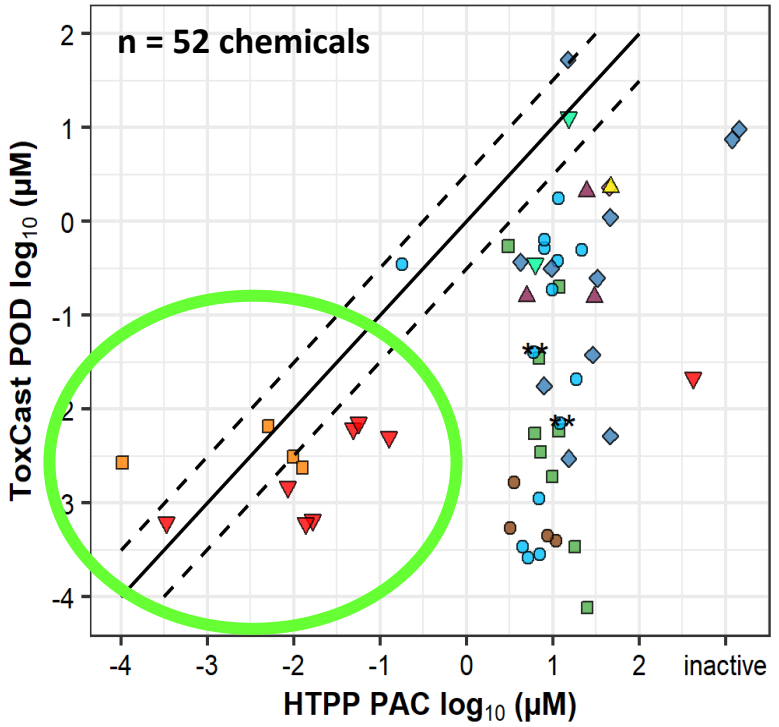
$$\text{Bioactivity exposure ratio (BER)} = \frac{\text{lower bound of HTPP bioactivity}}{\text{upper bound of exposure estimate}} = \log_{10}\left(\frac{\text{HTPP AED } 5^{th}}{\text{SEEM3 } 95^{th}}\right)$$



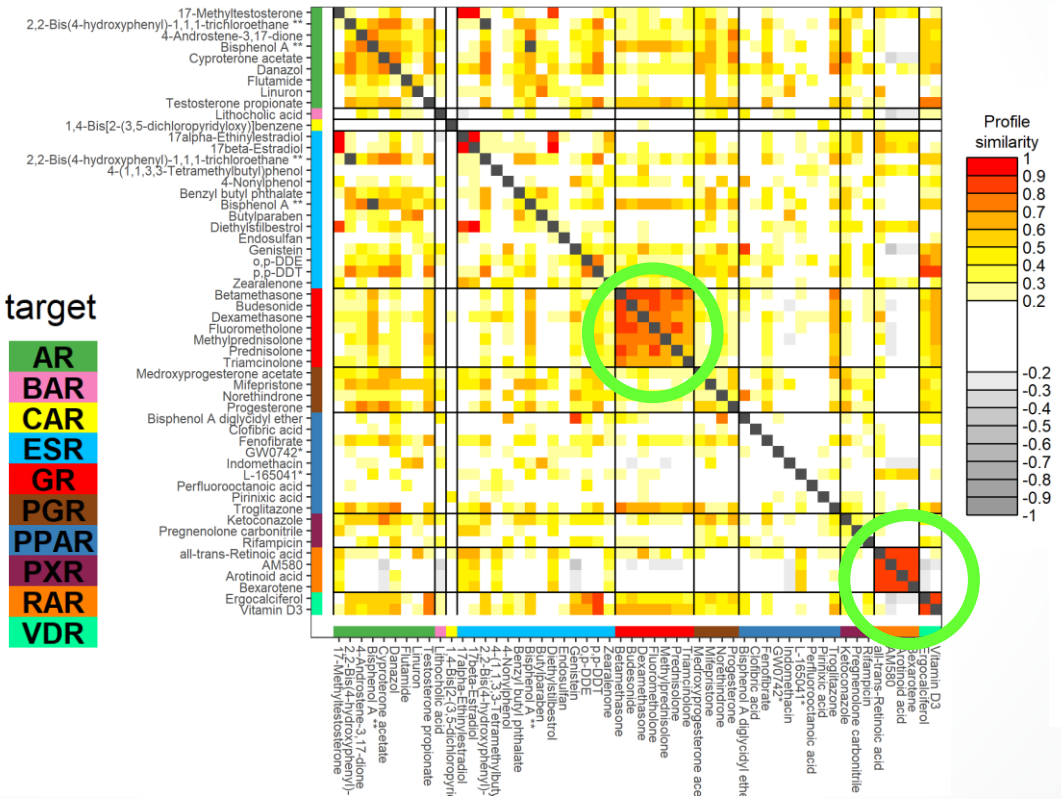
For some chemicals, the BER was negative, indicating a potential for humans to be exposed to bioactive concentrations of these chemicals

Contextual Response of Nuclear Receptor Modulators

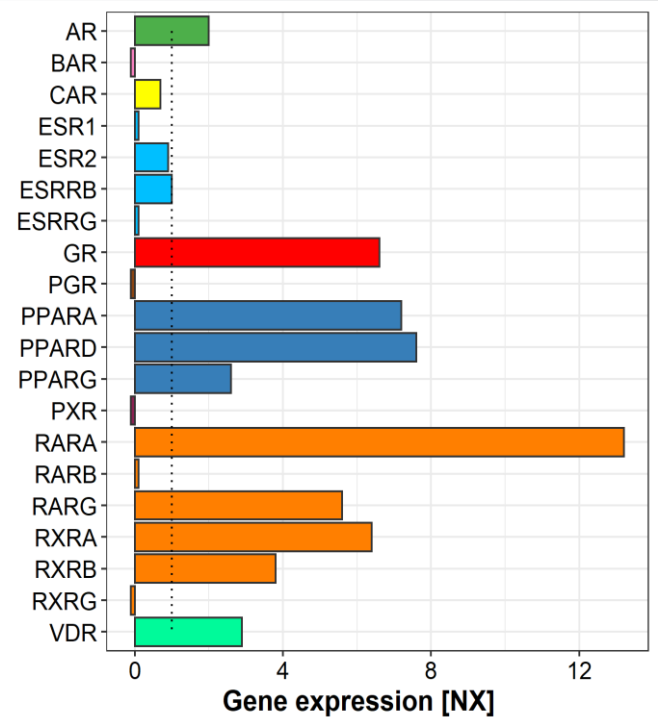
Comparison to ToxCast potencies



Profile Similarity

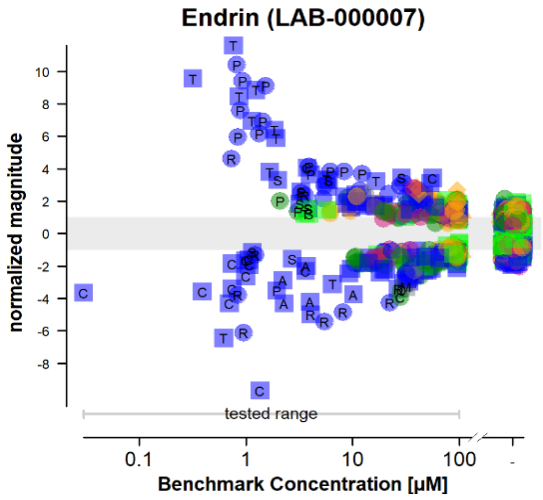


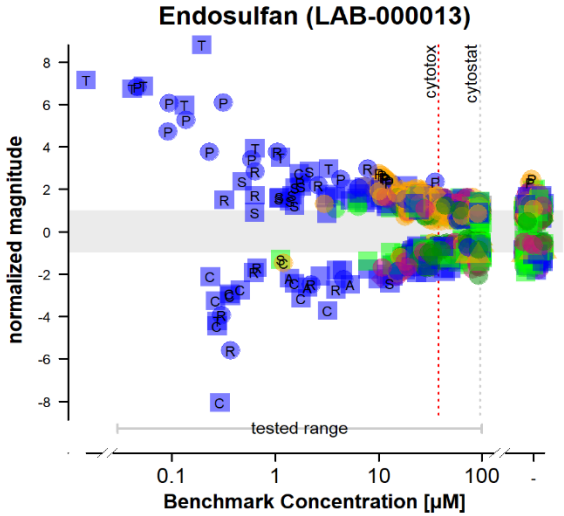
Gene expression in U-2 OS

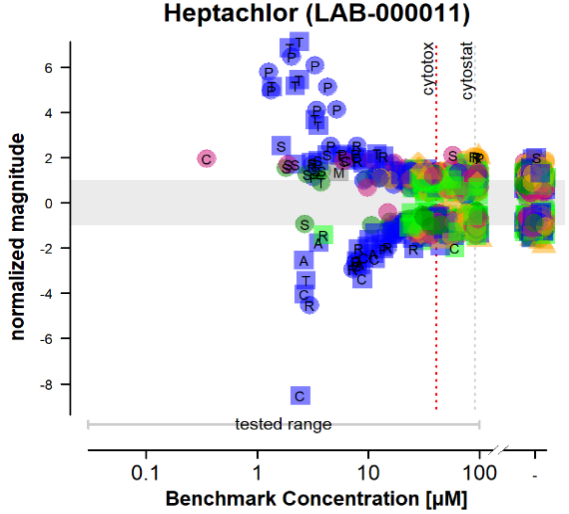


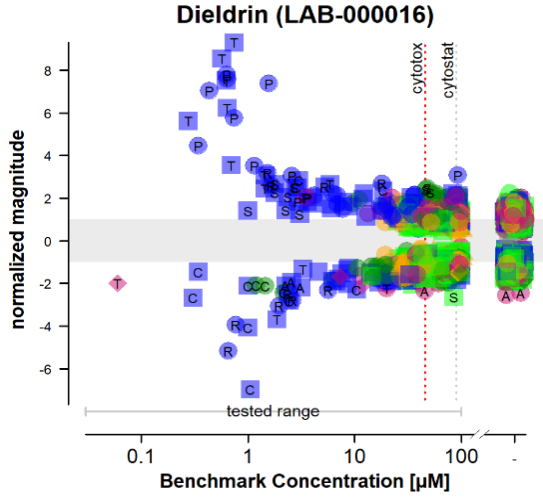
- For three receptor systems that are expressed in U-2 OS cells (GR, RAR/RXR, VDR) potencies were comparable with ToxCast.
- Phenotypic profiles for chemicals that affect these receptor systems are similar.

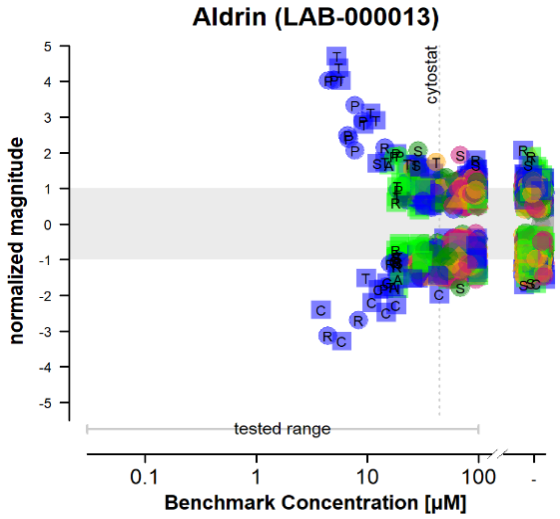
Structurally Similar Environmental Chemicals Can Produce Similar HTPP Profiles

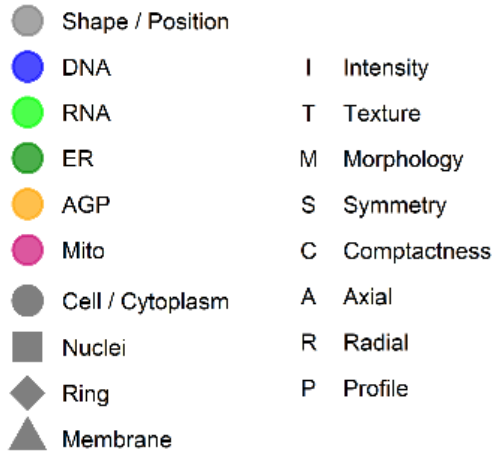


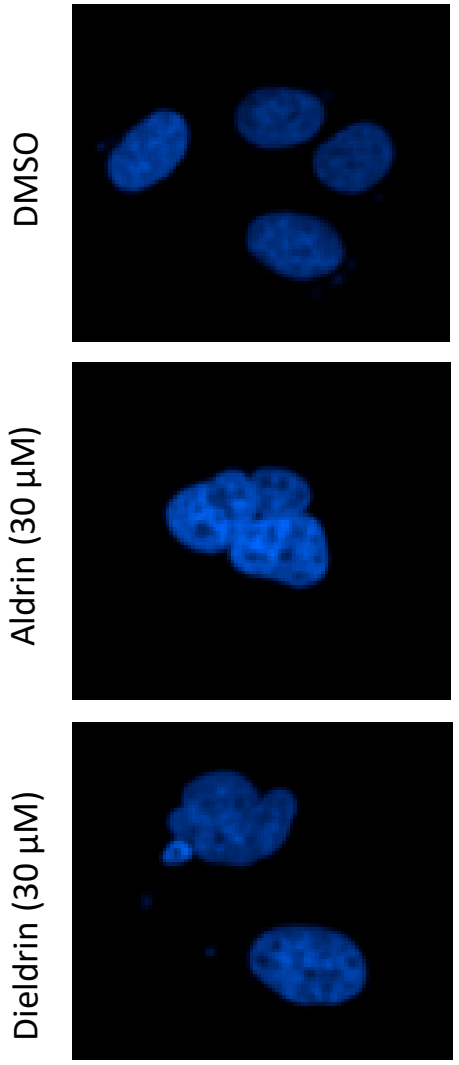








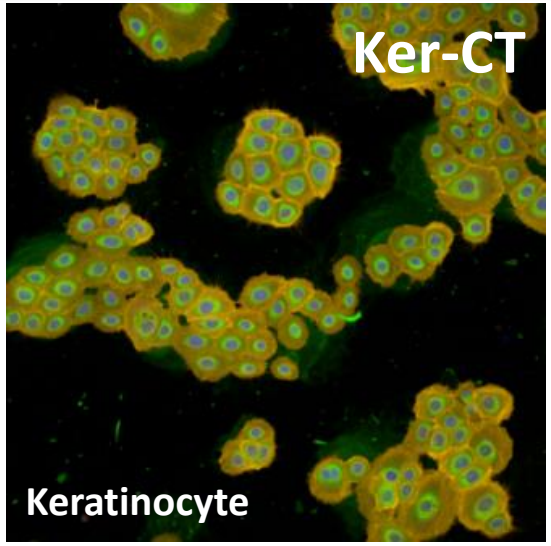


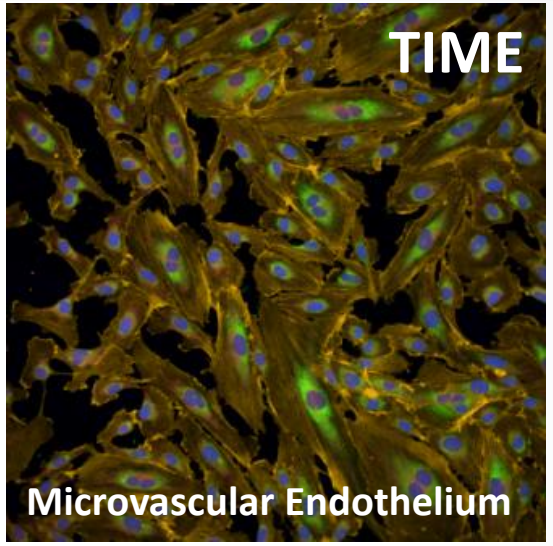


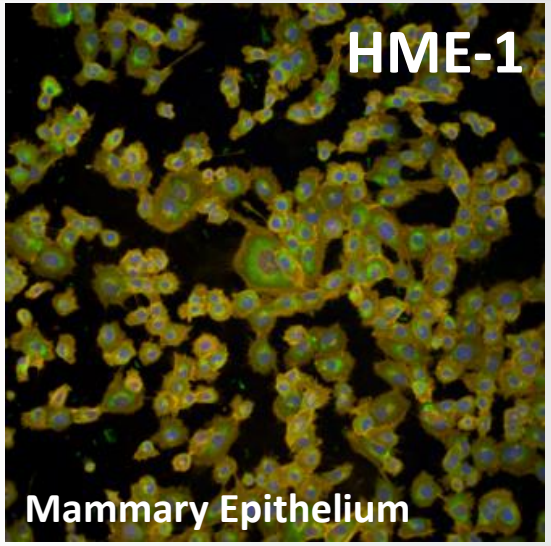
Preliminary results. Do not cite or quote.

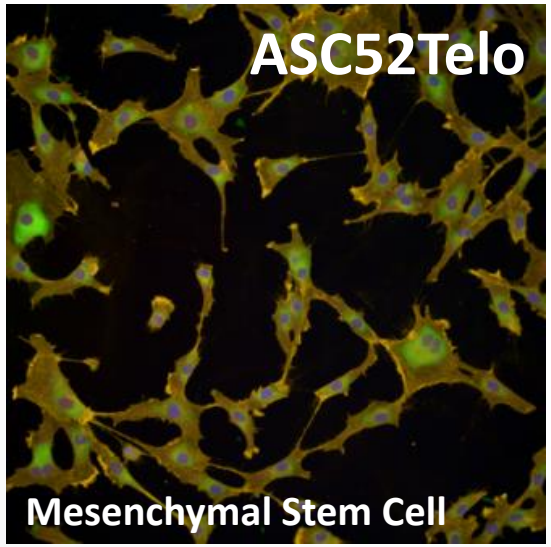
HTPP is Compatible with Biologically Diverse Cell Lines

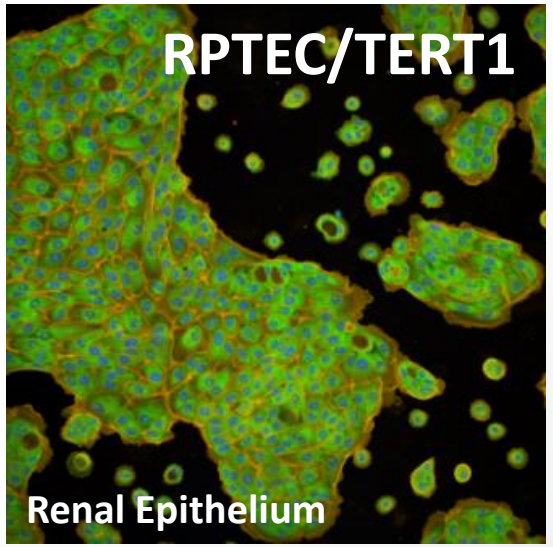
- HTPP is compatible with many human-derived cell culture models.
- Enables characterization of chemical effects across different domains of human biology.

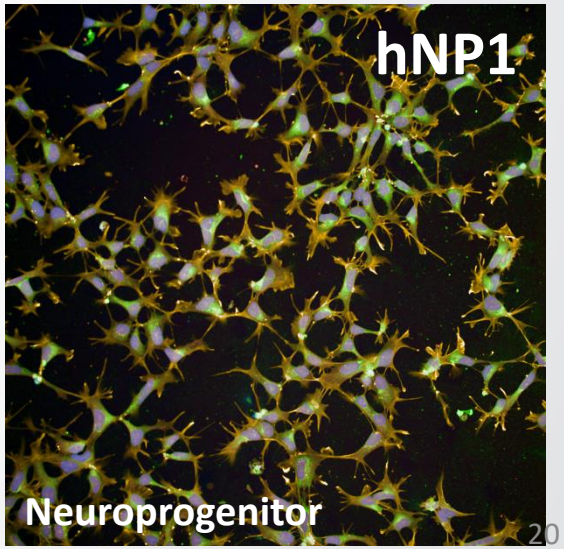












Preliminary results. Do not cite or quote.

Summary

- **Assay Reproducibility:** Demonstrated high assay reproducibility through the use of phenotypic reference chemicals and developed experimental designs that allow for evaluation of assay performance throughout large-scale screening campaigns.
- **Potency Estimation:** Developed a concentration-response modeling workflow to identify concentration thresholds for perturbation of cell morphology (e.g. phenotypic altering concentration, PAC).
- **Mechanistic Prediction:** Chemicals with strong and specific target mode associations can produce similar phenotypic profiles in U-2 OS cells. Strength of similarity varies according to baseline target expression.
- **Chemical Similarity:** Chemicals with similar chemical structures can also produce similar phenotypic profiles in U-2 OS cells.
- **Bioactivity to Exposure Ratio:** Phenotype altering concentrations (PACs) can be converted to administered equivalent doses (AEDs) and compared to human exposure predictions for chemical ranking and prioritization.
- **Biologically Diverse Cell Lines:** Compatibility of HTPP with many human-derived cell models permits characterization of chemical bioactivity across different domains of human biology.

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
- Russell Thomas
- Katie Paul-Friedman
- Logan Everett
- Imran Shah
- Richard Judson
- Woody Setzer
- Grace Patlewicz
- Derik Haggard

PerkinElmer

- Joe Trask
- Dana Hanes
- Jim Hostetter

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High-Throughput Transcriptomics

Logan J. Everett, Ph.D.

Computational Toxicology and Bioinformatics Branch

Biomolecular and Computational Toxicology Division

Center for Computational Toxicology and Exposure

Office of Research and Development, U.S. EPA

Disclaimer

The views expressed in this presentation are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Company or product names do not constitute endorsement by US EPA.

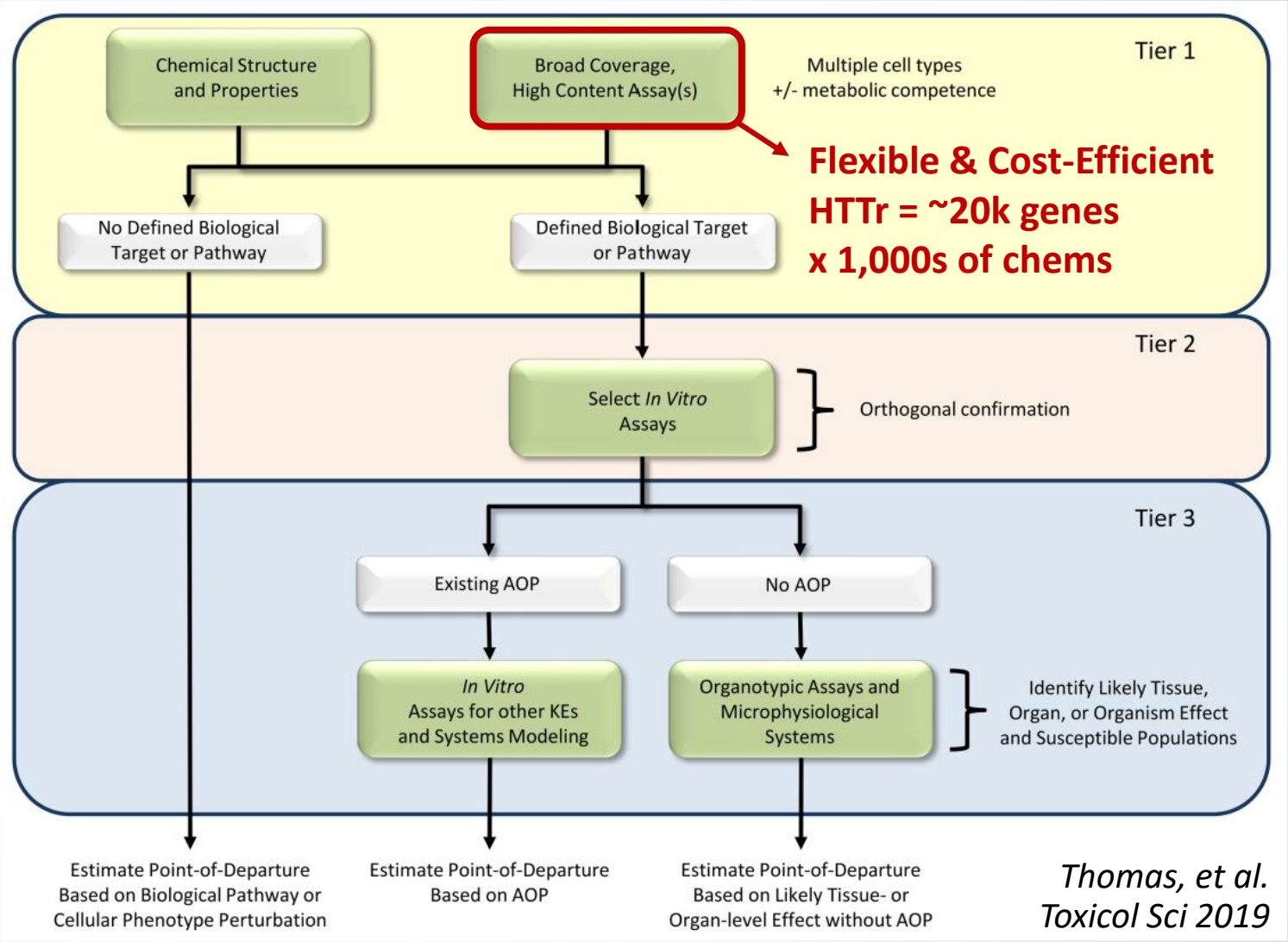
Tiered Chemical Safety Testing Strategy

Tier 1 Primary Goals:

- Prioritize chemicals by bioactivity & potency
- Predict biological targets for chemicals

HTTr Key Challenges:

- Curve-fitting on count-based data
- Summarization at pathway/chemical level



Thomas, et al. Toxicol Sci 2019

Regulatory Drivers: TSCA/Admin Memo Sep 2019; FY18-22 US EPA Strategic Plan, Obj 3.3

Automated *in vitro* Chemical Screening

Cryopreserved Cell Stocks

Cell Expansion

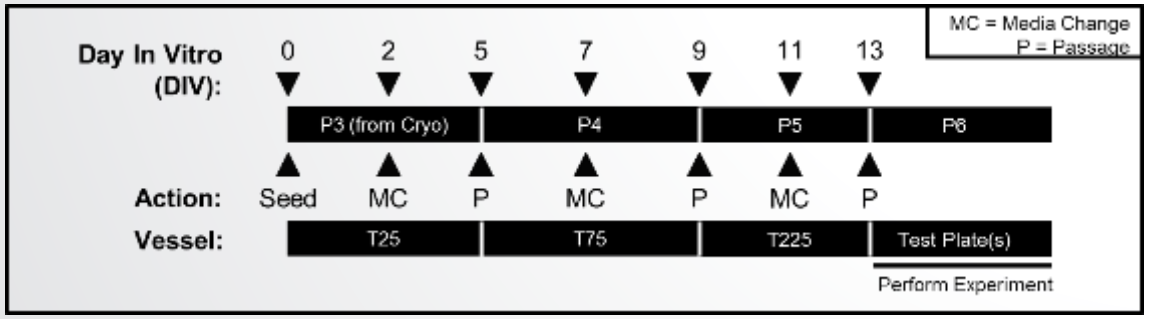
Cell Plating

BioTek MultiFlo ™ FX

Dispensing Test Chemicals

LabCyte Echo® 550 Liquid Handler

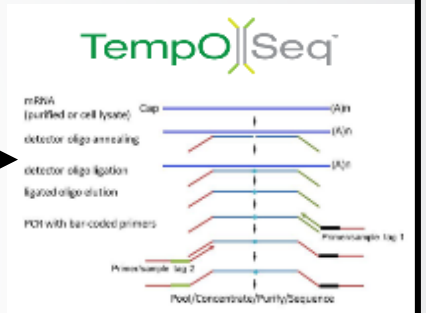
Standardized Expansion Protocol



Track 1: Targeted RNA-Seq

Generating Cell Lysates

TempO-Seq WT



Track 2: Apoptosis / Cell Viability

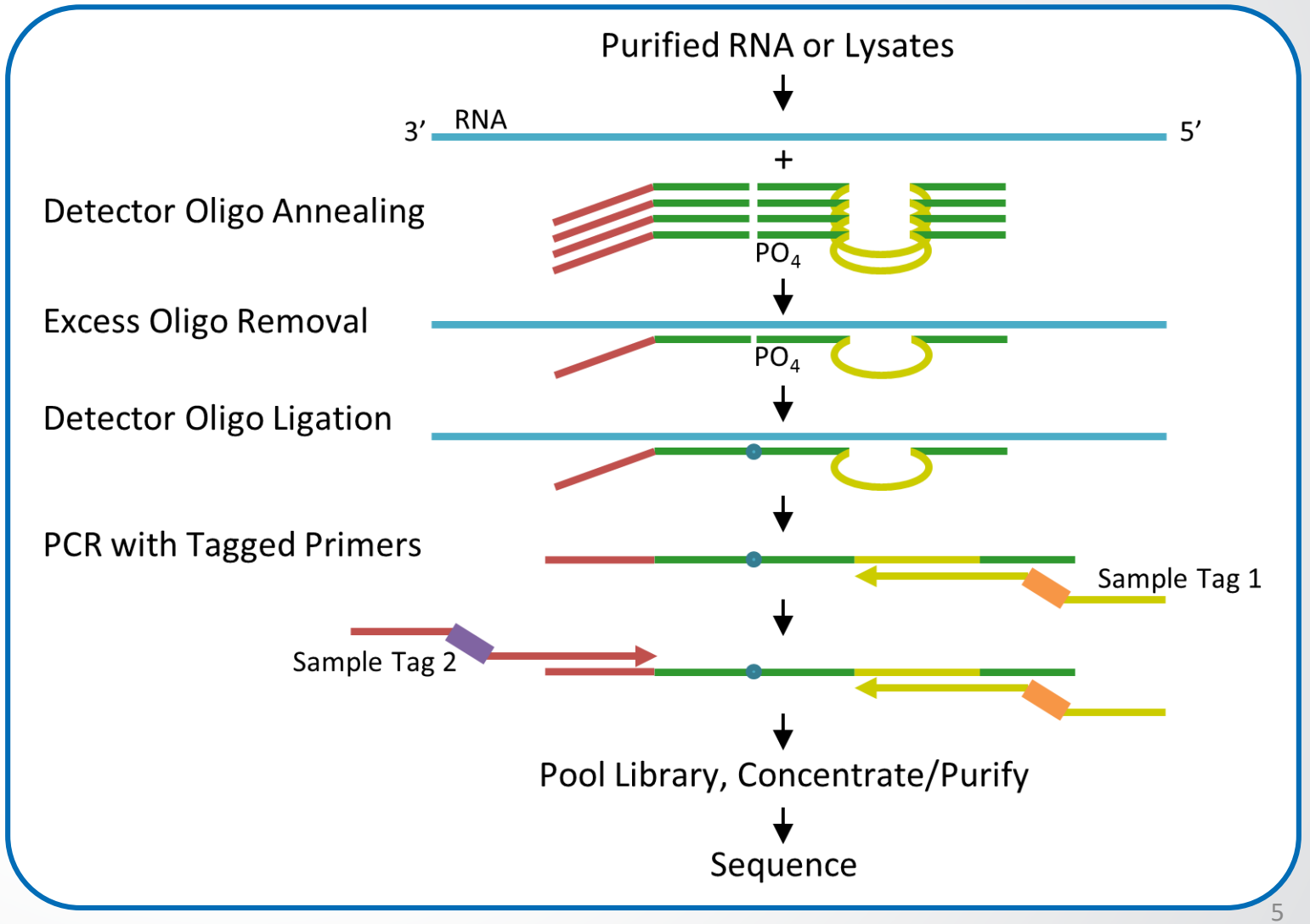
Reagent Dispensing

High Content Imaging

Perkin Elmer Opera Phenix™ High Content Screening System

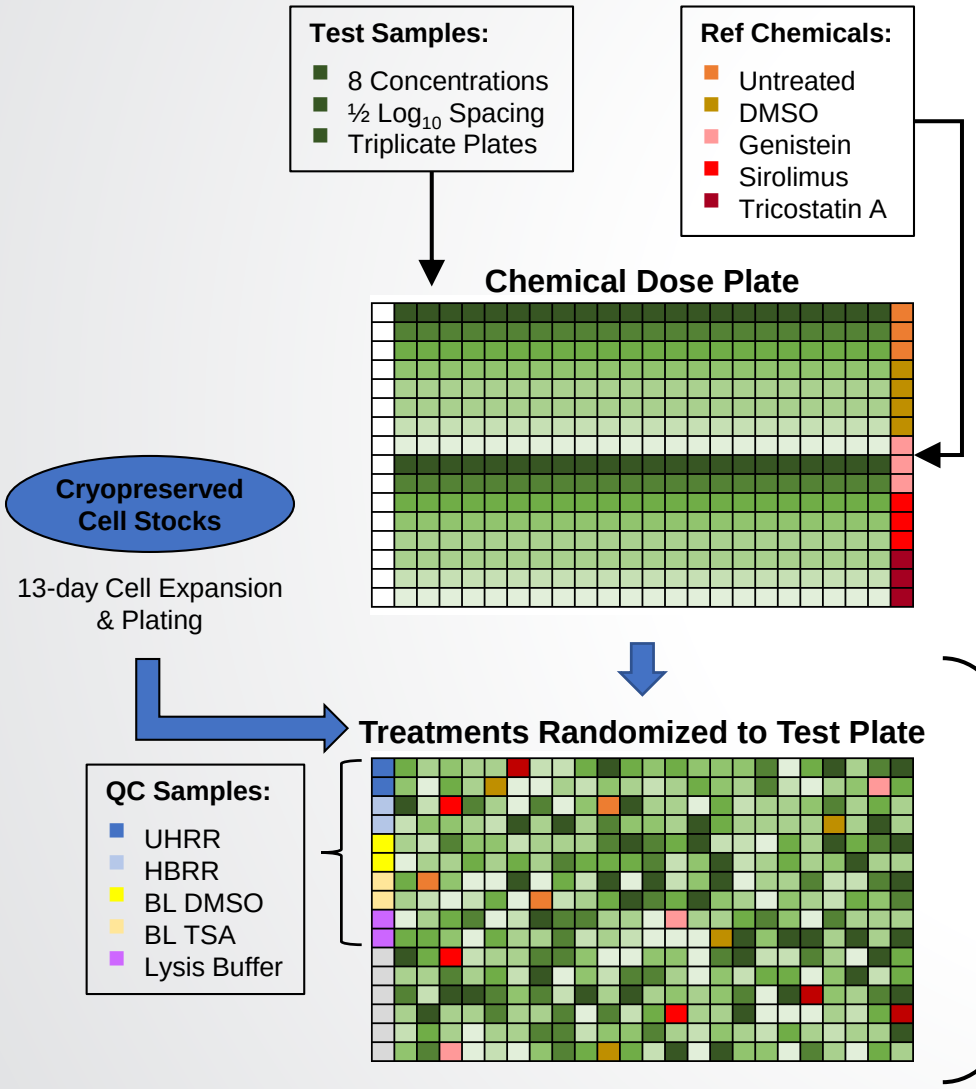
High-Throughput Transcriptomics Assay

- Targeted RNA-seq enables high-throughput profiling of **cell lysates** or purified RNA
- Probe set for whole human transcriptome targets ~21,000 human genes
- Captures majority of signal with much lower sequencing depth (~3M reads with attenuation)
- Barcoding and pooling allows multiplexing of hundreds of samples



Yeakley, et al. PLoS ONE 2017

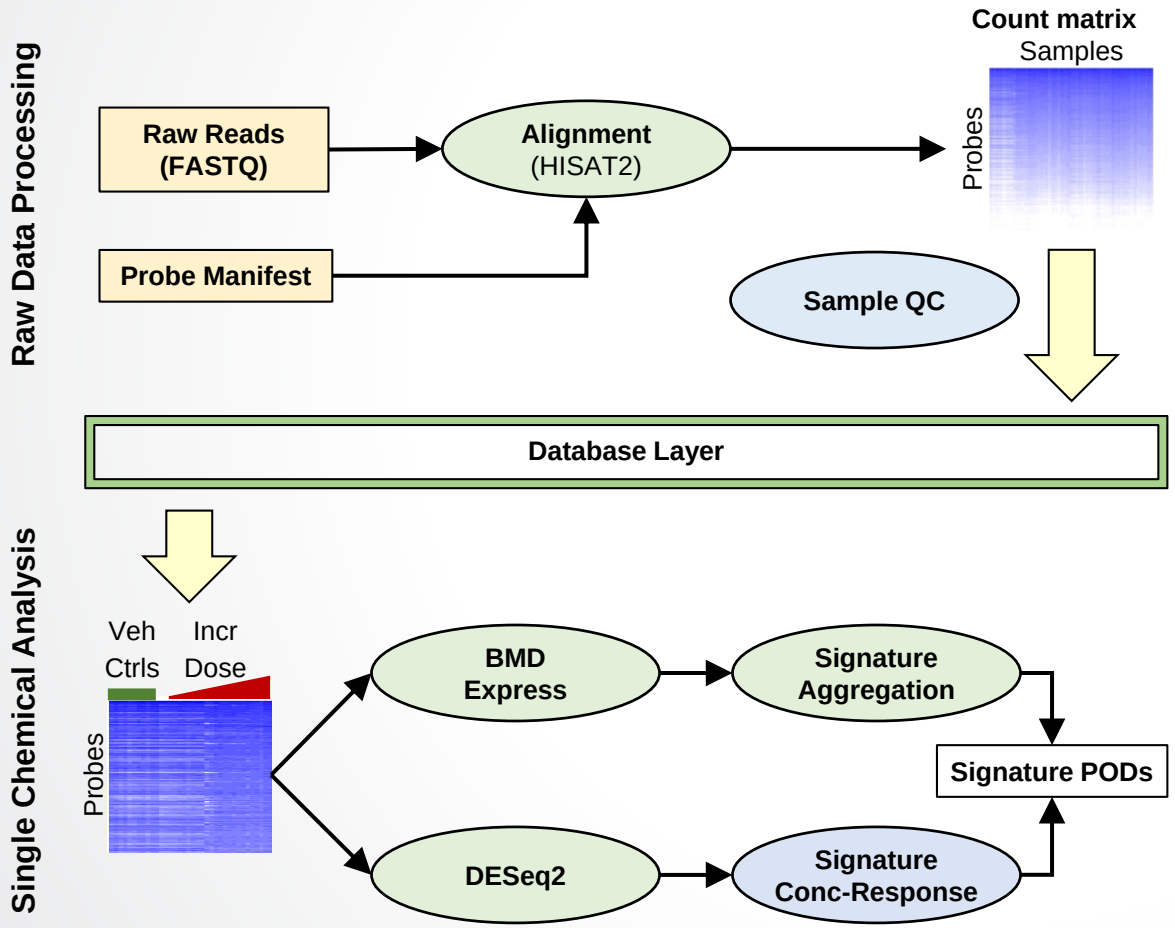
HTTr Study Design



- High-throughput *in vitro* screens performed on 384 well plates
- Standardized dilution series for every test sample
- Multiple QC and reference chemicals included on every plate to track assay performance
- Triplicate Test Plates:
  - Randomized independently
  - Separate cell culture batches

Harrill, et al. Toxicol Sci in press

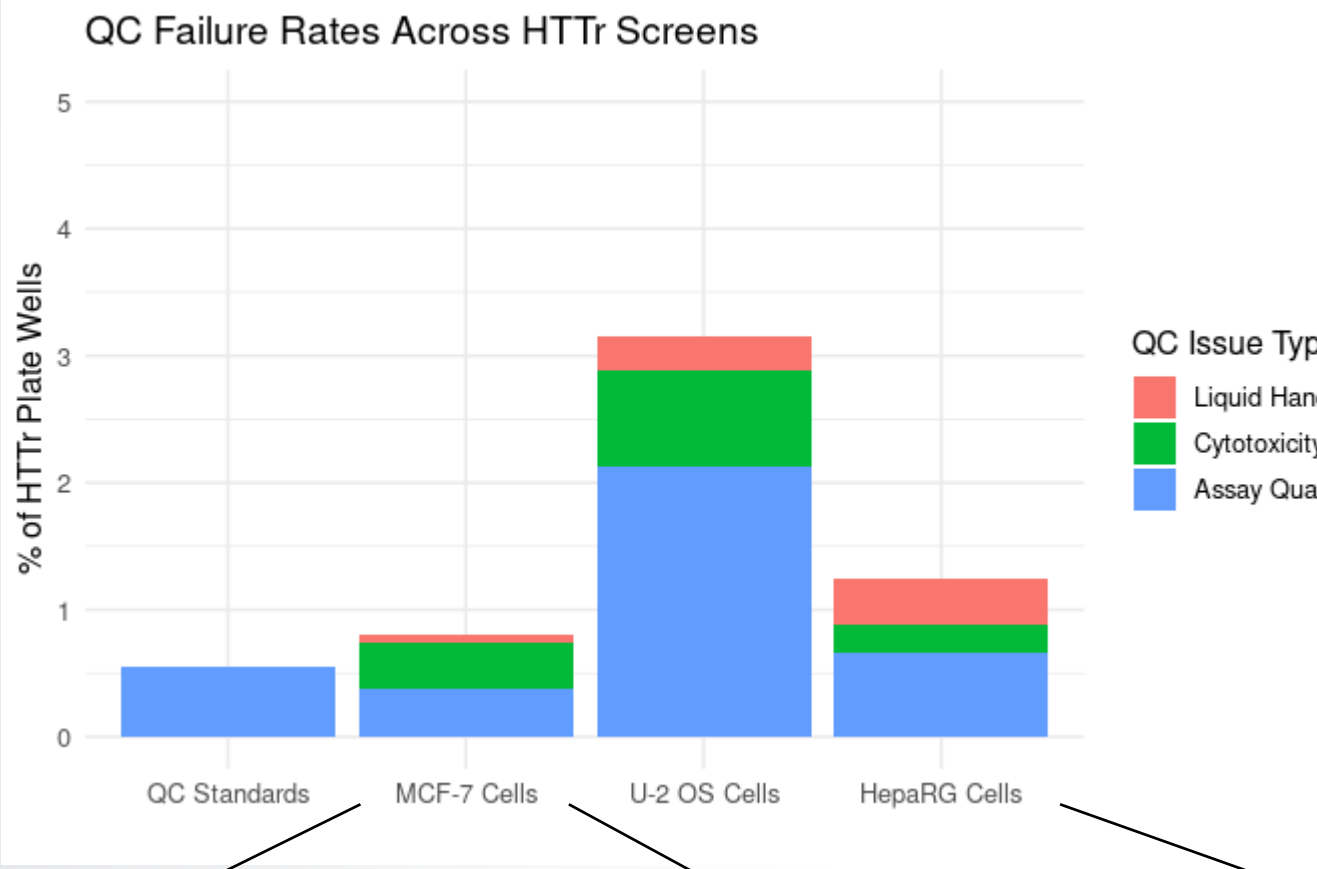
HTTr Bioinformatics Pipeline



- Rapid processing for large screens
- Many data steps performed independently for each test chemical:
  - Removal of low signal probes
  - Normalization
  - DESeq2 analysis
- Exploring multiple analysis strategies for curve-fitting and signature & chemical-level summarization

Harrill, et al. Toxicol Sci in press

HTTr Quality Control



Acoustic dispenser logs identify problems with chemical handling

Apoptosis/cell viability assays identify cytotoxic concentrations

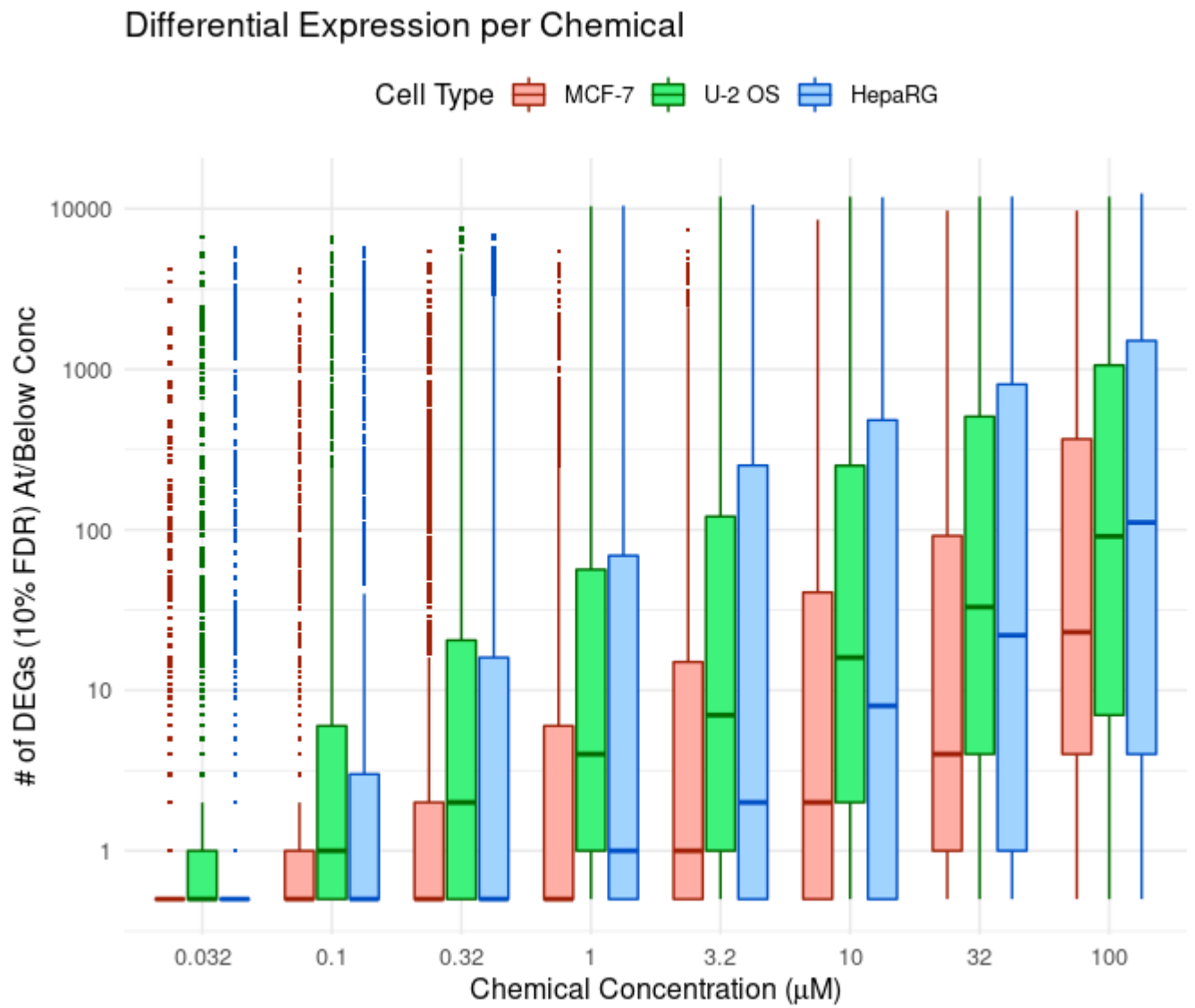
Bioinformatic QC checks remove:

- Low read depth samples
- High rate of alignment failure
- Samples with low gene coverage
- Samples with irregular count distributions

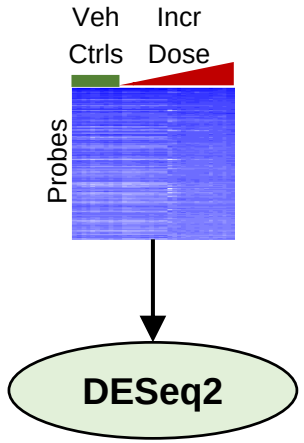
- 44 Chemical Pilot Study
- Screened 1,577 ToxCast chemicals

- Screened 1,201 ToxCast chemicals
- Screened 137 PFAS

Global View of Bioactivity



Count data for single chemical (vehicle controls + 8 concs x 3 reps)



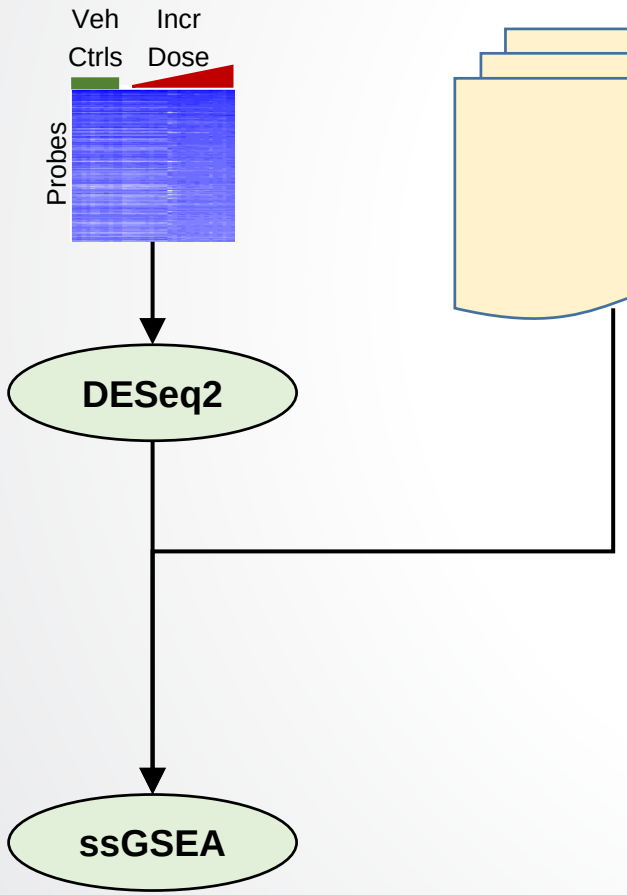
- Statistical model tailored to *-seq data
- Remove plate-level effects
- Smooths noise across depth & expression levels

(Love, et al. Genome Biol 2014)

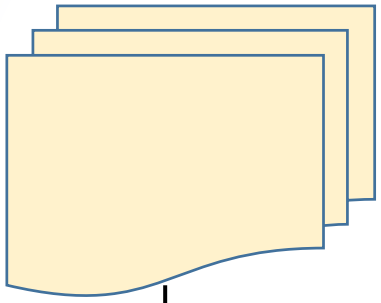
- Each boxplot shows distribution of DEG count per chemical
- **Primarily interested in transcriptional changes that:**
  - **Are coordinated across known pathways/gene sets**
  - **Fit standard curve-models across all concentrations**

Signature Scoring

Count data per chemical



Catalog of signatures with toxicological relevance, annotated for known molecular targets

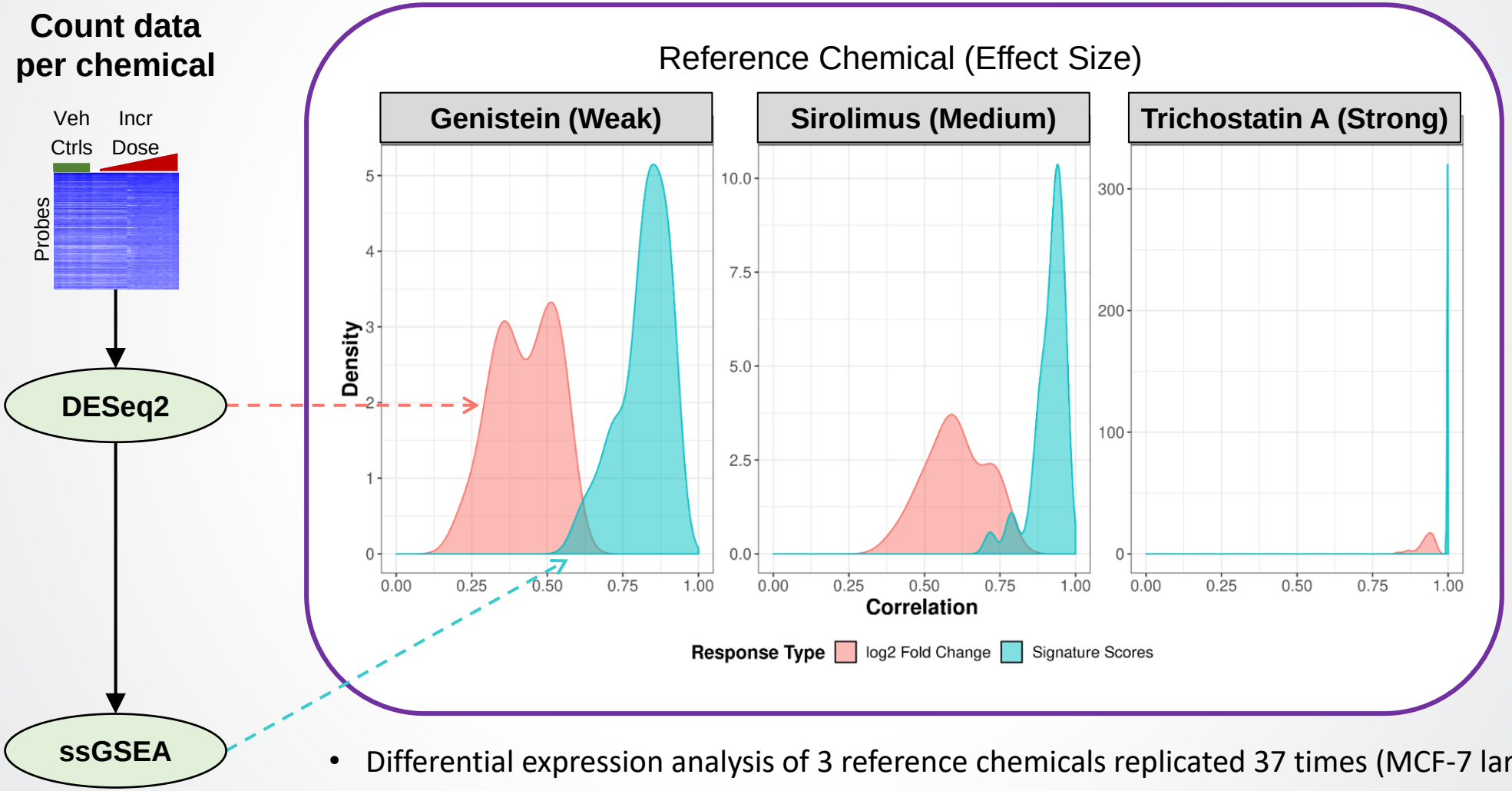


- **Bioplanet** *(Huang, et al. Front Pharmacol 2019)*
- **CMap** *(Subramanian, et al. Cell 2017)*
- **DisGeNET** *(Pinero, et al. Database 2015)*
- **MSigDB** *(Liberzon, et al. Cell Syst 2015)*

Single-Sample Gene Set Enrichment Analysis (ssGSEA) ***(Barbie, et al. Nature 2009)***

- Score coordinated responses at each concentration
- Use moderated log2 FC values from DESeq2 as input (no thresholds)
- Null distributions constructed by resampling log2 FC values from whole screen
- Alternate scoring function:
  $\text{mean}(\text{gene set log2FC}) - \text{mean}(\text{background log2FC})$

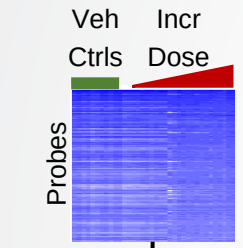
Signature Scoring



- Differential expression analysis of 3 reference chemicals replicated 37 times (MCF-7 large screen)
- Computed distribution of correlations between each replicate analysis

Signature Scoring

Count data per chemical



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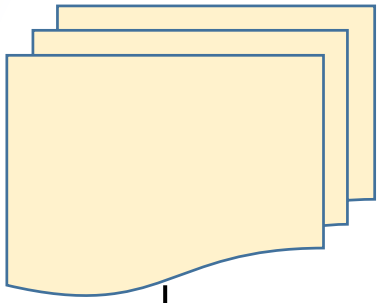

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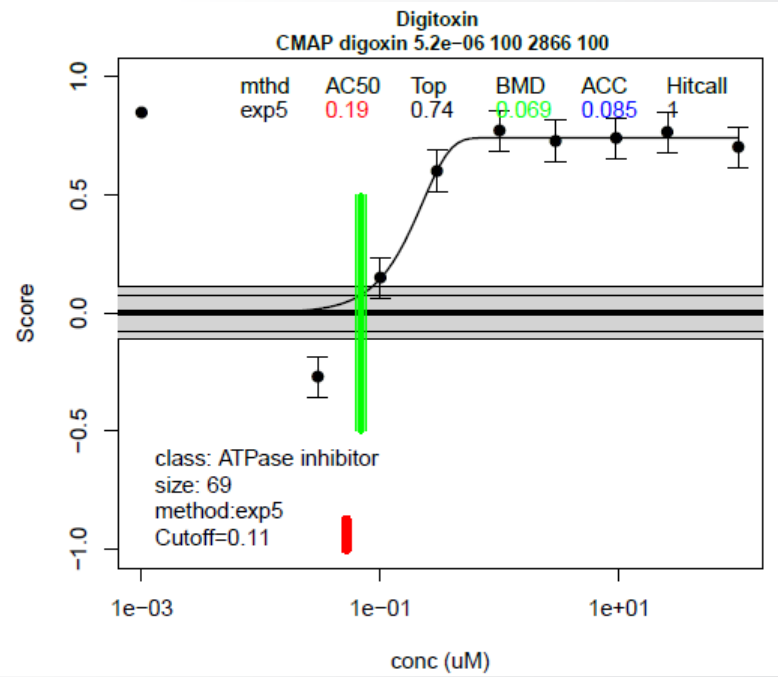
- **MSigDB** *(Liberzon, et al. Cell Syst 2015)*

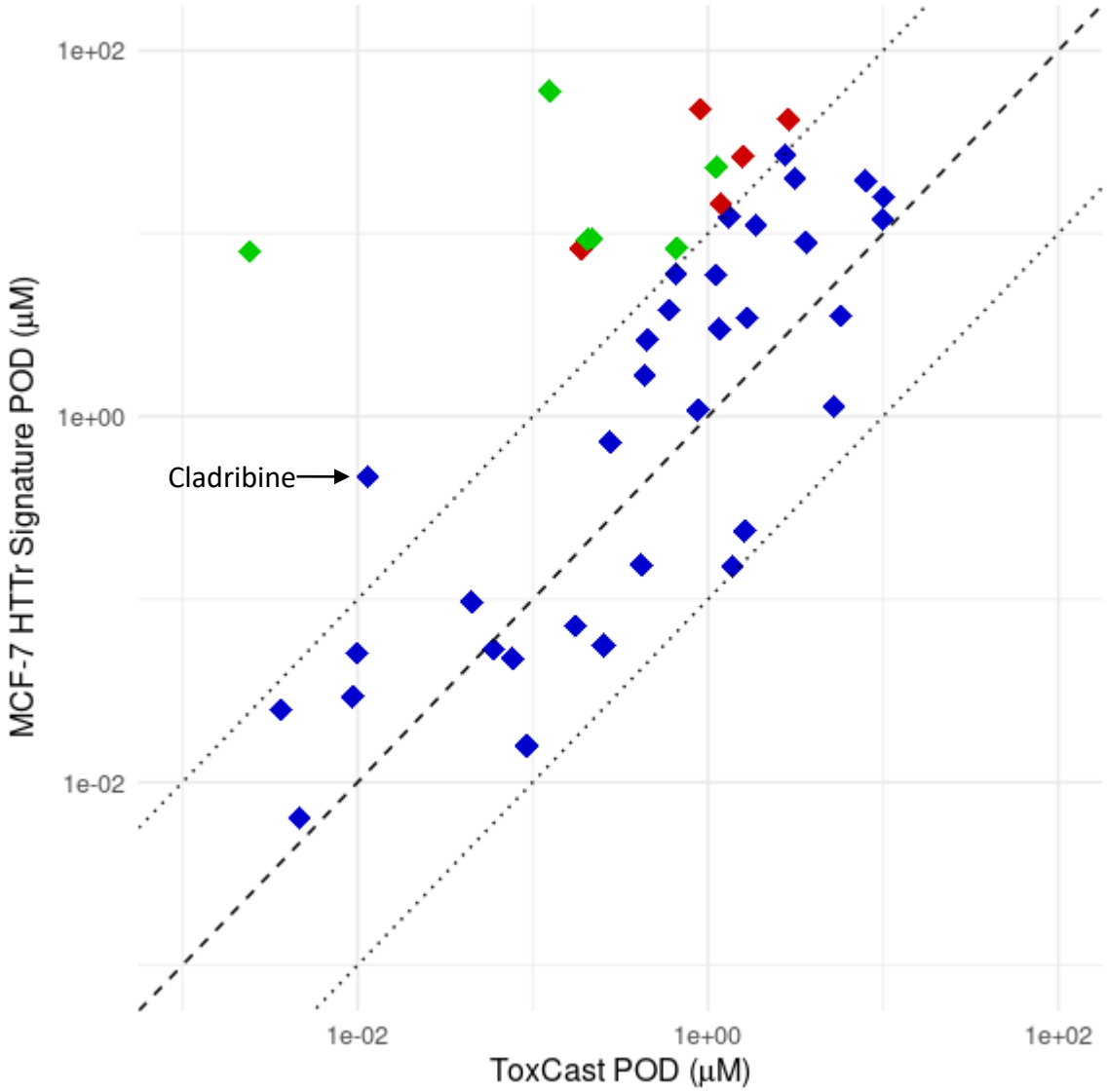
DESeq2

ssGSEA

Concentration-Response Curve Fitting (*tcplFit2*)

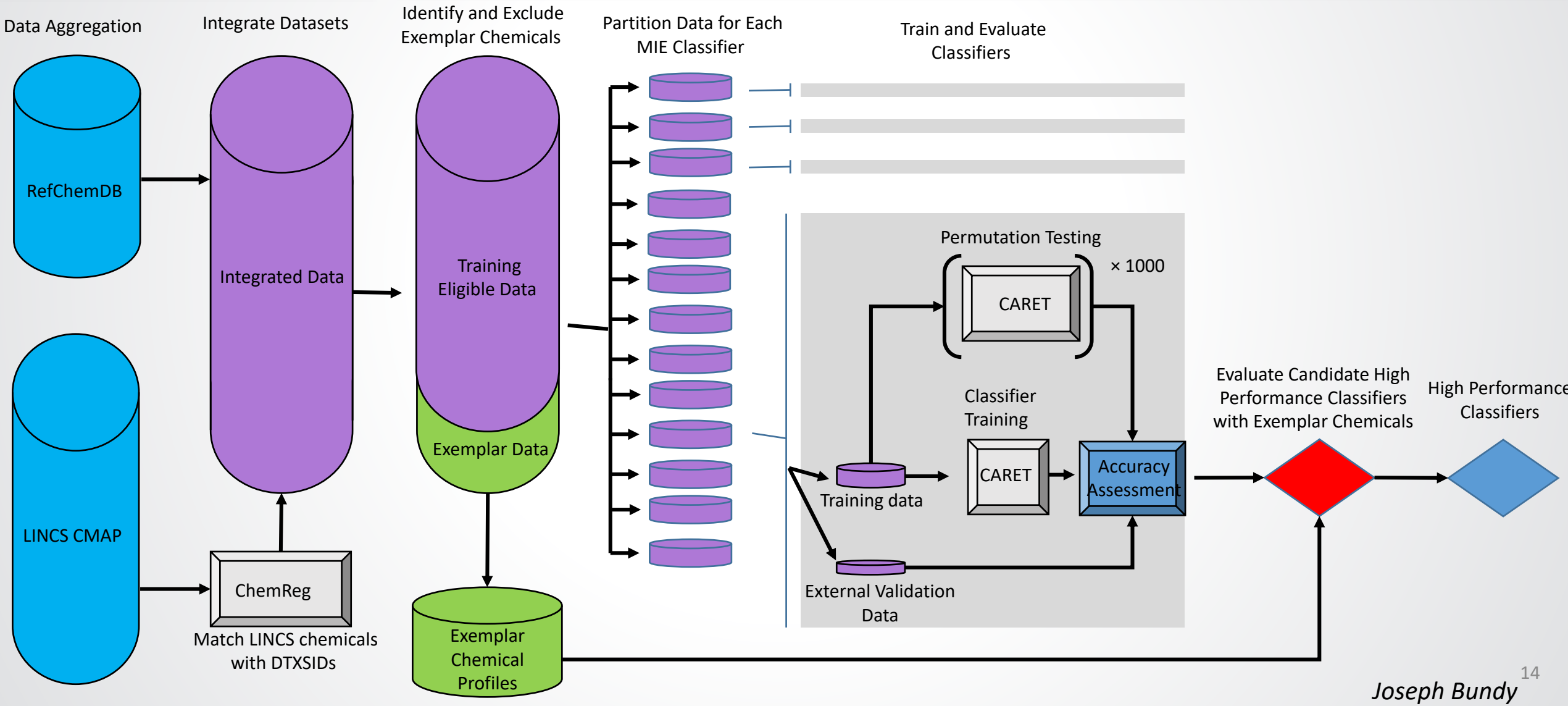


HTTr MCF-7 Pilot Analysis



- Pilot study of 44 well-characterized chemicals ***(Harrill, et al. Toxicol Sci, In Press)***
- Compared HTTr-derived PODs from MCF-7 cells to previous ToxCast HTS assay results (***Paul-Friedman, et al. Toxicol Sci 2020)***
- Signature-based POD are highly concordant with ToxCast results for the majority of test chemicals in pilot study
  - 6 chemicals with targets that have low/absent expression in MCF-7 cells
  - 5 chemicals show off-target hit as most potent assay in ToxCast
  - Cladribine is a non-specific DNA synthesis inhibitor

ML Models for MIE Classification



Joseph Bundy

Stress Response Gene Signatures

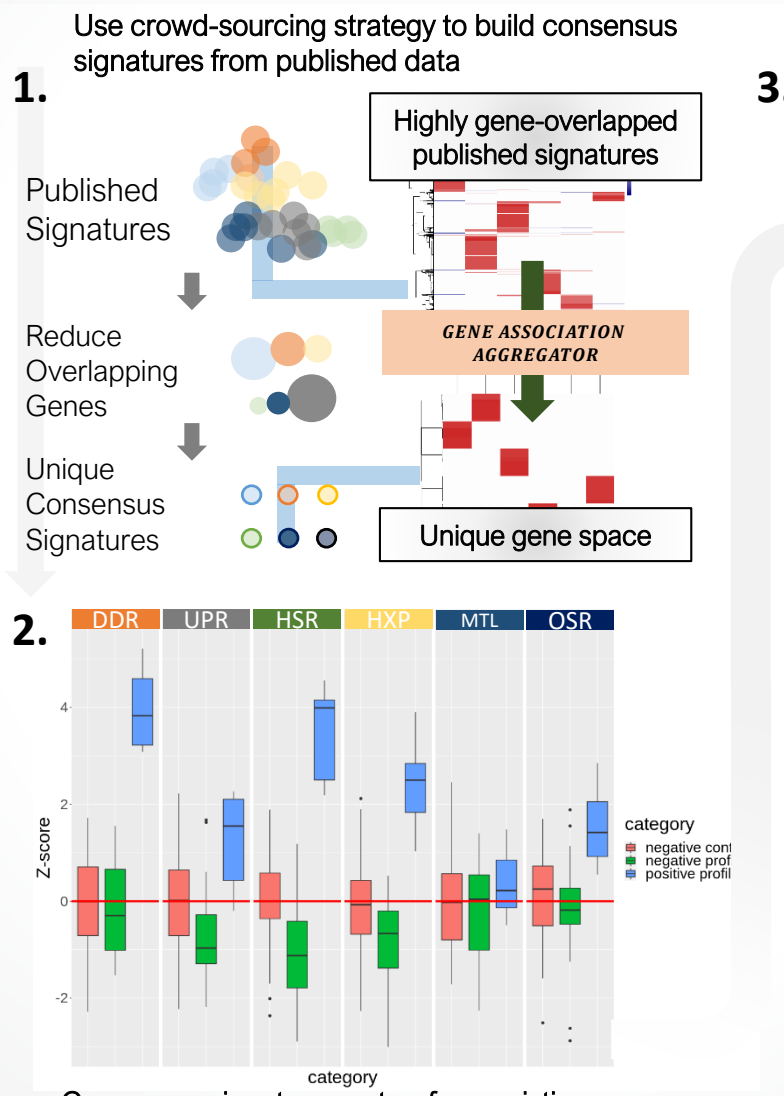
<u>Goal</u>: Develop NAMs to characterize non-specific environmental chemicals that activate stress response pathways (SRPs)

<u>Approach:</u> Characterize chemical hazards using HTTr data to assess SRP gene signature activity

<u>Challenges:</u> Cross-talk in signaling networks makes it difficult to find gene signatures of SRPs

<u>Results:</u> We have developed *consensus* SRP signatures for accurately classifying known stressors

<u>Future:</u> Use signatures to identify cellular states involved in adaptive stress responses and "tipping points" that lead to adversity



DNA Damage Response

Unfolded Protein Response

Heat Shock Response

Hypoxic Response

Response to Metals

Oxidative Stress Response

Bryant Chambers & Imran Shah

Conclusions

- CCTE has developed reliable and cost-efficient workflow for generating HTTr data from thousands of chemicals across multiple cell lines
- Preliminary/pilot analysis demonstrates that overall results are concordant with previous assays (ToxCast/HTS) and known chemical targets
- Upcoming research efforts will focus on:
  - Data generation in complementary cell models
  - Validation by orthogonal assays
  - Methods to summarize signature-level/overall PODs from high-dimensional data
  - Predictive models of MIEs/pathways relevant to toxicity
  - Coupling HTTr-derived PODs with HTTK/IVIVE work to predict *in vivo* safety levels

Acknowledgements

HTTr Team

Joshua Harrill

Richard Judson

Imran Shah

Woody Setzer

Derik Haggard

Beena Vallanat

Joseph Bundy

Bryant Chambers

Laura Taylor

Thomas Sheffield

Clinton Willis

CCTE Leadership

Rusty Thomas

Maureen Gwinn

John Cowden

Kimberly Slentz-Kesler

EPA Collaborators

Chris Corton

Mark Higuchi

Adam Speen

Johanna Nyffeler

National Toxicology Program

Scott Auerbach

Nisha Sipes (now at EPA)

Dahea You

HTTr Platform Selection

Matthew Martin

Agnes Karmaus

BioSpyder

References

- Thomas RS, Bahadori T, Buckley TJ, et al. *"The Next Generation Blueprint of Computational Toxicology at the U.S. Environmental Protection Agency"*, Toxicol Sci 2019
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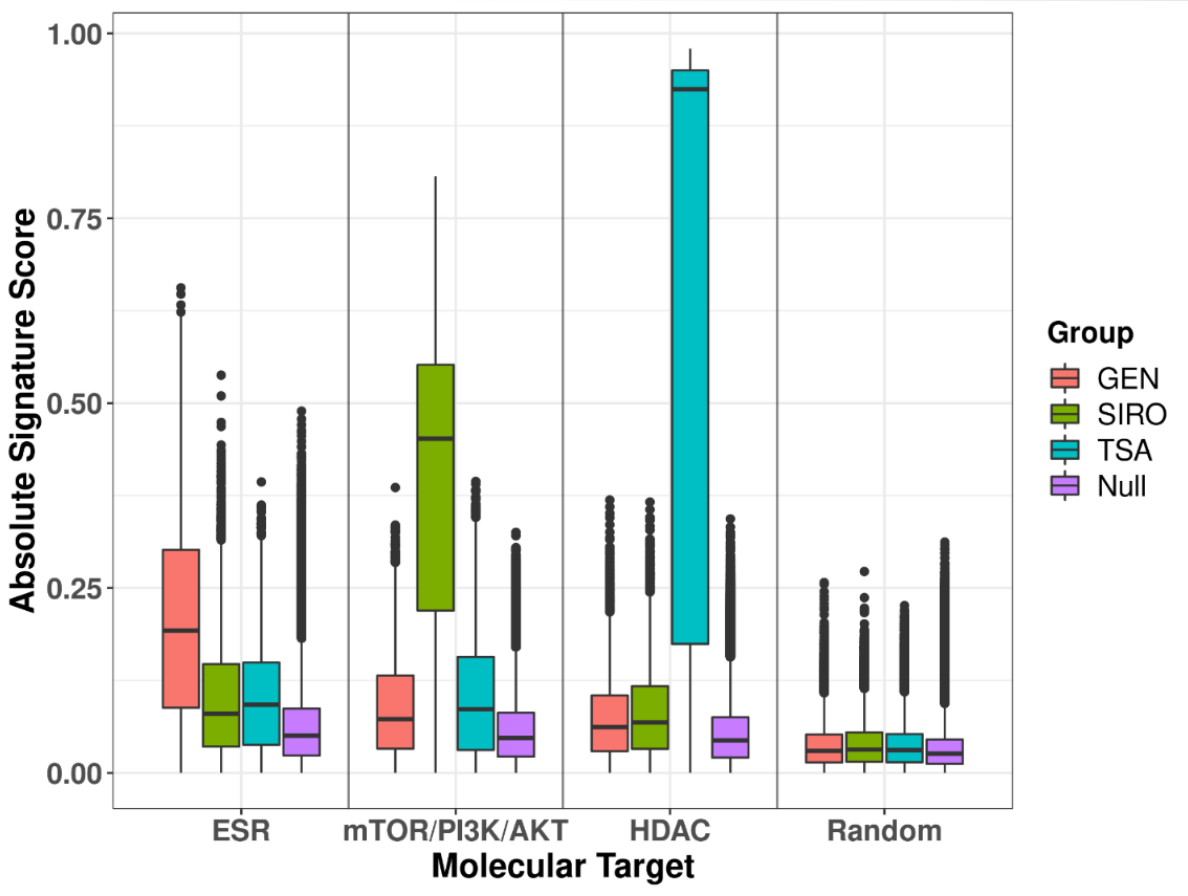
- Harrill J, Everett LJ, Haggard D, et al. *"High-Throughput Transcriptomics Platform for Screening Environmental Chemicals"*, Toxicol Sci 2021 *in press*
- Love MI, Huber W, and Anders S. *"Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2"*, Genome Biol 2014
- Barbie DA, Tamayo P, Boehm JS, et al. *"Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1"*, Nature 2009
- Huang R, Grishagin I, Wang Y, et al. *"The NCATS BioPlanet - An Integrated Platform for Exploring the Universe of Cellular Signaling Pathways for Toxicology, Systems Biology, and Chemical Genomics"*, Front Pharmacol 2019
- Subramanian A, Narayan R, Corsello SM, et al. *"A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles"*, Cell 2017
- Pinero J, Queralt-Rosinach N, Bravo A, et al. *"DisGeNET: a discovery platform for the dynamical exploration of human diseases and their genes"*, Database 2015
- Liberzon A, Birger C, Thorvaldsdottir H, et al. *"The Molecular Signatures Database (MSigDB) hallmark gene set collection"*, Cell Syst 2015
- Paul-Friedman K, Gagne M, Loo LH, et al. *"Utility of In Vitro Bioactivity as a Lower Bound Estimate of In Vivo Adverse Effect Levels and in Risk-Based Prioritization"*, Toxicol Sci 2020

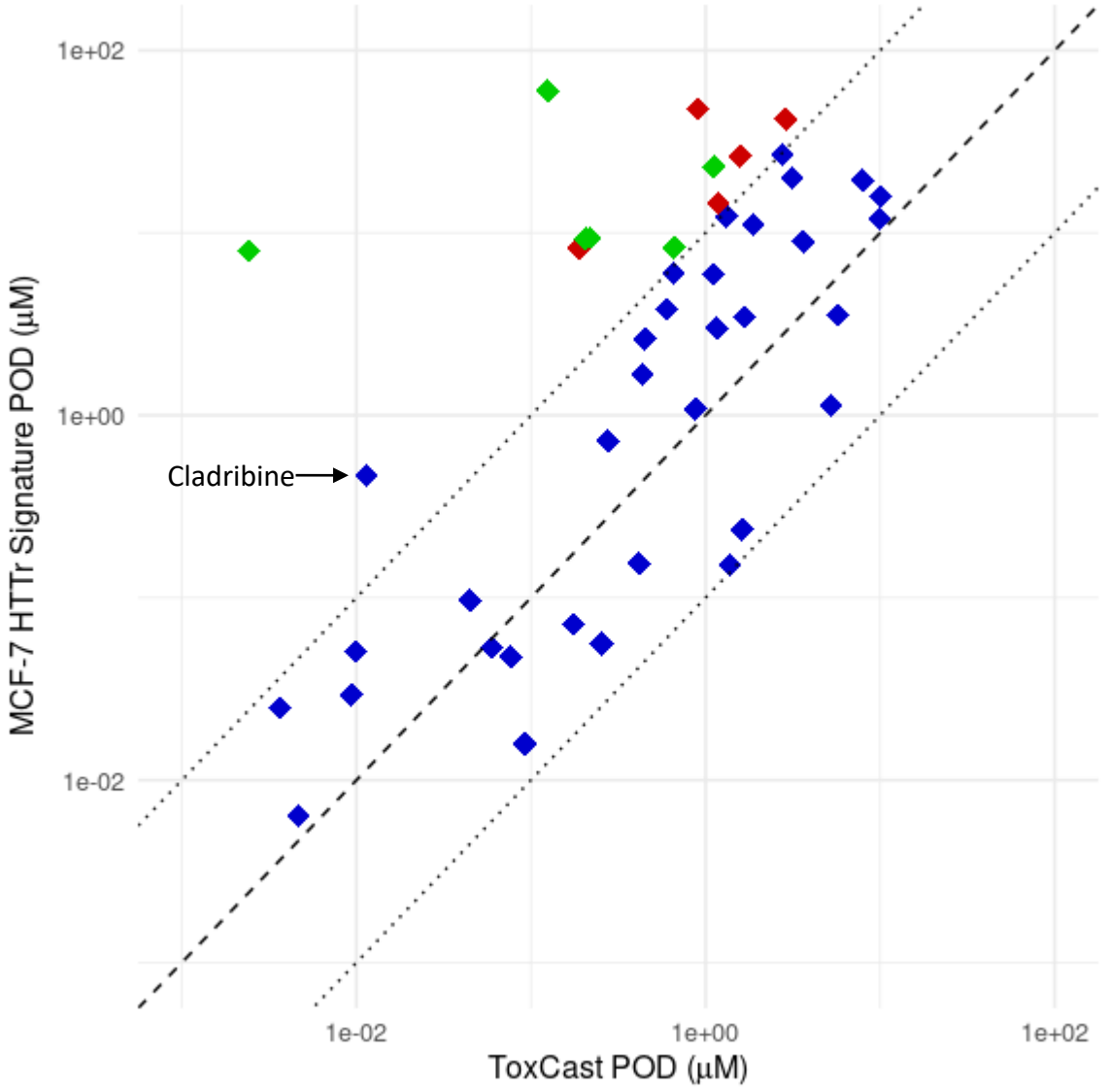
Extra Slides

Assay Reproducibility

- Analyzed differential expression response to 3 reference chemicals replicated 37 times throughout large screen (MCF-7)
  - GEN = Genistein (10uM)
  - SIRO = Sirolimus/Rapamycin (0.1uM)
  - TSA = Trichostatin A (1uM)
  - NULL = Signature scores derived from re-sampled log2 FC values
- Signatures were annotated for associated molecular targets
  - Random = Randomly selected gene sets with similar size to known signature gene sets
- Each reference chemical was enriched for higher scores from signature associated with correct molecular target
- Similar analysis and result found in MCF-7 pilot study ***(Harrill, et al. Toxicol Sci in press)***



HTTr MCF-7 Pilot Analysis



- 6 chemicals with targets that have low/absent expression in MCF-7 cells
  - 3,5,3'-triiodothyronine (Thyroid Receptor)
  - Cyproconazole (pan-CYP inhibitor)
  - Butafenacil (pan-CYP inhibitor)
  - Prochloraz (pan-CYP inhibitor)
  - Imazalil (pan-CYP inhibitor)
  - Propiconazole (pan-CYP inhibitor)
- 5 chemicals show off-target hit as most potent assay in ToxCast
  - Lovastatin
  - Clofibrate
  - Maneb
  - Lactofen
  - Vinclozolin
- Cladribine is a non-specific DNA synthesis inhibitor

(Harrill, et al. Toxicol Sci, In Press)

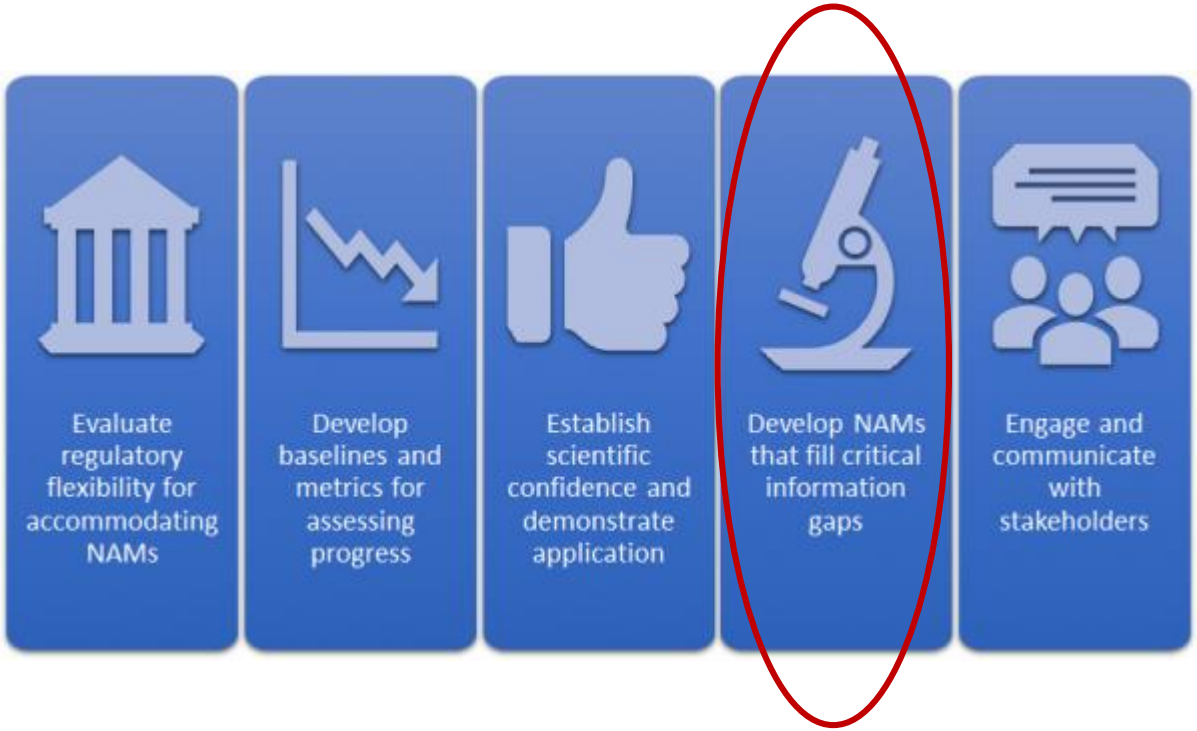
Retrofitting *in vitro* Systems with Metabolic Competence

Chad Deisenroth
Center for Computational Toxicology and Exposure
deisenroth.chad@epa.gov

US EPA CSS-HERA BOSC Chemical Safety Subcommittee Meeting
February 2nd, 2021

Disclaimer: The views expressed are those of the author and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

EPA New Approach Methods Work Plan: Reducing Use of Animals in Chemical Testing



Examples of information gaps

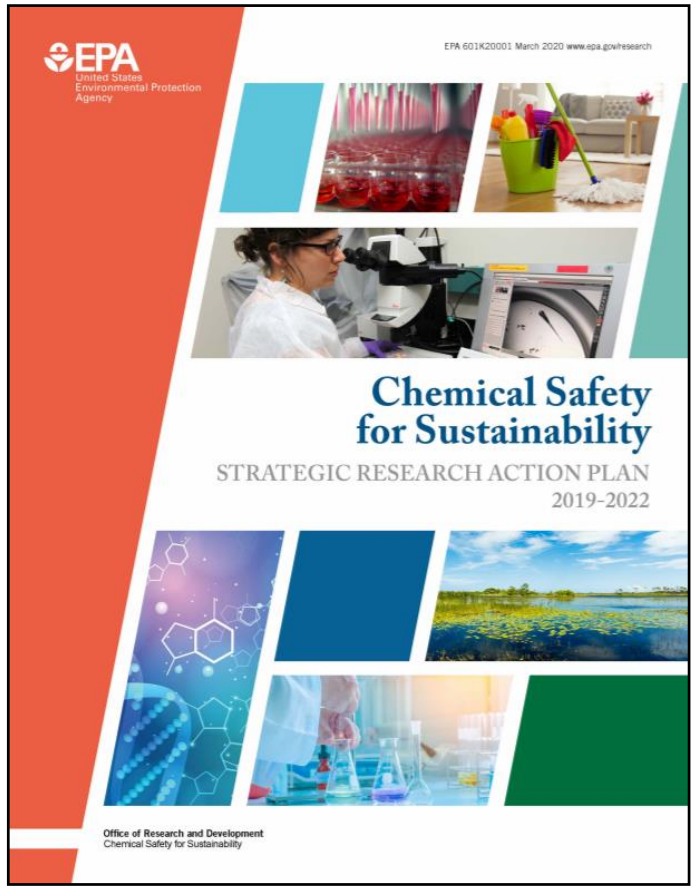
- Inadequate coverage of biological targets.
- Limited capability to address tissue- and organ-level effects.
- Lack of robust integrated approaches to testing and assessment (IATAs).
- Minimal capability for addressing xenobiotic metabolism in *in vitro* test systems.

https://www.epa.gov/sites/production/files/2020-06/documents/epa_nam_work_plan.pdf

Outline

Danica DeGroot
Steve Simmons
Todd Zurlinden
Andrew Eicher
James McCord
Kristen Hopperstad
Woody Setzer
Katie Paul-Friedman
Madison Feshuk
Rusty Thomas

Paul Carmichael
Mi-Young Lee



CSS.1.5 (High Throughput Toxicology): Develop and apply methods to incorporate endogenous and exogenous xenobiotic metabolism into high-throughput *in vitro* assays.

CSS.1.5.1: Application of the Alginate Immobilization of Metabolic Enzymes (AIME) method to incorporate hepatic metabolism into an Estrogen Receptor transactivation assay.

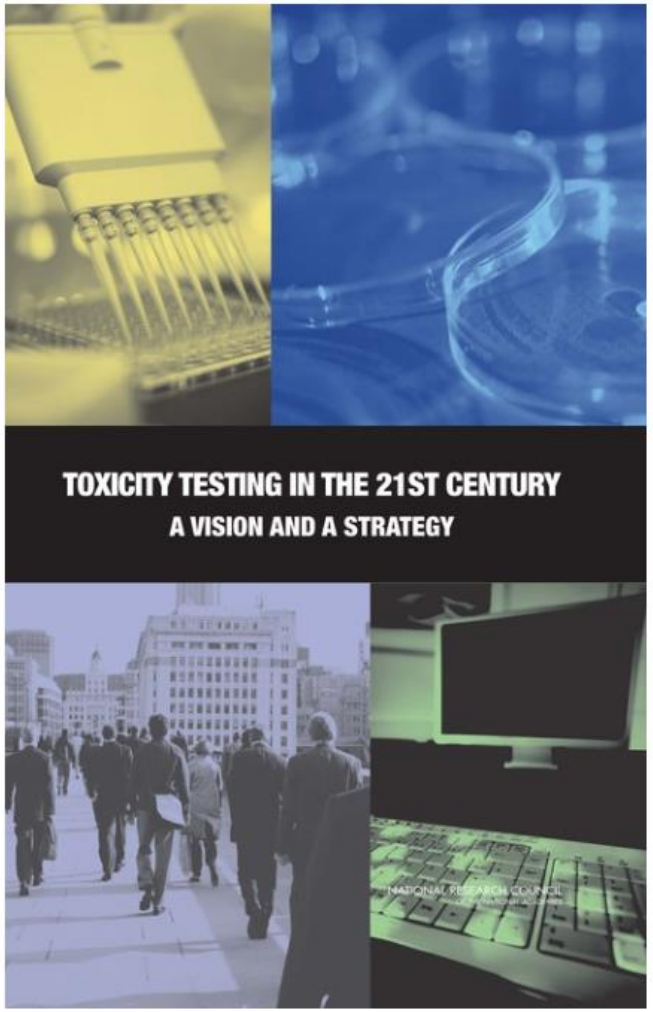
CSS.1.5.2: Development of a bioprinting approach to adapt the Alginate Immobilization of Metabolic Enzymes metabolism method for high-throughput screening applications.

Toxicity Testing in the 21st Century

National Research Council 2007 report calling for a genuine commitment to the reduction, refinement, and replacement of animal testing.

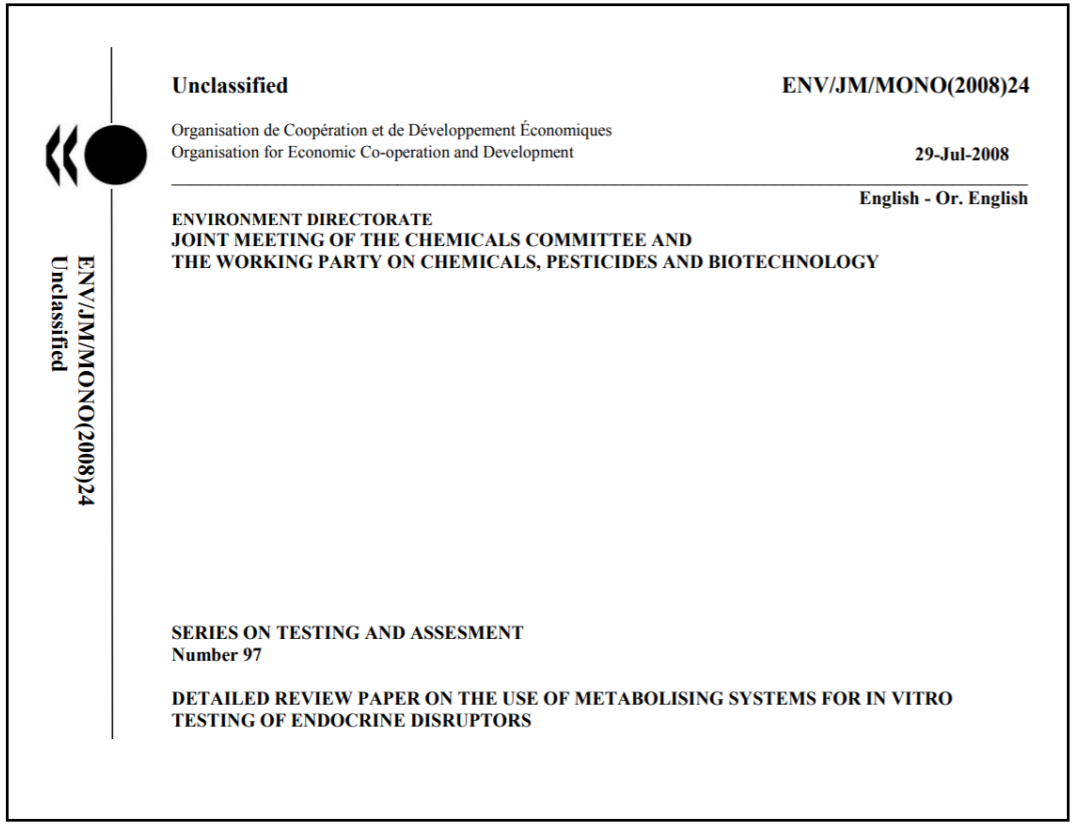
Key Questions for Implementation – Addressing Xenobiotic Metabolism

- "One of the challenges of developing an *in vitro* test system to evaluate toxicity is the current inability of cell assays to mirror metabolism in the integrated whole animal..."
- Methods to Predict Metabolism - How can adequate testing for metabolites in the high-throughput assays be ensured?
- Recommendations
  - Screening using computational approaches possible.
  - Limited animal studies that focus on mechanism and specific metabolites.



http://nap.edu/11970. DOI 10.17226/11970

OECD Detailed Review Paper (DRP 97) (2008) - *In Vitro* Metabolism Systems for Endocrine Disruptors



Unclassified ENV/JM/MONO(2008)24

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

29-Jul-2008

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

ENV/JM/MONO(2008)24
Unclassified

SERIES ON TESTING AND ASSESMENT
Number 97

DETAILED REVIEW PAPER ON THE USE OF METABOLISING SYSTEMS FOR IN VITRO TESTING OF ENDOCRINE DISRUPTORS

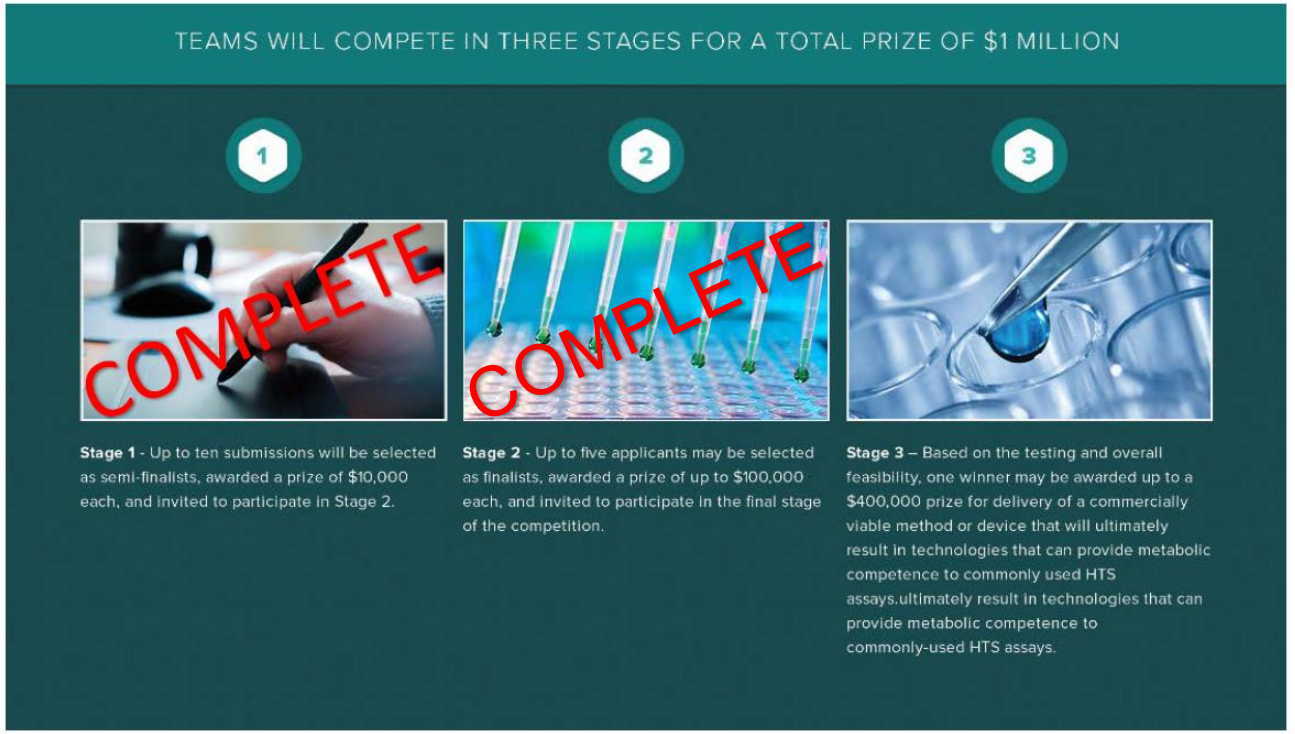
The Validation Management Group for Non-animal Testing (VMG-NA) meeting (2003)

- "…it was necessary to consider and preferably incorporate metabolism of compounds when considering the development of *in vitro* tests for endocrine active substances, to reflect the real *in vivo* situation, and so reduce the risks of false positives and false negatives."
- "Tests to detect EAS and tests that can predict the influence of chemicals on metabolism of endogenous or exogenous substances, or the influence metabolism of a chemical on its ultimate effect, are still being developed."
- "…the eventual need to combine the outcome of these developments will be an important component of the development of each field."

United States Environmental Protection Agency

TRANSFORM TOX TESTING CHALLENGE

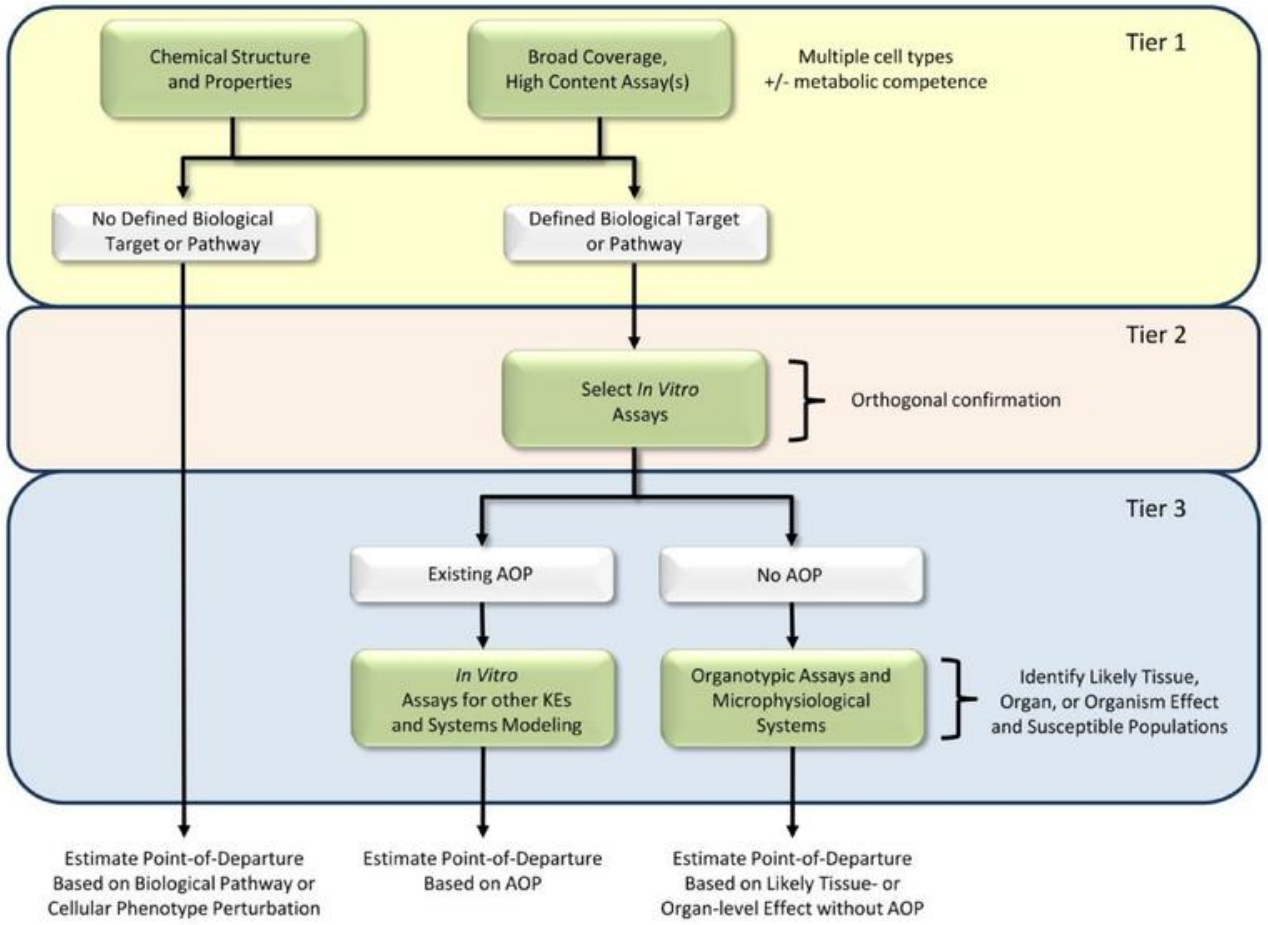
INNOVATING FOR METABOLISM

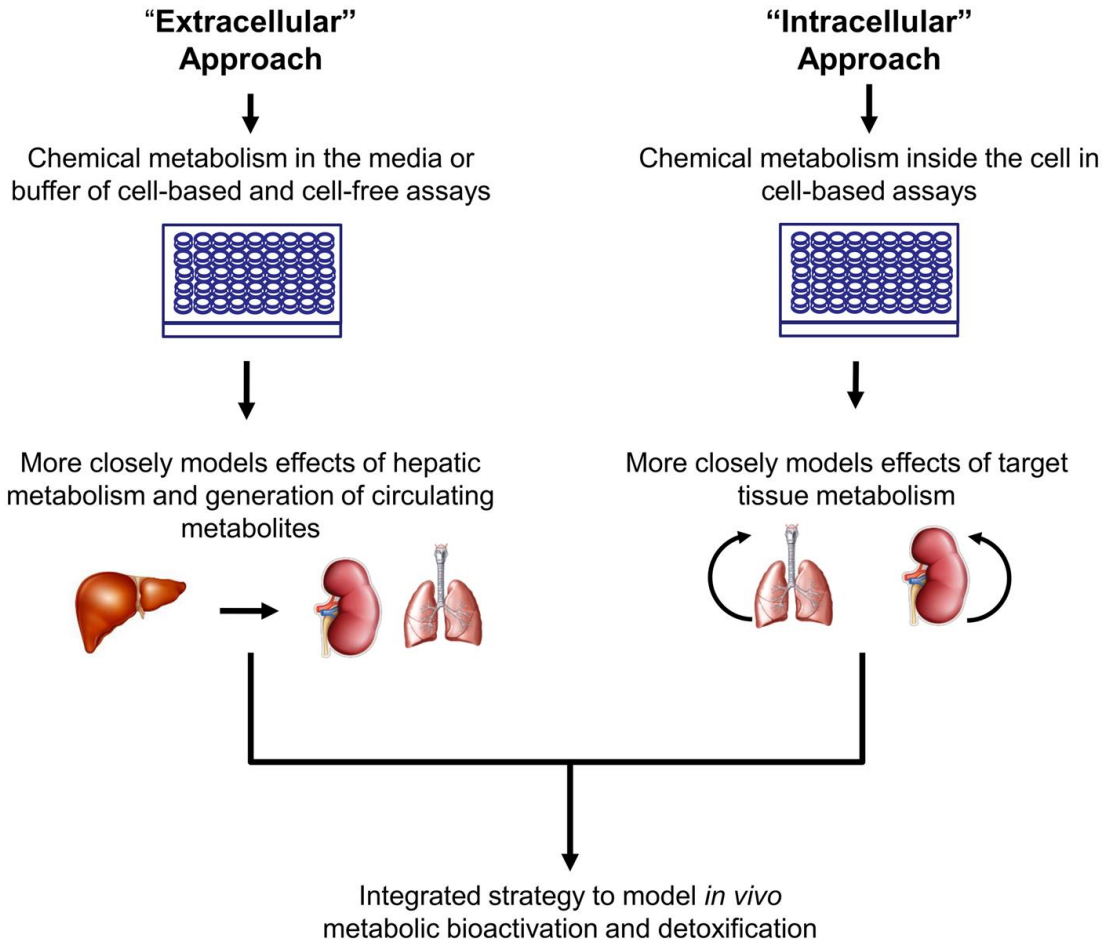


Identify innovative solutions to retrofit high-throughput assays with metabolic competence

(2016-2017) EPA, NTP, NCATS

The Next Generation Blueprint of Computational Toxicology at the U.S. Environmental Protection Agency

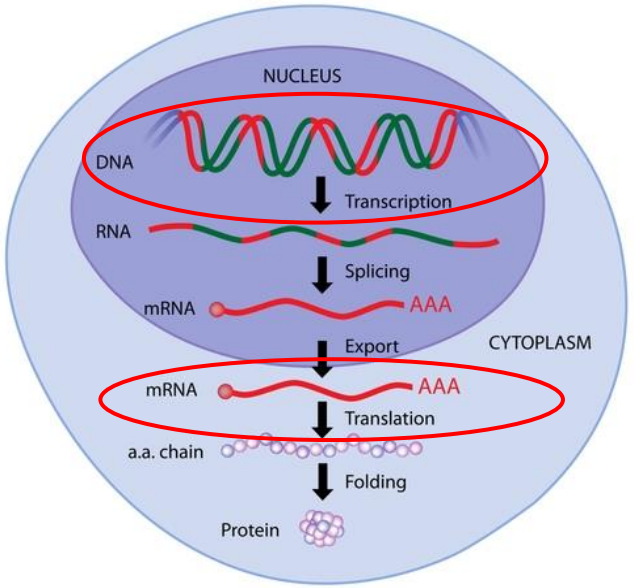


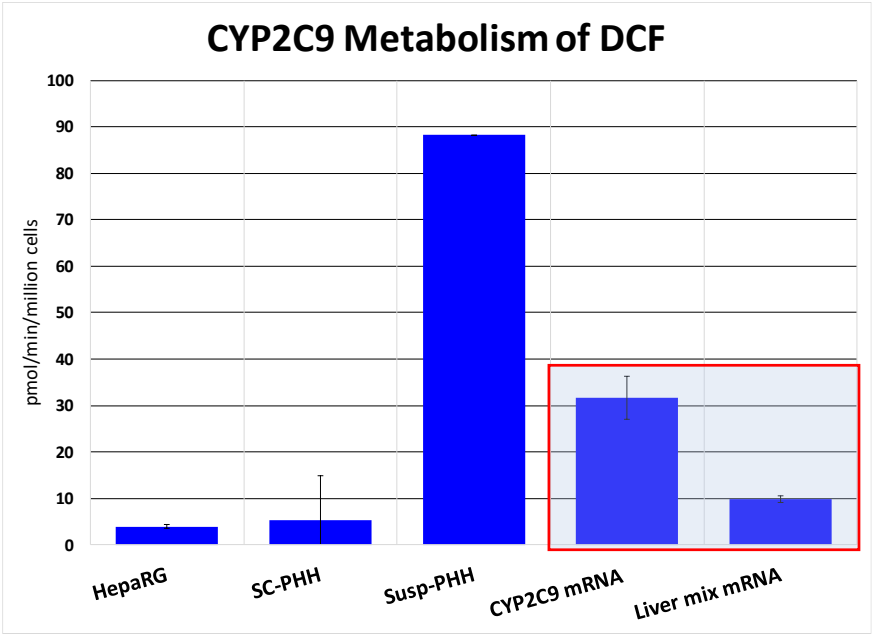


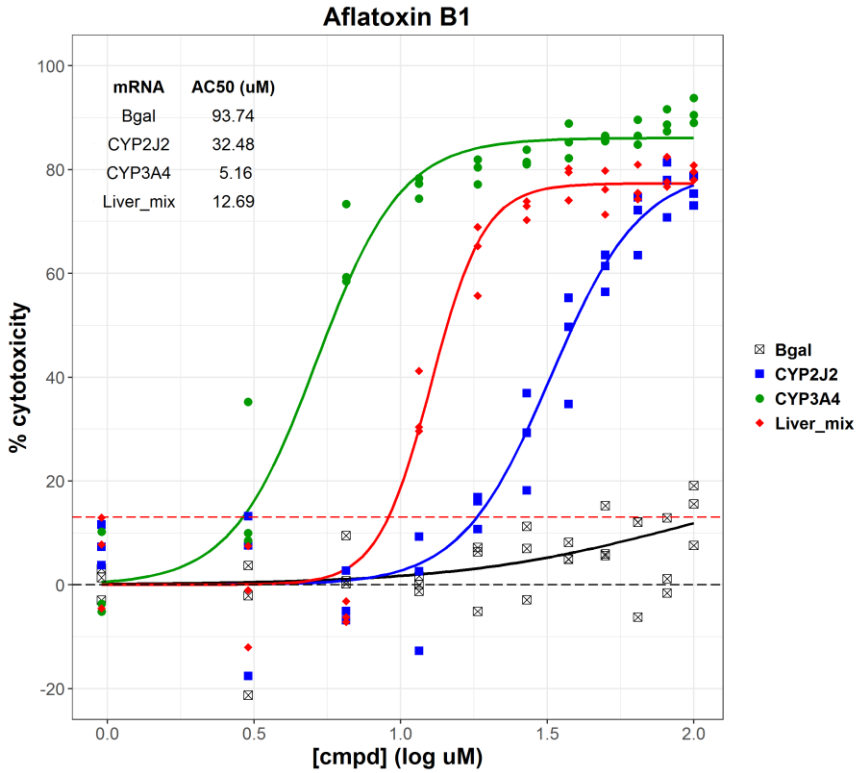
Intracellular Approach: Xenobiotic Metabolism by mRNA Transfection

Steve Simmons (EPA)

- Traditional DNA-based gene delivery methods use viral gene promoters to drive mRNA transcription.
- mRNA transfection is a novel approach that bypasses cellular DNA transcription.
- Rapid expression of metabolizing enzymes (steady state within 8-16 hours).
- User-defined composition and ratios of multiple input mRNAs.

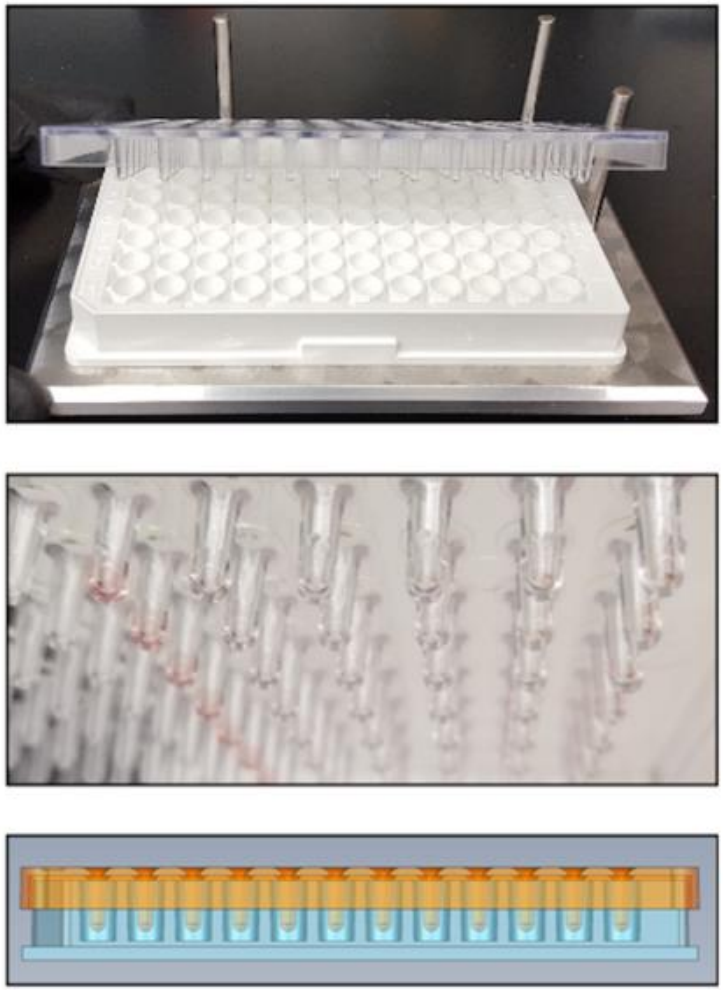


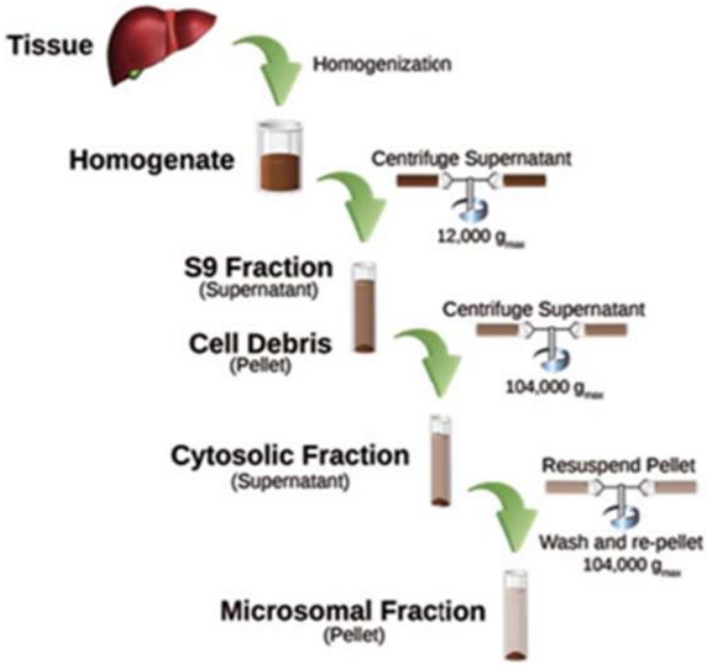




J Pharmacol Toxicol Methods. Jul-Aug 2018. DOI: 10.1016/j.vascn.2018.03.002

Extracellular Approach: Alginate Immobilization of Metabolic Enzymes (AIME)

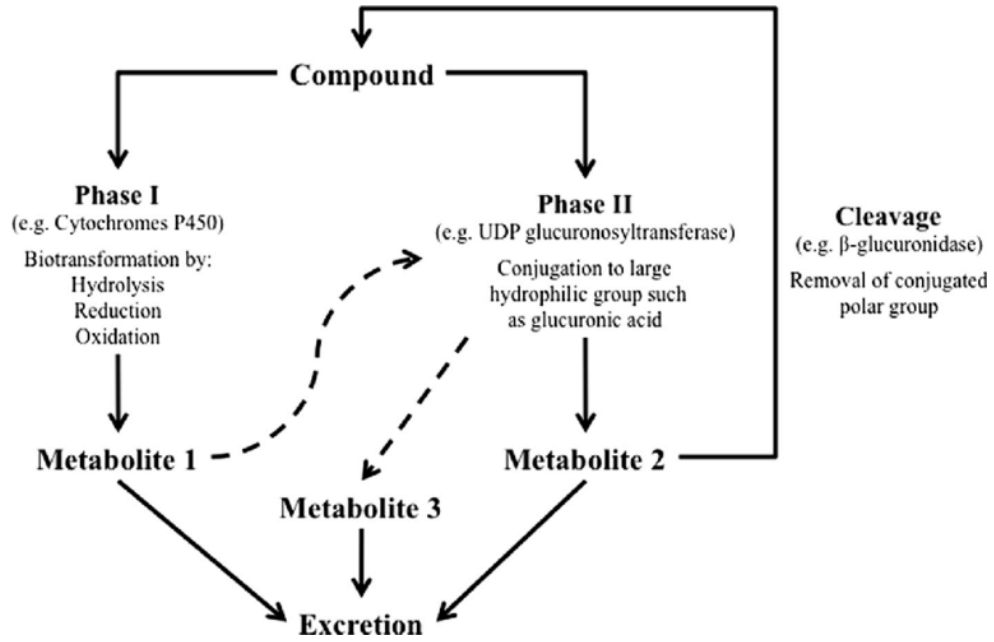


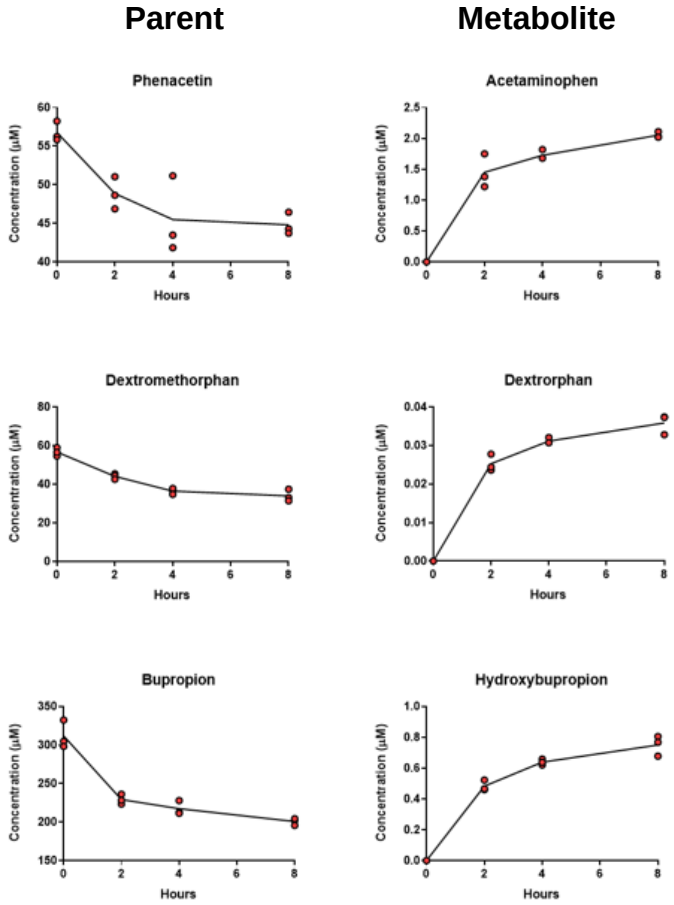


- **Liver Metabolism:** Phenobarbital/β-naphthoflavone-induced male Sprague Dawley rat hepatic S9.
- **Alginate Hydrogel:** Widely used in a variety of pharmaceutical and biomedical applications due to high biocompatibility, low toxicity, and mild gelation by divalent cations.
- **AIME:** The Alginate Immobilization of Metabolic Enzymes (AIME) platform consists of custom 96-well microplate lids containing solid supports attached to encapsulated hepatic S9-alginate microspheres.

Shimadzu Journal, 3(3), 64-68

Validation of Cytochrome P450 Metabolism





Key Points

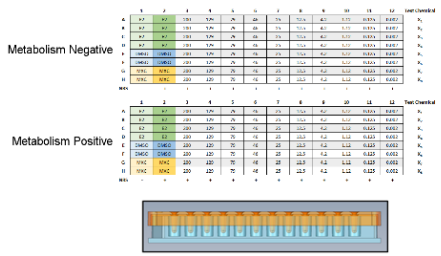
- AIME method optimized for Phase I metabolism.
- Metabolic activity validated across a diverse profile of CYPs with reference chemicals.

Substrate	Human	Rat
Phenacetin	CYP1A2	1A1, **1A2**
Bupropion	CYP2B6	**2B1**, 2B2, 2B3
Diclofenac	CYP2C9	**2C6**, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23
Dextromethorphan	CYP2D6	2D1, **2D2**, 2D3, 2D4, 2D5, 2D18
Chlorzoxazone	CYP2E1	**2E1**

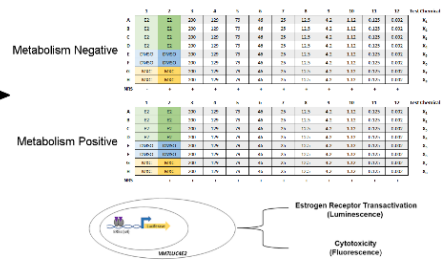
DOI:10.1007/s00338-014-1136-3

Retrofitting Metabolism to an Estrogen Receptor Transactivation Assay

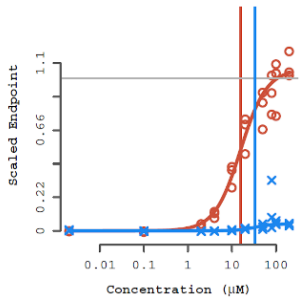
AIME Metabolism Assay



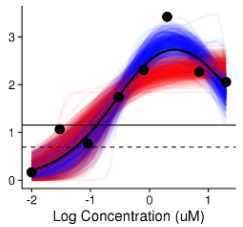
Estrogen Receptor Transactivation Assay



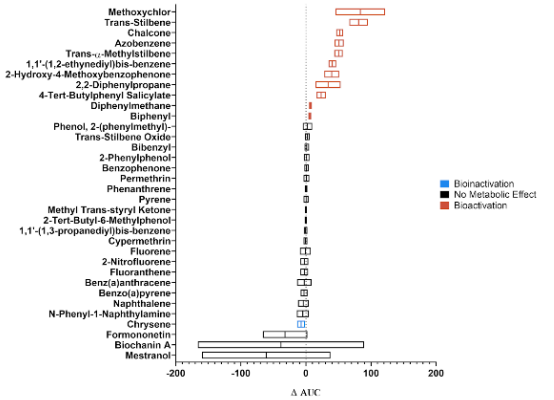
ToxCast Pipeline



Toxboot Uncertainty Quantification



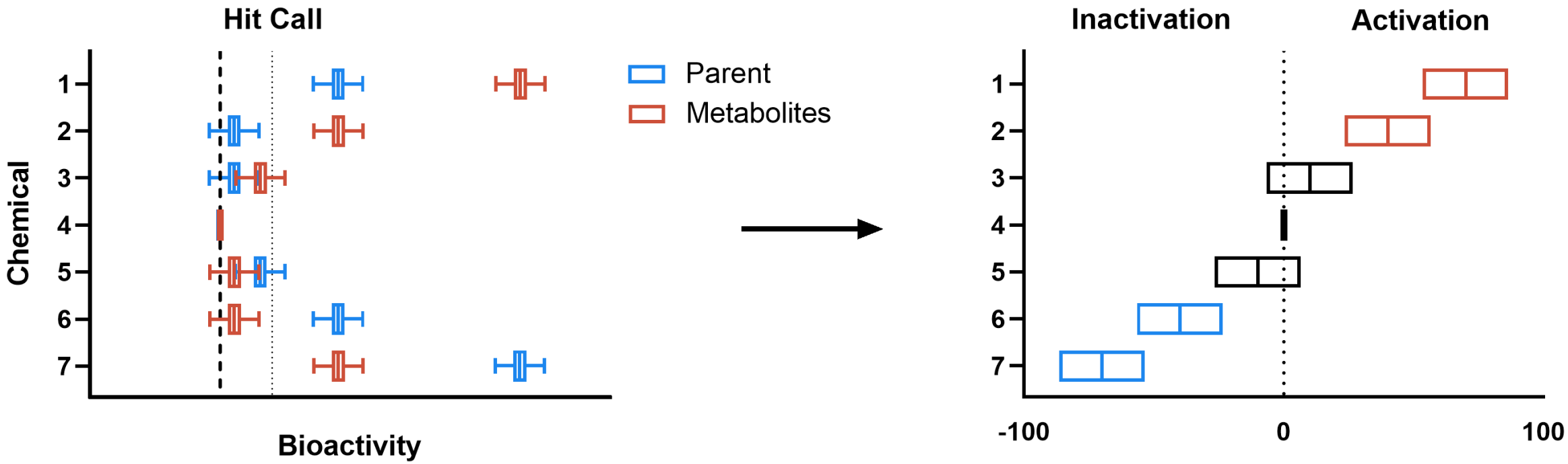
Rank Metabolism-dependent Bioactivity



Study Highlights

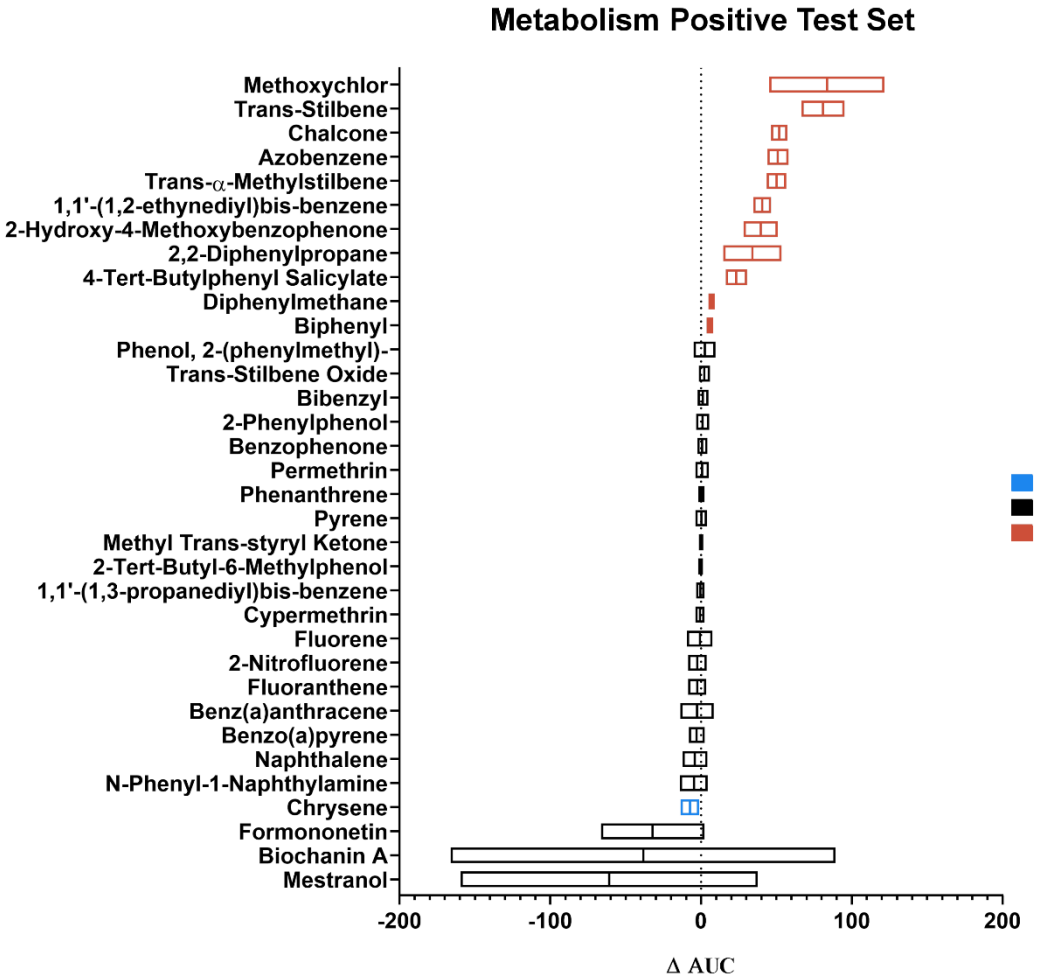
- Reprioritization of hazard based on metabolism-dependent bioactivity.
- Demonstrated utility of applying the AIME method for identification of false positive and false negative target assay effects.
- Enhanced *in vivo* concordance with the rodent uterotrophic bioassay.

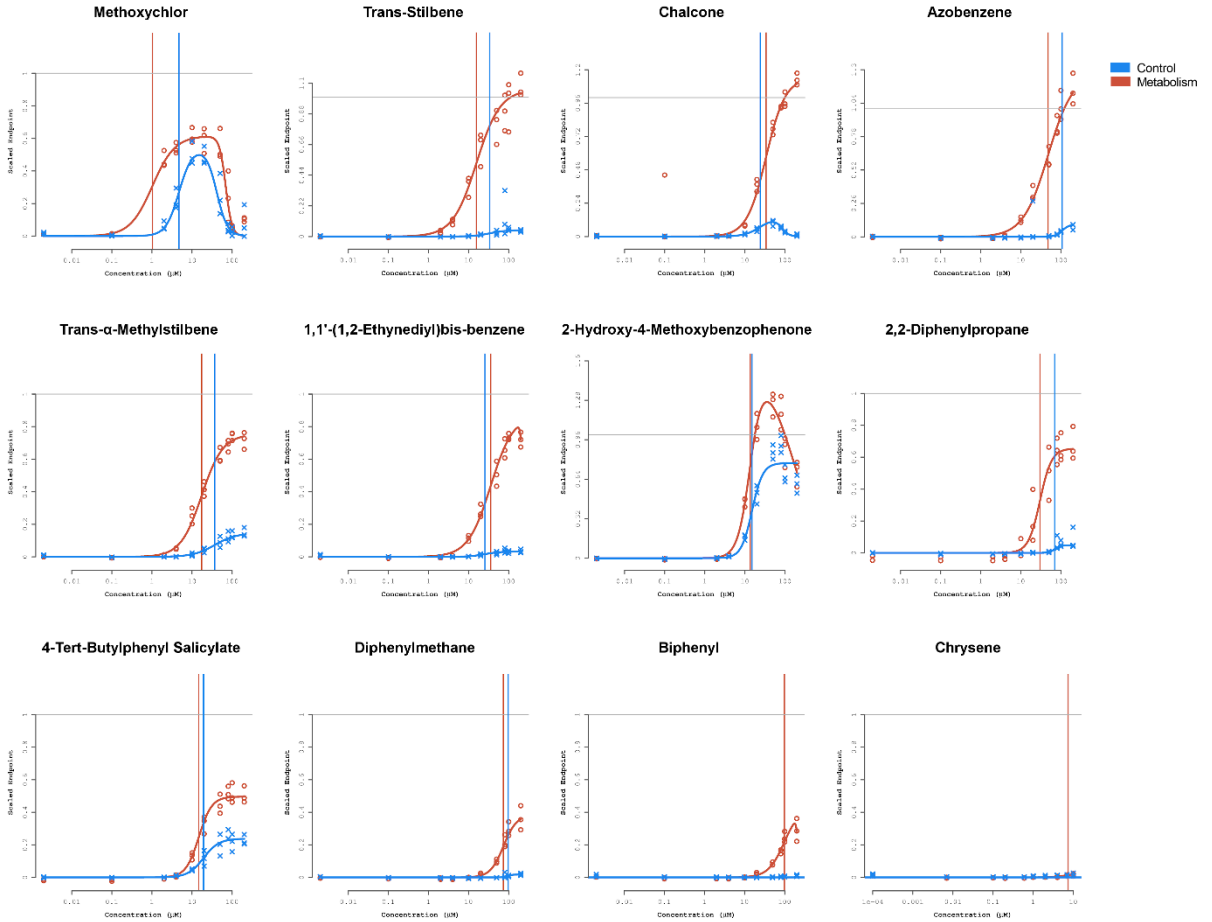
Toxboot Uncertainty Quantification: Statistical Analysis for Metabolism-dependent Effects



- A focus on false-positive and false-negative target assay effects alone omits a lot of important biology.
- Metabolism-dependent effects prioritized on a continuous scale to discriminate from target assay-dependent bioactivity thresholds.

AIME-coupled ERTA Metabolism Positive Test Set Screening

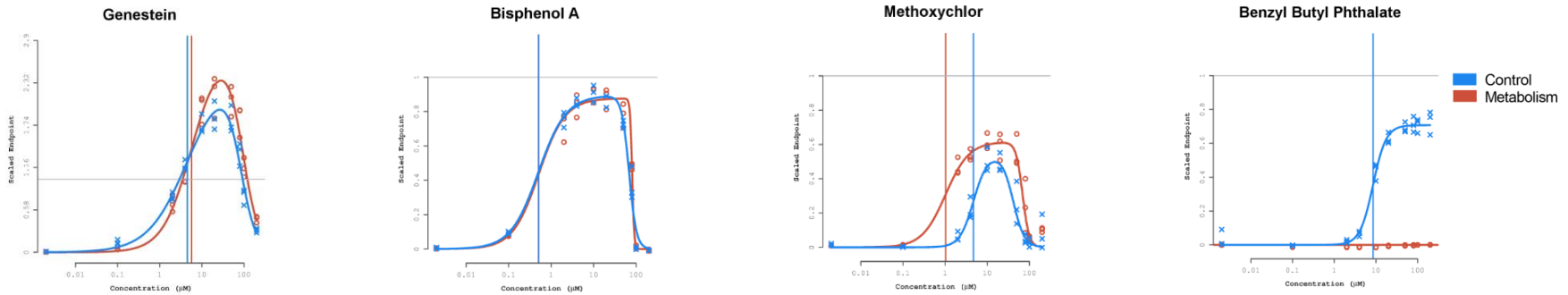




- 29/34 (85%) of parent chemicals from the positive test set were active in the absence of metabolism according to TCPL hit calls.
- 11/34 (32%) of chemicals exhibit significant metabolism-dependent bioactivation.

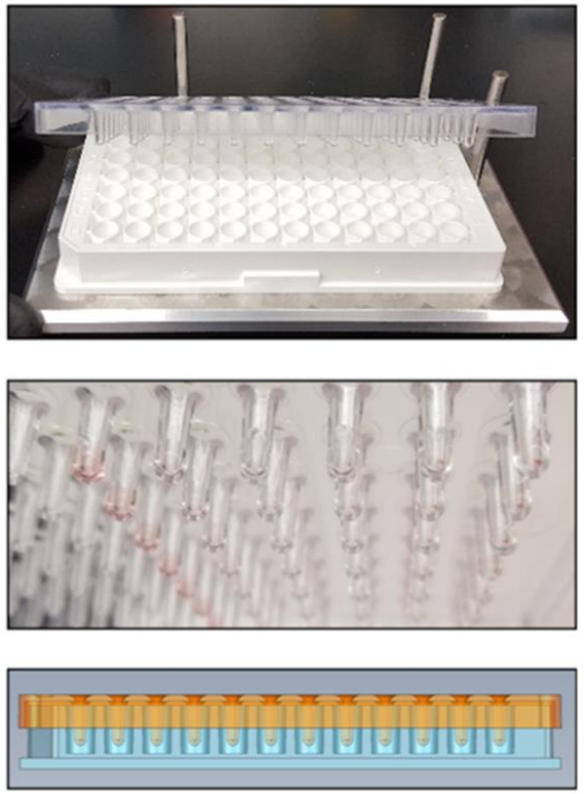
AIME - VM7Luc ERTA Assay: Relevance to the ToxCast ER Model and Uterotrophic Bioassay Data

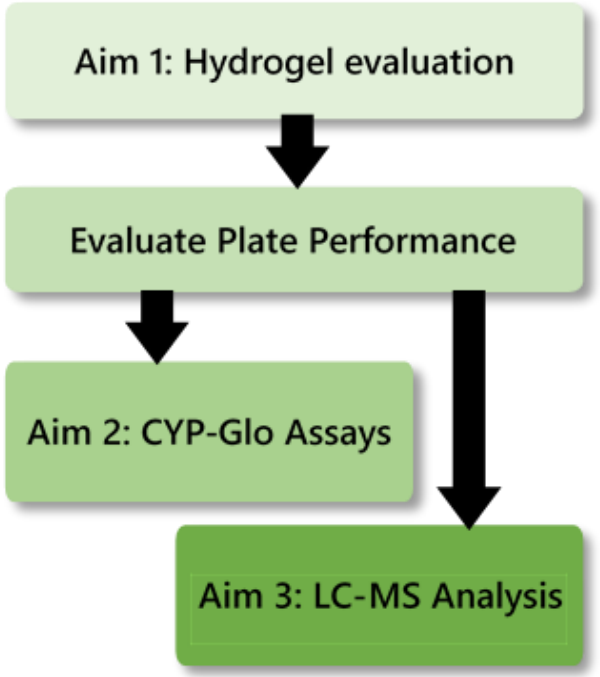
			ToxCast ER Model[a]	Uterotrophic Studies[b]			AIME - VM7Luc ERTA[c]						Concordance with In Vivo[d]		
CASRN	Chemical Name	Classification	AUC_Agonist	GL_Neg	GL_Pos	GL_WoE	Hitc_Met_Neg	Hitc_Met_Pos	$\Delta Hitc_{ER}$	ΔAUC	ΔAUC CI	Met_Effect	Met_Neg	Met_Pos	ΔMet
446-72-0	Genistein	Reference_Agonist	0.54	0	8	POS	1	1	0	27.96	[-1.37, 57.29]	NEG	1	1	0
80-05-7	Bisphenol A	Reference_Agonist	0.45	4	10	POS	1	1	0	1.57	[-46.01, 49.15]	NEG	1	1	0
72-43-5	Methoxychlor	Metabolism_Positive	0.25	1	3	POS	1	1	0	83.56	[45.44, 121.67]	POS	1	1	0
85-68-7	Benzyl butyl phthalate	Metabolism_Negative	0.18	1	0	NEG	1	0	-1	-73.48	[-78.91, -68.05]	POS	0	1	1

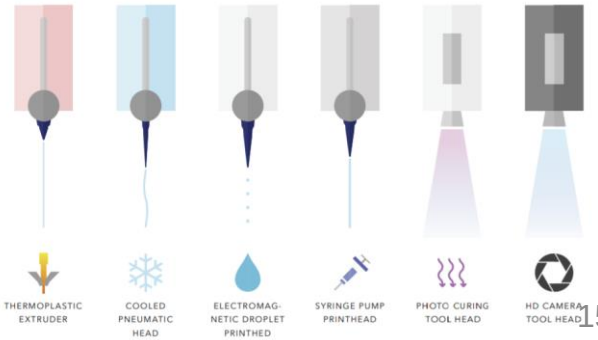


- Chemicals screened in the AIME-VM7Luc ERTA assay compared to ToxCast ER Model scores and Guideline-like Uterotrophic Studies (GL-UT) database.
- Comparison reveals cases of improved *in vitro* concordance with *in vivo* data.

Development of a Bioprinting Approach to Adapt the AIME Method for High-throughput Screening Applications







Goal: Adapt AIME method to an automated approach using bioprinting.

Approach: Evaluate various S9/hydrogel combinations, phase I and II optimization, and cross-linking approaches to increase workflow efficiency for metabolism screening.

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