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Retrofitting High-Throughput in vitro Assays for Metabolic Competence

Steven O. Simmons

Office of Research and Development
National Center for Computational Toxicology

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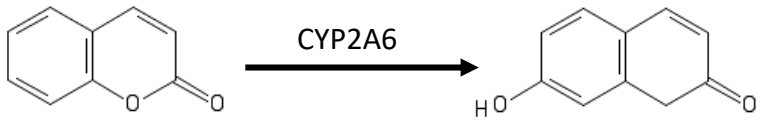
Why is Metabolic Competence Important for *in vitro* Assays?

Existing *in vitro* assays have limited or no metabolic capacity. This could lead to:

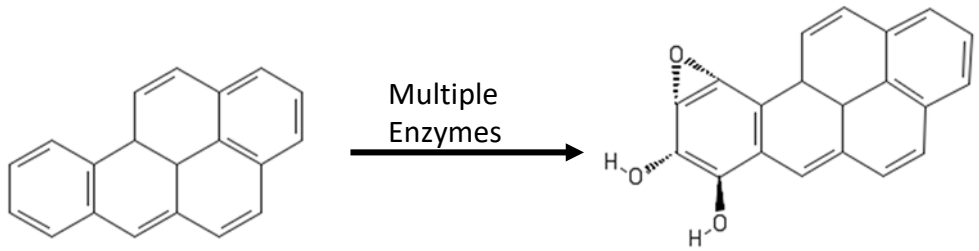
1. Overestimation of chemical hazard if the parent compound is detoxified to a less toxic or non-toxic metabolite

   Example: Coumarin



2. Underestimation of chemical hazard if the parent compound is activated to a more toxic metabolite

   Example: Benzo[a]pyrene



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ToxCast Assays Largely Lack Metabolic Competence

The ToxCast program has also faced scrutiny for the lack of metabolic competence in its *in vitro* assays.

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

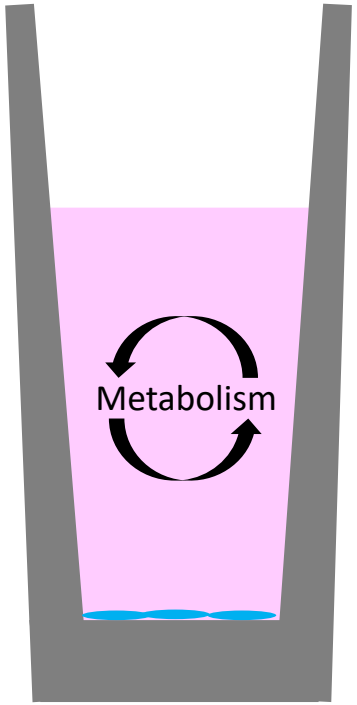
- The program consists of approximately **600 assays**
- The assays are comprised of both **cell-free** and **cell-based** technologies
- Assays are run in **high-throughput screening** platforms (~ 10,000 samples/day)

The Challenge:

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

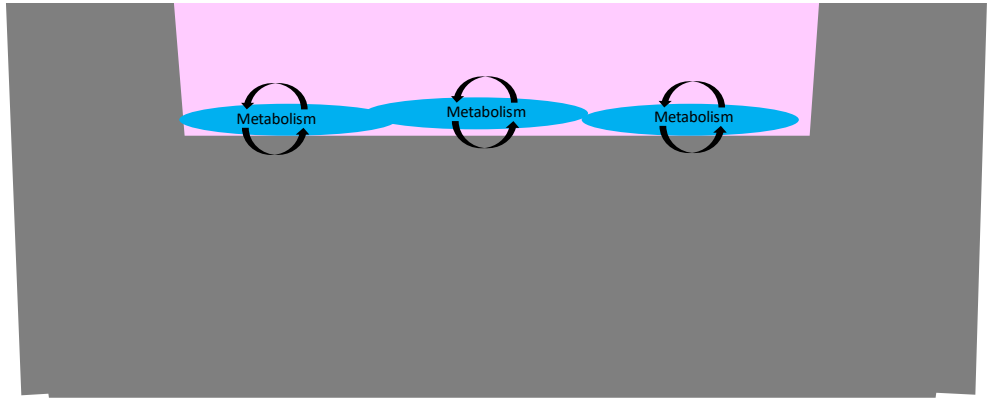
Two Strategies for Retrofitting *In Vitro* Assays for Metabolic Competence

"Extracellular" Strategy



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

"Intracellular" Strategy



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

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Extracellular Metabolism: Challenges with Direct S9 Addition

Direct addition of S9 to cells in culture has been used successfully to incorporate metabolic competence into existing assays. However, several challenges needed to be addressed in order to use S9 in our assays:

1. S9 is known to be cytotoxic with various cell types exhibiting differential sensitivities. This cytotoxicity limits the quantity of S9 that can be added per well.

2. S9 is not necessarily sterile, and lot-specific bacterial contamination can occur.

3. Direct addition of S9 is incompatible with some assay endpoints (e.g. fluorescence).

The Solution:

Encapsulate hepatic S9 so that test chemicals can freely interact with xenobiotic metabolizing enzymes and the assay constituents, while at the same time keeping the S9 contained and isolated from the assay matrix.

Alginate Immobilization of Metabolic Enzymes (AIME)

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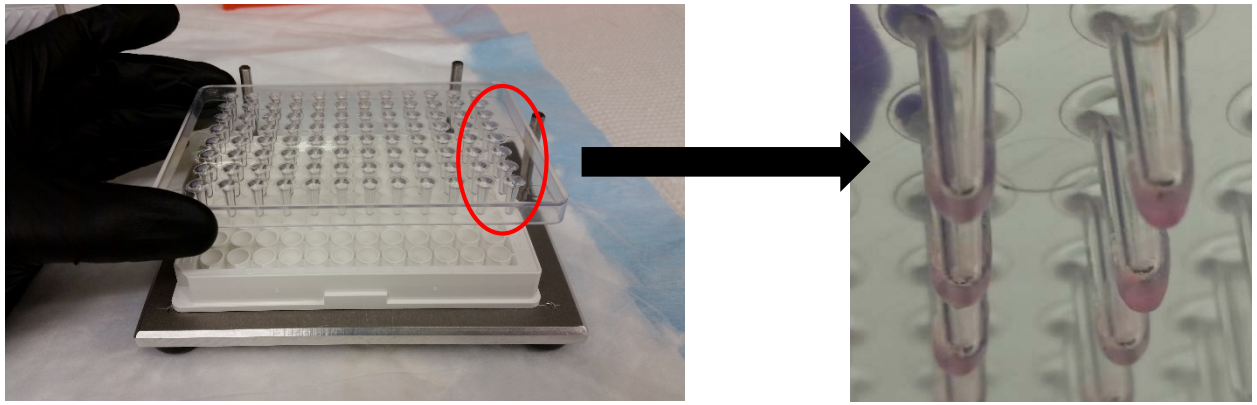
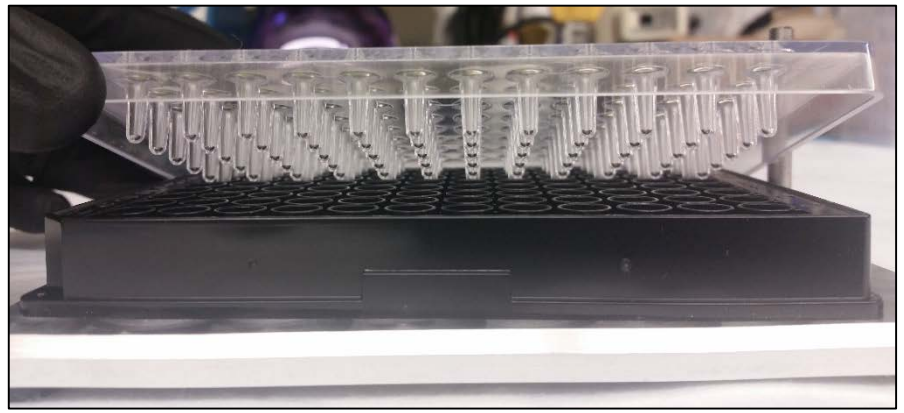
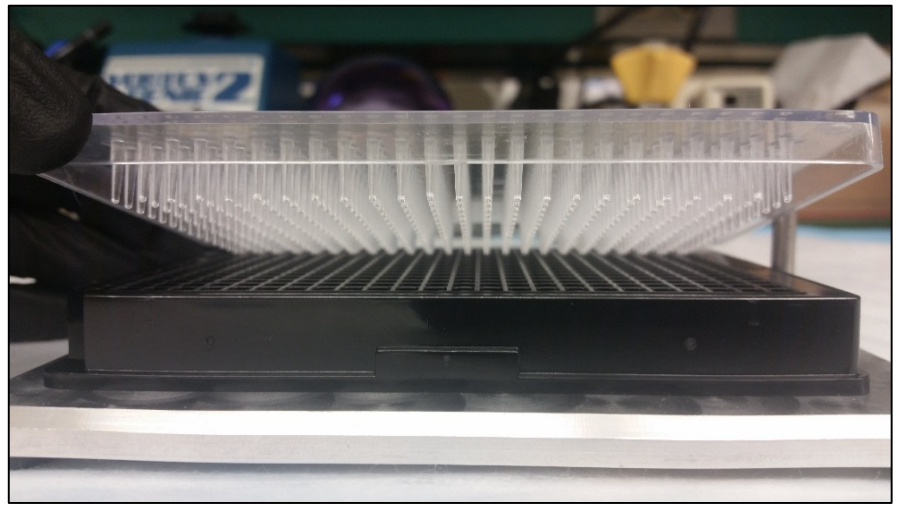
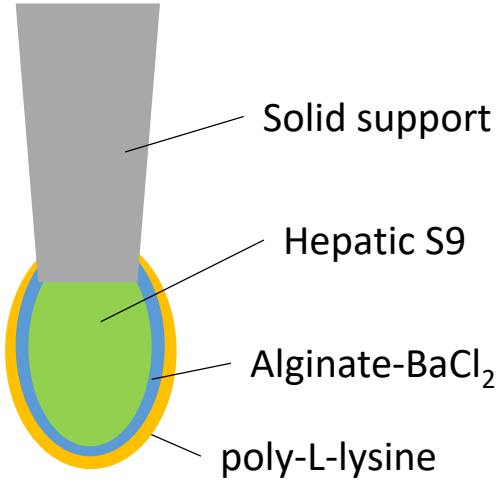
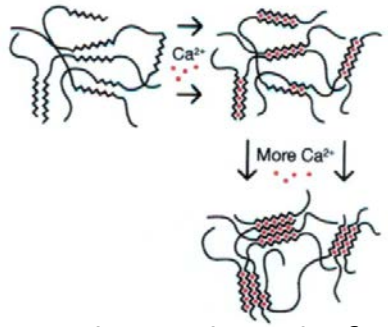
The AIME platform retrofits existing HTS assays with metabolic competence by encapsulating and attaching human liver homogenate (S9) to solid supports extending from custom microplate lids.







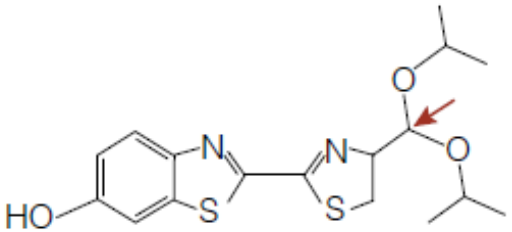




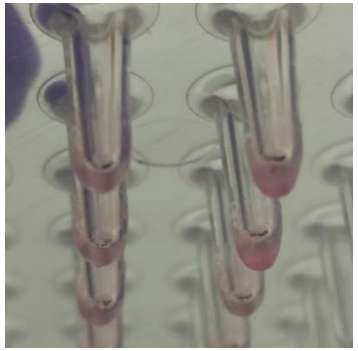
Alginate, a polysaccharide from seaweed, forms a gel network in the presence of multivalent cation ions

AIME Method Development Using Luminescent CYP3A4 Assay

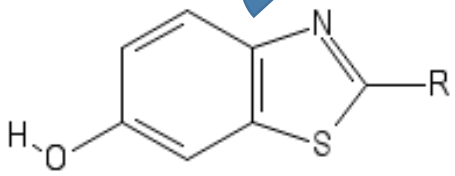
P450-Glo Assays from Promega



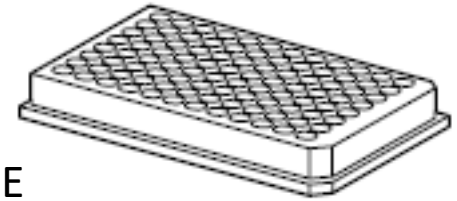
Luciferin-IPA



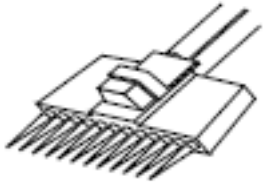
CYP3A4 with NADPH



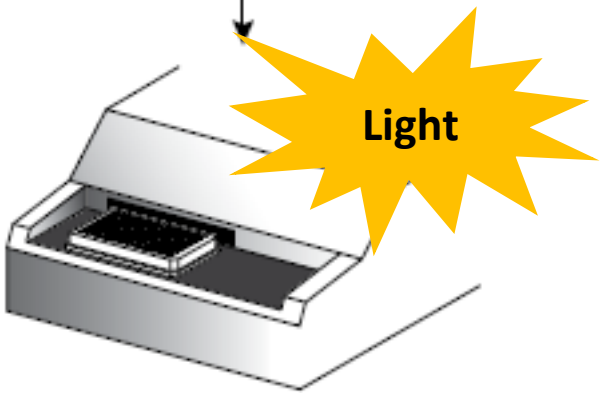
Luciferin



Remove AIME Lid

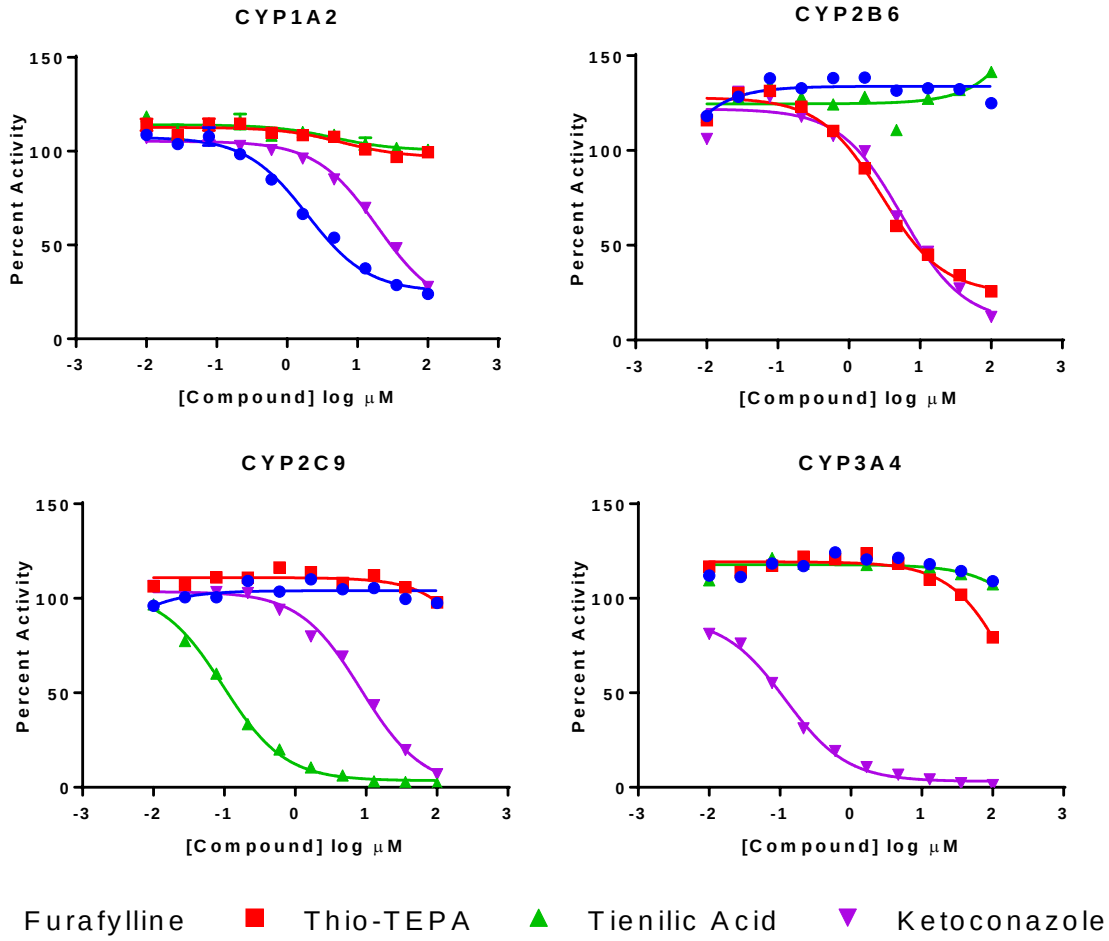


Luciferase



Provided first evidence that small molecules could diffuse into and out of the AIME microsphere

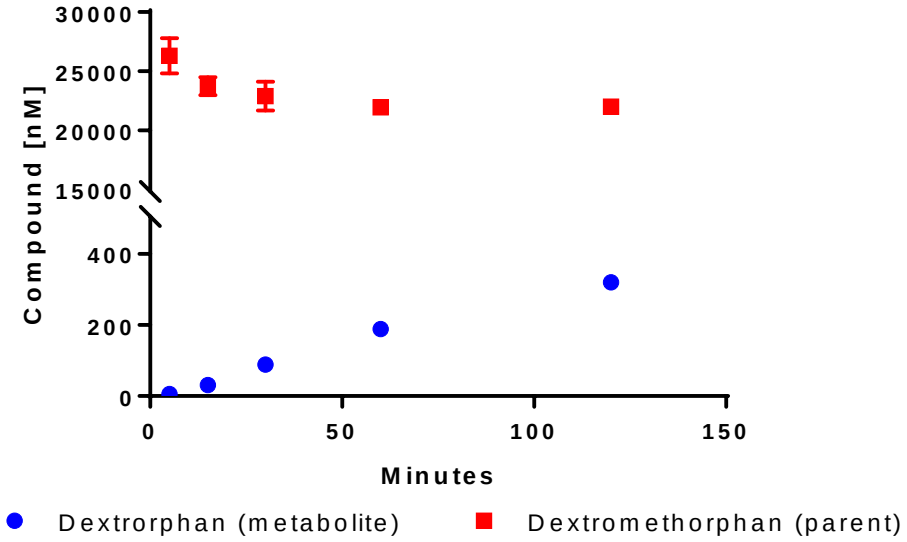
CYP Inhibition with Small Molecules: AIME vs. Free Human Hepatic S9



Compound	Mol. Wt. (g/mol)	Targeted P450	IC50 Free S9 (µM)	IC50 AIME (µM)
Furafylline	260.25	1A2	2.39	1.92
Thio-TEPA	189.22	2B6	7.46	2.86
Tienilic Acid	331.17	2C9	.053	.096
Ketoconazole	531.43	3A4	.086	0.12

AIME Forms Expected Metabolites from “Gold Standard” CYP Substrates

	CYP1A2	CYP2C9	CYP2D6	CYP2E1	CYP3A4
Parent	Phenacetin	Diclofenac	Dextromethorphan	Chlorzoxazone	Testosterone
Metabolite	Acetaminophen	4’-hydroxydiclofenac	Dextrorphan	6-hydroxychlorzoxazone	6β-Hydroxytestosterone



The AIME platform generates dextrorphan, a cytochrome P450 2D6 metabolite from the parent compound, dextromethorphan. Detection of the parent & metabolite was with LC-MS/MS.

Collaborators: Mark Strynar (NERL), Adam Swank (NHEERL)

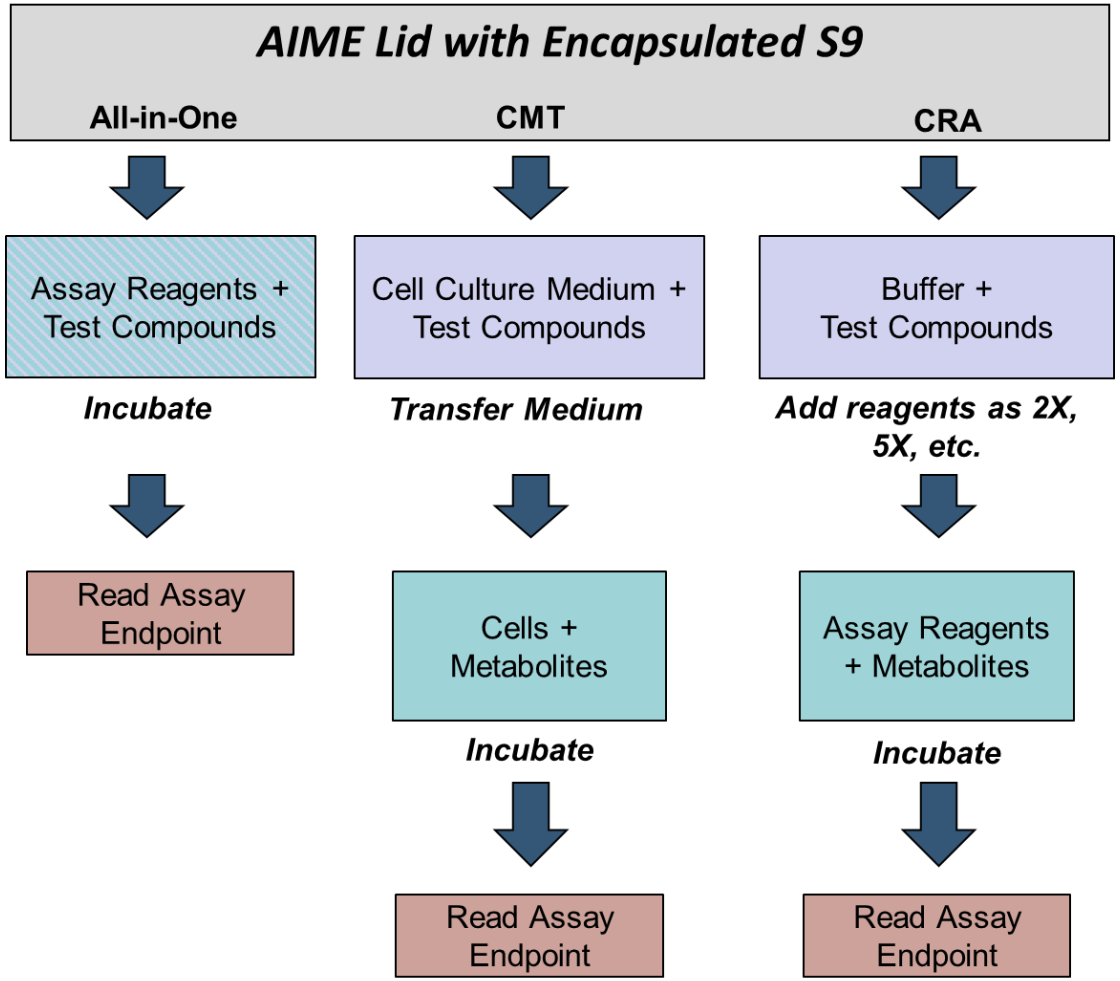
The Challenges of AIME Deployment

Question: How to couple two assays, that share some commonalities, but are fundamentally different, AND retain the complete or near-complete activity of each?

An analogy is to think of pairing a fettuccine alfredo recipe with a recipe for chocolate chip cookies.

Foodnetwork.com

AIME Deployment Strategies for Cell-Based & Cell-Free Assays



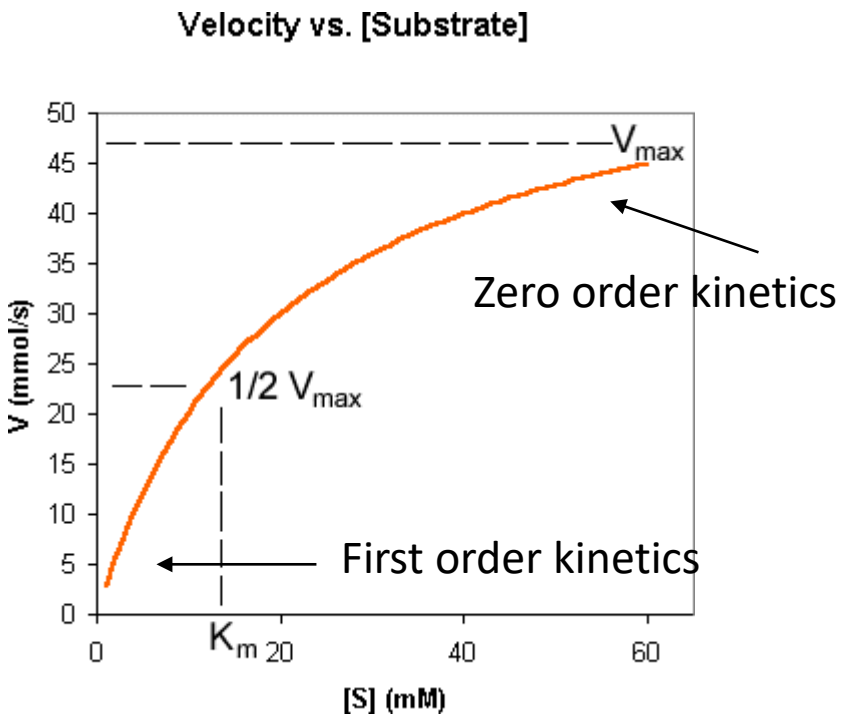
All-in-One Method: Metabolism of test compounds & assay are run simultaneously.

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.

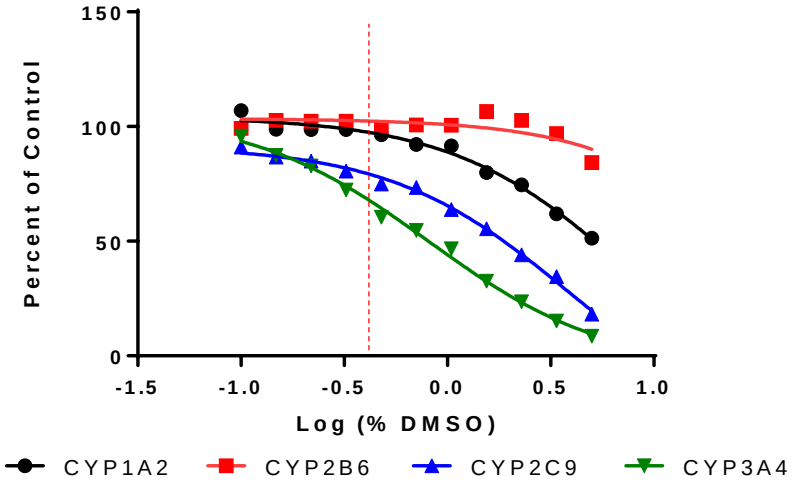
Km, Vmax, and Cytochrome P450s vs. DMSO levels

Michaelis-Menten Model: $v/V_{max} = [S]/Km + [S]$



- Metabolic reaction velocity is a function of substrate concentration.
- Testing at higher concentrations quickly becomes an issue because DMSO (library solvent) inhibits CYP activity.

DMSO Tolerance of CYP Enzymes in the AIME Platform

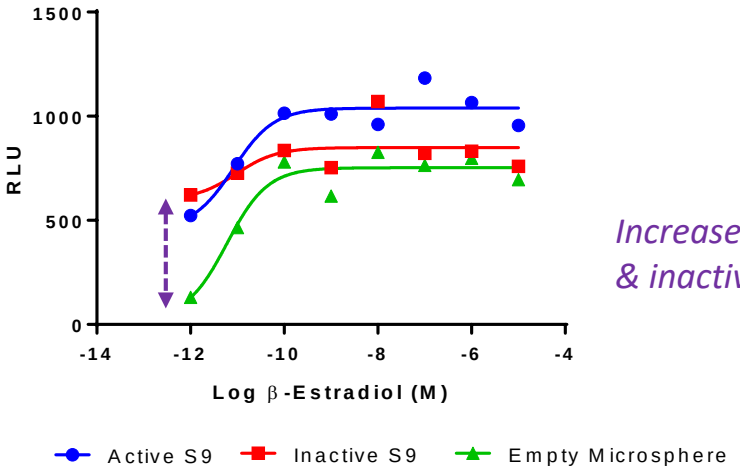


* DMSO inhibition in cell-free conditions

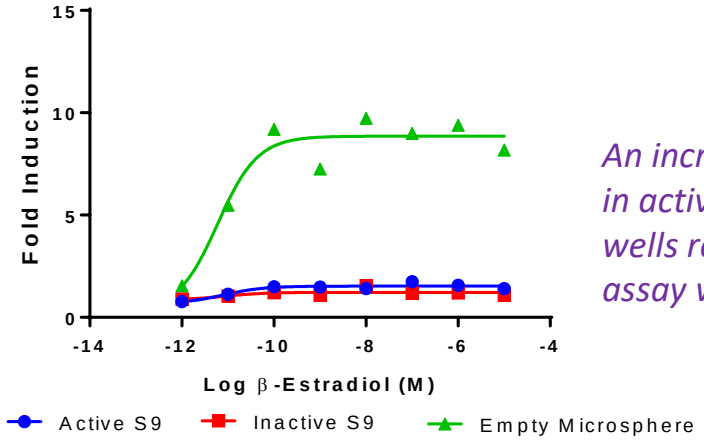
AIME Deployment – Unexpected Challenges

Human mixed gender hepatic S9, but not Aroclor-induced rat hepatic S9, interferes with the ER Transactivation Assay

Human S9

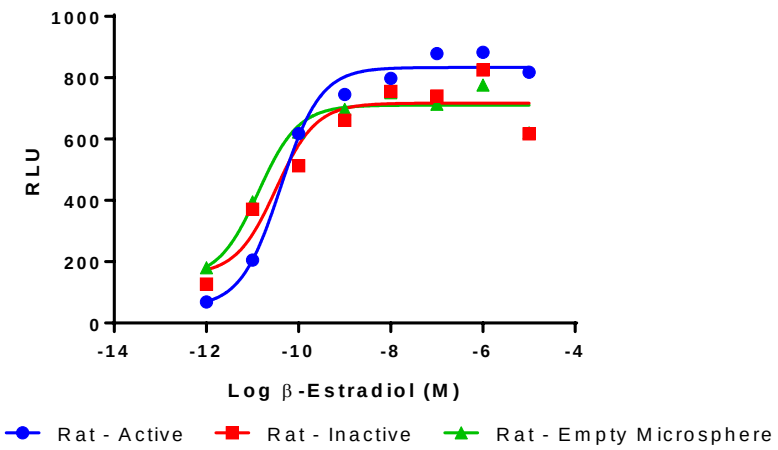


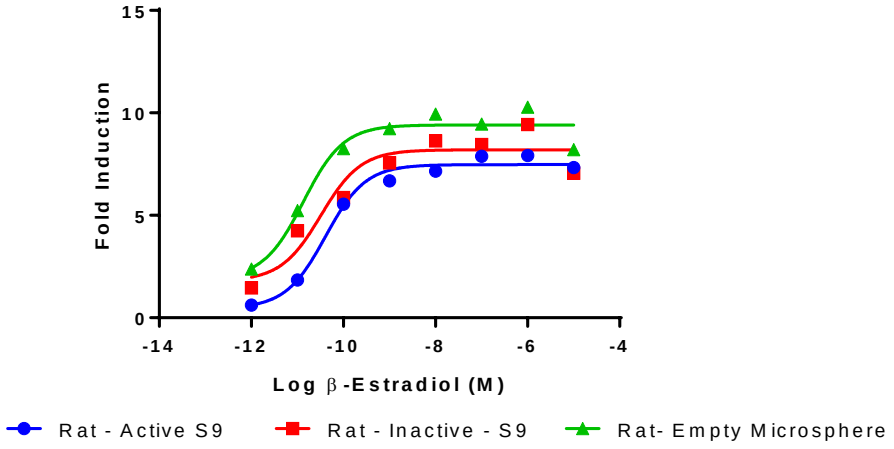
Increased E2 signal in active & inactive S9 wells



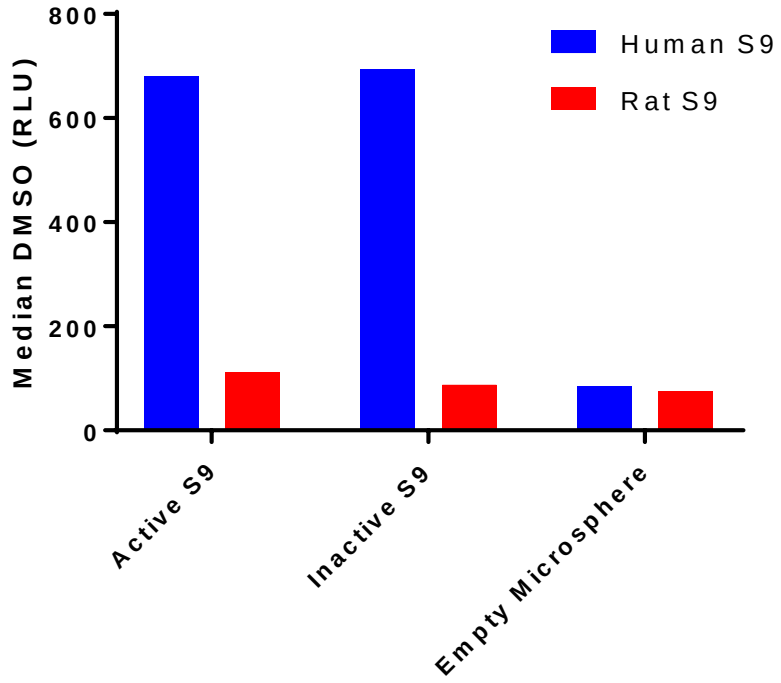
An increased DMSO signal in active and inactive S9 wells results in a diminished assay window.

Rat S9

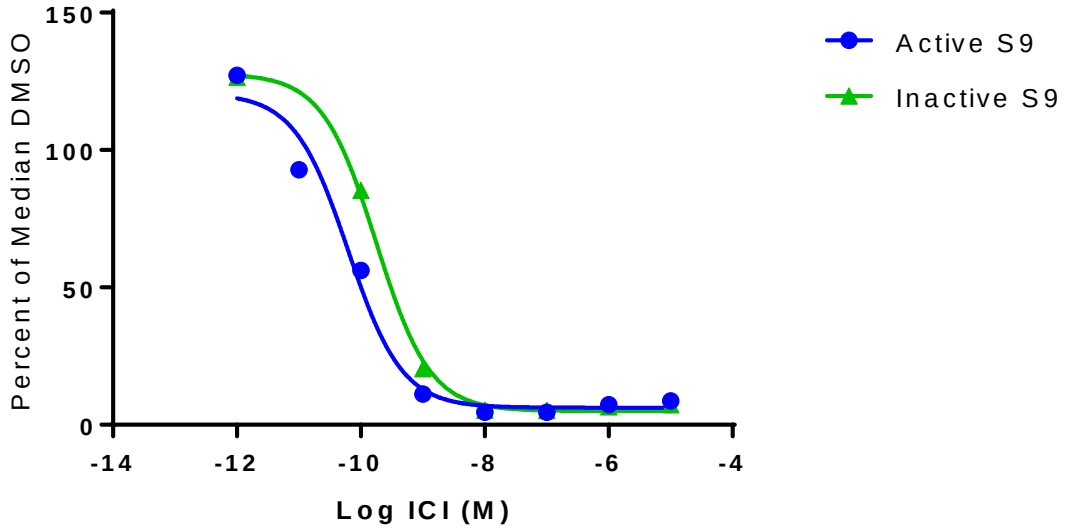




AIME Deployment – Unexpected Challenges

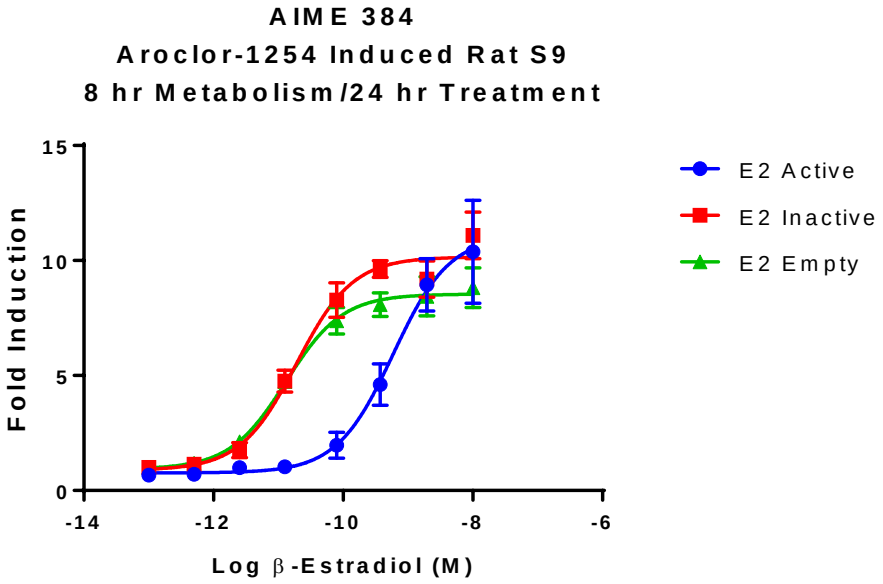


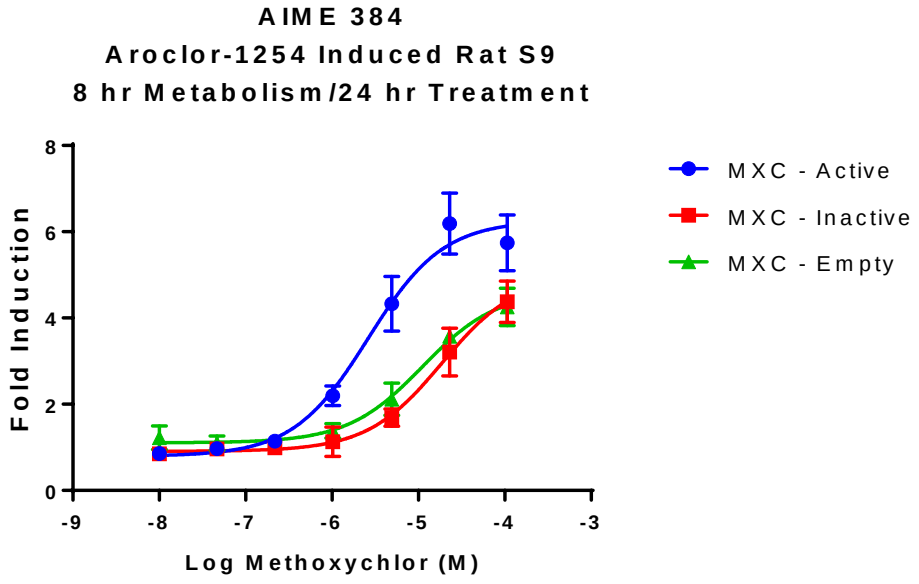
The difference appears to be mediated by ER-active compounds in the human hepatic S9 that increase the DMSO background

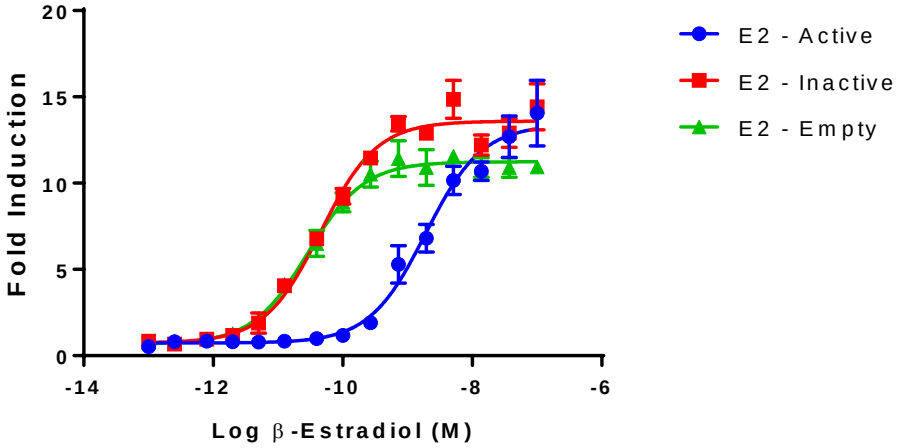


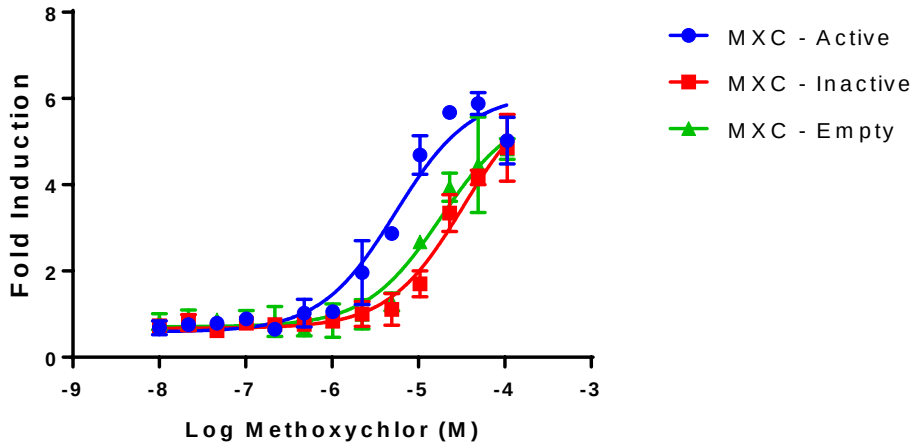
The ER antagonist ICI 182,780 can reduce the ERα – dependent luciferase activity from DMSO treatment in the presence of active or inactive S9.

AIME Deployment – ER Transactivation Assay









Acknowledgements

NCCT

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