Retrofitting an Estrogen Receptor Transactivation Assay with Metabolic Competence Using Alginate Immobilization of Metabolic Enzymes (AIME)

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Metabolic Competence is Not a Feature of Most ToxCast Assays

The ToxCast program has faced scrutiny as the majority of the HTS assays are deficient for xenobiotic metabolism.

However, the ToxCast program presents a unique challenge for incorporation of metabolism in that:

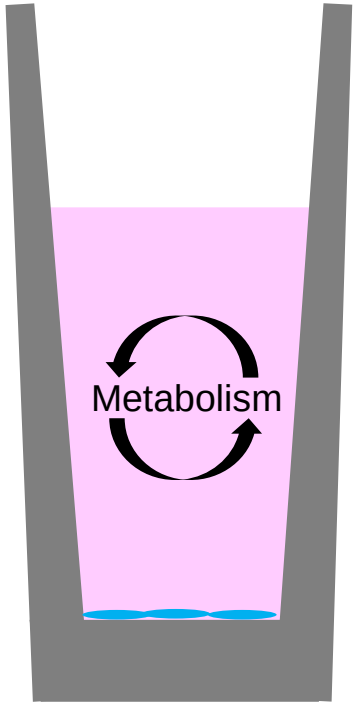
- The program consists of approximately **600 assays**
- The assays are comprised of both **cell-free** and **cell-based** technologies
- Assays are run in **high-throughput screening** platforms (~ 10,000 samples/day)

The Challenge:

To develop a method that is capable of retrofitting existing cell-free and cell-based high-throughput screening assays with metabolic competence

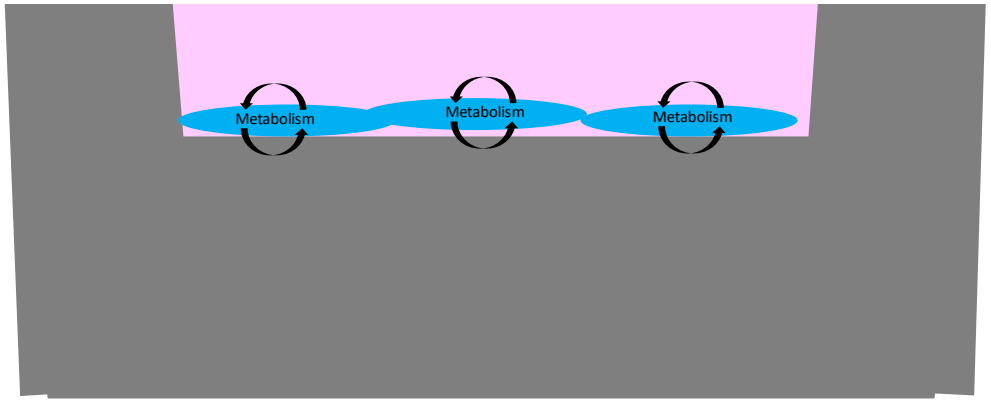
Developing Two Methods for Retrofitting HTS Toxicity Assays

Extracellular/Cell-free Method



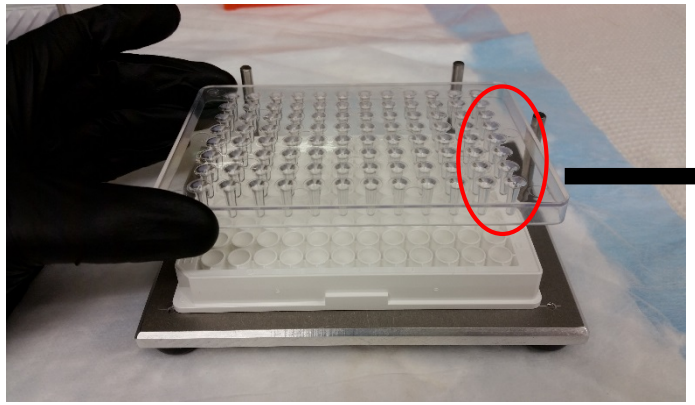
- Capable of metabolizing chemicals in the medium of both cell-based assays and cell-free assays
- More closely models hepatic metabolism and effects of circulating metabolites

Cell-based Method



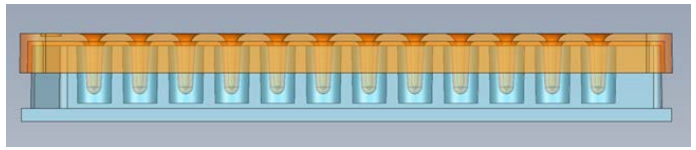
- Capable of metabolizing chemicals inside the cell, but only for cell-based assays
- More closely models effects of direct-acting metabolites

An Extracellular Metabolism Approach: Alginate Immobilization of Metabolic Enzymes (AIME)



AIME Lid & 96-Well Assay Plate

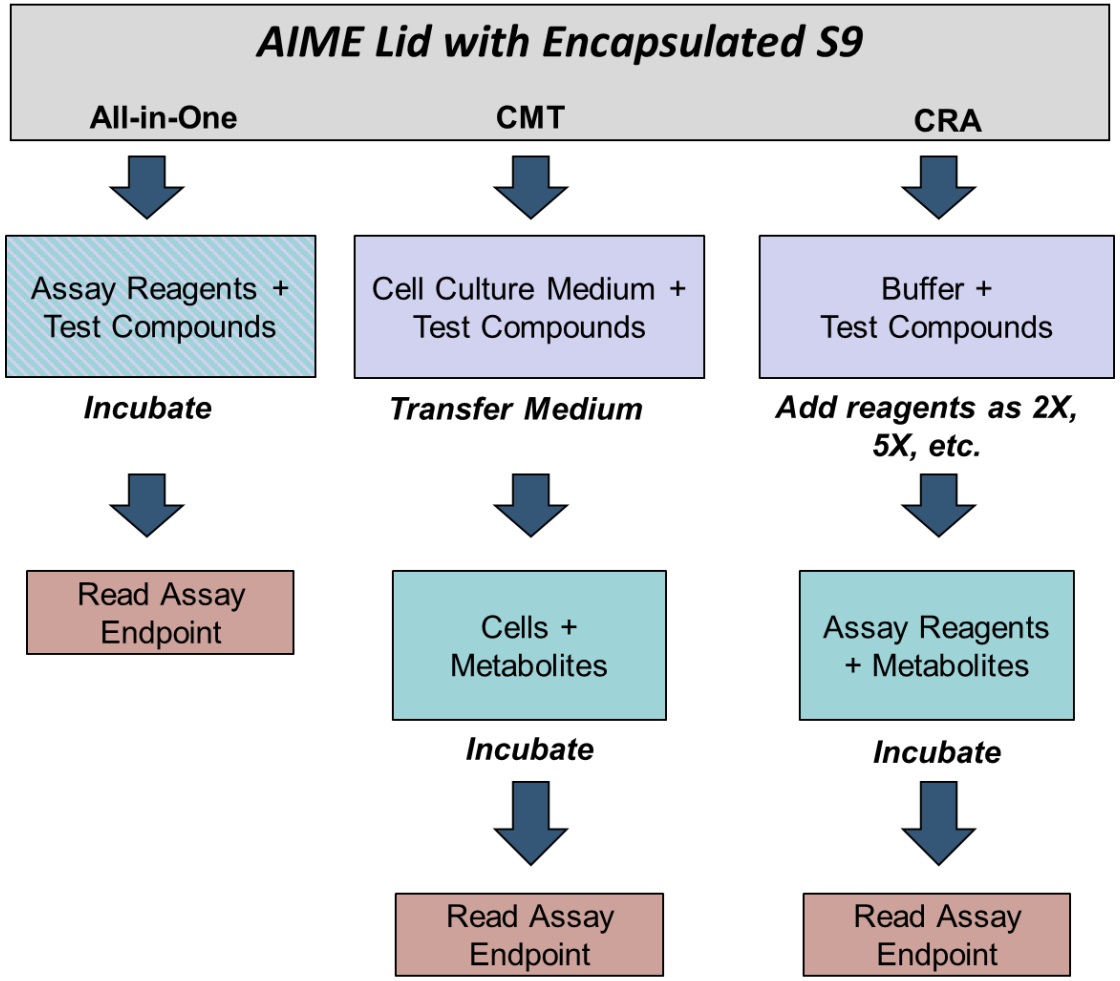
Enlargement of AIME alginate/S9 microspheres



Cross section of AIME Lid & Assay Plate

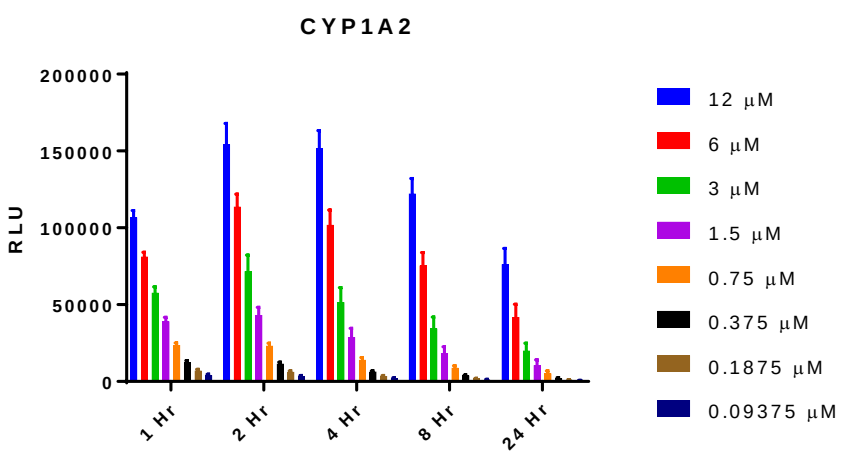
The AIME platform retrofits existing HTS assays with metabolic competence by encapsulating and attaching induced rat liver homogenate (S9) to solid supports extending from custom microplate lids.

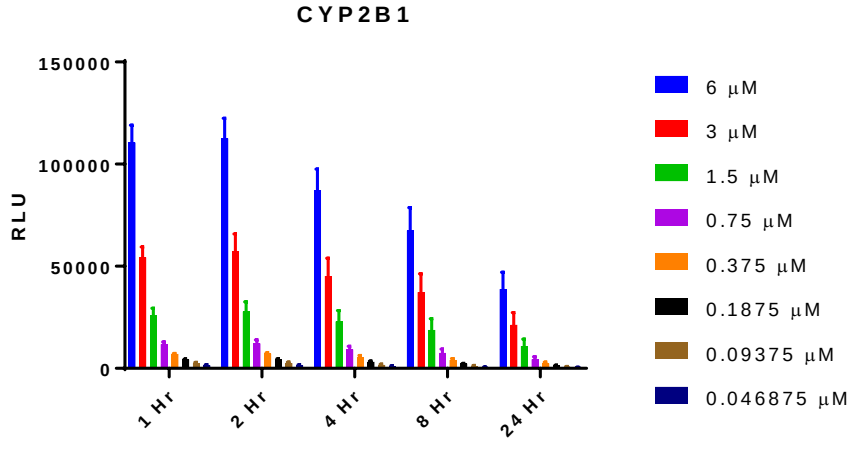
Alginate Immobilization of Metabolic Enzymes (AIME): Deployment Strategies for Cell-Based & Cell-Free Assays

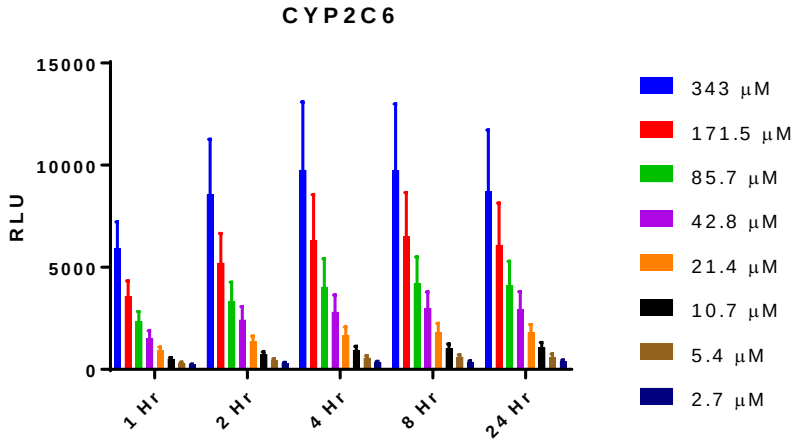


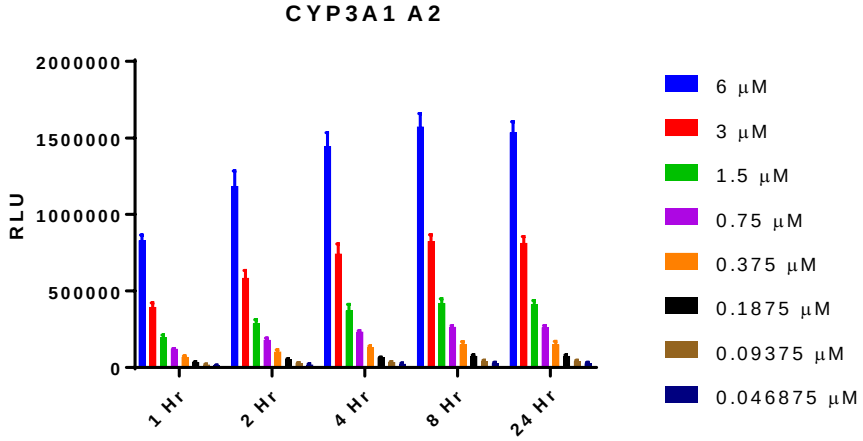
- **All-in-One Method (AIO):** Metabolism of test compounds & assay are run simultaneously.
- **Conditioned Medium Transfer (CMT):** Test compound metabolism occurs separately & prior to assay. Medium containing metabolites is then transferred to the assay plate.
- **Concentrated Reagent Addition (CRA):** Test compound metabolism occurs in the assay plate in conditions favorable to enzymatic activity. Following metabolite generation, concentrated reagents are added to initiate the assay.

Encapsulated Induced Rat Hepatic S9 Maintains Significant CypP450 Activity Over Time

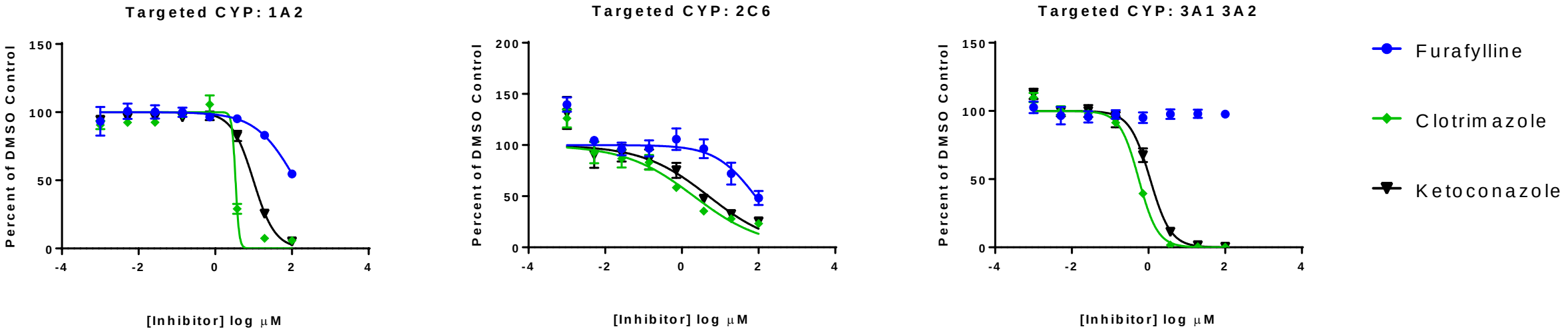






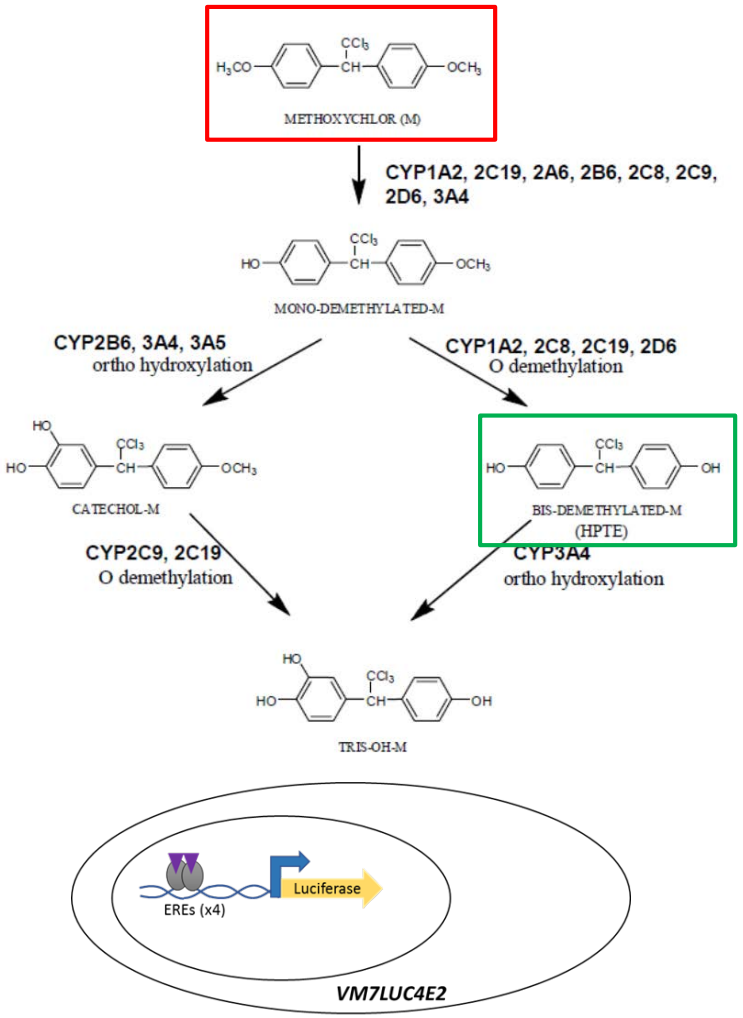


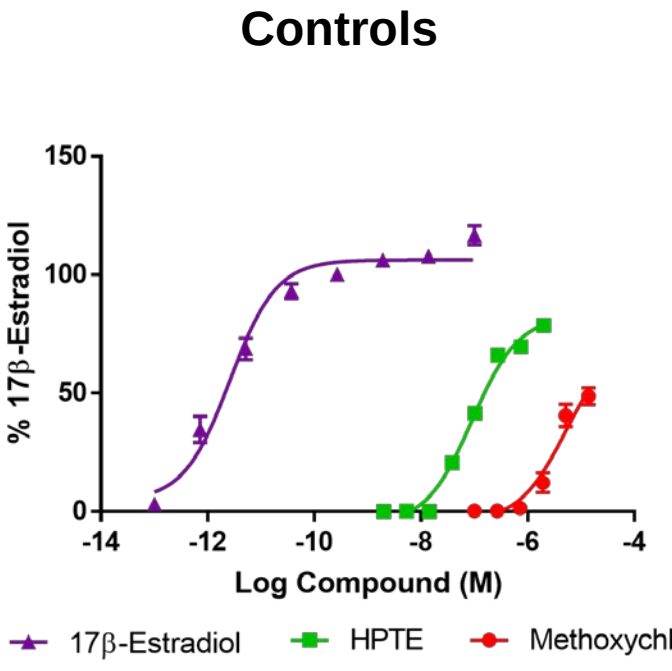
Small Molecules Freely Diffuse into the AIME Microsphere & Interact with P450 Enzymes

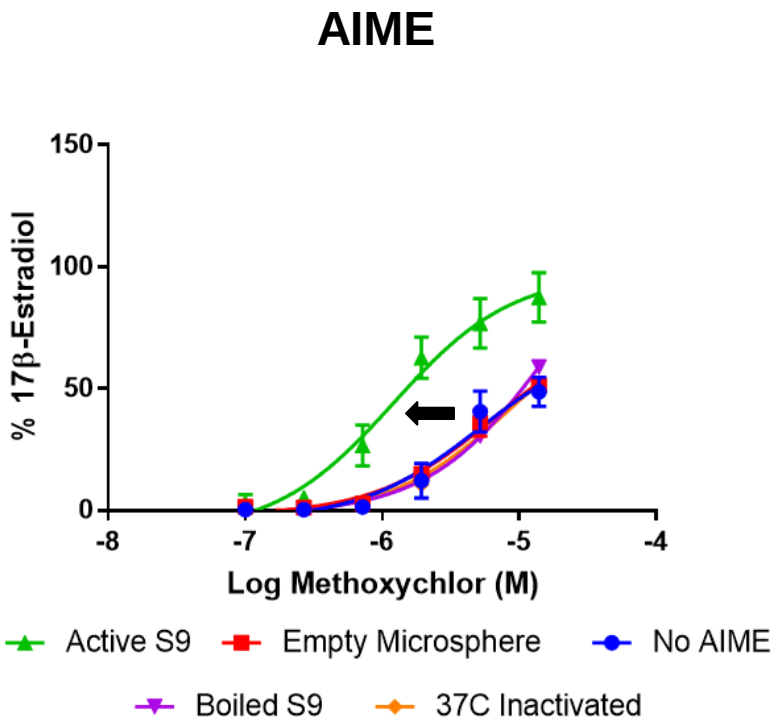


			IC50 (µM)		
Inhibitor	**Targeted Rat CYP**	**MW (g/mol)**	**CYP1A**	**CYP2C**	**CYP3A**
Furafylline	1A2	260.25	126.9	88.2	NA
Clotrimazole	3A1, 3A2	344.84	~ 3.4	2.1	0.56
Ketoconazole	Pan Inhibitor	531.43	9.9	5	1.1

VM7Luc4E2 Estrogen Receptor Transactivation Assay: A Model Assay for Evaluating Liver Metabolism

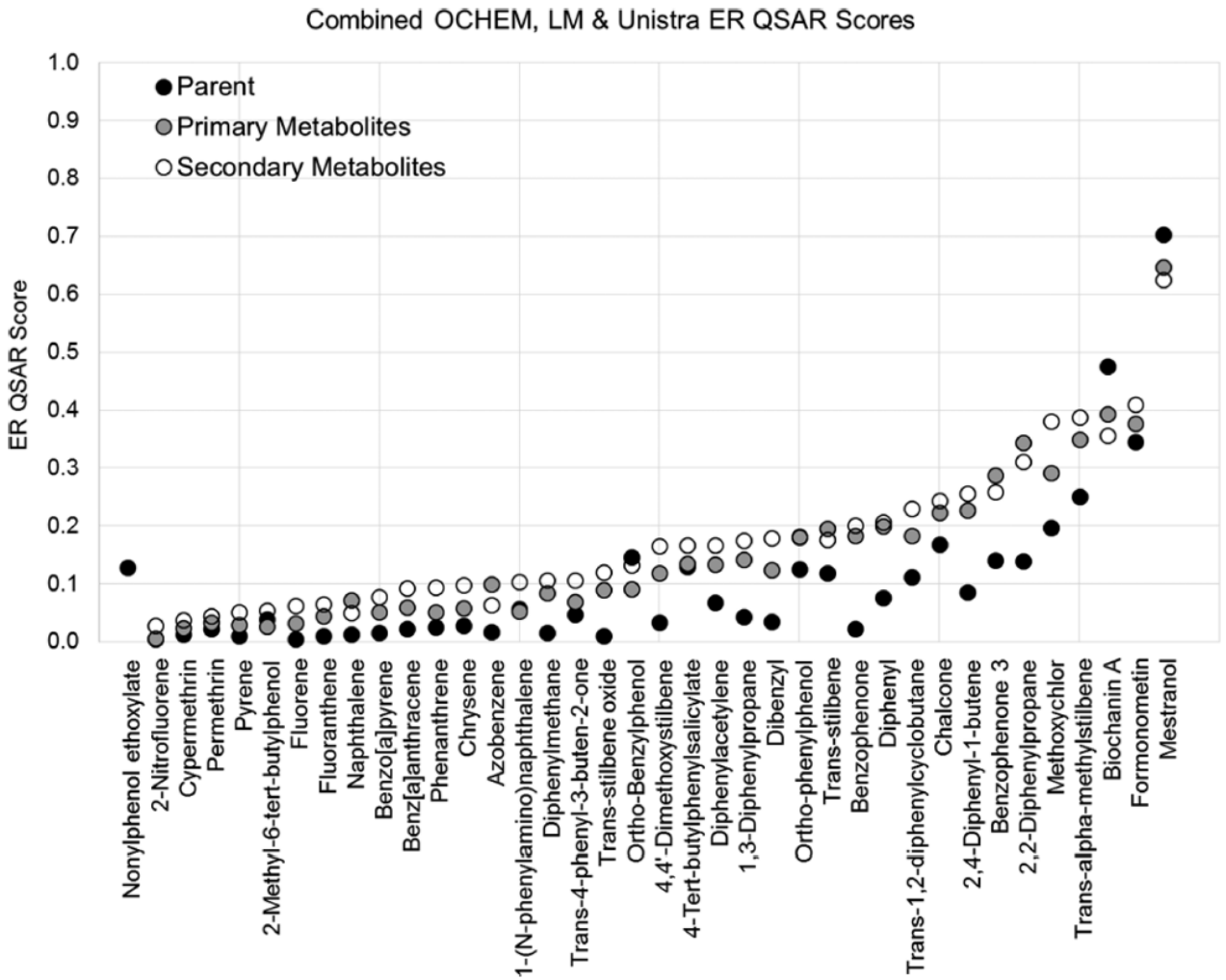






Potency shift between Methoxychlor and HPTE defines assay window for estrogenic metabolites

Screening for Predicted Estrogenic Metabolites in the AIME-coupled VM7Luc4E2 Assay



Current Study

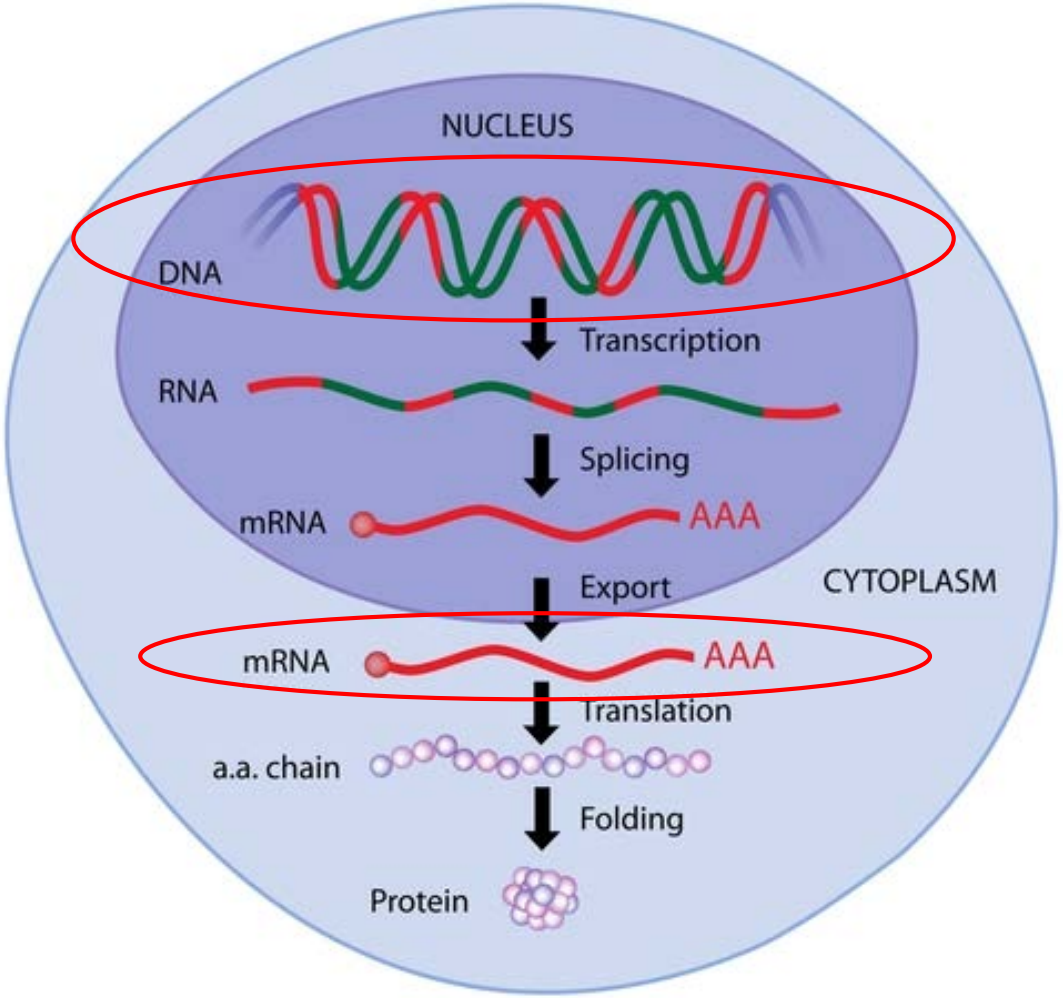
- Screen “Pinto” library
  - Pinto and Browne *et al* (2016) *Prediction of Estrogenic Bioactivity of Environmental Chemical Metabolites*. Chemical Research in Toxicology

- Assay run +/- metabolism to quantitatively evaluate potency shifts

Next Step

- Screen ~ 1,812 compounds +/- metabolism (August 2018)

Metabolic Gene Overexpression



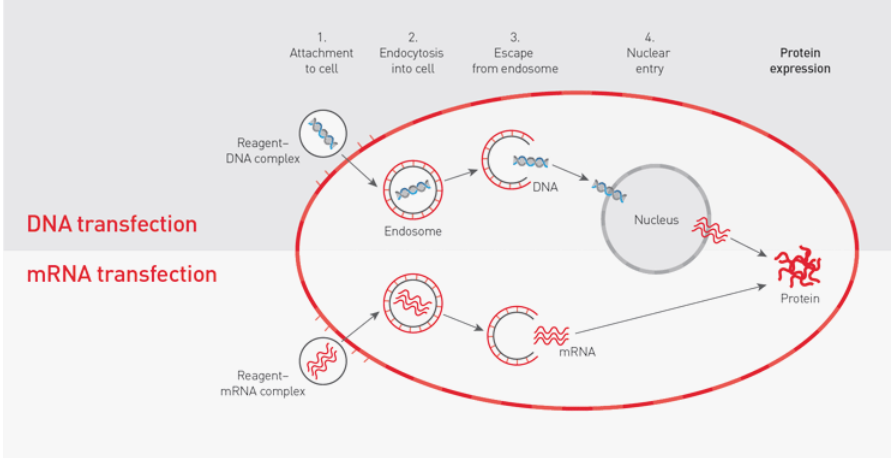
- Introducing xenobiotic-metabolizing enzyme (XME)-encoding genes back into cells with low/no expression is not novel
- Plasmid transfection, electroporation, and various viral vectors introduce XME-encoding genes (DNA) back into cells under control of gene promoters that drive strong expression (transcription)
- Transcription levels vary greatly between cell types and tightly controlled co-expression genes is difficult

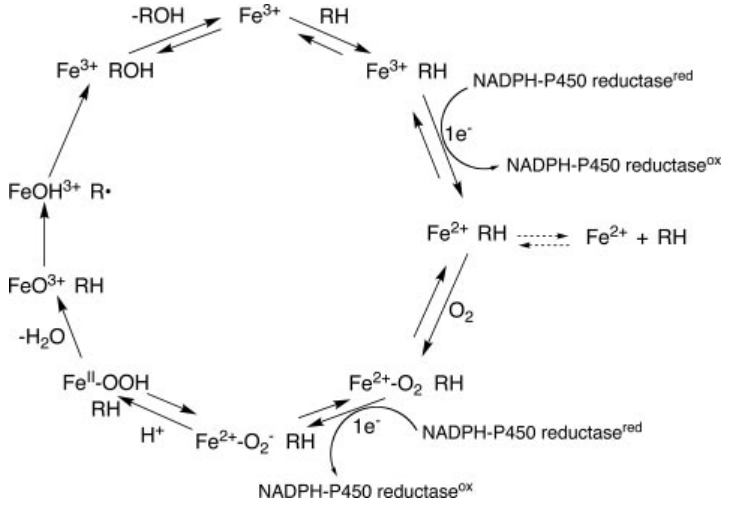
- Transfection of XME-encoding mRNAs is a novel approach that bypasses cellular transcription
- Chemically-modified nucleotides and cap eliminate the toxicity traditionally seen with RNA transfection
- Rapid XME expression and permits user to define composition and ratios of input mRNAs
- Method development focused on cytochrome P450 (CYP) enzymes, responsible for phase I metabolism

Method Optimization- Unique for Each Cell Line

- Nucleic acids (DNA or RNA) are large, charged molecules that do not readily cross the cell membrane
- Cationic lipid transfection
- The most popular transfection reagents in use today are cationic lipids (Lipofectamine ™, etc.)
- Lipid:RNA was optimized empirically using CYP3A4 activity
- Payload volume was optimized empirically using CYP3A4 activity
- POR required for the electron transfer from NADPH to cytochrome P450 enzymes in ER
- Although ubiquitously expressed, if POR is rate-limiting, CYP activity will be sub-optimal
- POR co-expression was optimized empirically using CYP3A4 activity

- Optimization nearly doubled CYP3A4 activity in HEK293T cells (over pre-optimized transfection conditions)



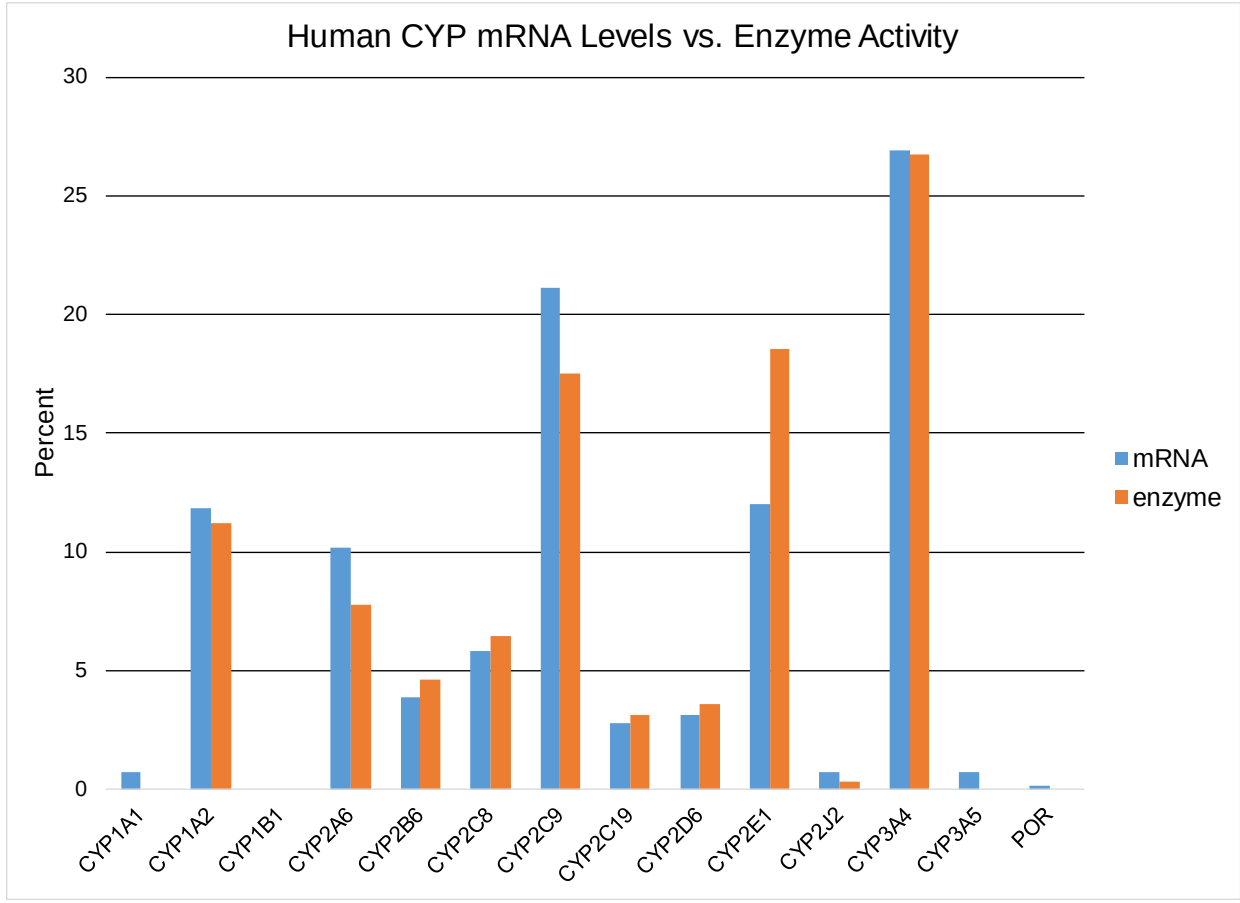


Characterizing a Panel of Human Liver CYP Enzymes

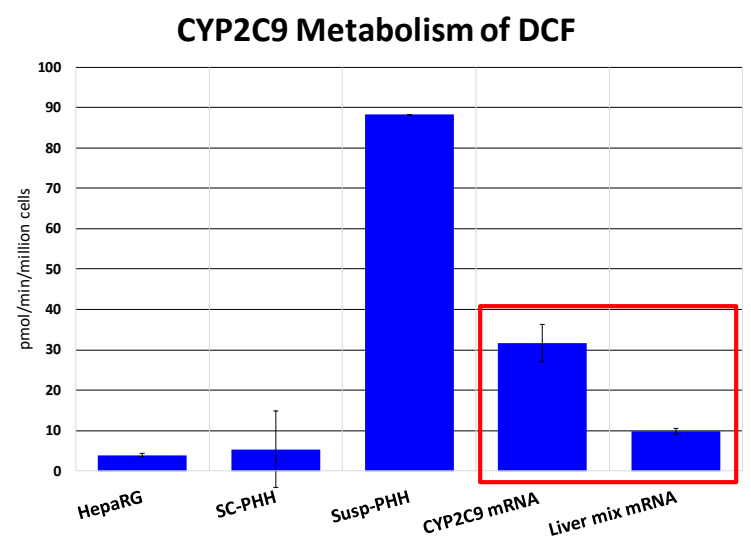
With optimization complete, next was to characterize the activity of the 10 most prevalent CYPs in human liver identified through a meta-analysis of over 700 subjects (Zanger and Schwab, 2013):

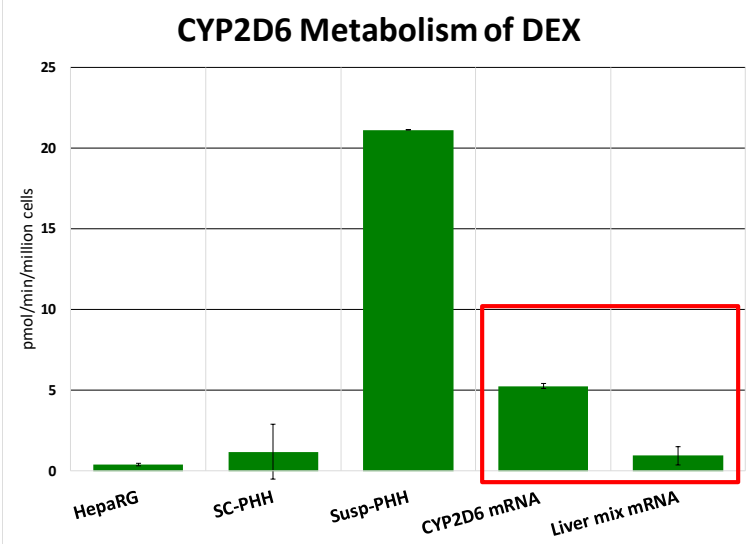
- CYP1A2 (12%)
- CYP2A6 (10%)
- CYP2B6 (4%)
- CYP2C8 (6%)
- CYP2C9 (21%)
- CYP2C19 (3%)
- CYP2D6 (3%)
- CYP2E1 (12%)
- CYP2J2 (1%)
- CYP3A4 (27%)

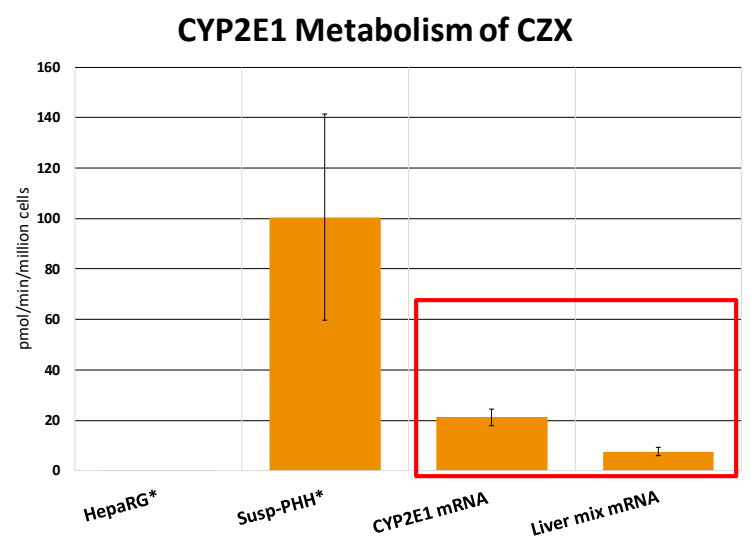
(% of pooled liver mRNA)

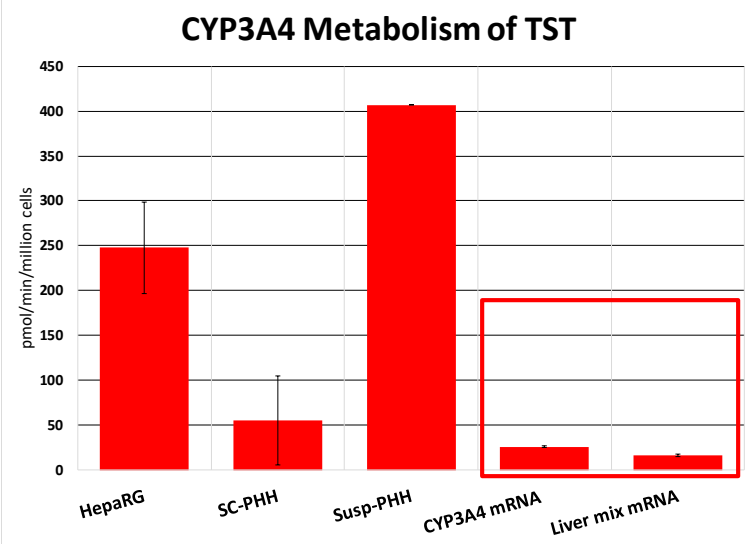


Comparison to “Gold-Standard” XM-Competent Cell Models









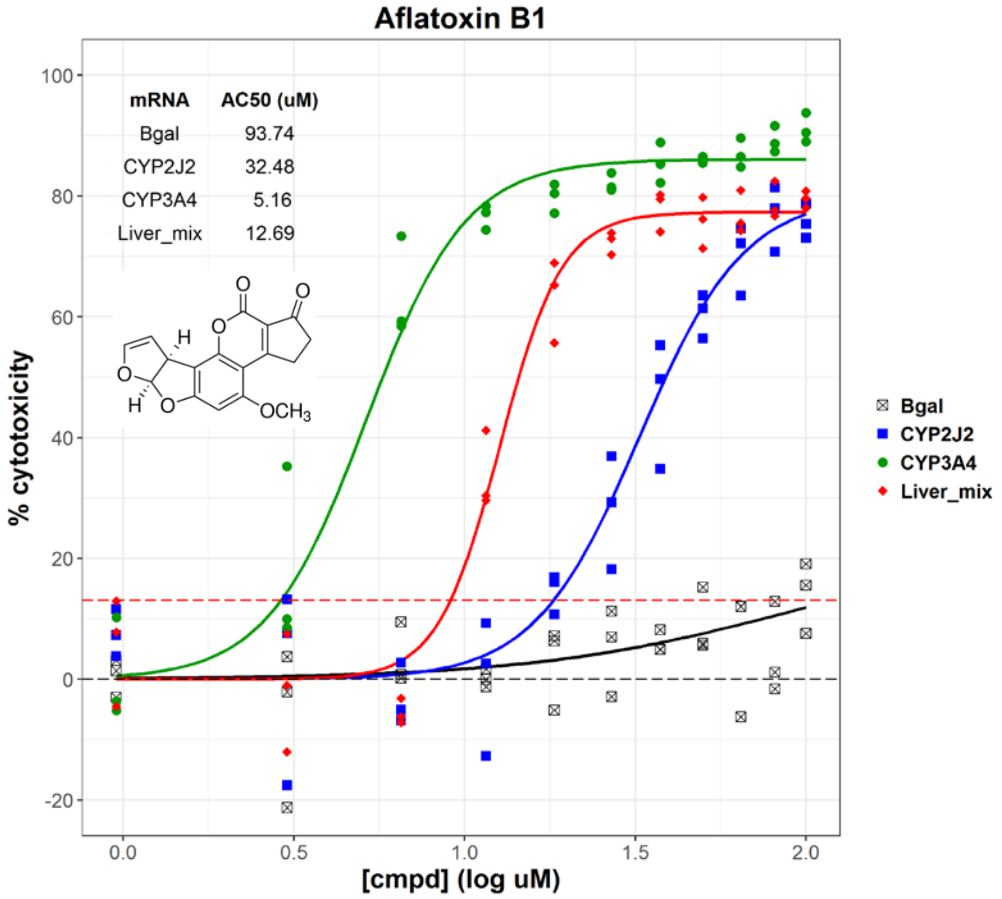
Proof-of-Principle: Deployment to a Cell-Based Assay

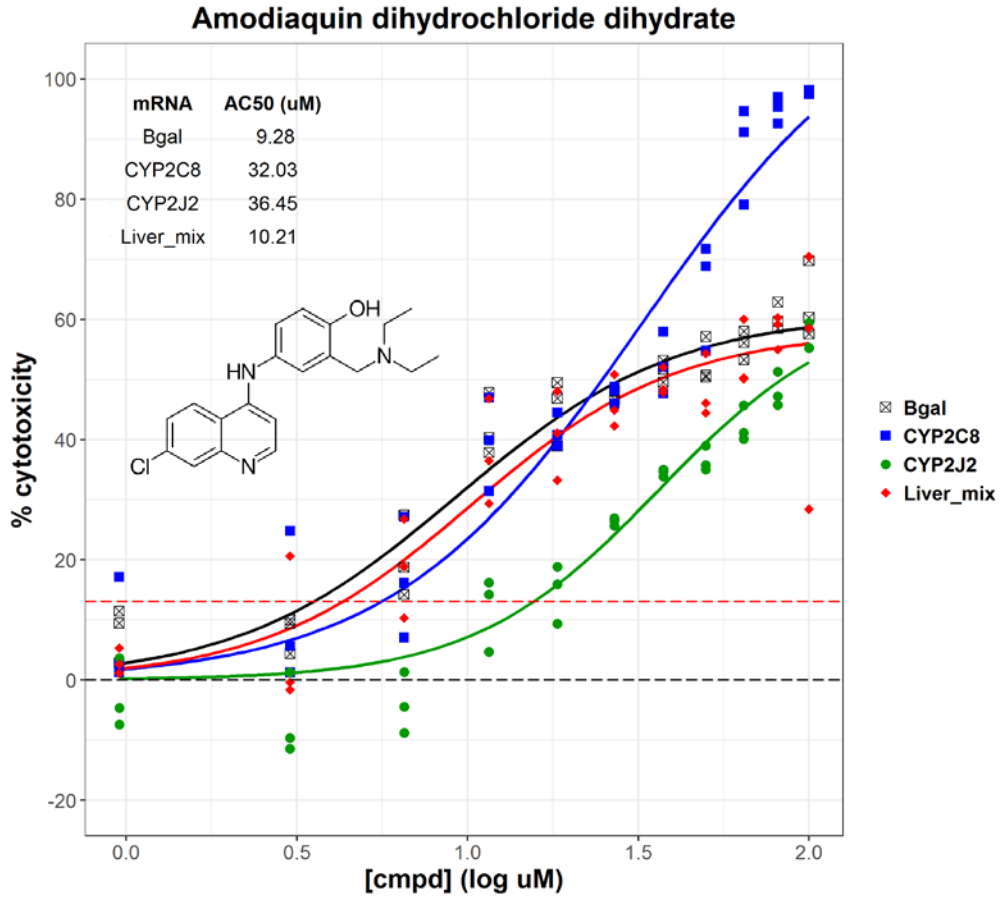
- mRNA transfection retrofits CYP-deficient cell model with robust CYP activity
- Onset of CYP activity is rapid (~6 hours post-transfection) and is sustained for at least 18 hours
- CYP enzymes produce predicted metabolites and at rates > than HepaRG and SC-PHH models, even when handicapped by HTS conditions

- **What happens when we couple this method with an existing cell-based assay?**
- **Can we observe CYP-dependent shifts in bioactivity?**

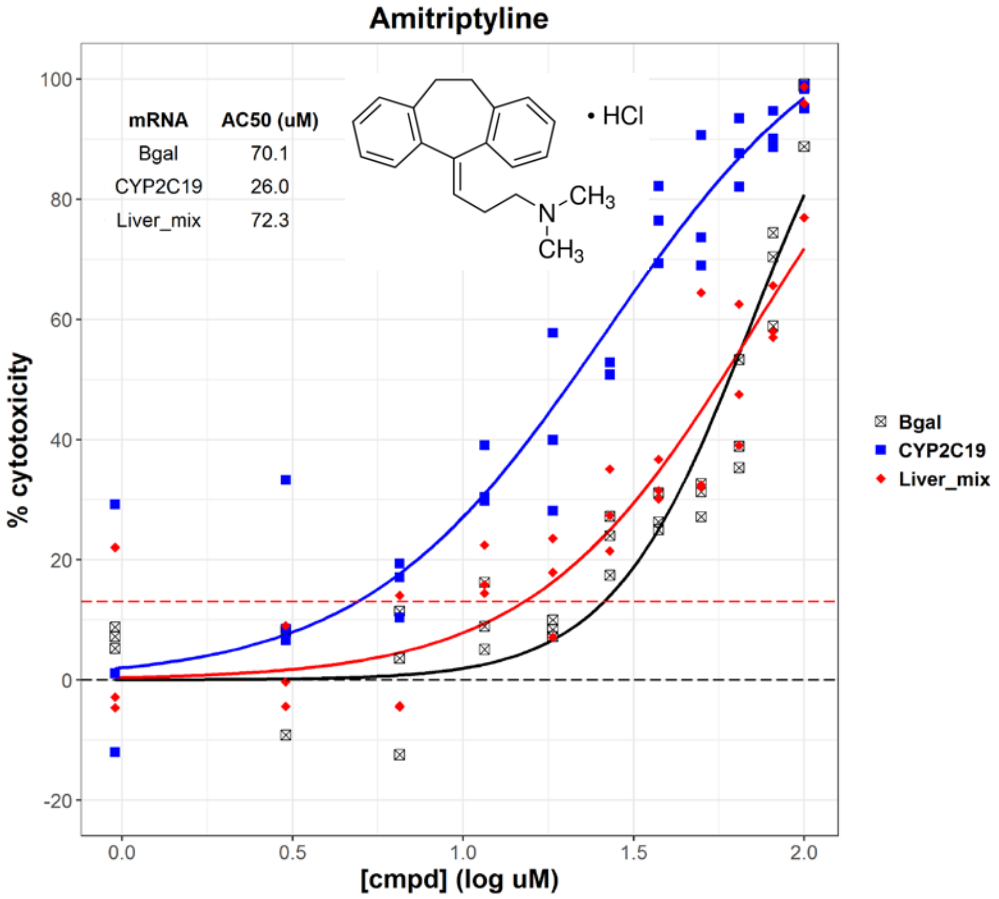
- HEK293T cells transfected with 10 x CYP singlets, Liver mix, and β-gal control (12 biogroups)
- 56 test compounds
- 11 concentrations
- 36 hour exposure
- N = 3
- Cytotoxicity measured using Cell Titer Glo™ Assay

Cytotoxicity Screening Results

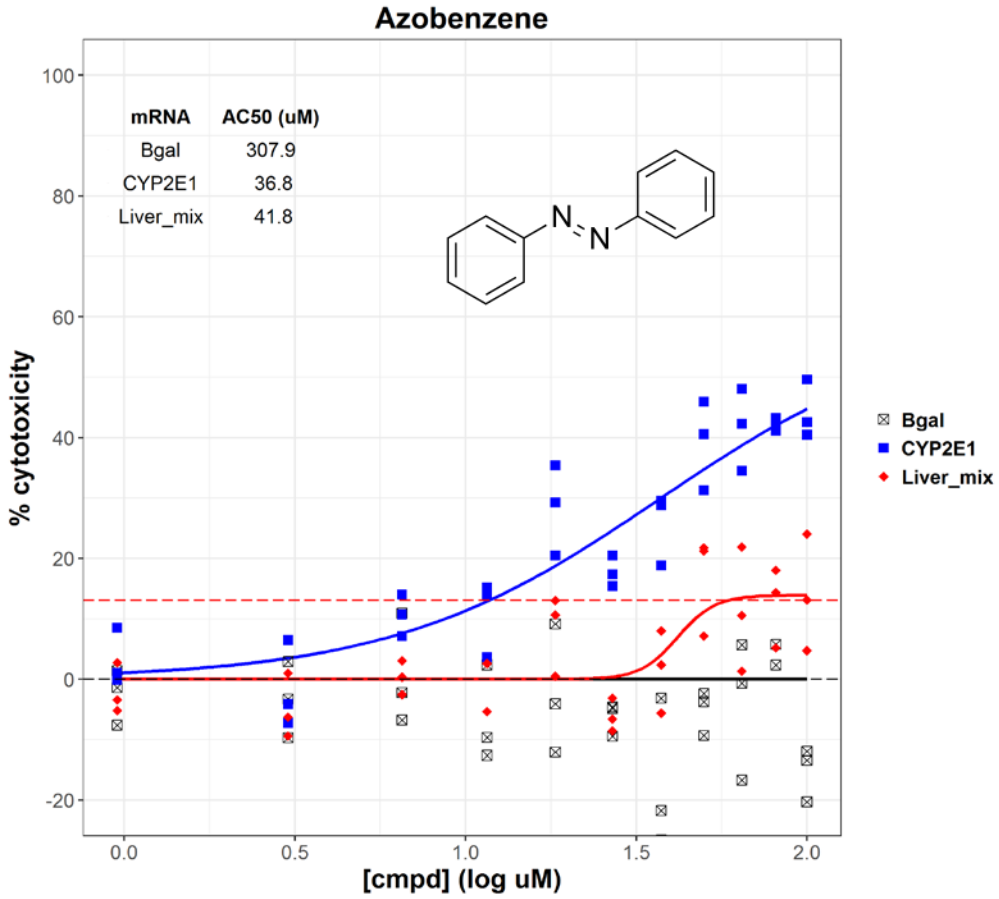




Cytotoxicity Screening Results (con't)

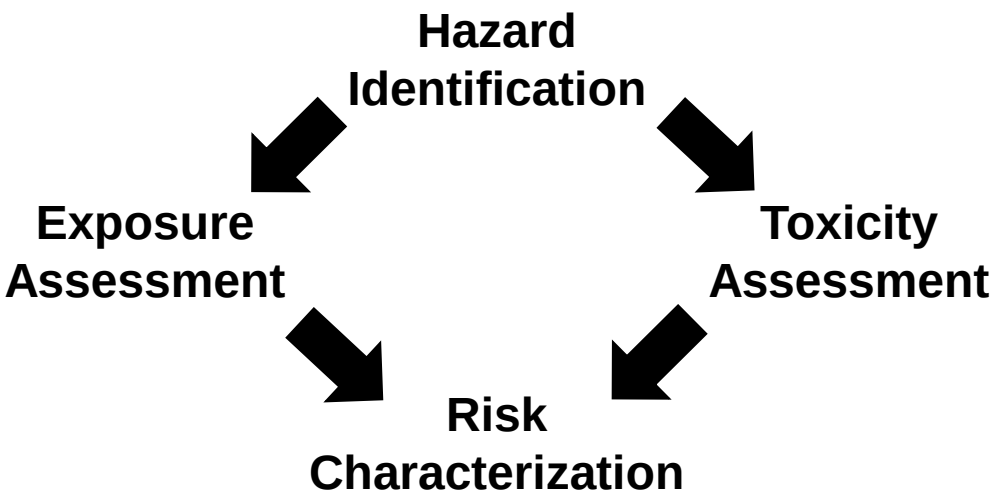


Active in 35 of 279 assays (12.5%)



Active in 14 of 882 assays (1.6%)

Tying Back to Risk Assessment



- Risk Assessments are based upon being able to:
  1. Identify **hazards**: linking agent to an adverse outcome
  2. Quantify **toxicity**: dose-dependent level of adversity
  3. Measure **exposure**

- Retrofitting HTS assays with xenobiotic metabolism improves:
  - Hazard: ability to detect bioactivated parents (false negatives)
  - Toxicity: dosimetric data for bioactive metabolites (left-shifting)
  - Toxicity: better potency estimates for detoxified parents (right-shifting)
  - Toxicity: use of polymorphic mRNAs to quantify adversity in genetic diverse populations

- Increasing the capabilities of alternative methods to identify hazards and assess toxicities is a critical step to incorporating these methods into risk assessment