

# TOWARD VALIDATION OF A HUMAN IN VITRO ASSAY FOR DEVELOPMENTAL TOXICITY ASSESSMENT

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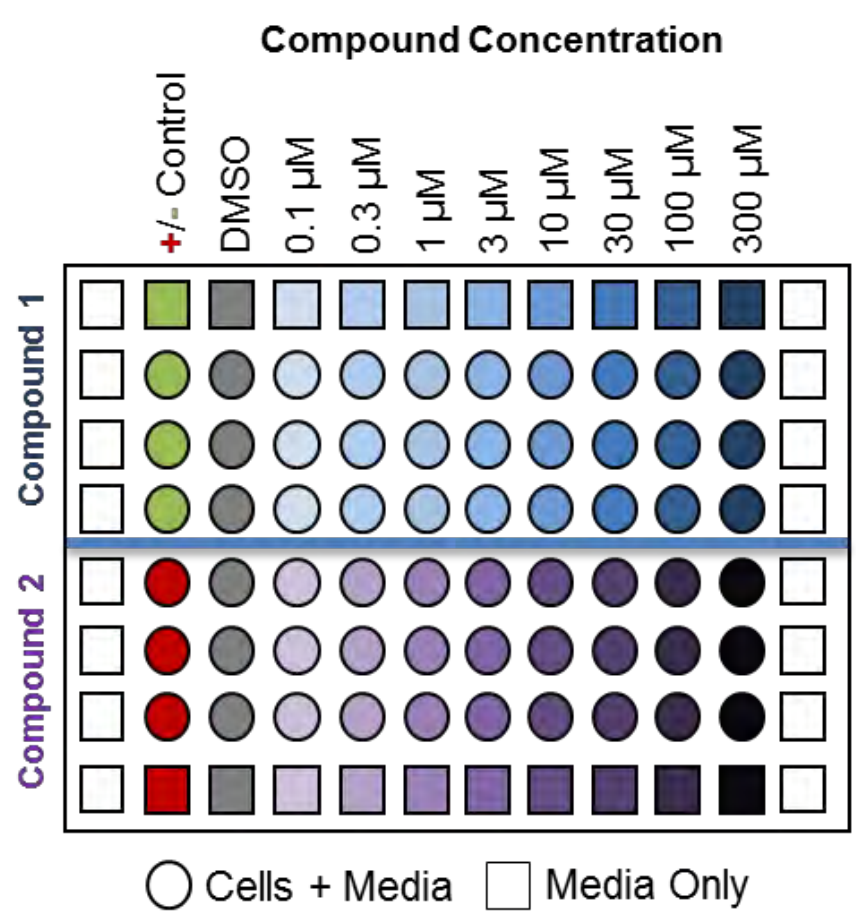
## INTRODUCTION

Innovative *in vitro* toxicity screening assays aimed at reducing or replacing the use of animal models are required for the REACH initiative (Europe) and Tox21 initiative (US) to evaluate thousands of chemicals for safety. We have created a predictive, human *in vitro* pluripotent stem cell based developmental toxicity assay that can reduce costs, animal and compound use, and has potential to increase pharmaceutical and chemical safety. The goal of the current study was to assess the assay's biomarkers of teratogenicity potential across a broader range of chemicals and migrate the method from a liquid chromatography high resolution mass spectrometry (LC-HRMS) based analysis to a simpler platform. Induced pluripotent stem (iPS) cells were exposed to 8 concentrations of 66 pharmaceutical, environmental and industrial compounds that have been associated with developmental toxicity or considered to be free of developmental toxicity. Spent media was collected and analyzed by LC-HRMS for biomarker discovery and confirmation. The assay was then migrated to a triple quadrupole (QqQ)-LC-MS platform, enabling targeted biomarker analysis and providing for simpler quantification. The results from initial comparisons of the two LC/MS platforms are highly correlated indicating similar assay performance across a broad series of chemical compounds tested.

## METHODS



- Cell Culture**
  - Expand iPS cells on Matrigel in mTeSR1
  - iPS cell line: HYR103 (ATCC)
  - Plate in 96-well assay plates at 100,000 cells/well
- Compound Exposure**
  - Expose iPS cells to compound for 48 hours
  - Collect spent media for LC-MS analysis
  - Analyze viability
- Sample Preparation**
  - Filter samples to remove molecules >10KDa
  - Dry and resubitize
- Mass Spectrometry**
  - Analyze spent media using LC coupled to a HR or QqQ MS with an ESI interface to determine levels of ornithine and cystine
  - Include stable labeled internal standards
- Data Analysis**
  - Calculate change for each metabolite in treated samples relative to DMSO controls
  - Determine o/c ratio
  - Fit a 4 parameter log-logistic model
  - Predict developmental toxicity potential



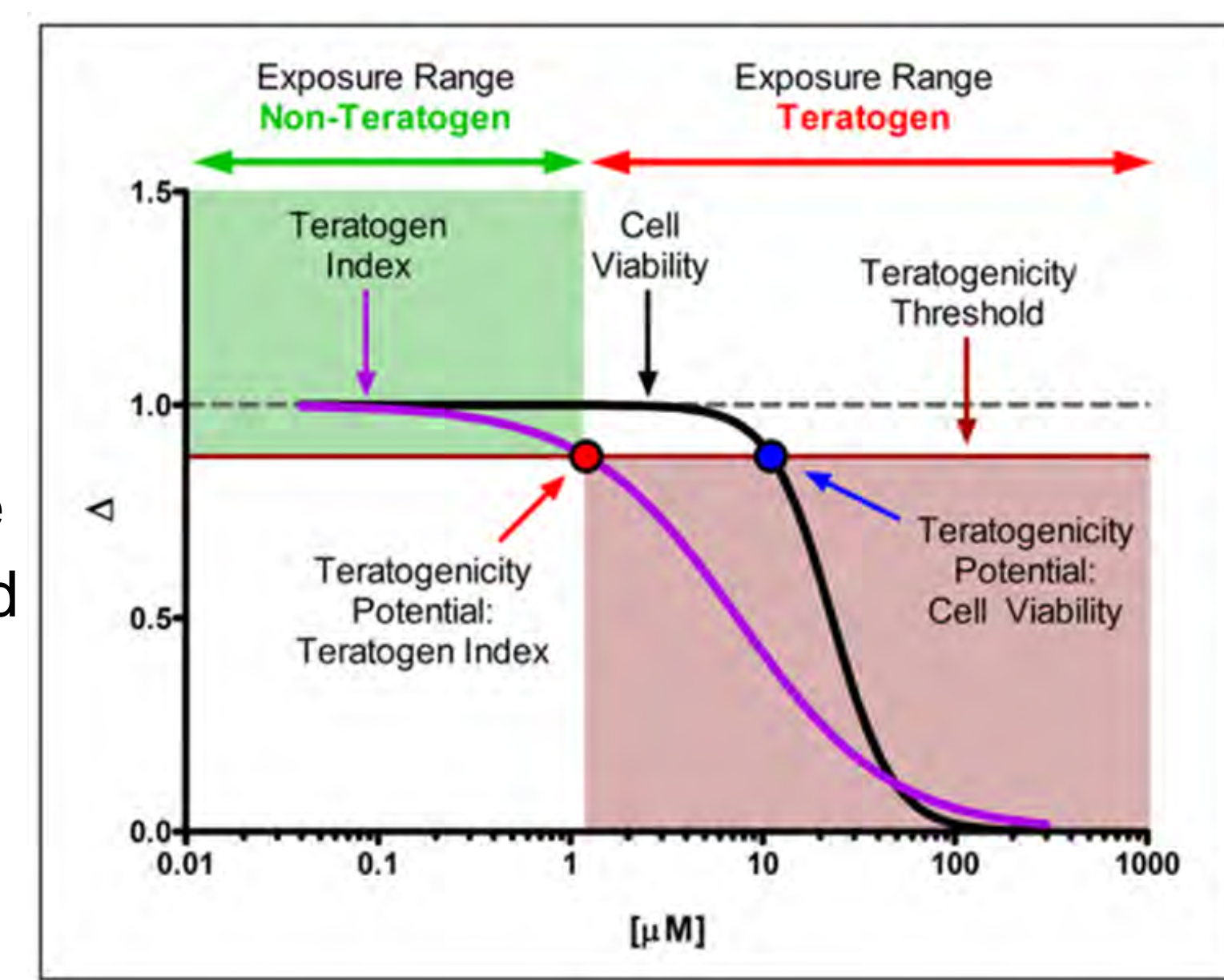
96-well plate layout for test compound evaluation. Each plate contains two test compounds (blue and purple colors) treated at 8 concentrations each, a reference treatment (grey) of 0.1% DMSO, and positive and negative control treatments to track assay performance (red and green, respectively).

## Exposure Based Prediction of Developmental Toxicity

Prediction of teratogenicity for unknown test compounds is based on where the dose-response curve for the o/c ratio crosses the teratogenicity threshold.<sup>1</sup>

### Biomarker-Based Endpoint

- Ornithine/Cystine (o/c) Ratio
- Dose-response curve (purple line) fit to a 4 parameter log-logistic non-linear model
- Concentration where a compound shows teratogenicity potential (red dot, TP) is where the curve crosses the teratogenicity threshold (red line)



### Cytotoxicity Endpoint

- Dose-response curve (black line) fit to a 4 parameter log-logistic non-linear model

### Used in combination with all other available data

- Prioritization of compound development
- Reduction in animal testing (REACH, Tox 21)

## RESULTS

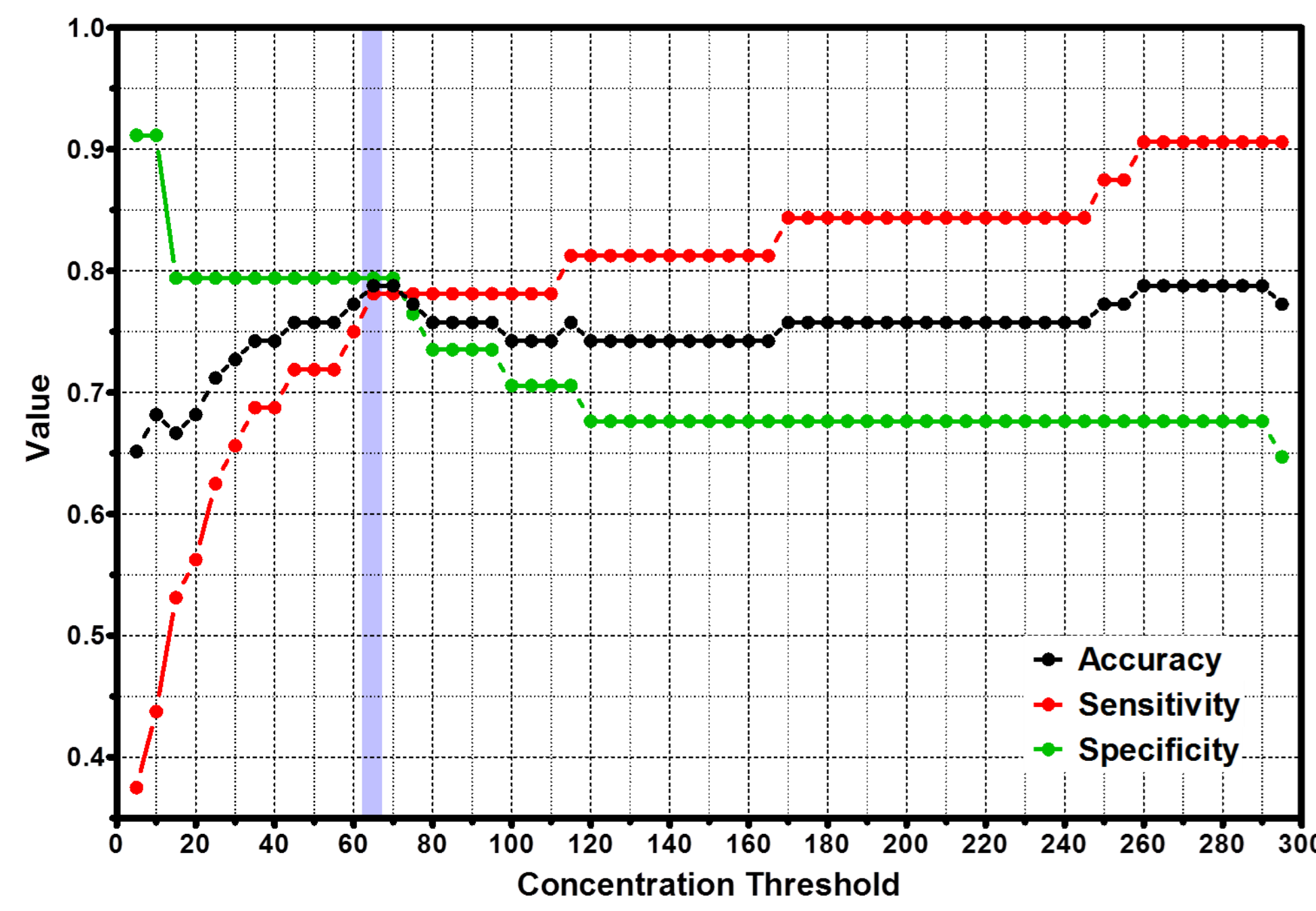
### The Assay Performs Well Across Compound Types

**Challenge:** Limited human exposure data for most environmental and industrial compounds to assess assay performance

### To Enable Assessment of the Assay:

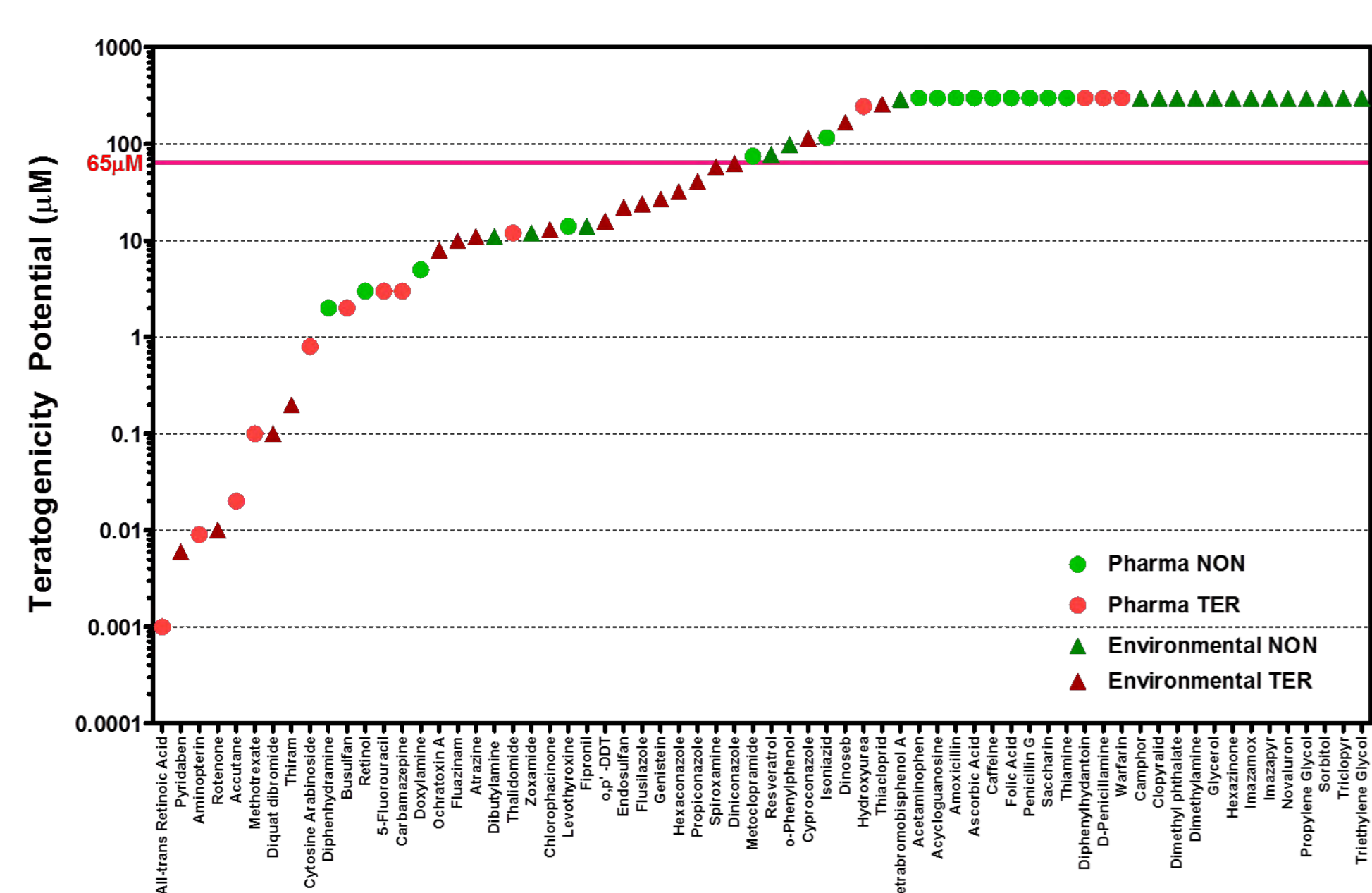
An optimal concentration threshold (Tx) for compound prediction was determined based on:

- Data from a combined training set of pharmaceutical, environmental and industrial compounds
- Known *in vivo* compound classifications
- Optimization to maximize sensitivity and specificity using the measured Teratogenicity Potential (TP) concentrations



### Performance Assessment using the Concentration Threshold:

- Tx = 65 μM
- Compound teratogenicity predictions were based on TP concentration.
- Magnitude of observed TP for known strong and weak teratogens supports development of a three class system for classification.
- For example, the previously classified strong teratogens 5-fluorouracil, all-trans retinoic acid, busulfan, cytosine arabinoside, methotrexate and ochratoxin A all have TP values <10 μM.
- This approach is not required for application of the assay to unknown compounds.



	# Correct/# Tested	Value
Accuracy	52/66	0.79
Sensitivity	25/32	0.78
Specificity	27/34	0.79

NON-TERATOGENS				TERATOGENS			
Compound	Chemical Classification	Prediction	Teratogenicity Potential (μM)	Compound	Chemical Classification	Prediction	Teratogenicity Potential (μM)
Camphor	Environmental	NON	300	Atrazine	Environmental	TER	11
Clopyralid	Environmental	NON	300	Chlorophacinone	Environmental	TER	13
Dibutylamine	Environmental	TER	11	Cyproconazole	Environmental	NON	115
Dimethyl phthalate	Environmental	NON	300	Diniconazole	Environmental	TER	63
Dimethylamine	Environmental	NON	300	Dinoseb	Environmental	NON	168
Flupyrrolid	Environmental	TER	14	Diquat dibromide	Environmental	TER	0.1
Glycerol	Environmental	NON	300	Endosulfan	Environmental	TER	22
Hexazinone	Environmental	NON	300	Fluazinam	Environmental	TER	10
Imazamox	Environmental	NON	300	Flusilazole	Environmental	TER	24
Imazapyr	Environmental	NON	300	Genistein	Environmental	TER	27
Novaluron	Environmental	NON	300	Hexaconazole	Environmental	TER	32
o-Phenylphenol	Environmental	NON	99	o,p'-DDT	Environmental	TER	16
Propylene Glycol	Environmental	NON	300	Ochratoxin A	Environmental	TER	8
Resveratrol	Environmental	NON	78	Propiconazole	Environmental	TER	41
Sorbitol	Environmental	NON	300	Pyridaben	Environmental	TER	0.006
Tetrabromobisphenol A	Environmental	NON	293	Rotenone	Environmental	TER	0.01
Tripropyl	Environmental	NON	300	Spiroxamine	Environmental	NON	58
Triethylene Glycol	Environmental	NON	300	Thiacloprid	Environmental	NON	260
Zoxamide	Environmental	TER	12	Thiram	Environmental	TER	0.2
Acetaminophen	Pharmaceutical	NON	300	5-Fluorouracil	Pharmaceutical	TER	3
Acycloguanosine	Pharmaceutical	NON	300	Accutane	Pharmaceutical	TER	0.02
Amoxicillin	Pharmaceutical	NON	300	All-trans Retinoic Acid	Pharmaceutical	TER	0.001
Ascorbic Acid	Pharmaceutical	NON	300	Aminopterin	Pharmaceutical	TER	0.009
Caffeine	Pharmaceutical	NON	300	Busulfan	Pharmaceutical	TER	2
Diphenhydramine	Pharmaceutical	TER	2	Carbamazepine	Pharmaceutical	TER	3
Doxylamine	Pharmaceutical	TER	5	Cytosine Arabinoside	Pharmaceutical	TER	0.8
Folic Acid	Pharmaceutical	NON	300	Diphenylhydantoin	Pharmaceutical	NON	300
Isoniazid	Pharmaceutical	NON	116	D-Penicillamine	Pharmaceutical	NON	300
Levothyroxine	Pharmaceutical	TER	14	Hydroxyurea	Pharmaceutical	NON	246
Metoprolamide	Pharmaceutical	NON	75	Methotrexate	Pharmaceutical	TER	0.1
Penicillin G	Pharmaceutical	NON	300	Thalidomide	Pharmaceutical	TER	12
Retinol	Pharmaceutical	TER	3	Warfarin	Pharmaceutical	NON	300
Saccharin	Pharmaceutical	NON	300				
Thiamine	Pharmaceutical	NON	300				

## CONCLUSIONS AND FUTURE DIRECTIONS

- The current study demonstrates that the biomarker-based assay performs well in across a diverse set of chemicals representing the pharmaceutical and chemical industries.
- A concentration threshold was determined to assess assay performance, however it is not necessary to use this threshold in application of the assay to unknown compounds. A test compound's teratogenic potential is based on the exposure level at which the test compound perturbs metabolism in a manner indicative of teratogenicity. This can be used in combination with all other available data for compound prioritization.
- The assay was 79% accurate in classifying potential developmental toxicants across a wide range of chemistries and applications when a concentration threshold of 65 μM was applied.
- The assay was easily transferred from a high resolution LC-MS platform to a triple-quadrupole platform, more commonly used in bio analytical laboratories worldwide.
- Results indicate that strong teratogens are more potent and easily identified using this assay. Many of the compounds have not yet been classified as strong or weak/moderate; however, the assay performs well in identification of teratogens overall.
- Future activities include determining predictivity and exposure windows for compound classification as strong, weak or non-teratogens based on the calculated teratogenicity potential.

<sup>1</sup>Our recent publication in *Birth defects research. Part B, Developmental and reproductive toxicology* describes development of this assay in hES cells.

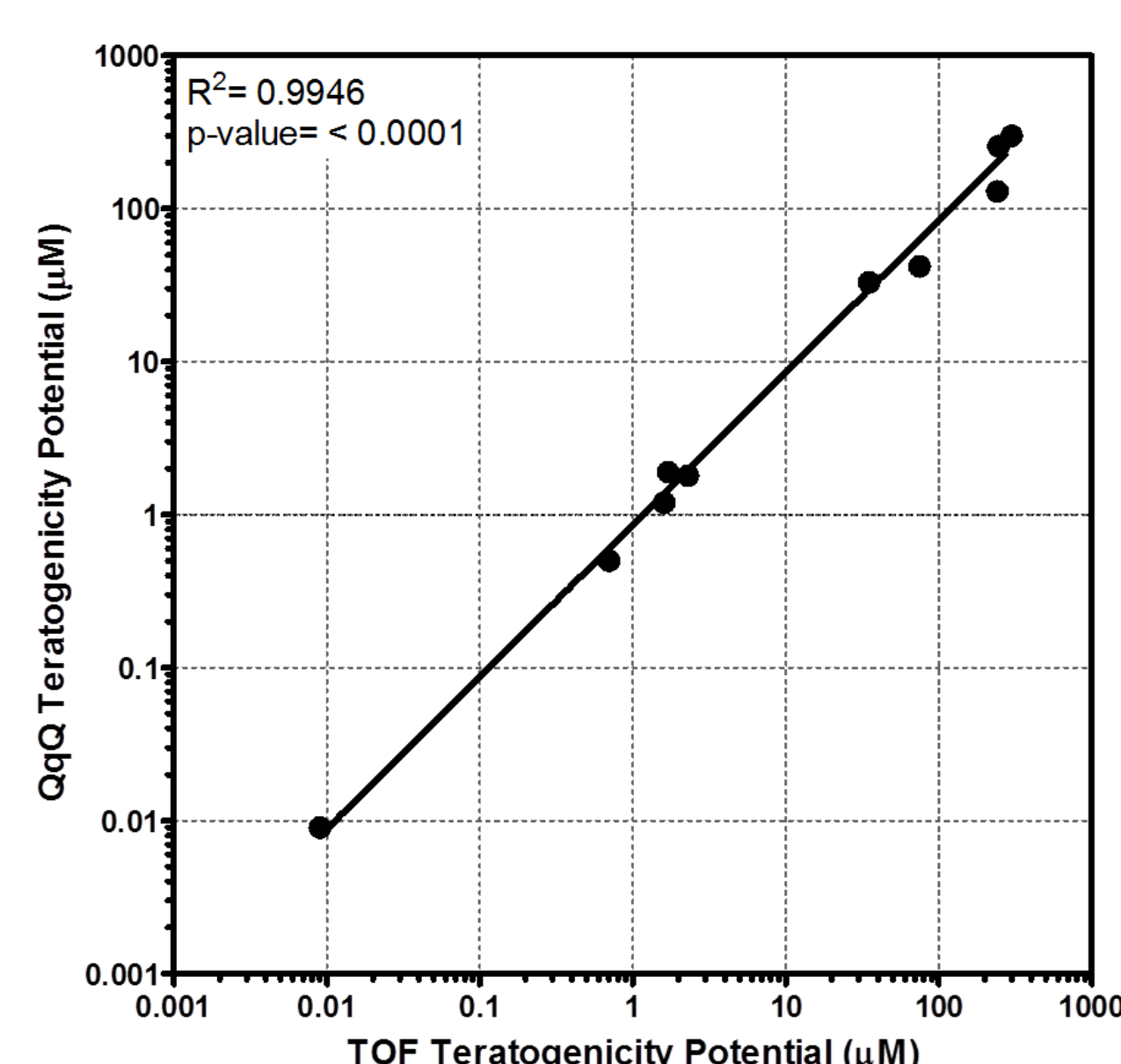
Palmer JA, et al. Establishment and assessment of a new human embryonic stem cell-based biomarker assay for developmental toxicity screening. *Birth Defects Res B Dev Reprod Toxicol.* 2013;**98**(4):343-363.

## ACKNOWLEDGEMENTS

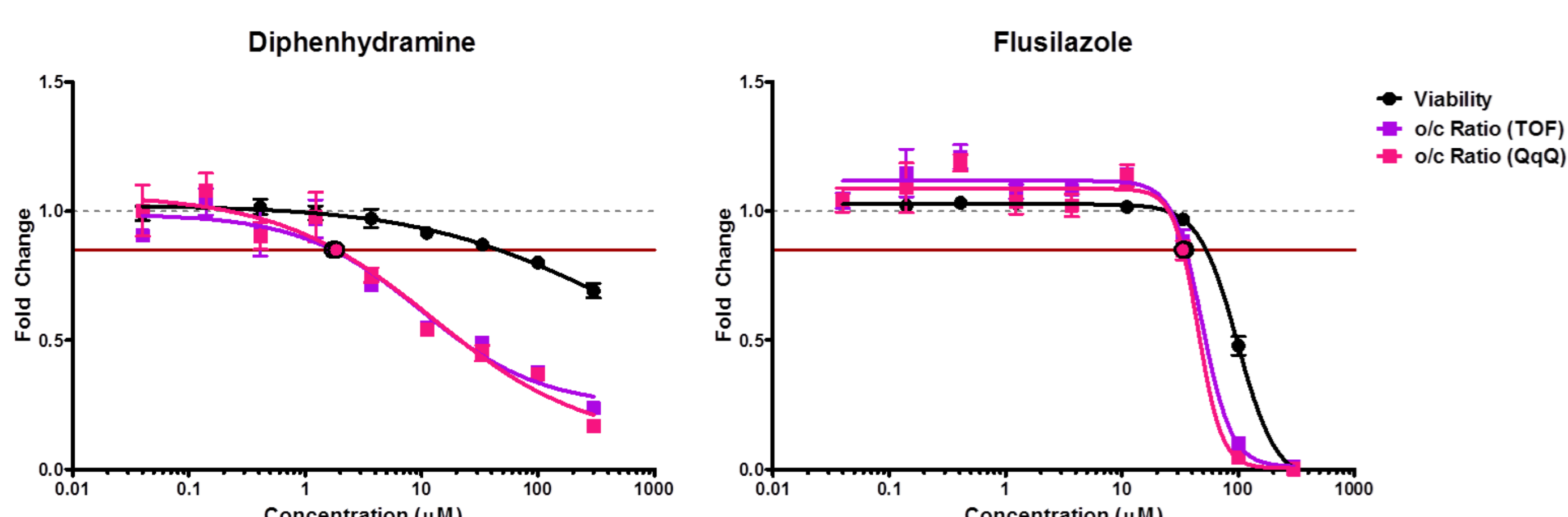
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### Excellent Performance Across Two Analytical Platforms

Representative teratogenicity potential concentrations from assay results measured using the TOF (x-axis) vs the QqQ (y-axis) for a subset of the training set compounds (n=12). Each point represents one compound.



### Predictions are the Same Independent of LC-MS Platform



Representative results comparing the performance of the o/c ratio between the TOF (purple points and curve) and QQQ (pink points and curve) platforms. Cell viability (black points and curve) is shown for reference. The points are the mean values and the error bars are the standard error of the mean.