United States Environmental Protection Agency

EPA's ToxCast Program: Covering the Mechanistic Space

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CBCRP
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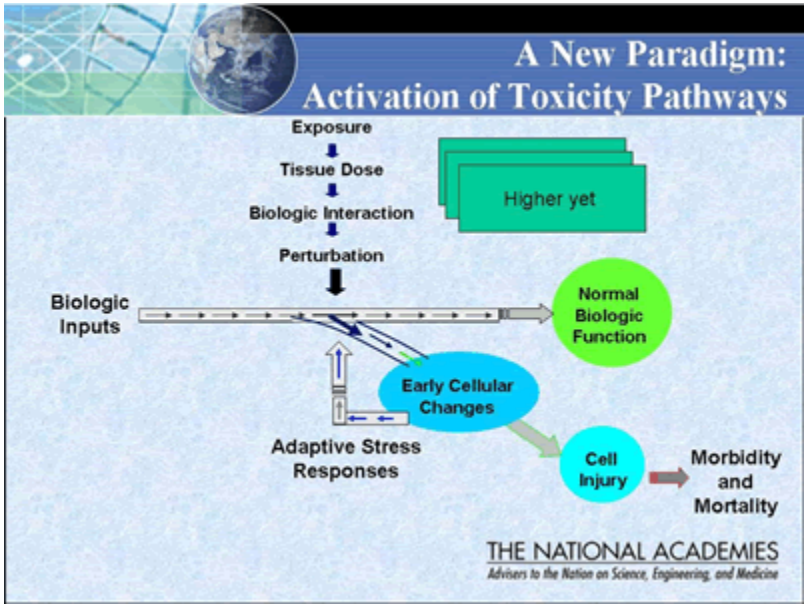
ToxCast / Tox21 Overall Strategy

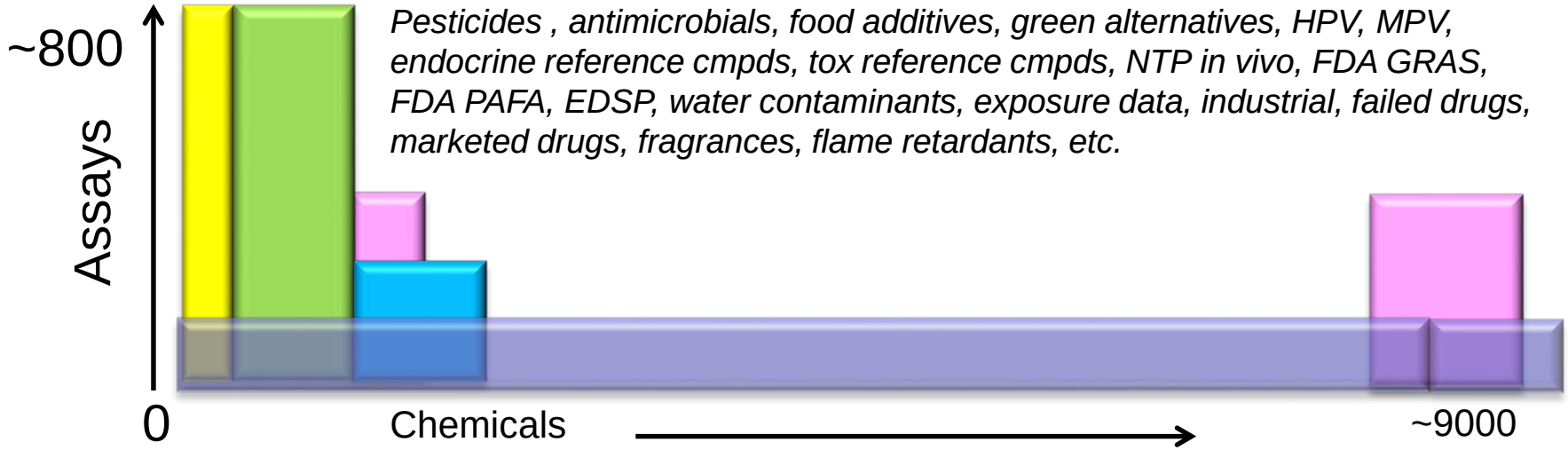
- Identify targets or pathways linked to toxicity (AOP focus)
- Identify/develop high-throughput assays for these targets or pathways
- Develop predictive systems models
  - *in vitro/in silico→ in vivo*
  - human focus
- Use predictive models (qualitative):
  - Prioritize chemicals for targeted testing
  - Suggest / distinguish possible AOP / MOA for chemicals
- *High-throughput Exposure Predictions*
- *High-throughput Risk Assessments*

TOXICITY TESTING IN THE 21ST CENTURY: A VISION AND A STRATEGY, NRC, 2007.



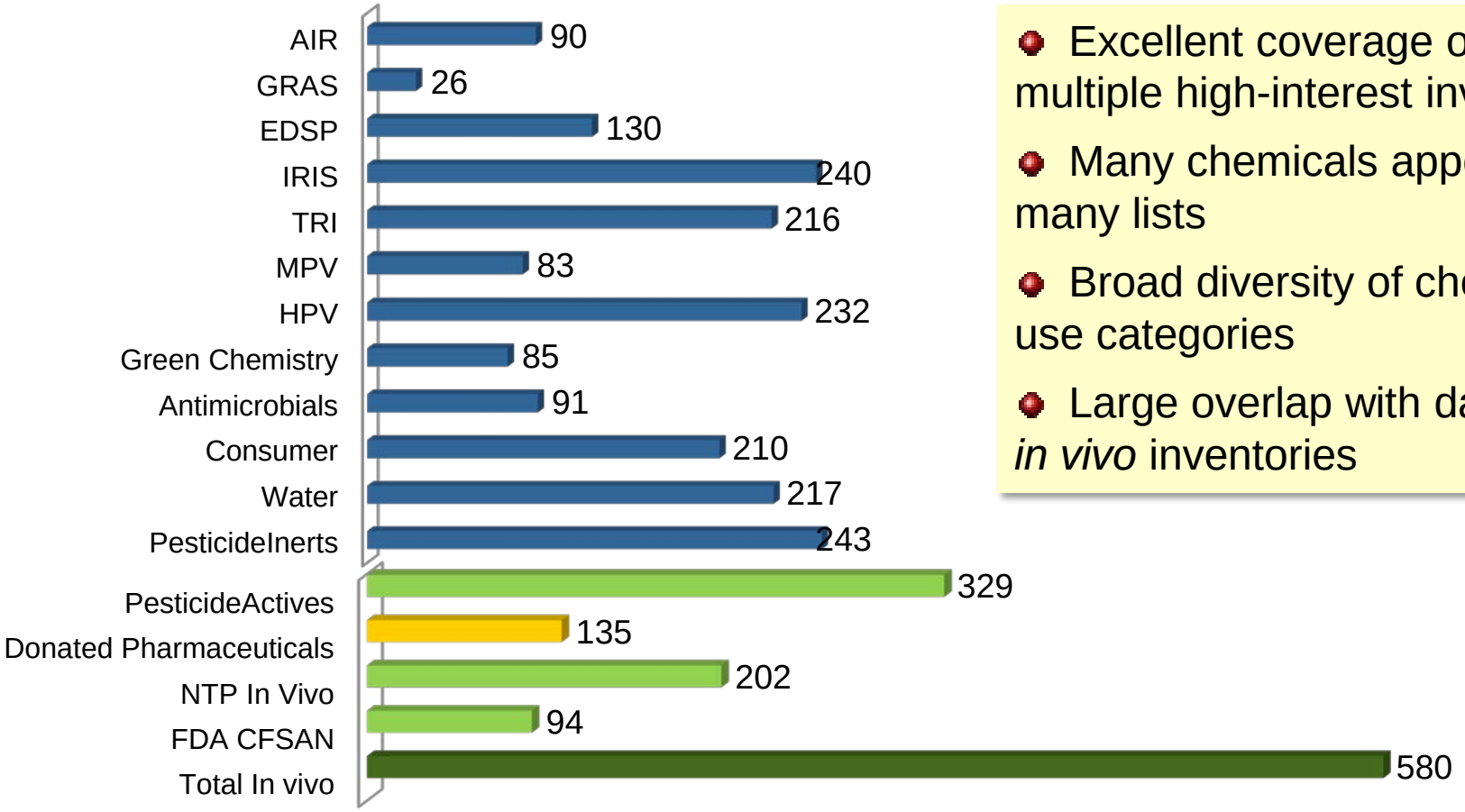
ToxCast & Tox21: Chemicals, Data and Release Timelines

Set	Chemicals	Assays	Endpoints	Completion	Available
ToxCast Phase I	293	~600	~700	2011	Now
ToxCast Phase II	767	~600	~700	03/2013	Now
ToxCast E1K	800	~50	~120	03/2013	Now
Tox21	~9000	~80	~150	In progress	Ongoing



Pesticides , antimicrobials, food additives, green alternatives, HPV, MPV, endocrine reference cmpds, tox reference cmpds, NTP in vivo, FDA GRAS, FDA PAFA, EDSP, water contaminants, exposure data, industrial, failed drugs, marketed drugs, fragrances, flame retardants, etc.

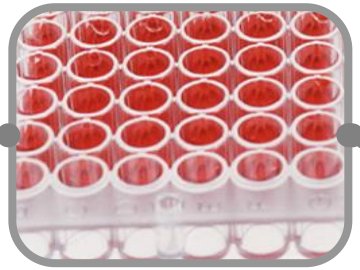
ToxCast PhI & PhII 1060: # Compounds per Inventory



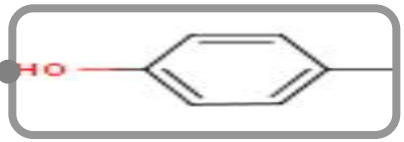
- Excellent coverage of multiple high-interest inventories
- Many chemicals appear on many lists
- Broad diversity of chemical-use categories
- Large overlap with data-rich *in vivo* inventories

Hazard Predictions for Prioritization: High-Throughput Screening (HTS)

Robots

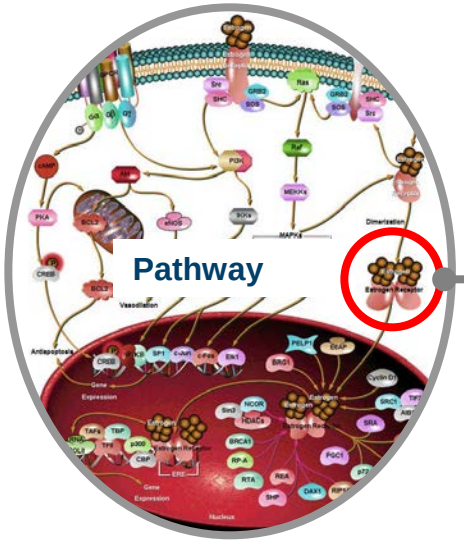


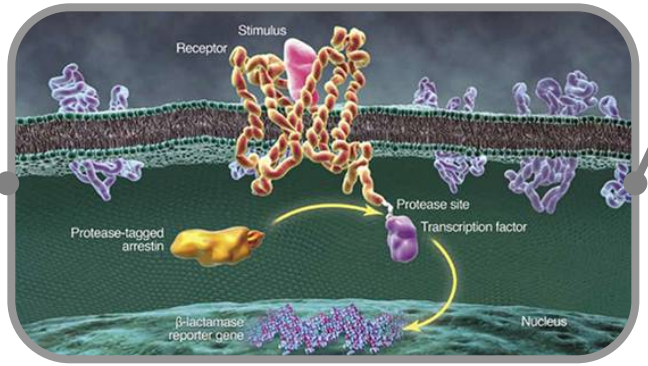
96-, 384-, 1536 Well Plates



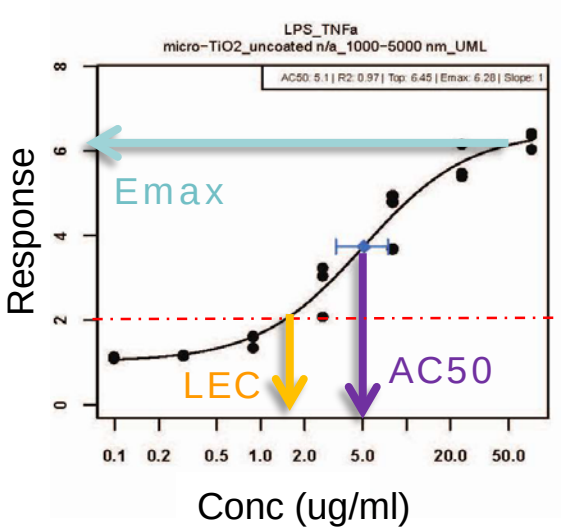
Chemical Exposure

Cell Population

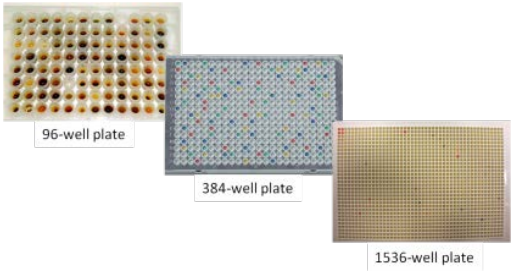




Target Biology (e.g., Estrogen Receptor)



ToxCast Assays (>700 endpoints)



Assay Provider
ACEA
Apredica
Attagene
BioReliance
BioSeek
CeeTox
CellzDirect
Tox21/NCATS
NHEERL MESC
NHEERL Zebrafish
NovaScreen (Perkin Elmer)
Odyssey Thera
Vala Sciences

Biological Response
cell proliferation and death
cell differentiation
Enzymatic activity
mitochondrial depolarization
protein stabilization
oxidative phosphorylation
reporter gene activation
gene expression (qNPA)
receptor binding
receptor activity
steroidogenesis

Target Family
response Element
transporter
cytokines
kinases
nuclear receptor
CYP450 / ADME
cholinesterase
phosphatases
proteases
XME metabolism
GPCRs
ion channels

Assay Design
viability reporter
morphology reporter
conformation reporter
enzyme reporter
membrane potential reporter
binding reporter
inducible reporter

Readout Type
single
multiplexed
multiparametric

Cell Format
cell free
cell lines
primary cells
complex cultures
free embryos

Species
human
rat
mouse
zebrafish
sheep
boar
rabbit
cattle
guinea pig

Tissue Source

Lung	Breast
Liver	Vascular
Skin	Kidney
Cervix	Testis
Uterus	Brain
Intestinal	Spleen
Bladder	Ovary
Pancreas	Prostate
Inflammatory	Bone

Detection Technology
qNPA and ELISA
Fluorescence & Luminescence
Alamar Blue Reduction
Arrayscan / Microscopy
Reporter gene activation
Spectrophotometry
Radioactivity
HPLC and HPEC
TR-FRET

List of assays and related information at: **http://www.epa.gov/ncct/**

ToxCast Results: 1051 Chemicals x 791 Assay Readouts

United States Environmental Protection Agency

ACEA: red
Attagene: orange
Apredica: black
BioSeek: green
Novascreen: gray
Tox21: violet
OT: blue

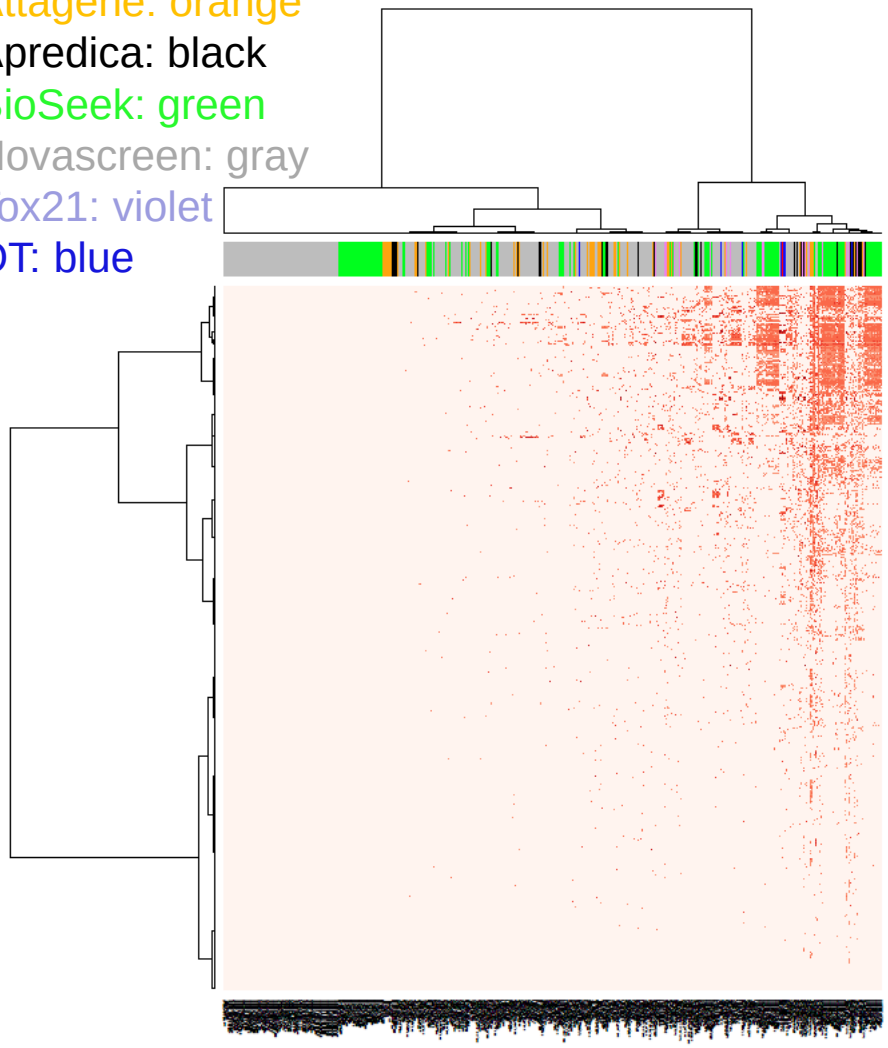
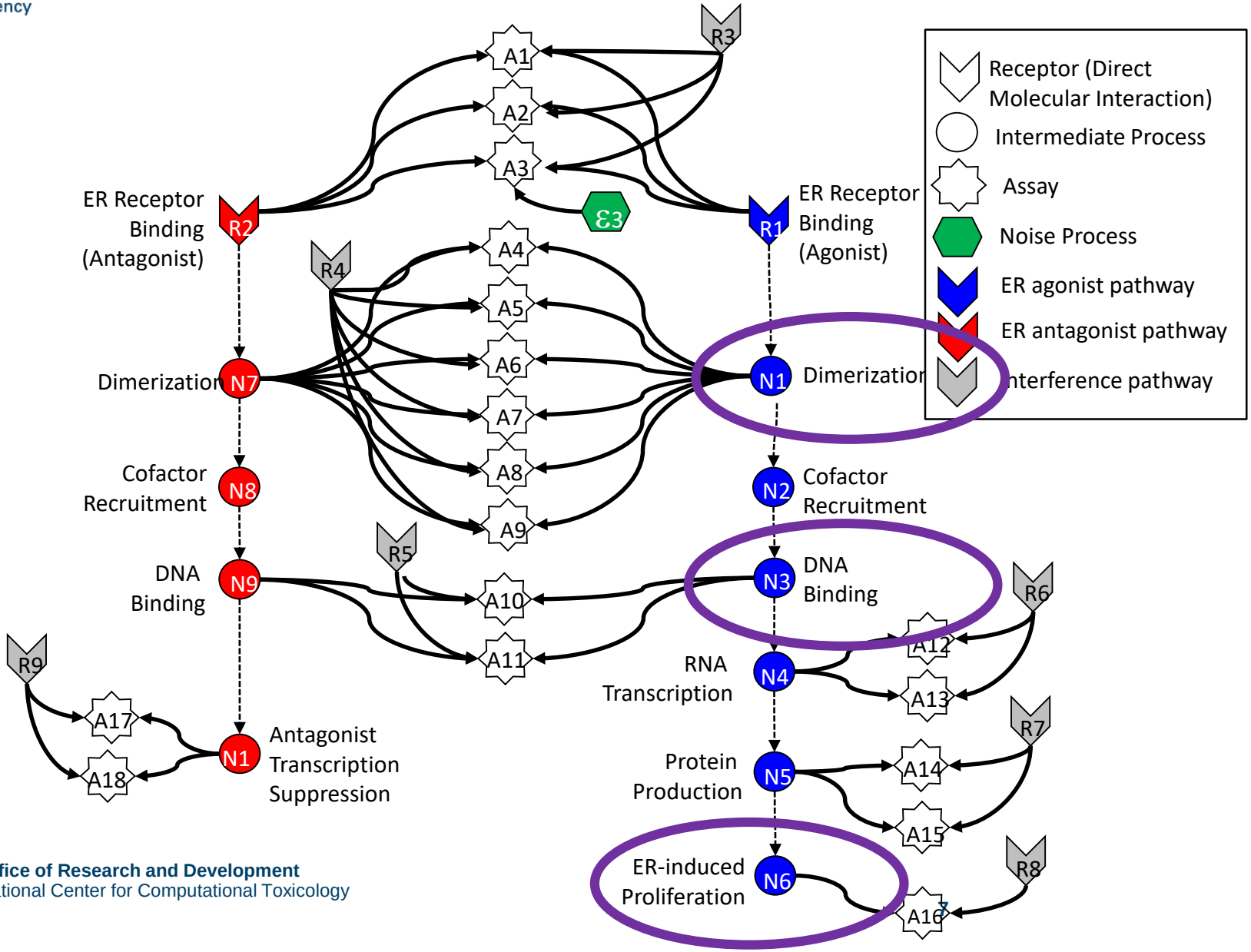


Table 2 Top 20 most promiscuous chemicals[a]			
		AC50s	
Chemical Name	Total	<=10µM	<=1µM
Phenylmercuric acetate	90	47	20
Mancozeb	88	41	13
Gentian violet	86	51	5
Sodium dodecylbenzenesulfonate	82	19	0
Tributyltin methacrylate	79	48	12
Tributyltin chloride	77	45	9
Mercuric chloride	73	45	14
Perfluorooctane sulfonic acid	72	13	2
{4-[3-(aminomethyl)phenyl]piperidin-1-yl}{5-[(2-fluorophenyl)ethynyl]furan-2-yl}methanone (pharma)	71	25	4
Dodecylbenzene sulfonate triethanolamine (1:1)	66	7	1
SSR241586 (pharma)	66	30	8
Emamectin benzoate	65	14	2
{4-[5-(aminomethyl)-2-fluorophenyl]piperidin-1-yl}(4-bromo-3-methyl-5-propoxythiophen-2-yl)methanone hydrochloride (pharma)	64	19	2
(1R)-1-[(ethoxycarbonyl)oxy]ethyl 1-{[5-(5-chlorothiophen-2-yl)-1,2-oxazol-3-yl]methyl}-2-{[1-(propan-2-yl)piperidin-4-yl]carbamoyl}-1H-indole-5-carboxylate hydrochloride(pharma)	63	29	2
Maneb	62	31	16
SSR150106 (pharma)	62	41	13
Didecyl dimethyl ammonium chloride	62	30	2
Zamifenacin (pharma)	60	27	11
SSR125047 (pharma)	59	16	3
Metiram	56	16	4

Sipes *et al*., Chem Res Toxicol. 26:878-95, 2013

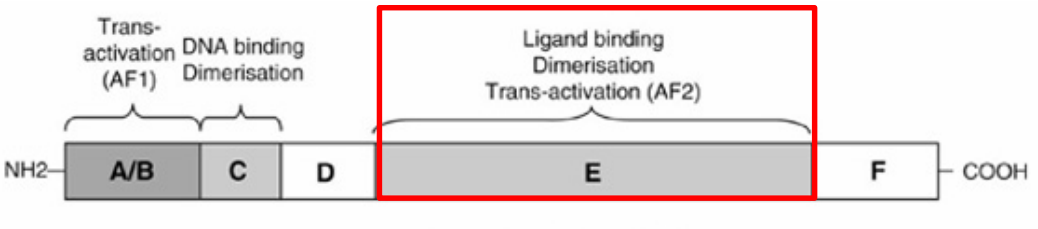
ER Pathway Model



ER LBD PCAs: novel tools for quantitatively assessing the effects of estrogenic and anti-estrogenic compounds on estrogen receptor homo- and heterodimers

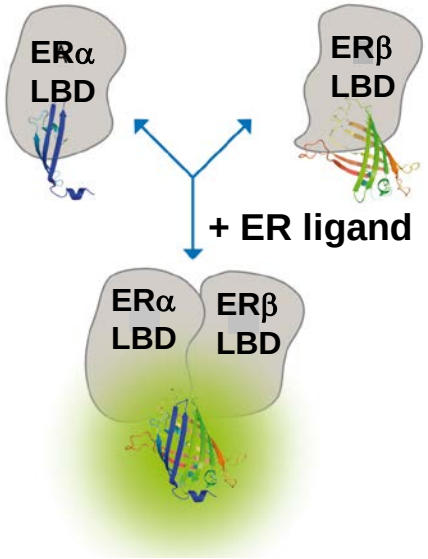
United States Environmental Protection Agency

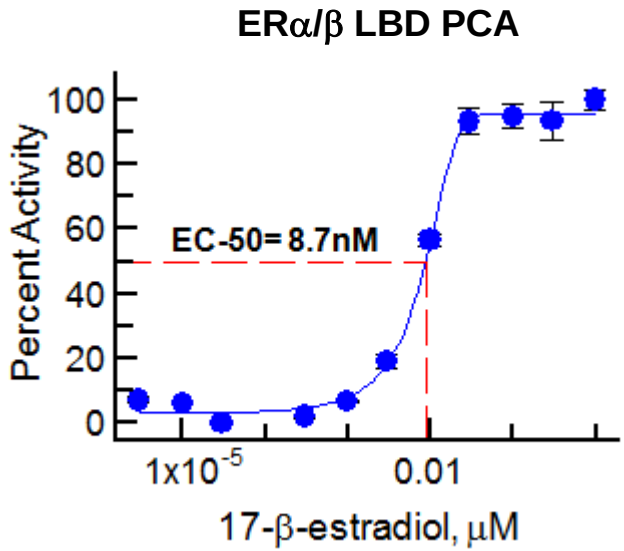
Domain structure of Estrogen Receptor



Concept: Ligand binding domain of ER α and/or β fused to fragments of YFP

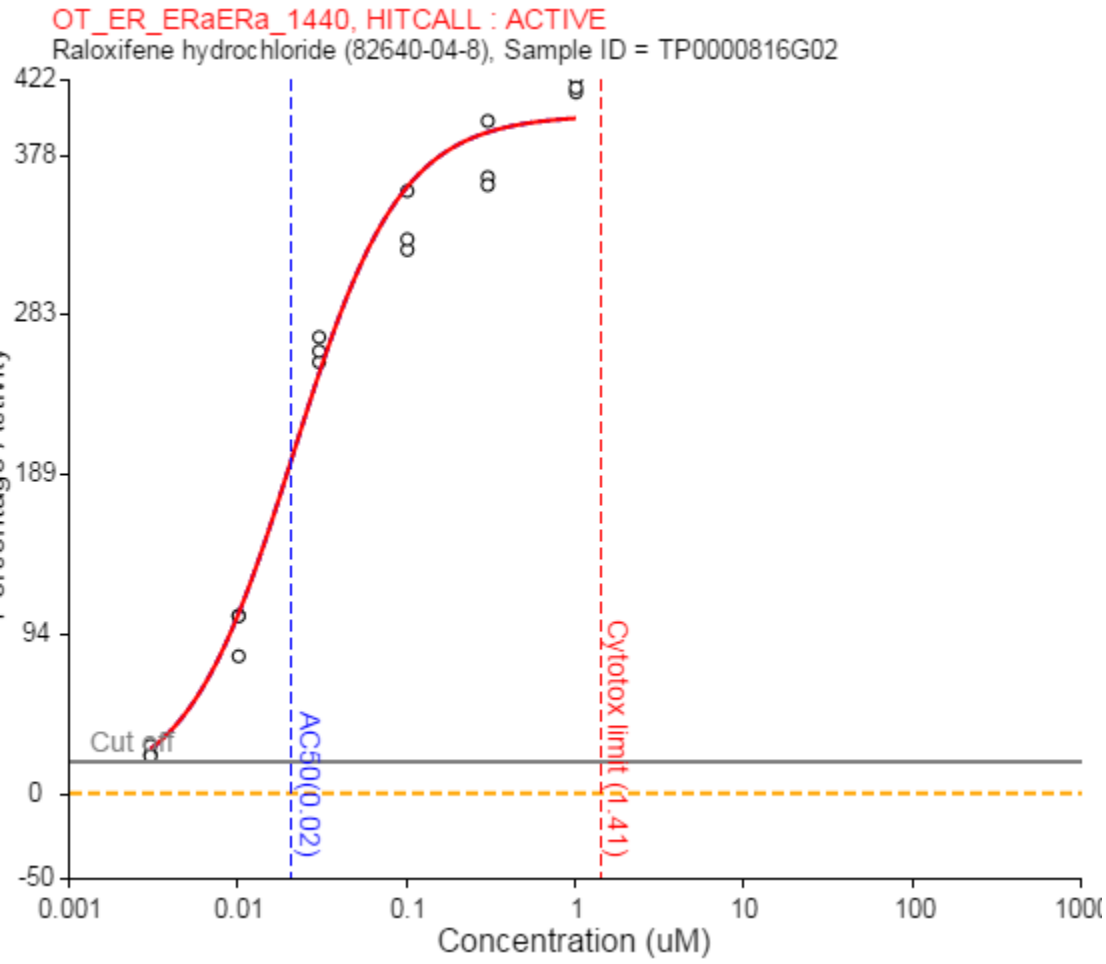
ER homo- and heterodimers display ligand-selective activity leading to a unique but overlapping set of dimer-mediated effects

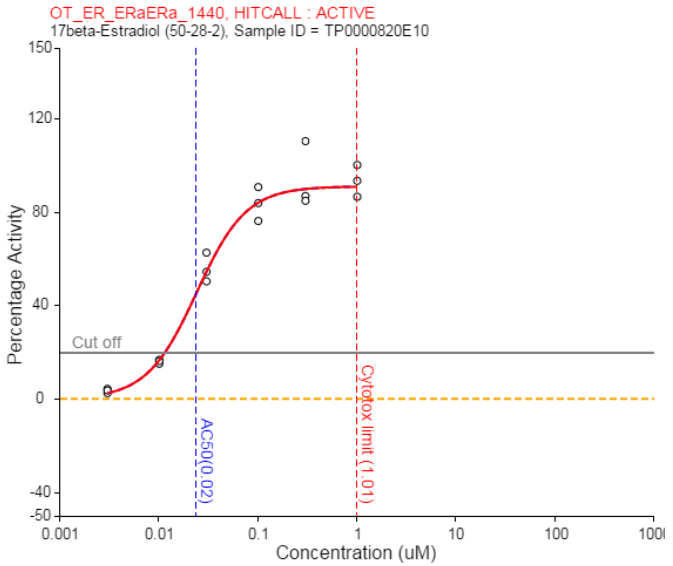




All ER LBD assays performed in phenol red-free medium containing 10% charcoal-stripped FBS.

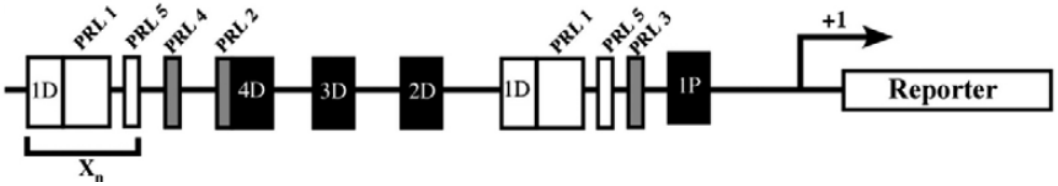
Example Data: Raloxifene & β-estradiol ERα:ERα 24 hr

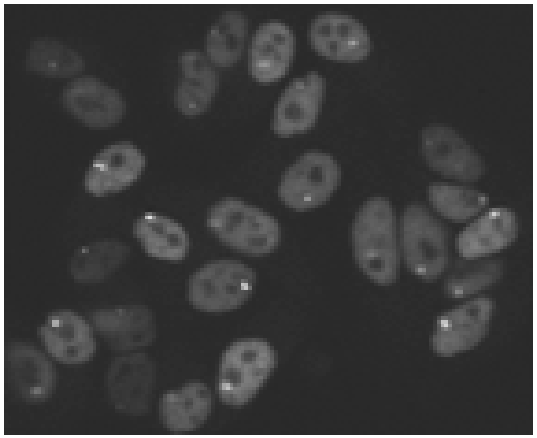


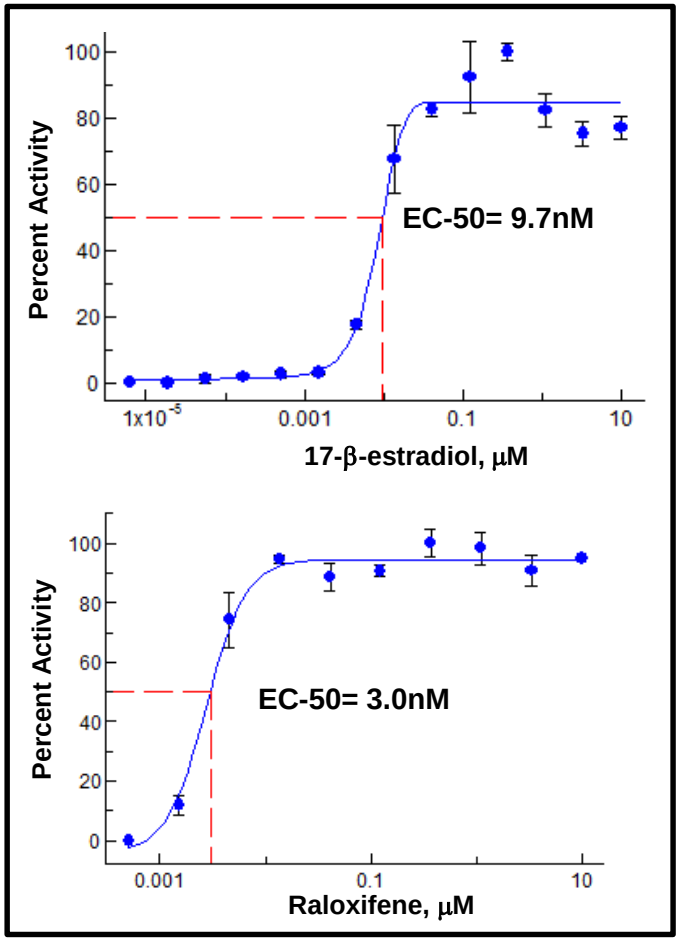


High Content Analysis of Estrogen Receptor Binding to EREs

Macroscopic prolactin array allows for the visualization of full length GFP-ER loading onto a genomically incorporated promoter in HeLa cells





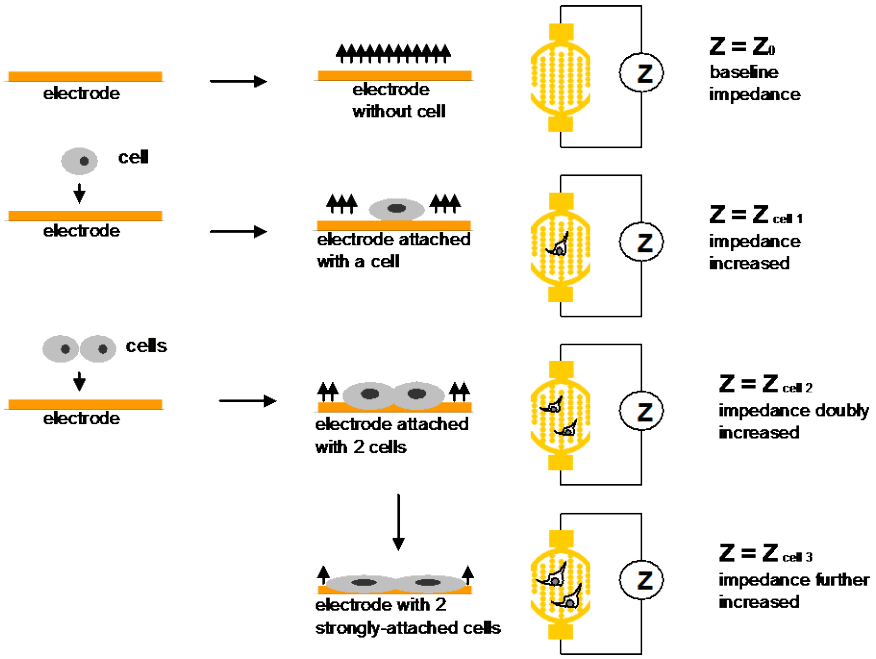


- Stable cell line (in HeLa cells) expressing very low levels of GFP-tagged full length ERα and an integrated locus of many prolactin repeats (ERE)
- GFP-ERα loads onto the array in response to ER agonists and antagonists (typically at low nanomolar concentrations), detected as a discrete spot (see image above) in the nucleus
- 'Number of Spots' is used as the metric to quantify responses to compounds in this assay
- Assay detects both agonists and antagonists; post-processing to differentiate mode of action is possible
- Assay can also be formatted to quantify recruitment of co-regulators (e.g. SRC-3

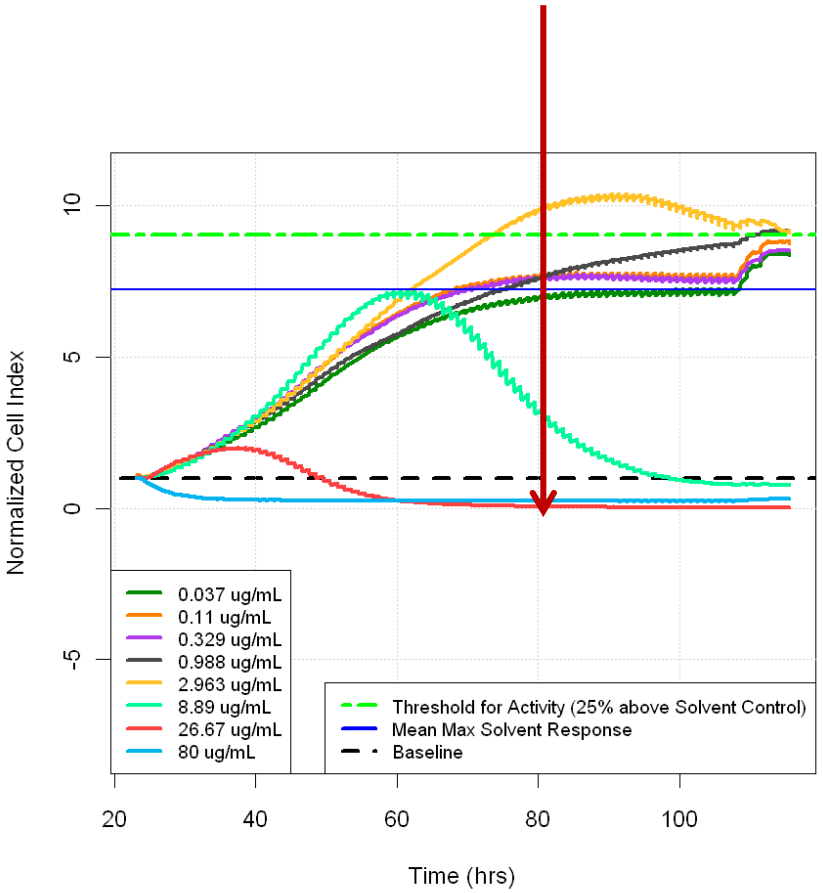
ACEA: Real Time Cell Growth Kinetics

United States Environmental Protection Agency

- Human T47D breast carcinoma cell line
  - Estrogen-responsive
  - Measured both increased and decreased proliferation
- Concentration-response testing
  - 8 conc/3-fold serial dilutions
  - Duplicate wells
- Positive controls: E2 and MG132
- Real-time measurements during exposure (0-72 hr)
- AC50s calculated for both increased and decreased proliferation using one time point (80 hr)

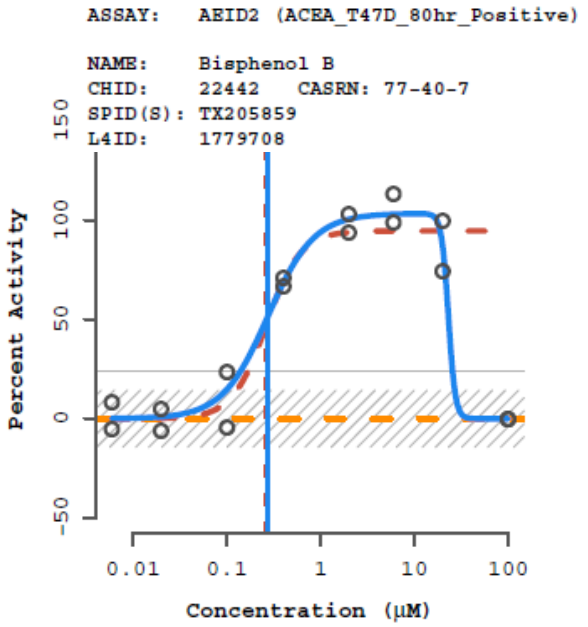


Proliferation and Cytotoxicity Measured

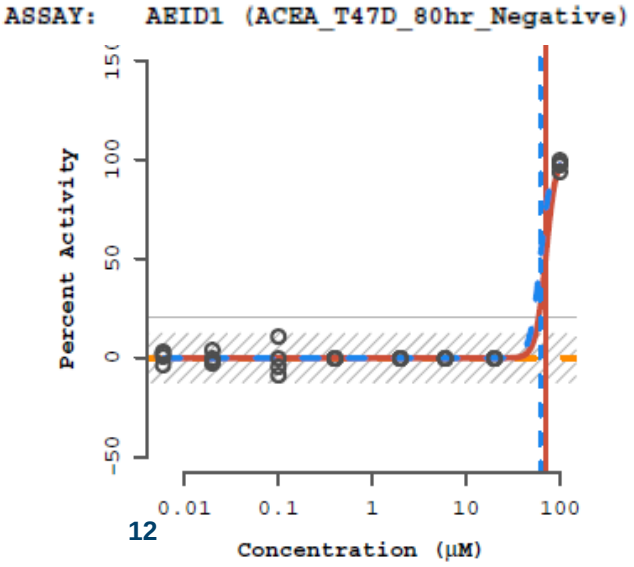


T47D Human Mammary Cancer Cell Line

Proliferation measured and scaled to E2 positive control

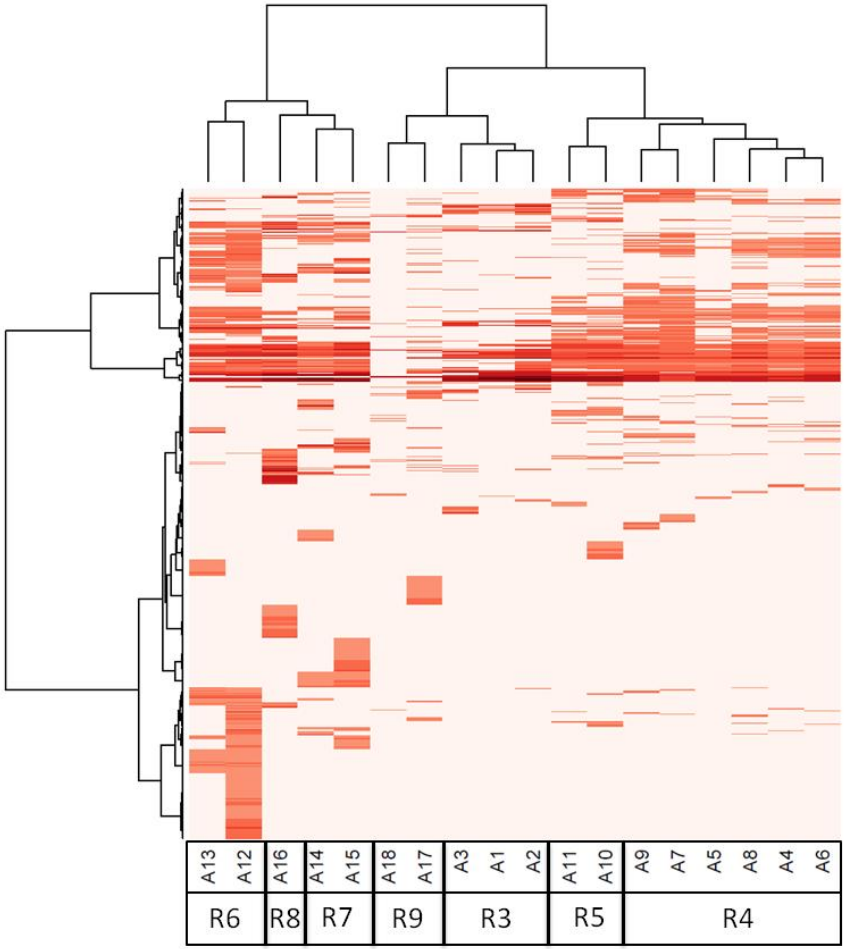


Cytotoxicity measured and scaled to MG132



Major theme – all assays have false positives and negative

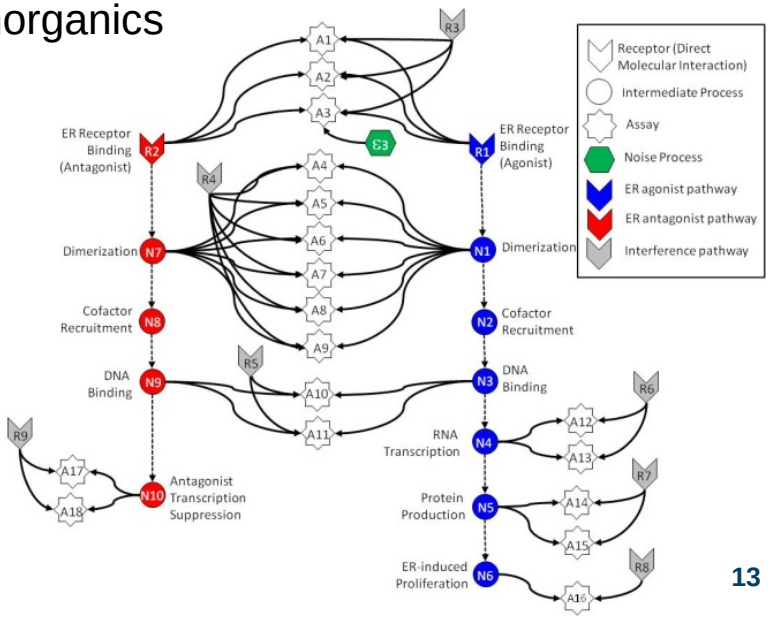
Assays cluster by technology, suggesting technology-specific non-ER activity



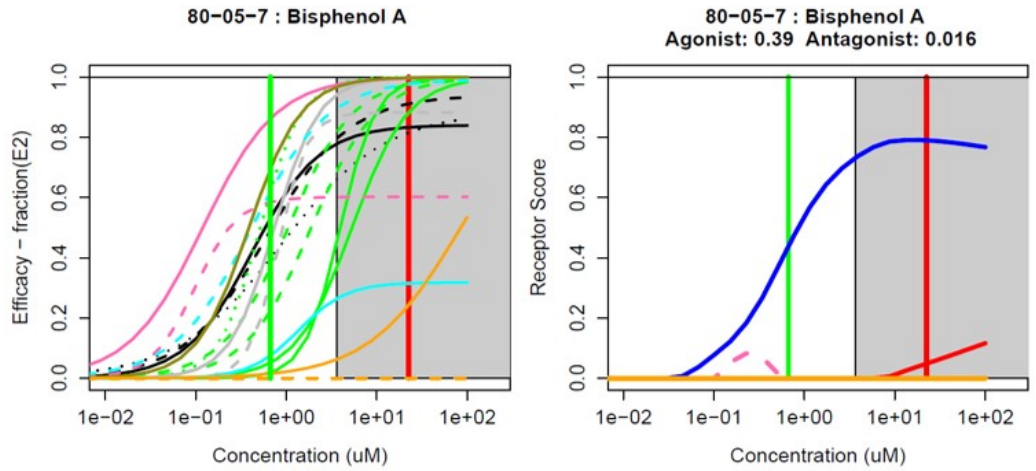
Much of this “noise” is reproducible, i.e. it is “assay interference”

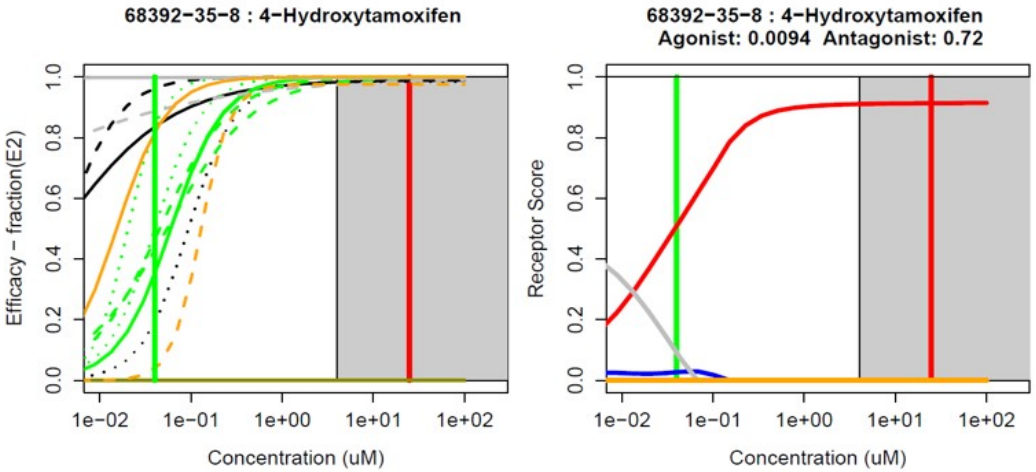
Result of interaction of chemical with complex biology in the assay

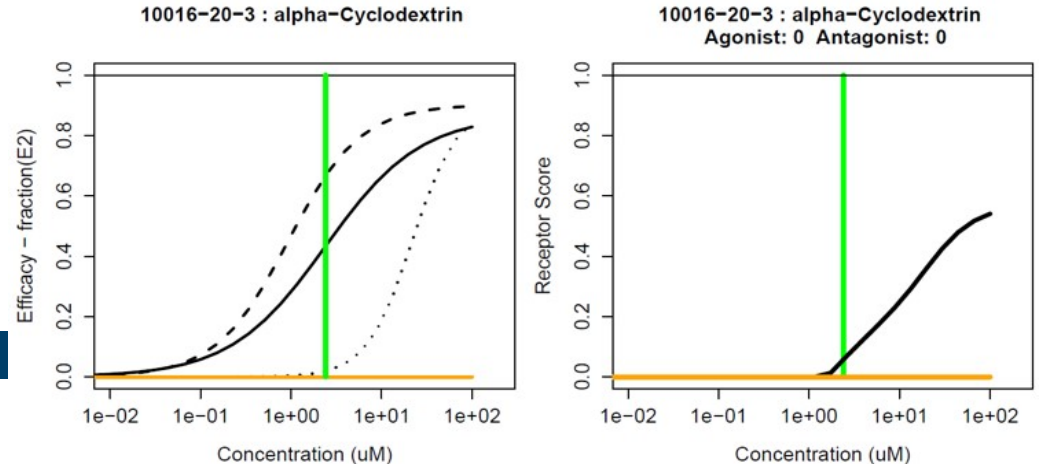
Our chemical library is only partially “drug-like”
-Solvents
-Surfactants
-Intentionally cytotoxic compounds
-Metals
-Inorganics

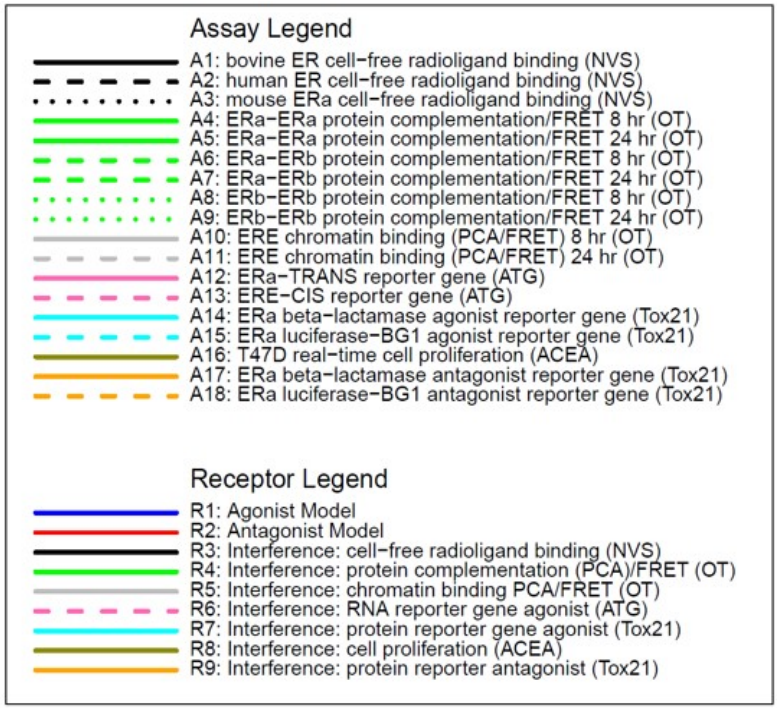


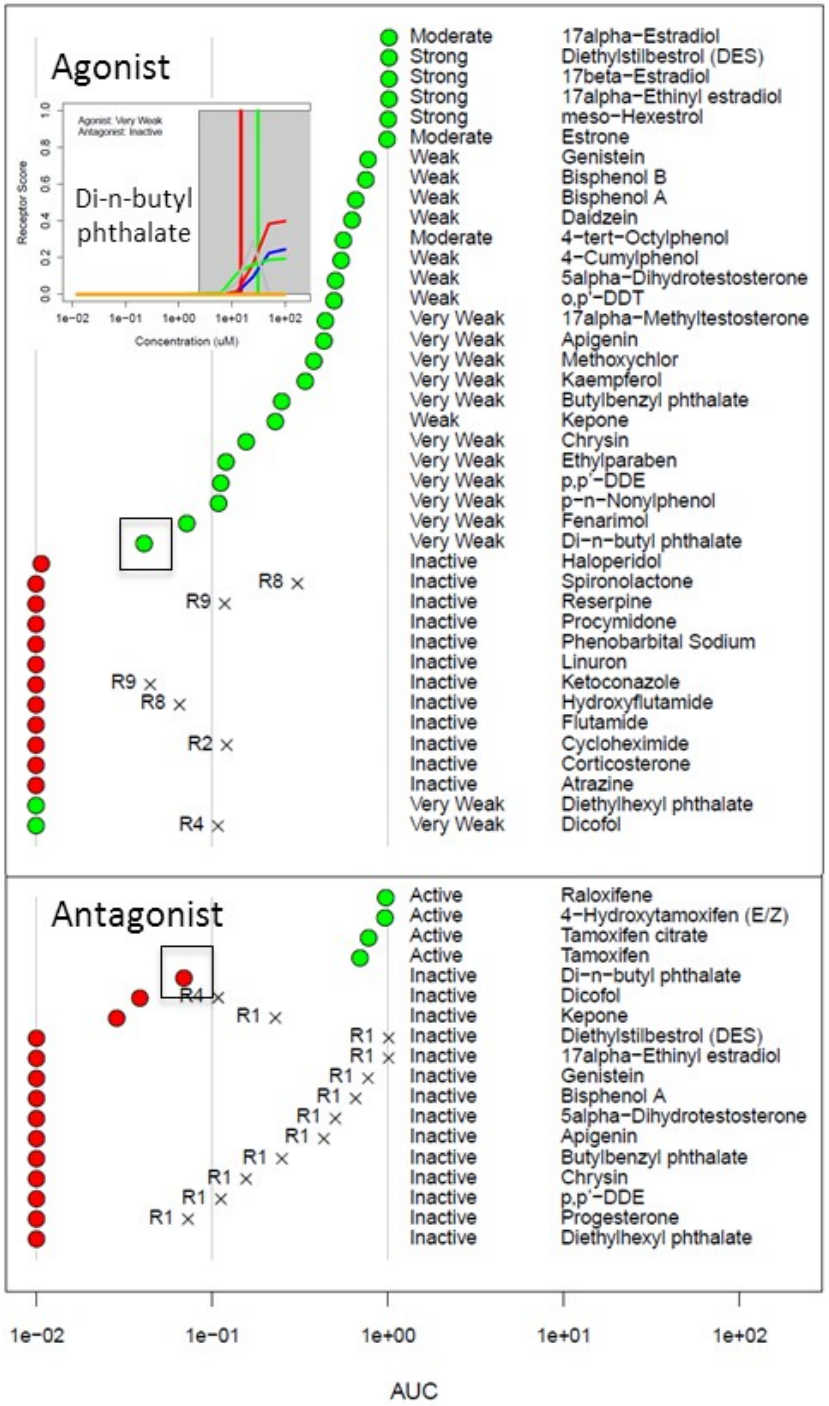
Example Agonist, Antagonist, Interference Chemicals



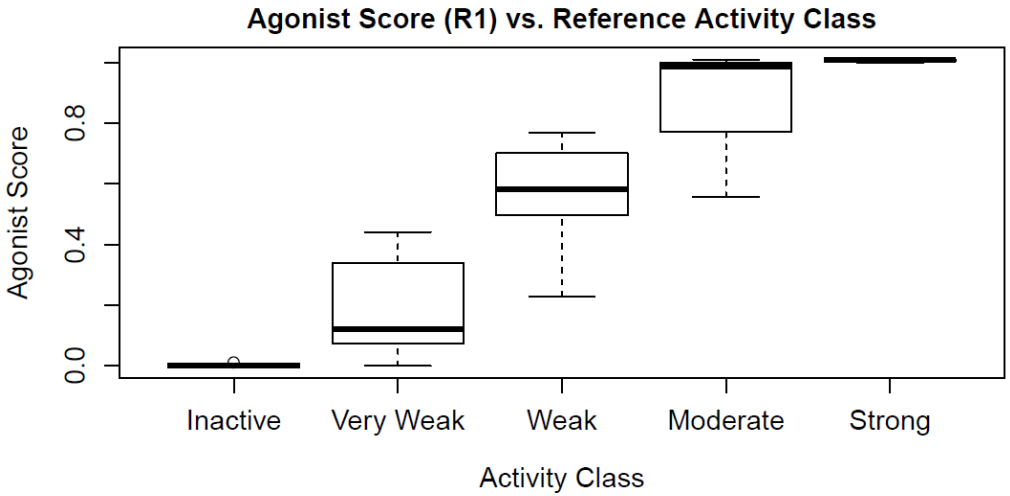






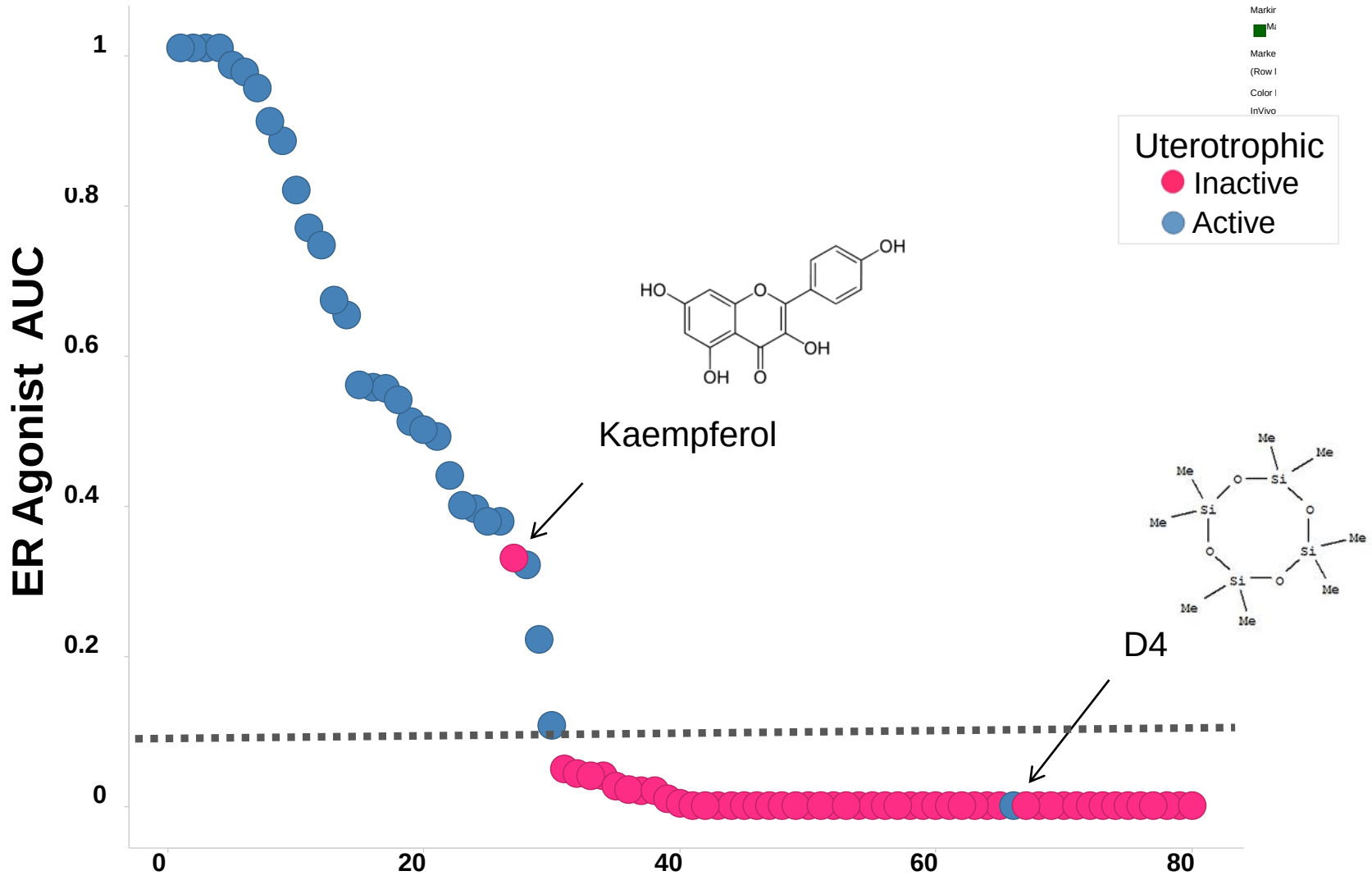


In Vitro Reference Chemical Performance



Judson et al., Tox Sci 2015

ER Agonist AUC vs Uterotrophic Outcomes



Browne et al., Environ. Sci. Technol., **2015**

United States Environmental Protection Agency

"The approach incorporates validated high-throughput assays and a computational model and, based on current research, can serve as an alternative for some of the current assays in the Endocrine Disruptor Screening Program (EDSP) Tier 1 battery."

Public Data Access using iCSS Dashboard

www.actor.epa.gov/dashboard/

ToxCast/Tox21 Relevant Assays

- ER pathway assays (functional cellular and binding)
- AR pathway assays (functional cellular and binding)
- PR binding
- Aromatase inhibition (functional cellular and enzyme inhibition)
- T47D cell proliferation
- H295R cell steroidogenesis
- NRF2/ARE oxidative stress (multiple cell lines and formats)

Breast Cancer-Relevant Gaps

- Targeted Testing
  - PR functional assay (existing hPR and bPR binding assay for ToxCast chemicals)
  - Others?

- Non-Targeted Testing
  - Global transcriptomics
    - pilot work in progress (SOT presentation)
    - multiple cell types (to be determined)
    - Multiple exposure times, multi-concentration
    - NGS (RNA-seq) approach

- Phenotypic Screening
  - Suppression of apoptosis
  - Alteration of cell cycle
  - Proliferation in physiologically relevant cell model

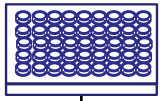
Assay Nominations Welcome!

- **Tox21 Assay Nomination Form:**
- **Date:**
- **Name:**
- **Organization:**
- **Contact Information:**
- **Assay Name:**
- **Biological/Toxicity Pathway:**
- **Relevance to Tox21:**
- **Critical Factors for Assay Success:**
- **Assay Technology:**
- **Assay Source:**
- **Assay Format:**
- **Reference Compounds:**
- **Validation Status:**
- **Estimated Major Costs:**

Multi-Path Strategy for Retrofitting ToxCast Assays with Metabolic Competence

"Extracellular" Strategy

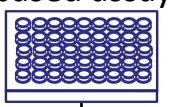
Capable of metabolizing chemicals in the media or buffer of cell-based and cell-free assays



More closely models effects of hepatic metabolism and generation of circulating metabolites

"Intracellular" Strategy

Capable of metabolizing chemicals inside the cell in cell-based assays



More closely models effects of target tissue metabolism

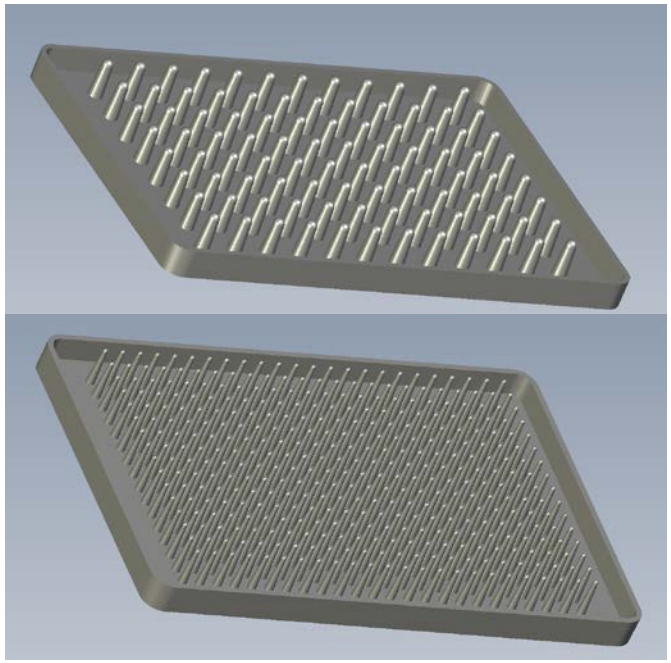
Integrated approach to model *in vivo* metabolic bioactivation and detoxification

Metabolic Competence Project Group:

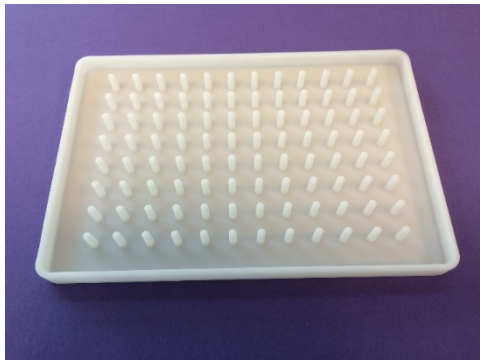
Steve Simmons (PI)

Danica DeGroot (Postdoc)

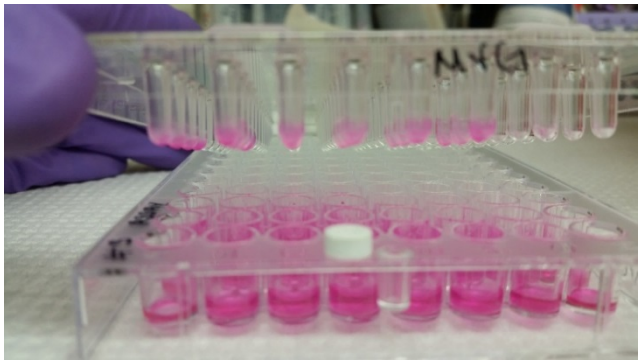
Progress on Retrofitting In Vitro Assays for Metabolic Competence – Extracellular Strategy



CAD Drawings of First Generation Plate Lids

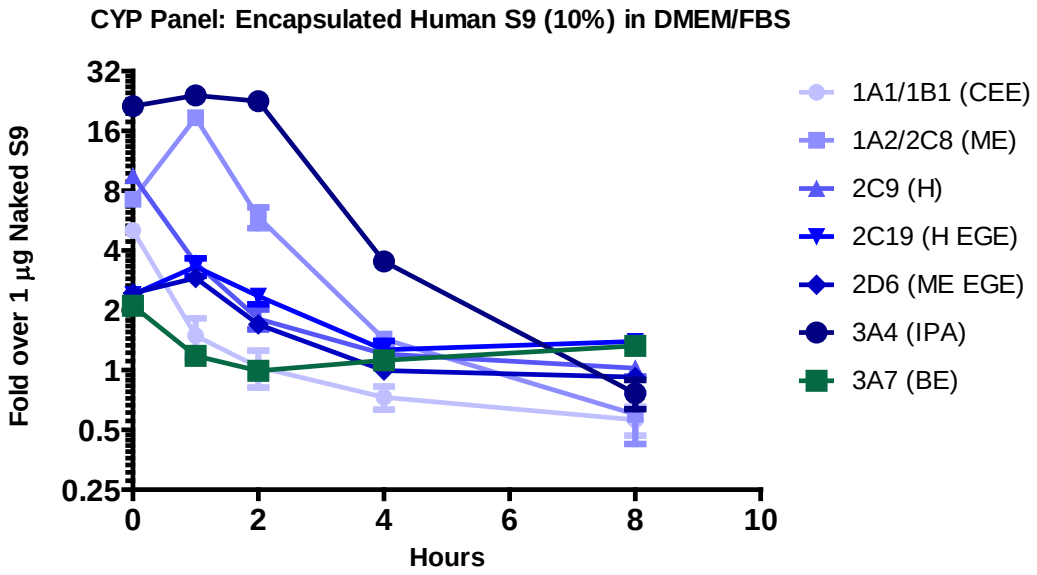


Prototype lids constructed

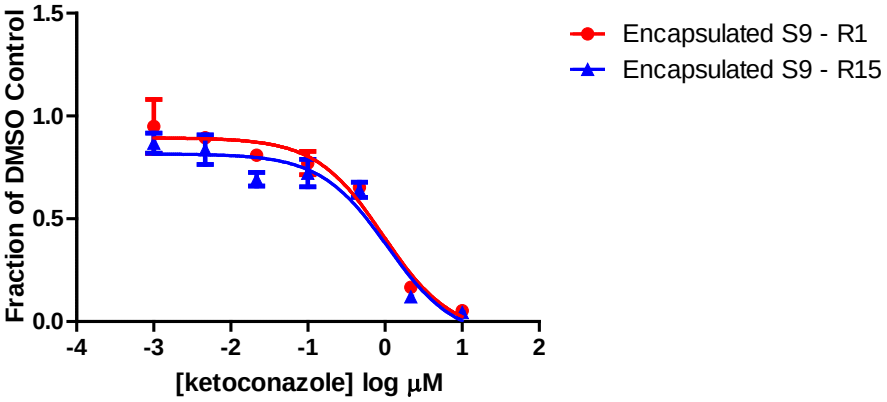


Alginate microspheres bound to polystyrene posts

Human liver S9 can be encapsulated in alginate and is metabolically active for hours under typical cell culture conditions

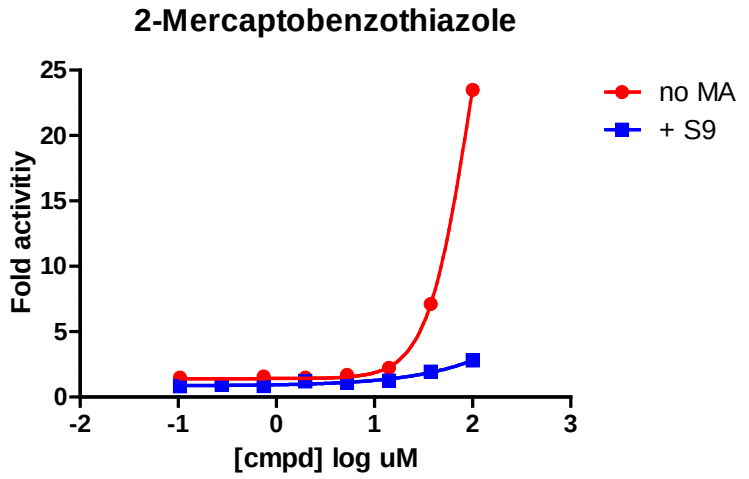


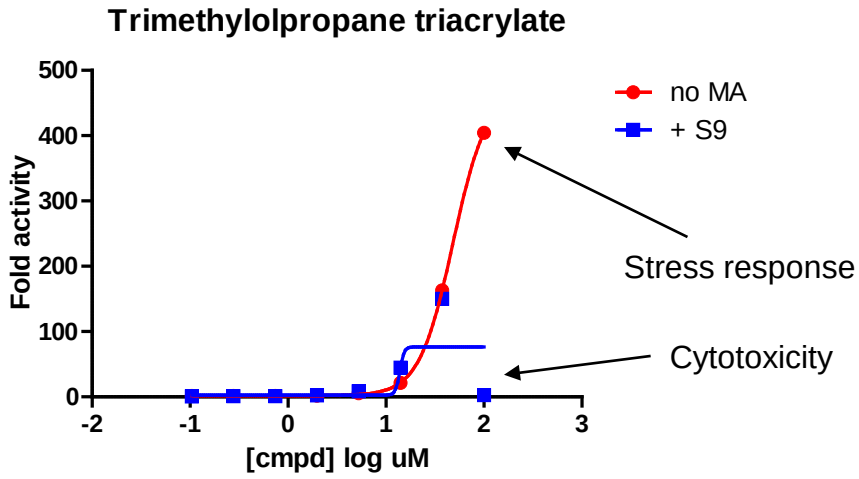
Proof of Concept Studies



1. Pro-luciferin substrate (MW ~ 350) enters microsphere
2. Metabolized by CYP3A4 to D-luciferin (MW ~280)
3. Must the microsphere to be detected

- Demonstrates that small molecules and their metabolites can freely diffuse through the microsphere pores
- Note that CYP3A4 inhibitor ketoconazole (MW ~ 530) can also freely diffuse into microsphere inhibit reaction

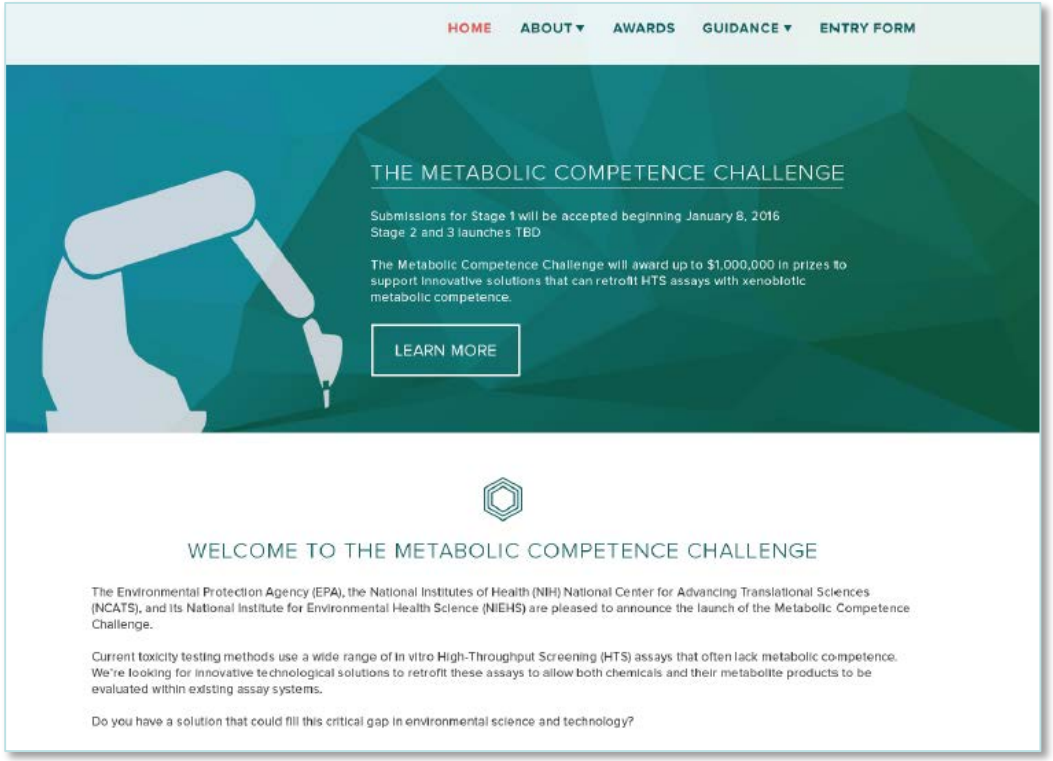


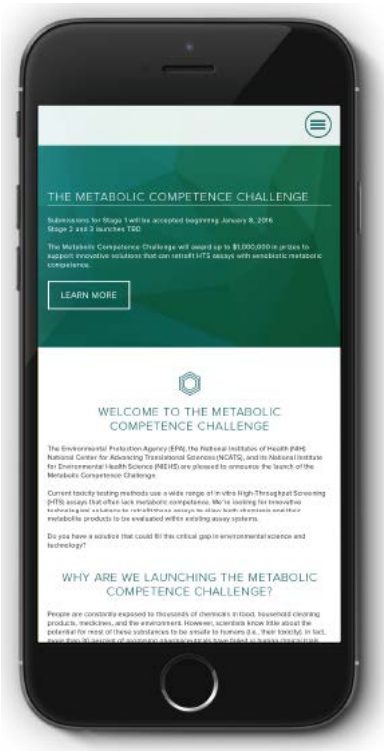


Using a cell-based stress reporter assay, we observe that the presence of S9 is often detoxifying (left), but in some instances enhances the toxicity of the test compound (right)

EPA, NTP, and NCATS Soliciting Solutions from the Broader Scientific Community

The Metabolic Competence Challenge





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