United States Environmental Protection Agency

Retrofitting an Estrogen Receptor Transactivation Assay with Metabolic Competence

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Center for Computational Toxicology and Exposure
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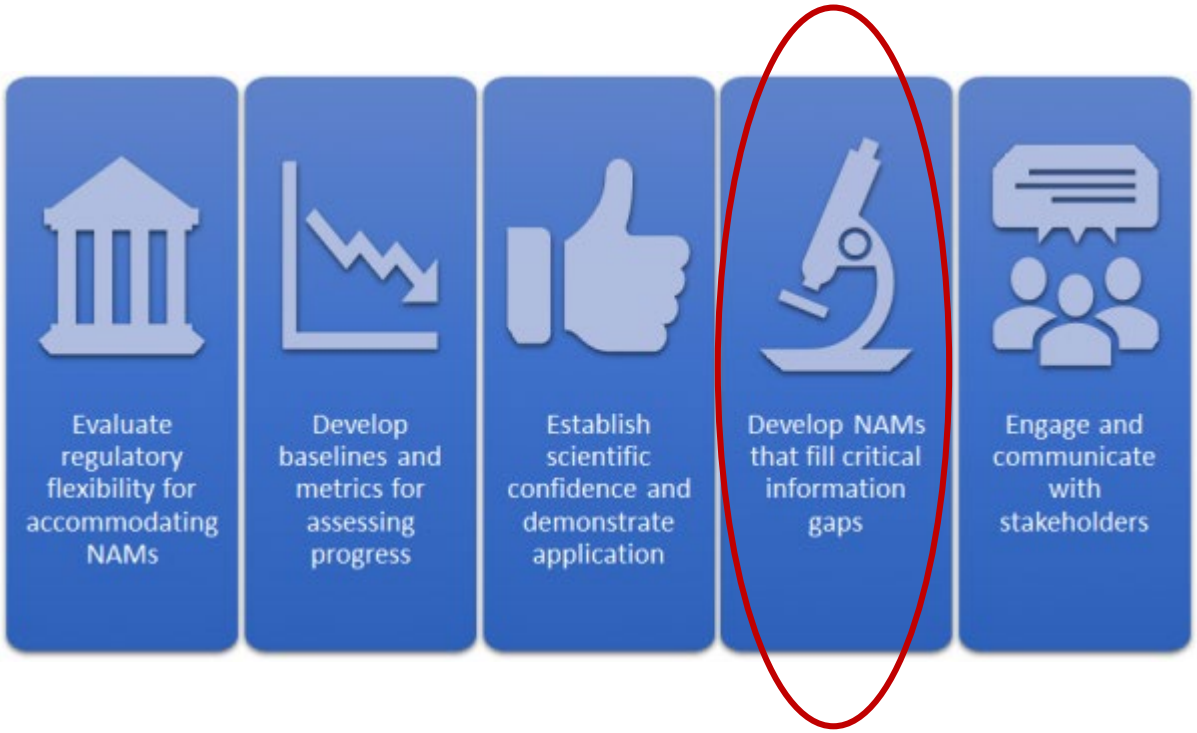
EPA NAMs Conference 2020: State of the Science on Development and Use of NAMs for Chemical Safety Testing

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

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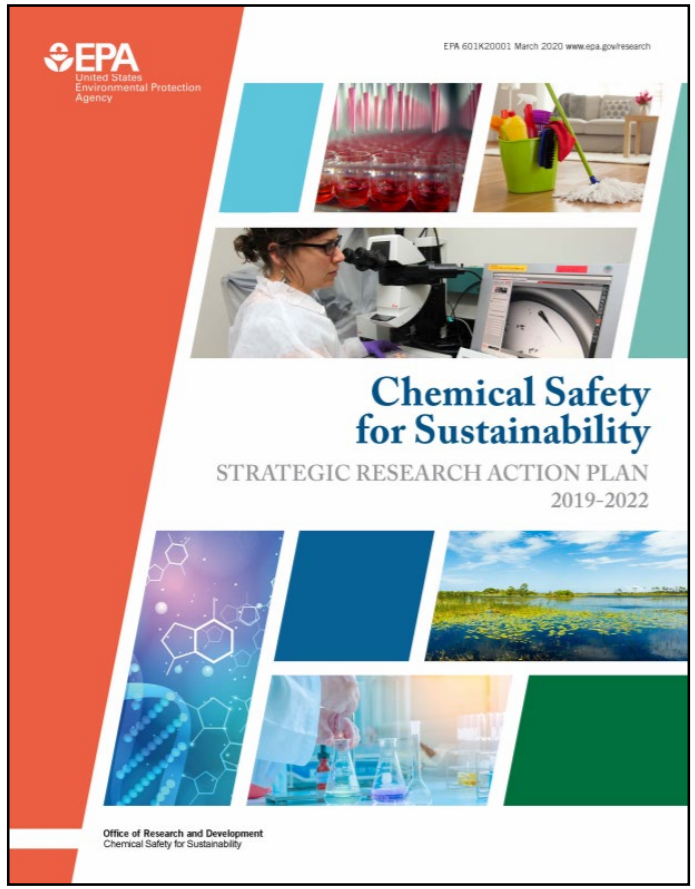


CSS.1.5 (High Throughput Toxicology): Develop and apply methods to incorporate endogenous and exogenous xenobiotic metabolism into high-throughput *in vitro* assays.

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

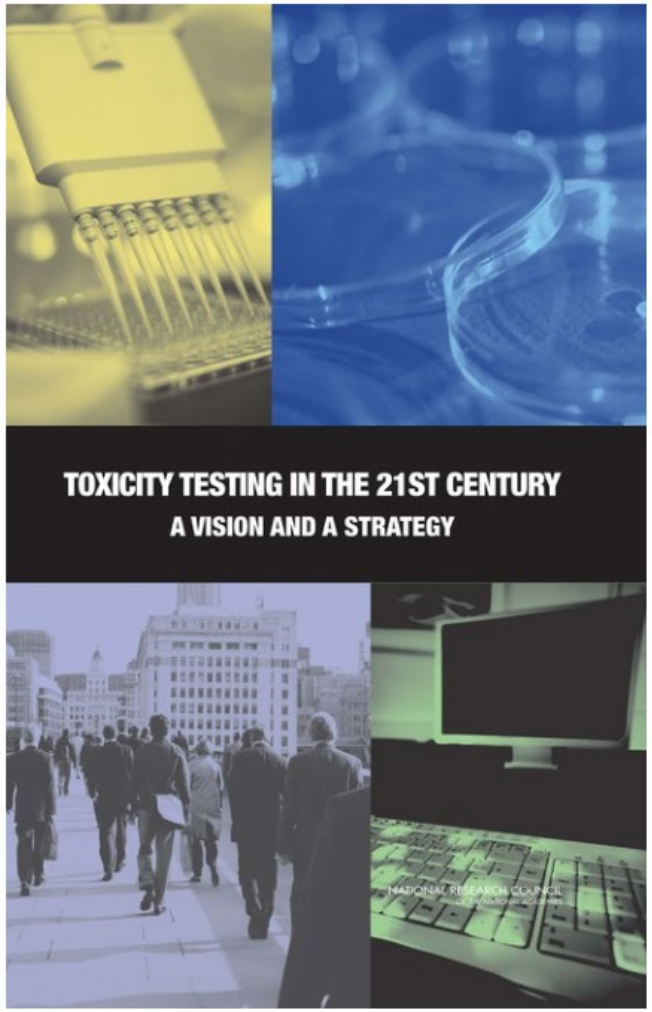
CSS.1.5.2: Development of a bioprinting approach to adapt the Alginate Immobilization of Metabolic Enzymes metabolism method for high-throughput screening applications.

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.

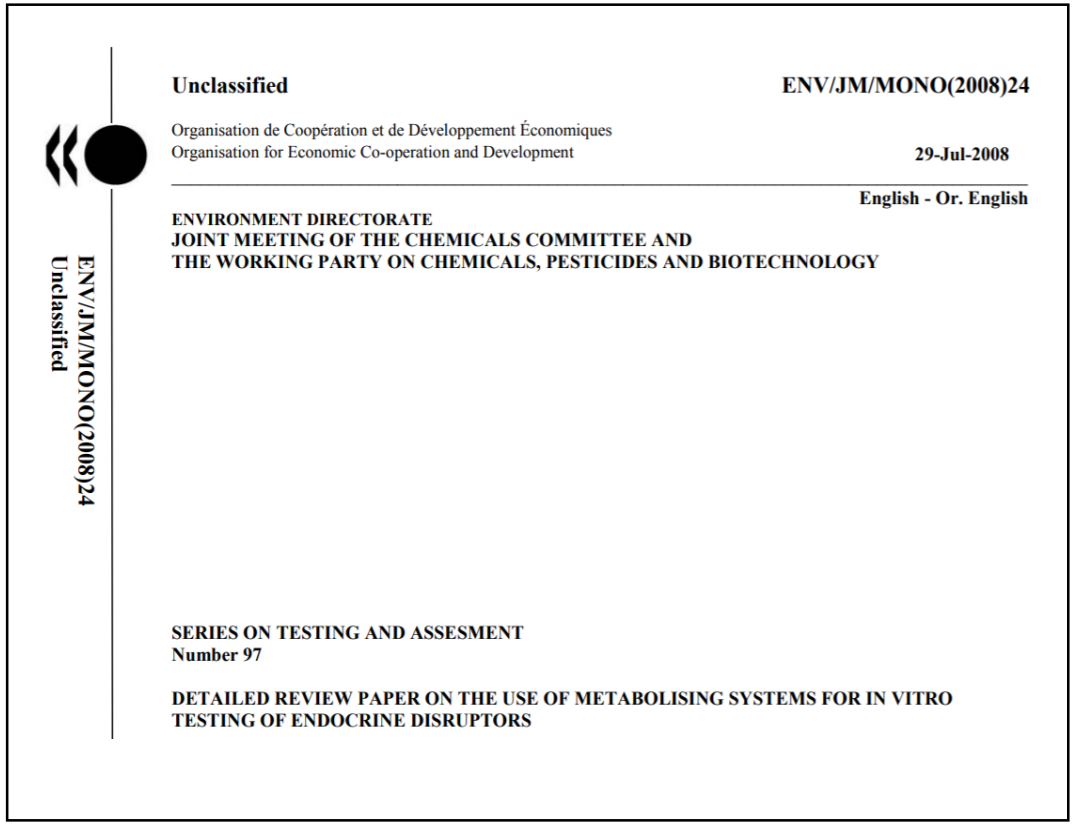
Key Questions for Implementation – Addressing Xenobiotic Metabolism

- "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."

EPA United States Environmental Protection Agency

TRANSFORM TOX TESTING CHALLENGE

INNOVATING FOR METABOLISM

TEAMS WILL COMPETE IN THREE STAGES FOR A TOTAL PRIZE OF $1 MILLION

1

Stage 1 - Up to ten submissions will be selected as semi-finalists, awarded a prize of $10,000 each, and invited to participate in Stage 2.

2

Stage 2 - Up to five applicants may be selected as finalists, awarded a prize of up to $100,000 each, and invited to participate in the final stage of the competition.

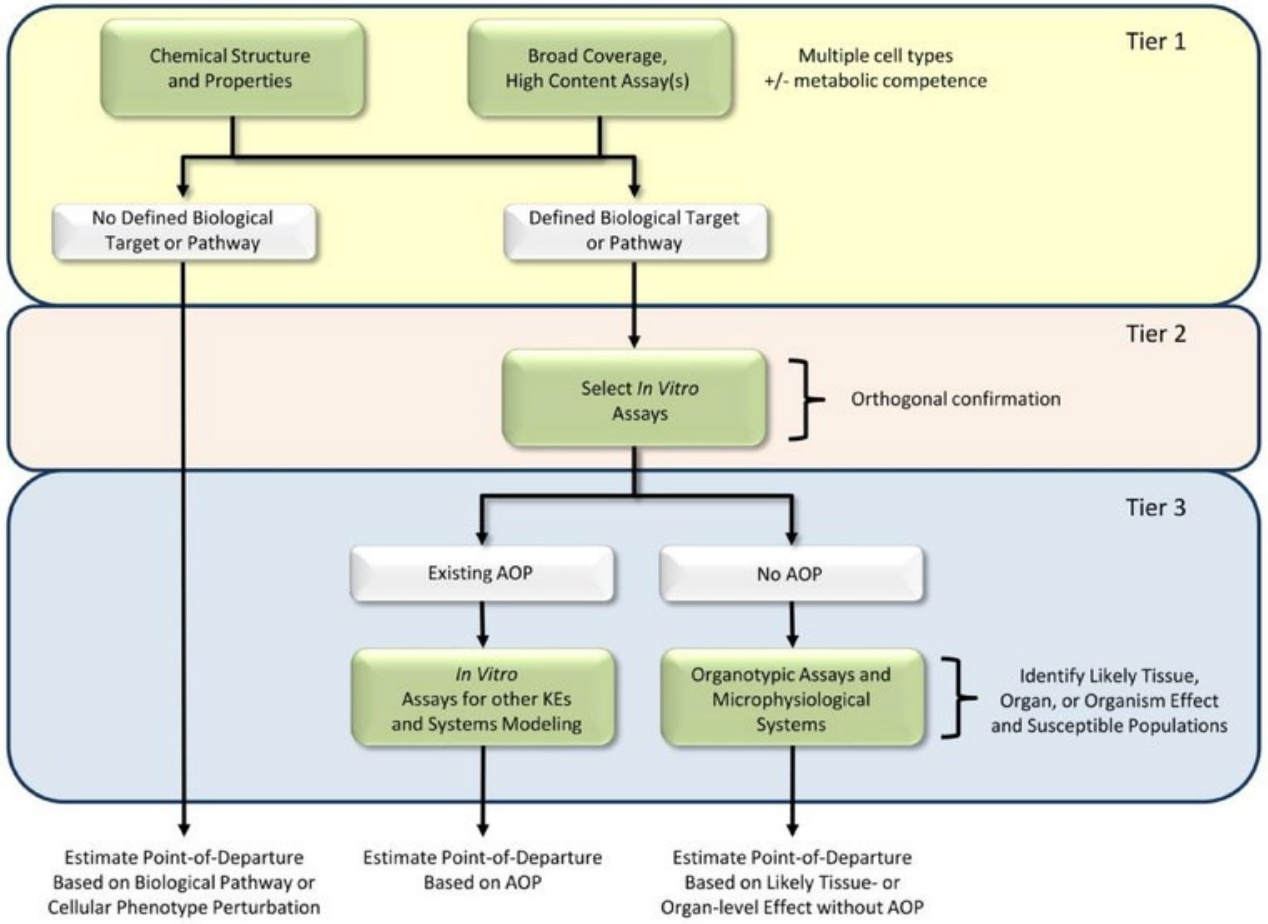
3

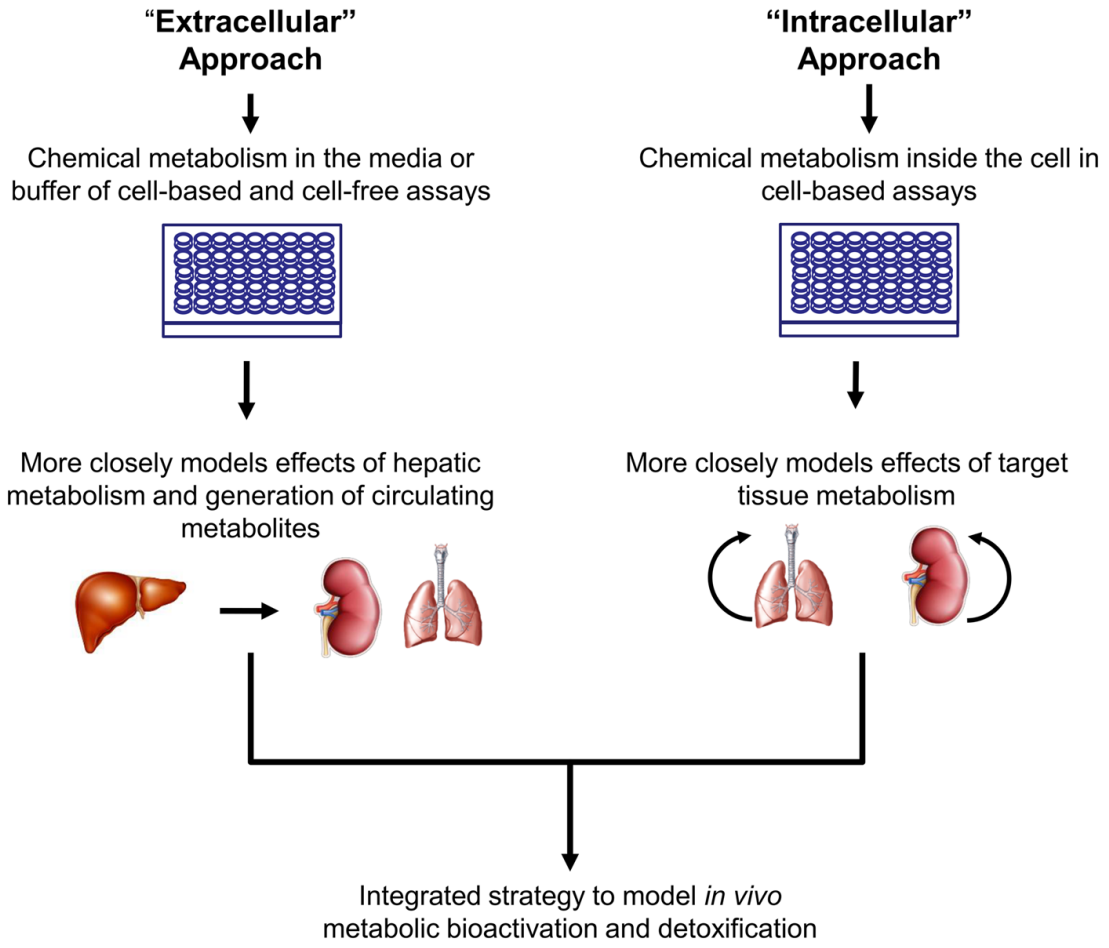
Stage 3 – Based on the testing and overall feasibility, one winner may be awarded up to a $400,000 prize for delivery of a commercially viable method or device that will ultimately result in technologies that can provide metabolic competence to commonly used HTS assays.ultimately result in technologies that can provide metabolic competence to commonly-used HTS assays.

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



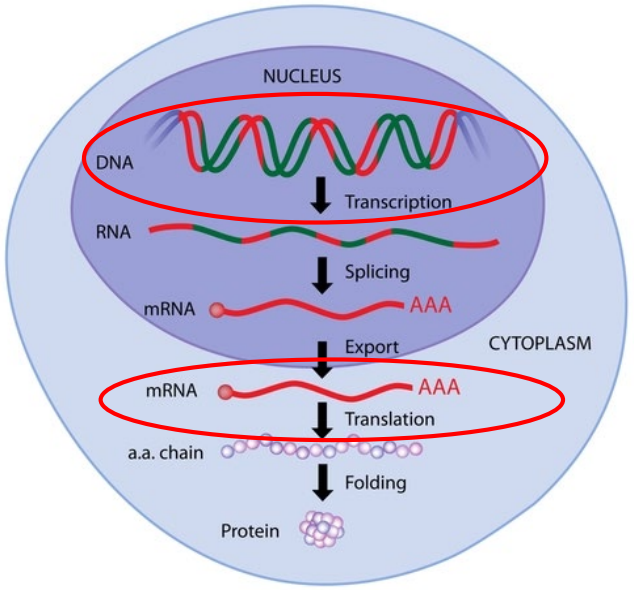


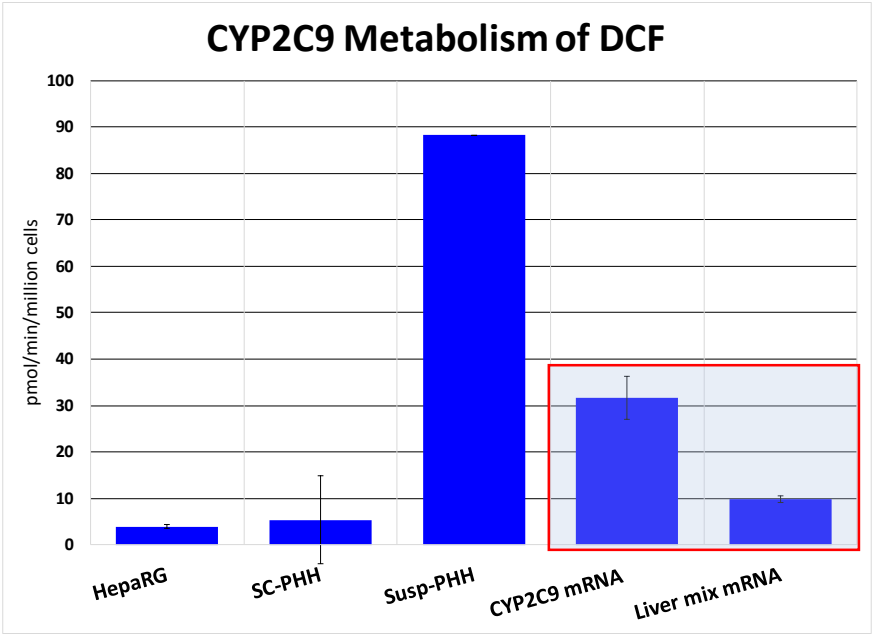
Toxicol Sci, June 2019, DOI:10.1093/toxsci/kfz058

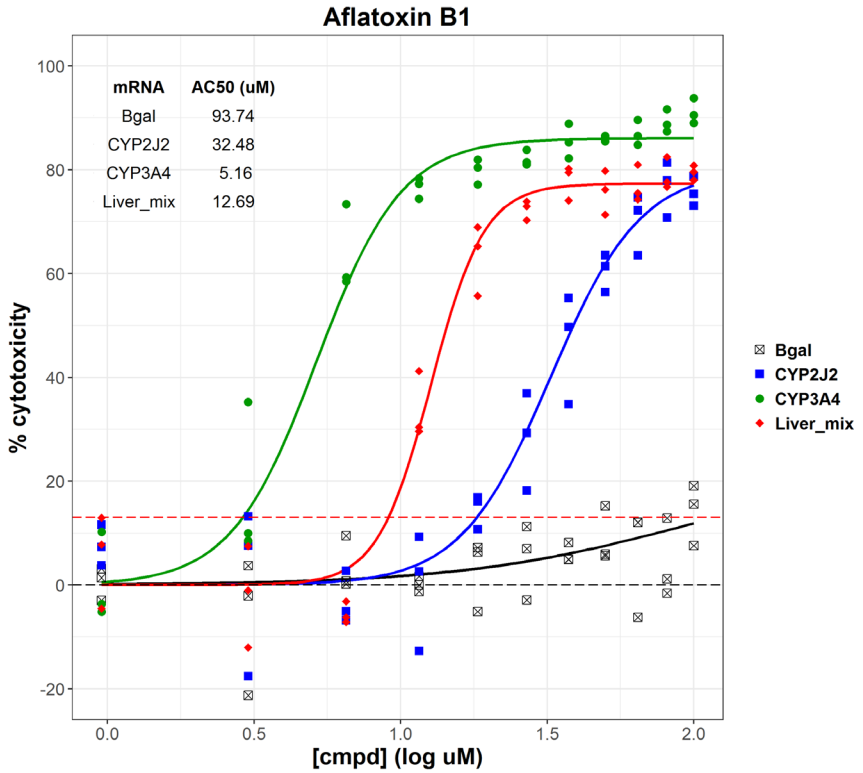
Intracellular Approach: Xenobiotic Metabolism by mRNA Transfection

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.

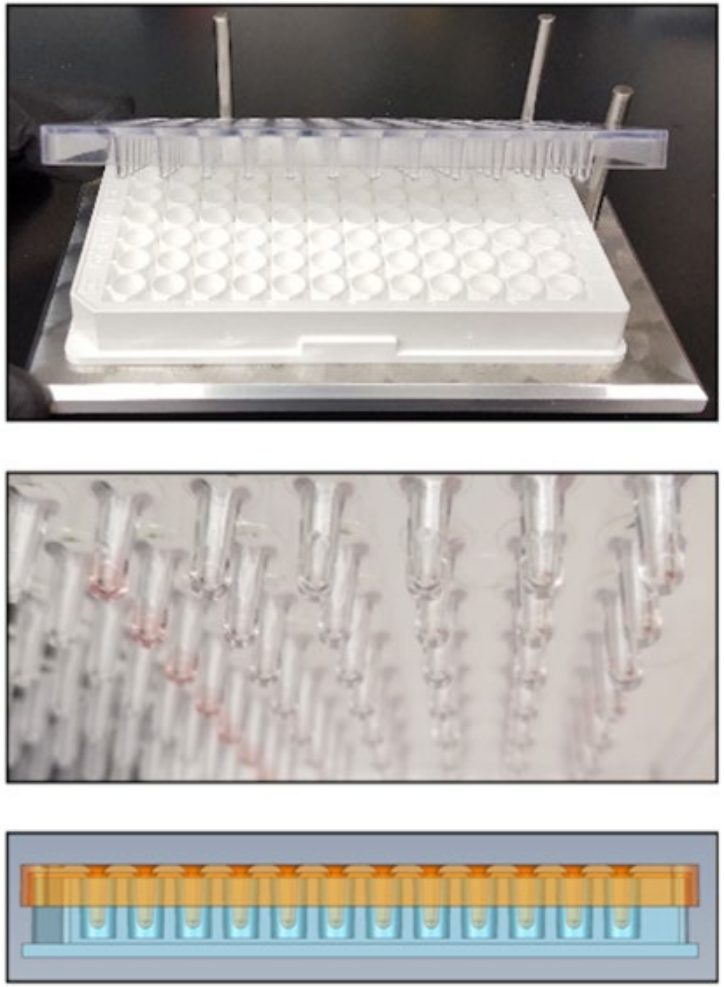


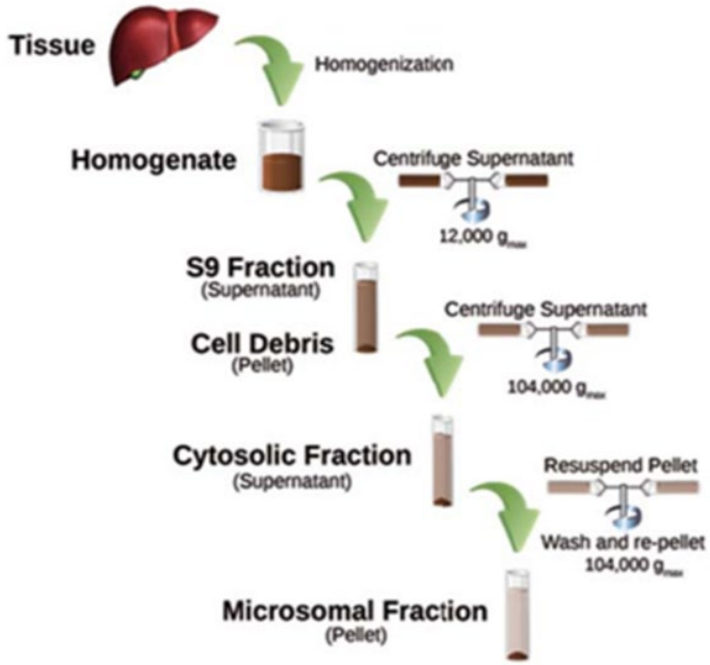




J Pharmacol Toxicol Methods. Jul-Aug 2018. DOI: 10.1016/j.vascn.2018.03.002

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.

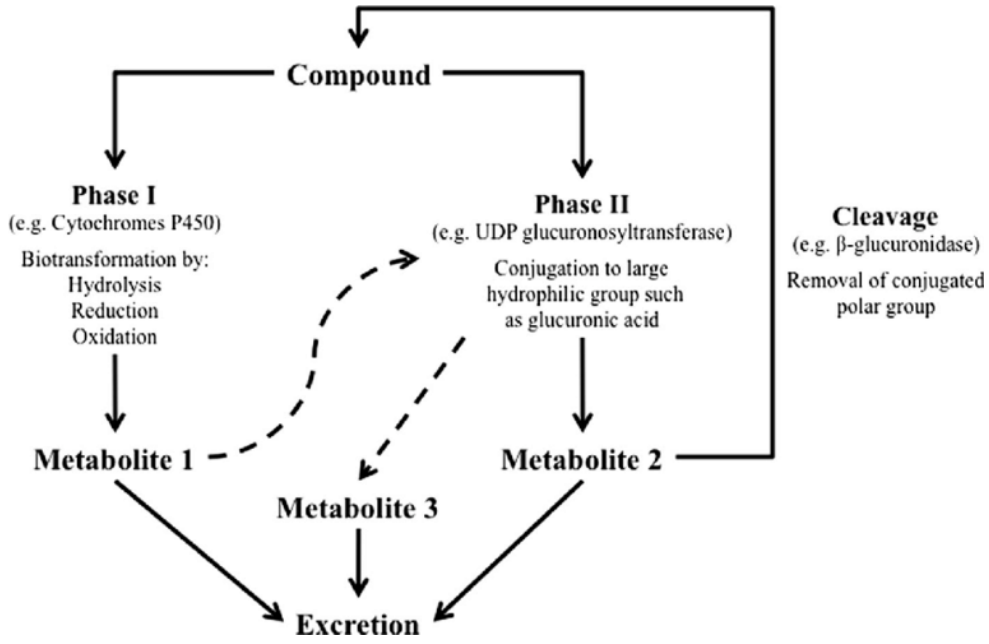
Shimadzu Journal, 3(3), 64-68

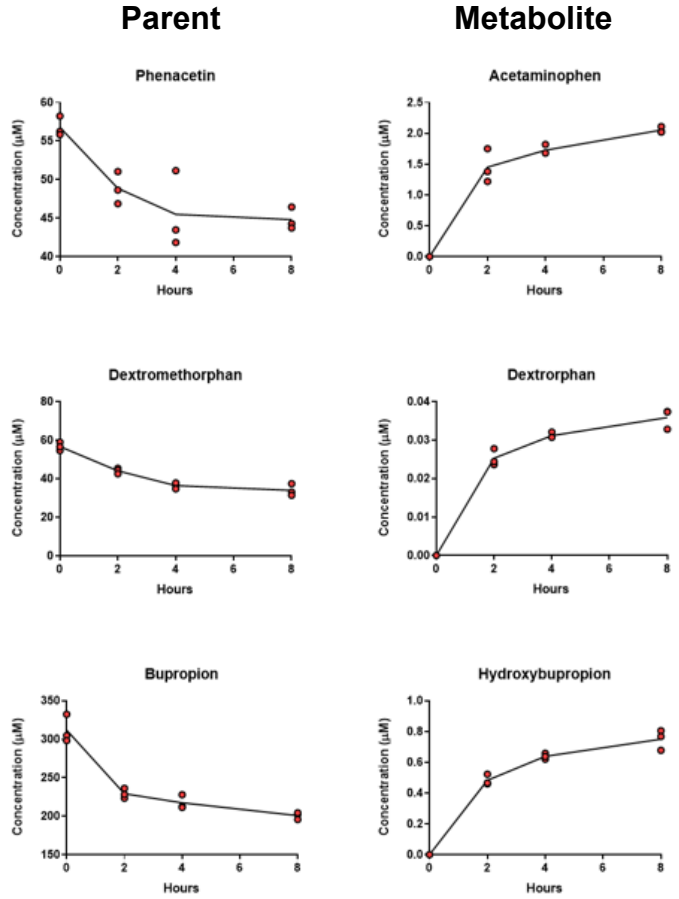
Evaluation of Cytochrome P450 Metabolism

S9 fractions are dependent on co-factor supplementation for optimal performance.

Phase I metabolism co-factors
- NADPH (CYPs)

Phase II metabolism co-factors
- UDPGA (Glucuronidation)
- PAPS (Sulfation)
- GSH (Glutathione)





Key Points

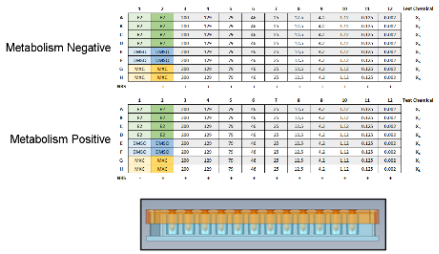
- AIME method optimized for Phase I metabolism.
- Metabolic activity validated across a diverse profile of CYPs with reference chemicals.
- 2-hour incubation period suitable for parent compound depletion and metabolite accumulation.

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**

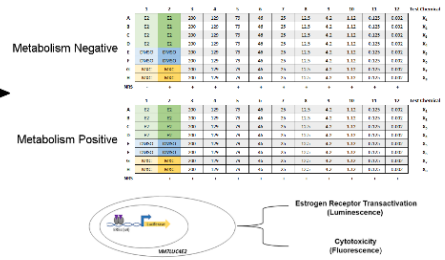
DOI:10.1007/s00338-014-1136-3

Retrofitting Metabolism to an Estrogen Receptor Transactivation Assay

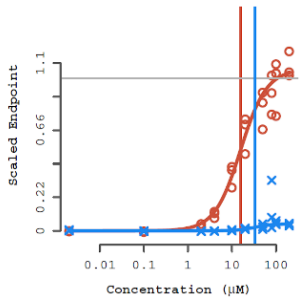
AIME Metabolism Assay



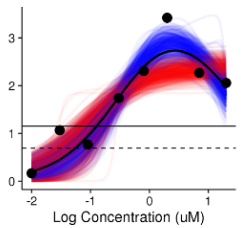
Estrogen Receptor Transactivation Assay



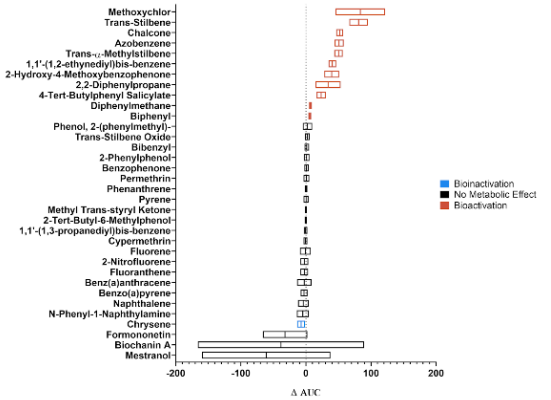
ToxCast Pipeline



Toxboot Uncertainty Quantification



Rank Metabolism-dependent Bioactivity



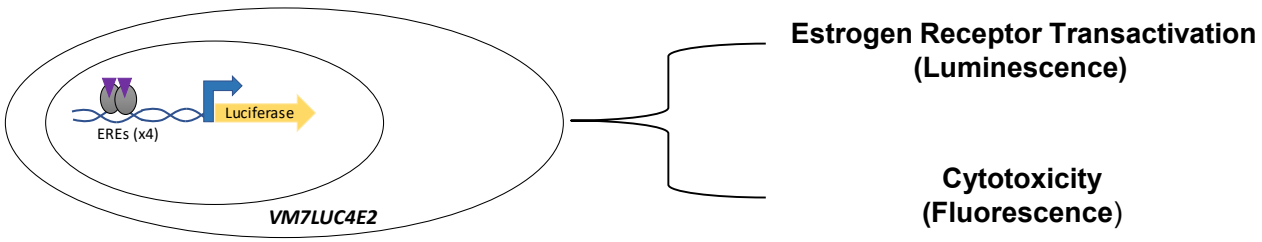
Study Highlights

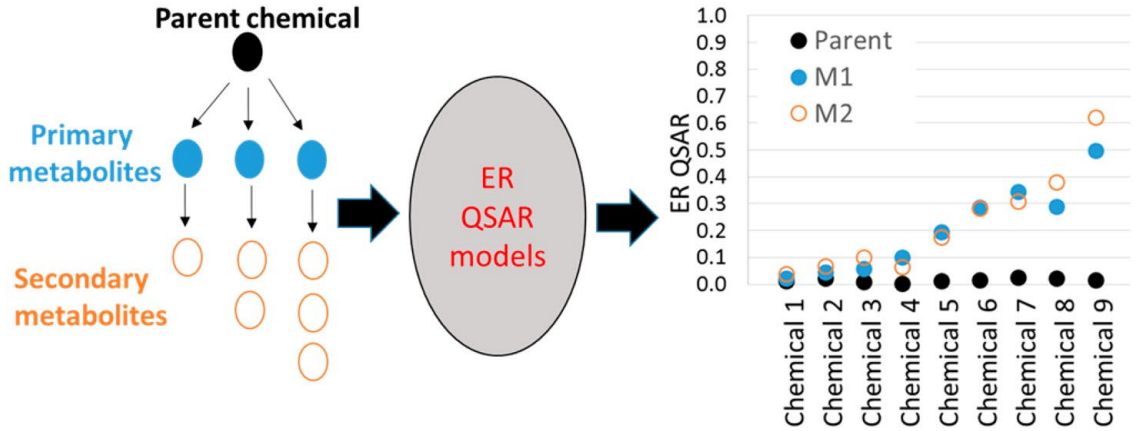
- Reprioritization of hazard based on metabolism-dependent bioactivity.
- Demonstrated utility of applying the AIME method for identification of false positive and false negative target assay effects.
- Enhanced *in vivo* concordance with the rodent uterotrophic bioassay.

Toxicol Sci, Sep 2020, DOI:10.1093/toxsci/kfaa147

Retrofitting Metabolism to an Estrogen Receptor Transactivation Assay

Assay Design Specifications	
Assay	VM7Luc4E2 (Formerly BG1Luc4E2 of TG 457)
Metabolism	AIME (PB-βNF Induced Rat S9); 2 Hours
Matrix	Alginate + 10% S9
NADPH Regeneration System (NRS)	Optimized concentrations of NADP+, G6P, G6PDH for cell-based assay
Format	Metabolism Negative (Alginate Only)
	Metabolism Positive (Alginate + S9)
Endpoints	ER Transactivation (Luciferase) and Viability (Fluorescence)
Plate Format	96-well
Dose Spacing	10 pt; alternative dose spacing
Concentration Range	2 nM - 200 µM
Controls	17β - Estradiol (ER Transactivation)
	DMSO (Vehicle)
	Methoxychlor (Bioactivation)
Data Analysis	ToxCast Pipeline





Chemical Selection	n	Classification
Assay Controls	8	ER Agonist (OECD TG 455)
	3	ER Antagonist (OECD TG 455)
	3	Negative (OECD TG 455)
Pinto Library	34	Metabolism Positive
	14	Metabolism Negative

Pinto et al, Chem. Res. Toxicol. 2016, DOI: 10.1021/acs.chemrestox.6b00079

Retrofitting Metabolism to an Estrogen Receptor Transactivation Assay

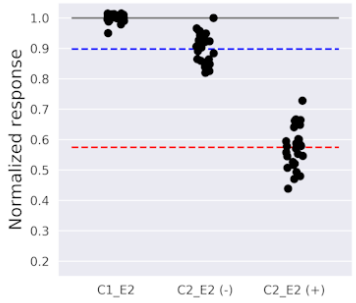
Metabolism Negative

	1	2	3	4	5	6	7	8	9	10	11	12	Test Chemical
A	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_1
B	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_2
C	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_3
D	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_4
E	DMSO	DMSO	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_5
F	DMSO	DMSO	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_6
G	MXC	MXC	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_7
H	MXC	MXC	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_8
NRS	-	+	+	+	+	+	+	+	+	+	+	+	

Metabolism Positive

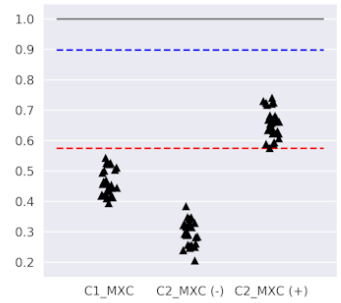
	1	2	3	4	5	6	7	8	9	10	11	12	Test Chemical
A	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_1
B	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_2
C	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_3
D	E2	E2	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_4
E	DMSO	DMSO	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_5
F	DMSO	DMSO	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_6
G	MXC	MXC	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_7
H	MXC	MXC	200	129	79	46	25	12.5	4.2	1.12	0.125	0.002	X_8
NRS	-	+	+	+	+	+	+	+	+	+	+	+	

17β - Estradiol



(100 pM)

Methoxychlor



(10 µM)

Paired Plate Format: Test compounds run +/- metabolism in parallel

Plate Design

- Column 1: No AIME. Guideline-like test conditions
- Column 2: AIME. +/- Metabolism test conditions
- Column 3-12: Alternative dose spacing of test compound
- Cell culture medium: +/- NADPH Regeneration System (NRS)

Reference Compounds

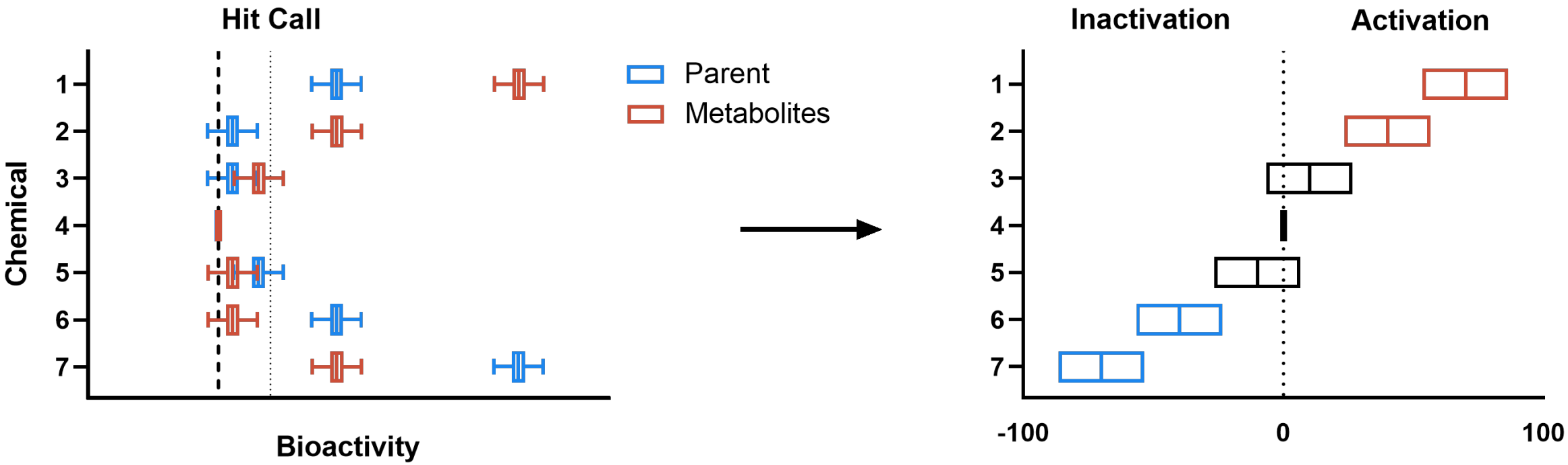
- Target Assay - 17β-estradiol (E2)
- Metabolism Assay - Methoxychlor (MXC)
- Solvent – DMSO (0.2%)

Assay Performance

- Z'-factor, coefficient of variation (CV)
- +/- Metabolism
- +/- NADPH Regeneration System (NRS)

		Metabolism							
		Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
NRS	Neg	0.90	NA	6.75	NA	2.77	NA	5.39	NA
	Pos	0.91	0.69	8.93	17.17	2.82	8.51	2.98	5.23
		Z'		CV: DMSO		CV: E2		CV: MXC	

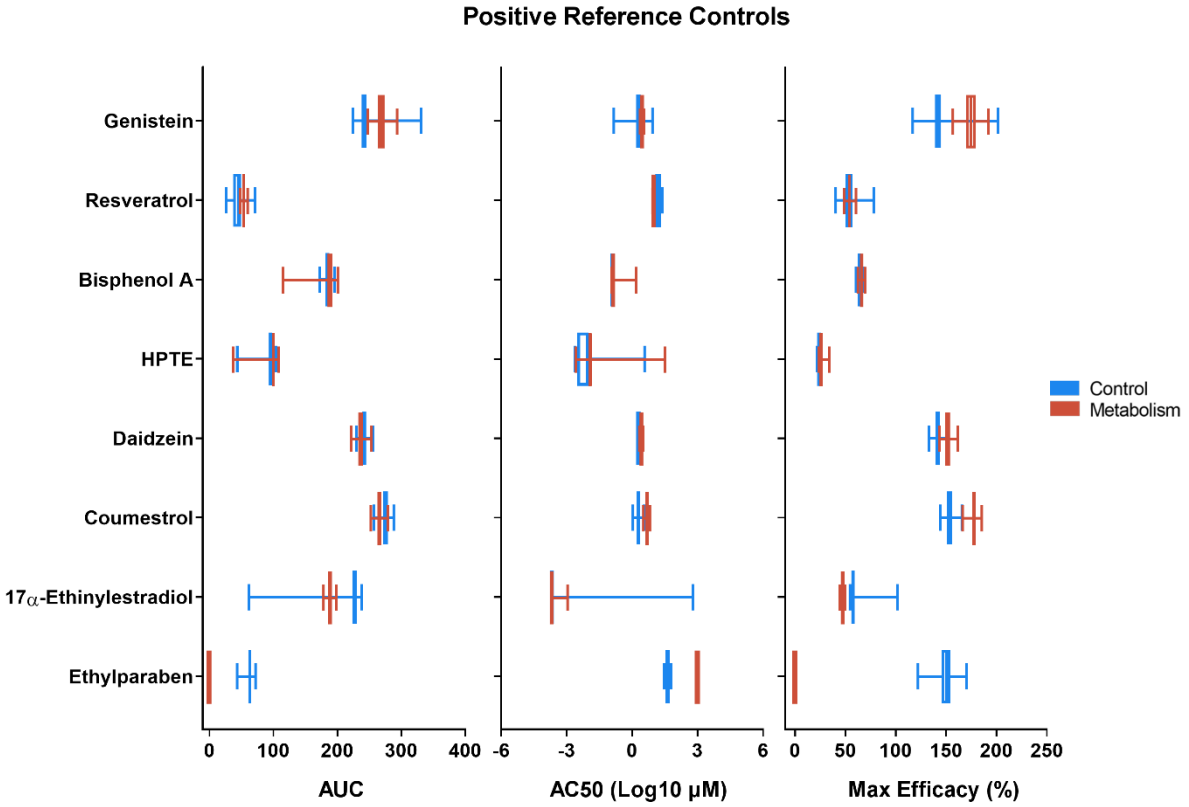
Toxboot Uncertainty Quantification: Statistical Analysis for Metabolism-dependent Effects

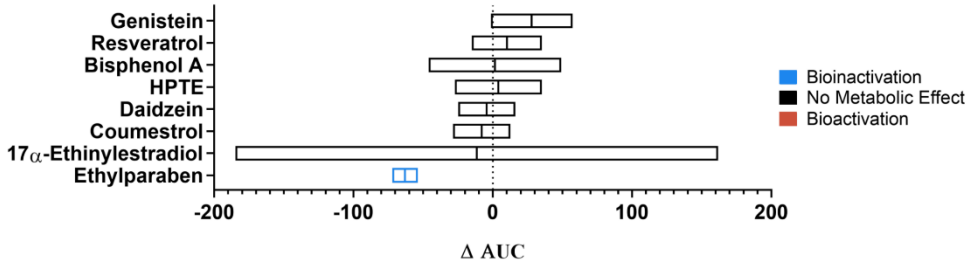


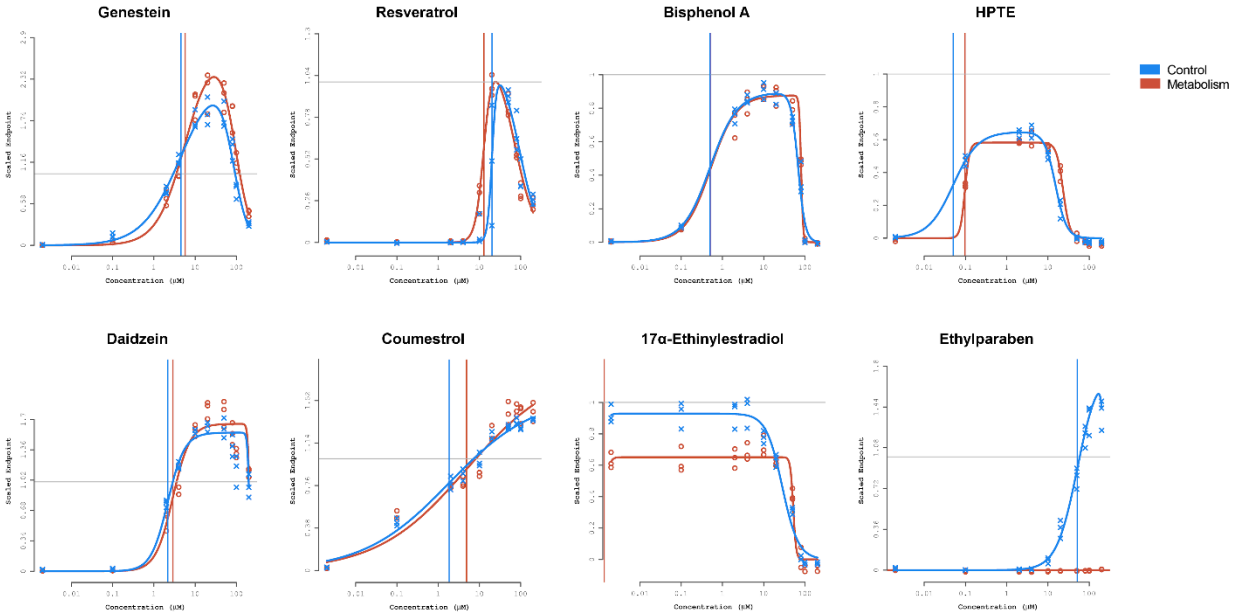
- **Problem**: Focus on false-positive and false-negative target assay effects alone omits a lot of important biology.
- **Objective**: Discriminate metabolism-dependent effects from target assay-dependent effects.
- **Solution**: Prioritize metabolism-dependent effects on a continuous scale using ΔAUC.
  - *CI:* confidence interval
  - μ_p and μ_n: mean ERTA AUC signal in metabolism positive and negative modes
  - *q:* quantile of the standard normal distribution
  - σ_p and σ_n: standard deviation for the ERTA AUC signal in metabolism positive and negative modes

$$CI = (\mu_p - \mu_n) \pm q \times \sqrt{\sigma_p^2 + \sigma_n^2}$$

AIME-coupled ERTA Positive Reference Compound Screening

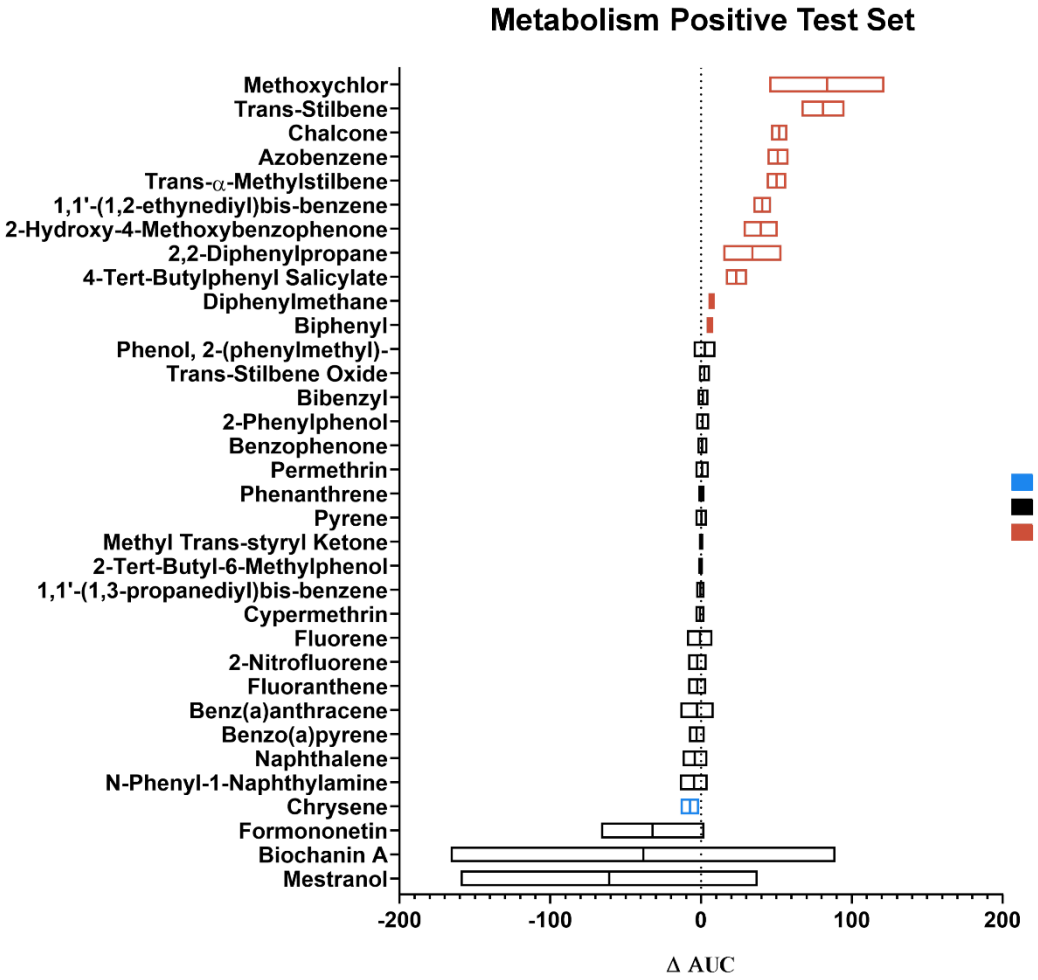


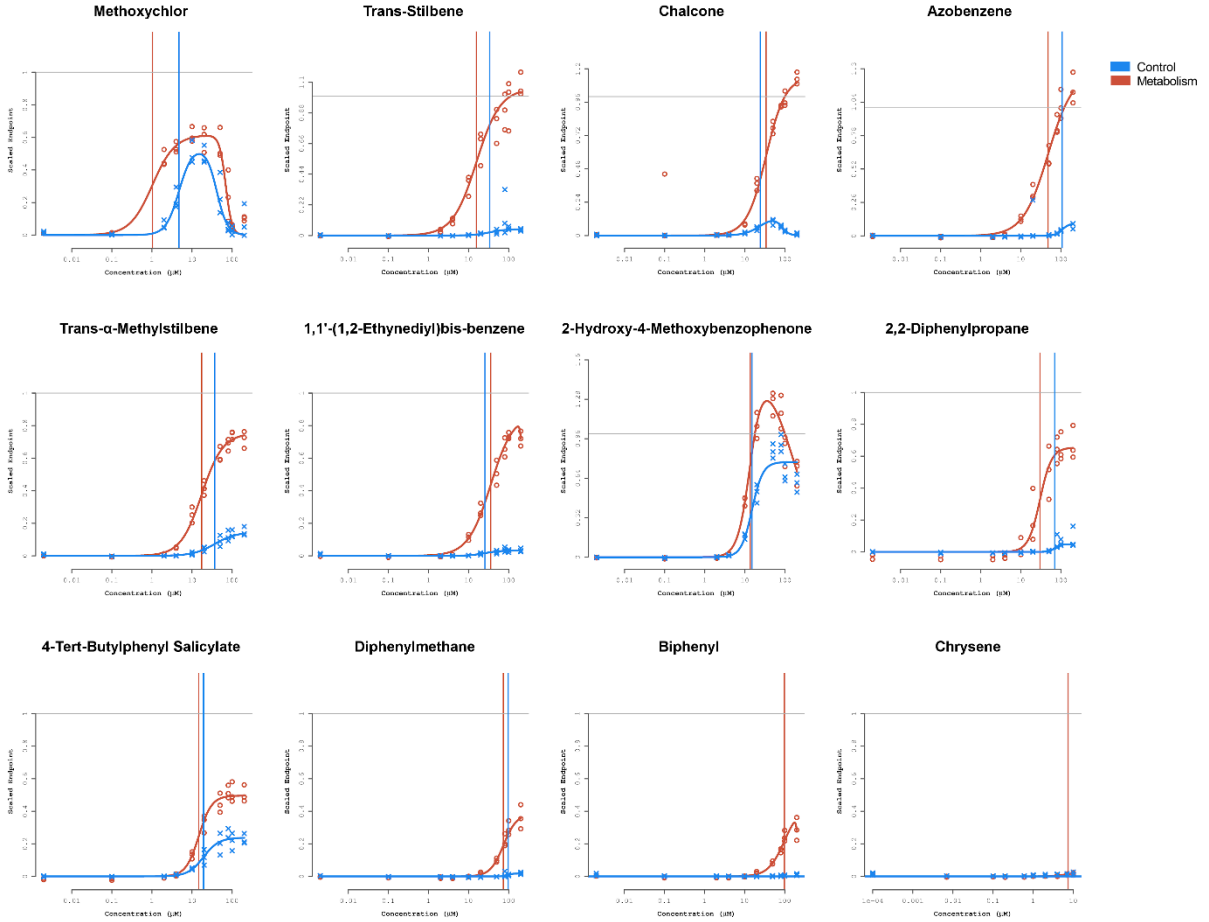




- Toxboot analysis of Area Under the Curve (AUC), potency (AC50), and efficacy.
- Positive ERTA reference chemicals perform primarily as expected.
- Ethylparaben is significantly bioinactived (false-positive).

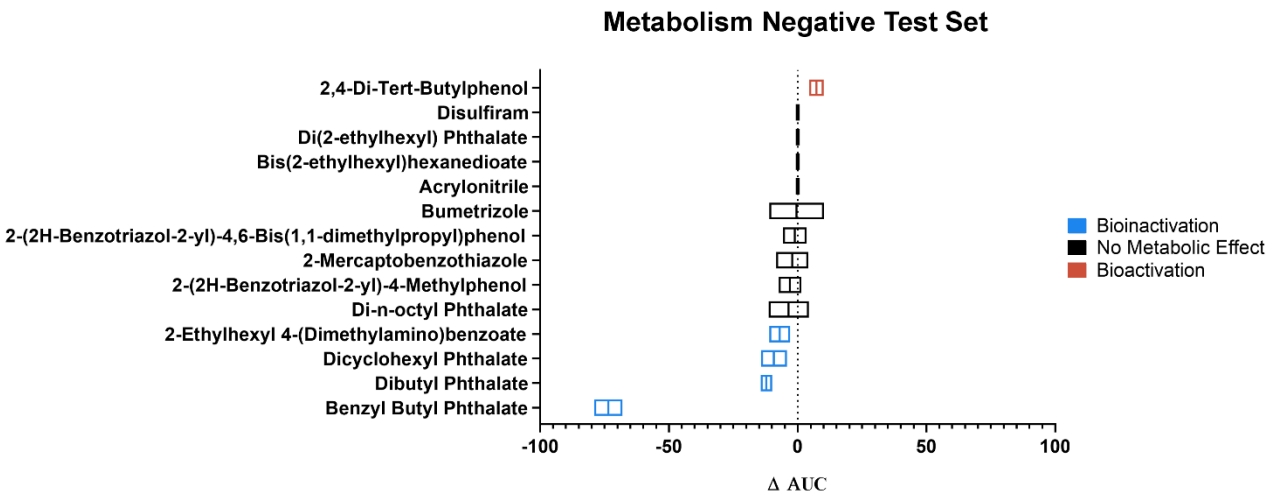
AIME-coupled ERTA Metabolism Positive Test Set Screening

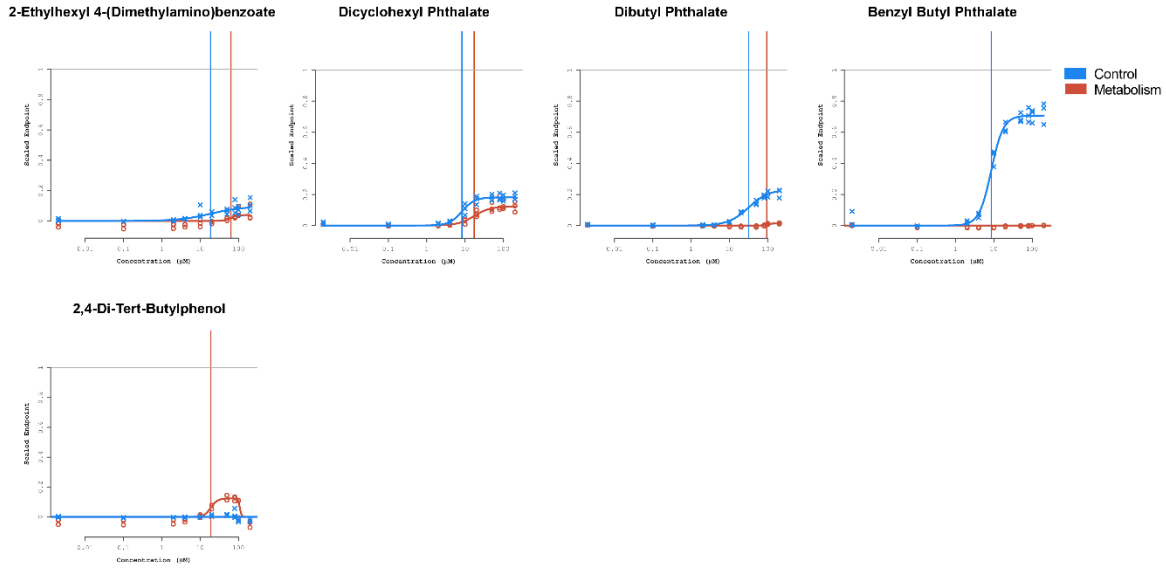




- 29/34 (85%) of parent chemicals from the positive test set were active in the absence of metabolism according to TCPL hit calls.
- 11/34 (32%) of chemicals exhibit significant metabolism-dependent bioactivation.

AIME-coupled ERTA Metabolism Negative Test Set Screening

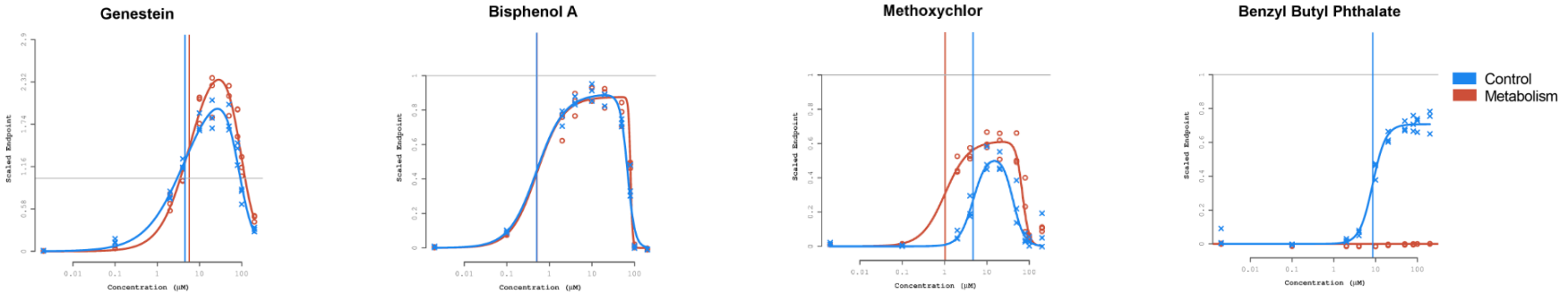




- 6/14 (43%) of parent chemicals from the negative test set were active in the absence of metabolism according to TCPL hit calls.
- 4/16 (25%) of chemicals exhibit significant metabolism-dependent bioinactivation.

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



- The 63 chemicals screened in the AIME-VM7Luc ERTA assay compared to ToxCast ER Model scores and Guideline-like Uterotrophic Studies (GL-UT) database.
- [a]ToxCast ER Model (Browne et al. 2015) scores for agonist mode (AUC_Agonist).
- [b]Uterotrophic data derived from guideline-like (GL) studies in the curated uterotrophic database (Kleinstreuer et al. 2016).
- [c]Results for binary TCPL hit calls in metabolism negative (Hitc_Met_Neg) and positive (Hitc_Met_Pos) modes.
- [d]AIME-VM7Luc ERTA concordance (1) or non-concordance (0) to *in vivo* uterotrophic study data (GL_WoE).

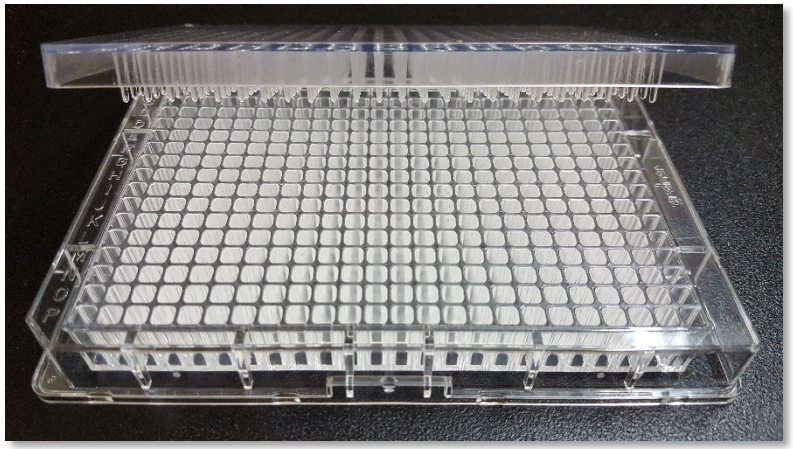
AIME – VM7Luc ERTA ToxCast Screening

AIME/Assay Destination Plate (uM): dest plate_1-12

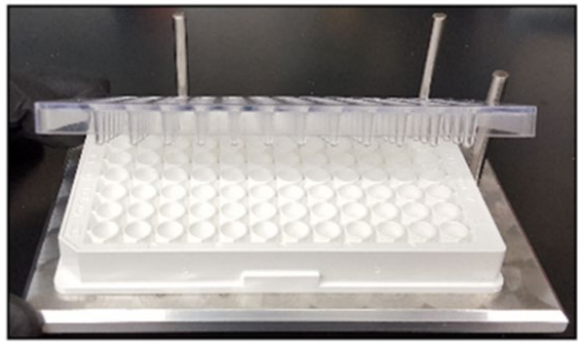
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	DMSO	DMSO	DMSO	DMSO	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
B	DMSO	DMSO	DMSO	DMSO	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
C	DMSO	DMSO	DMSO	DMSO	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
D	DMSO	DMSO	DMSO	DMSO	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
E	DMSO	DMSO	DMSO	DMSO	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
F	E2	E2	E2	E2	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
G	E2	E2	E2	E2	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
H	E2	E2	E2	E2	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
I	E2	E2	E2	E2	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
J	E2	E2	E2	E2	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
K	TSB	TSB	TSB	TSB	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
L	TSB	TSB	TSB	TSB	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
M	TSB	TSB	TSB	TSB	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
N	EPB	EPB	EPB	EPB	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
O	EPB	EPB	EPB	EPB	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
P	EPB	EPB	EPB	EPB	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002	200	129	79	45.7	25	12.5	4.16	1.12	0.125	0.002
AIME	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

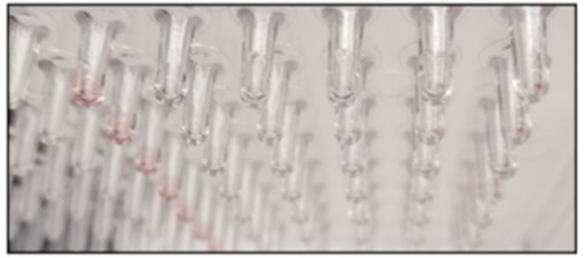
Design Specifications	
Chemical Library	768 compounds (ph1_v2, ph2, e1K)
Assay	VM7LUC4E2
Metabolism	AIME (induced rat S9)
Endpoints	ER Transactivation (Luciferase) and Viability (Fluorescence)
Plate Format	384 +/- Metabolism
Dose Spacing	10 pt; alternative dose spacing
Concentration Range	2 nM - 200 µM
Controls	17-β Estradiol (ER Transactivation)
	DMSO (Vehicle)
	trans-Stilbene (Bioactivation)
	Ethylparaben (Bioinactivation)
Data Analysis	ToxCast Pipeline

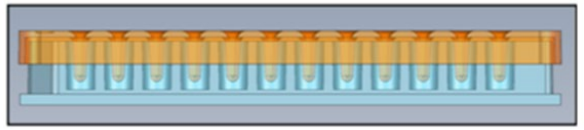
Plate Stats	S/B	Z'	Metabolism	Control Mode
E2:DMSO	10.1	0.7	Negative	ER Assay Dynamic Range
TSB(Pos):TSB(Neg)	2.8	0.7	Positive	Bioactivation
EPB(Neg):EPB(Pos)	21.1	0.8	Positive	Bioinactivation

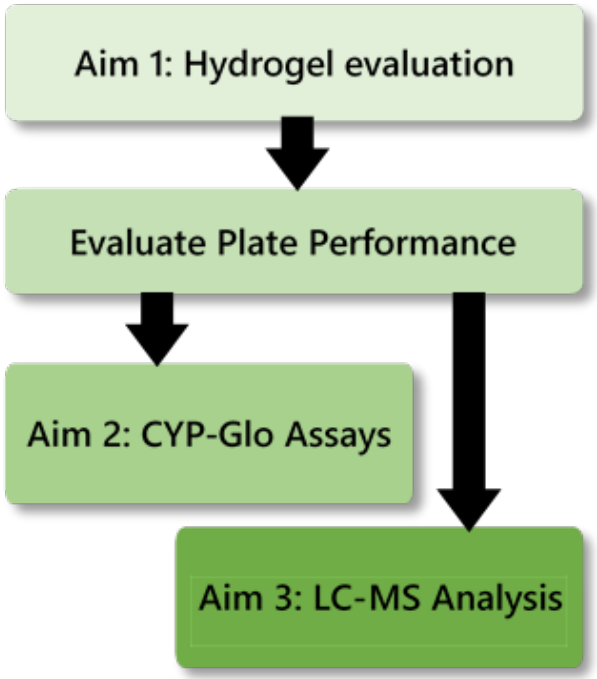


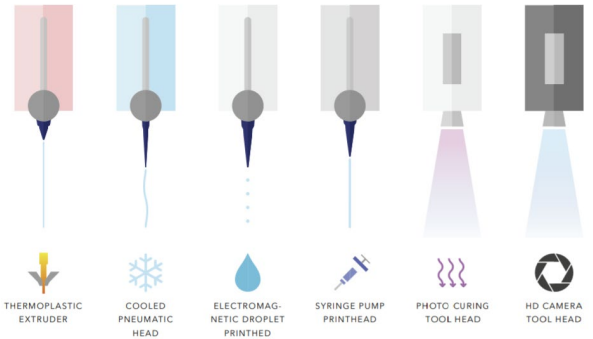
Development of a Bioprinting Approach to Adapt the AIME Method for High-throughput Screening Applications











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

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

EPA United States Environmental Protection Agency

Questions?

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