Chemical Screening for Bioactivated Electrophilic Metabolites Using Alginate Immobilization of Metabolic Enzymes (AIME)

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Objective

To develop a platform to retrofit existing high-throughput screening assays with metabolic competence.

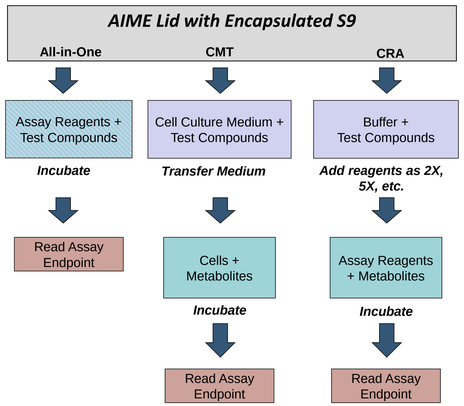
Introduction

The EPA's ToxCast program utilizes a wide variety of high-throughput screening assays (HTS) to assess chemical perturbations of molecular and cellular endpoints. A key limitation of many HTS assays used for toxicity assessment is the lack of xenobiotic metabolism which precludes the detoxification as well as toxic bioactivation of chemicals tested *in vitro*, thereby mischaracterizing the potential hazard posed by these chemicals. To address this deficiency, we have developed the AIME platform to retrofit existing HTS assays with extracellular xenobiotic metabolism. By encapsulating hepatic S9 in alginate microspheres, cytotoxicity and assay interference associated with direct addition of S9 is reduced. Here we describe deployment strategies used with the AIME platform and present our data from three different assay deployments to illustrate the advantages and disadvantages of each strategy.

Materials & Methods

Chemicals – All chemicals were purchased from Sigma-Aldrich and stock solutions were prepared in DMSO.

Alginate Immobilization of Metabolic Enzymes (AIME) – Human hepatic S9 (pooled, mixed gender) or Aroclor-induced male rat hepatic S9 was encapsulated in alginate microspheres using a modification of a cell encapsulation protocol by Lee et *al.* (1).



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

All-in-One Method

Metabolism of test compounds & assay are run simultaneously

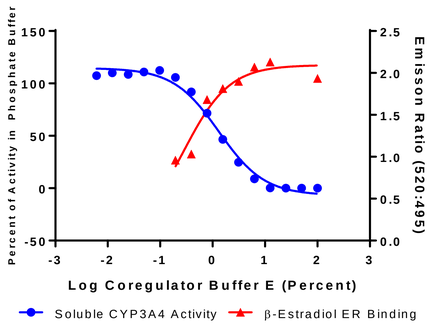


Figure 1: The All-in-One method requires a compromise between enzyme activity and assay performance in a cell-free ER ligand binding assay.
CYP3A4 activity in human hepatic S9 (P450-Glo; Promega) and the emission ratio from the LanthaScreen TR-FRET ER Alpha Coactivator Assay (ThermoFisher) were examined in 2-fold dilutions of Coregulator Buffer E into 100 mM phosphate buffer (pH 7.4).

Advantages

Fast & easy
Increases throughput

Disadvantages

Assay conditions often inhibit enzymatic activity

Concurrent metabolism can confound interpretation of results

Conditioned Medium Transfer (CMT)

Test compound metabolism occurs separately & prior to assay. Medium containing metabolites is then transferred to the assay plate.

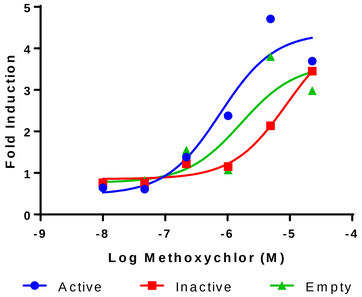


Figure 2: Conditioned Medium Transfer (CMT) used to deploy AIME to a cell-based estrogen receptor transactivation assay.
An AIME lid was prepared with active rat hepatic S9, heat-inactivated hepatic S9, and empty microspheres (no S9) and immediately added to a microplate containing methoxychlor in phenol red-free DMEM with an NADPH regeneration system. Metabolism was allowed to occur for 2 hours after which the medium was transferred to a new microplate containing VM7Luc4E2 cells which have been stably transfected with an ER-responsive luciferase reporter gene (3). Following treatment for 24 hours, the cells were lysed and luciferase activity measured.

Advantages

Allows for xenobiotic transformation prior to assay initiation

Disadvantages

Transfer (and aspiration step) increases time & decreases throughput

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.

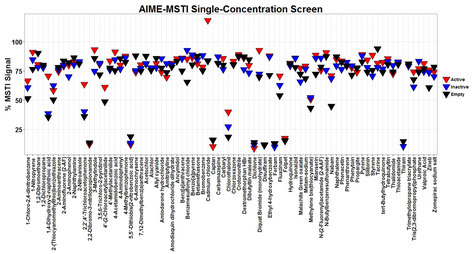


Figure 3: Concentrated Reagent Addition (CRA) used to deploy AIME to the MSTI Fluorescence-Based Thiol-Reactive Assay™.
AIME lids containing active human hepatic S9, heat-inactivated S9 or an empty microsphere (no S9) were added to microplates containing test compounds in phosphate buffer and an NADPH regeneration system. Metabolism was allowed to occur for 1 hour followed by the addition of a 2X MSTI reagent. The plates were incubated at room temperature for 30 minutes followed by quantitation of the fluorescent signal (excitation: 510 nm; emission 650 nm). *An increase in electrophilicity was detected as a decrease in the fluorescent signal.* Z' scores: active (0.66), inactive (0.82) and empty (0.77)

Advantages

Eliminates medium aspiration & transfer steps of CMT. Relies only on reagent additions.

Disadvantages

Proprietary assay buffers may not be available in concentrated forms

Results & Conclusions

- We have successfully produced functional AIME microspheres on lids compatible with 96- and 384-well microplates.
- Three strategies for deployment of the AIME platform include the All-in-One method, conditioned medium transfer (CMT) and concentrated reagent addition (CRA).
- The All-in-One method is preferred for retrofitting existing HTS assays due to its speed and ease of deployment; however, assays utilizing buffers or other reagents which are incompatible with xenobiotic metabolizing enzymes may dictate use of an alternative method.
- Conditioned Medium Transfer (CMT) has been successful used to deploy the AIME platform to a cell-based estrogen receptor transactivation assay.
- Using Concentrated Reagent Addition (CRA), the AIME platform was coupled to the MSTI Fluorescence-Based Thiol-Reactive Assay™. However, AIME-mediated metabolic activity had limited effect on enhancing the electrophilicity of compounds screened with this assay.
  - For several compounds (2,2',4'-trichloroacetophenone, 1,4-dihydroxy-2-naphthoic acid, and diquat bromide) AIME-mediated metabolic activity resulted in a suppression of electrophilicity that was not due to protein binding, suggesting a possible detoxification.
  - Overall, coupling the AIME platform to the MSTI assay did not identify any novel electrophiles in the tested chemical library. Possible explanations include assay incompatibility with AIME, low formation of metabolites, and/or low electrophile capture due to the highly-reactive nature of these compounds.

Future Directions

- Encapsulation of human hepatocytes and/or Supersomes to increase the metabolic capacity of the AIME platform
- Identification and quantitation of metabolites using LC-MS/MS to quantify metabolic output of the AIME platform. This will provide information needed to determine the minimum metabolite levels required for detection in retrofitted assays as well as the ratio of parent/metabolite(s) produced.
- Functional evaluation of AIME platform using a training set of chemicals known to be detoxified and bioactivated by various human liver CYP enzymes
- Deployment of the AIME platform to additional cell-free and cell-based assays with expanded set of ToxCast chemicals

References

1. Lee et al. (2013) Sens. Actuators, B 177: 78-85
2. McCallum et al. (2013) J. Biomol. Screening, 18(6): 705-713
3. Rogers and Denison (2000) In Vitro Mol. Toxicol. 13(1): 67-82

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