

High-Throughput Toxicogenomic Screening of Chemicals in the Environment using Metabolically Competent Hepatic Cell Cultures

The ToxCast LTEA Assay

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ThermoFisher

(formerly Life Technologies)

Jessica A. Bonzo

Raf Witek

NIEHS

Stephen Ferguson

Quintiles

(formerly Expression Analysis)

Patrick Hurban

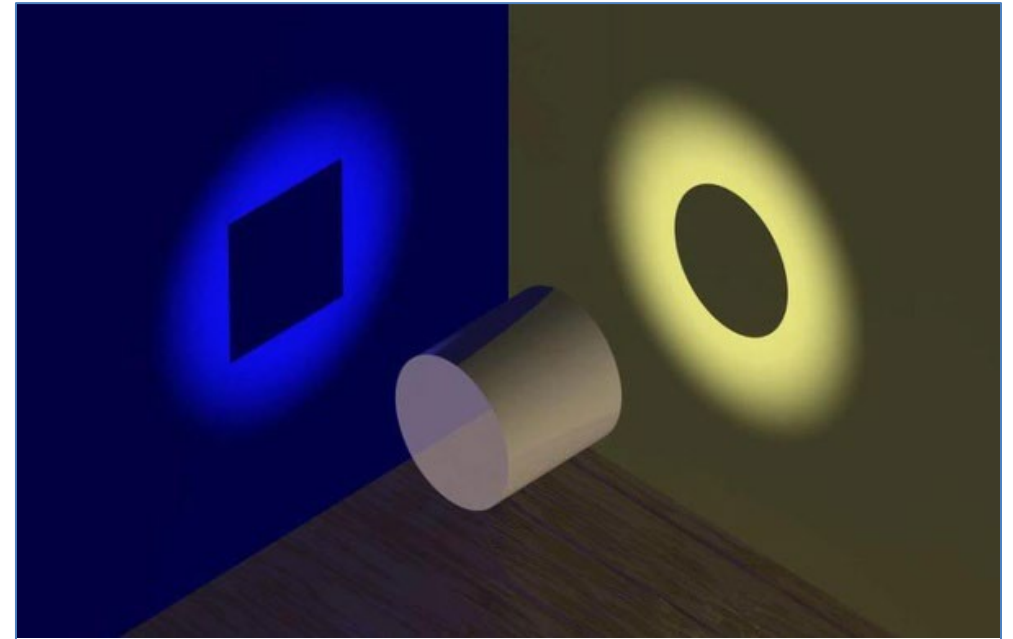
Stephen Siferd

The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the U.S. EPA

Introduction to ToxCast

- There are thousands of chemicals in commerce and the environment that lack the hazard data needed to assess risk posed to the public health (Judson, 2008)
- Chemicals that are not foods, drugs, or pesticides are regulated by the Toxic Substances Control Act (TSCA) which is administered by the EPA
- The EPA launched the **ToxCast** (*toxicity forecaster*) project in 2008 to develop data allowing prioritization of chemicals based on potential hazard

In Nate Silver's (<https://fivethirtyeight.com/>) terminology:
a ***prediction*** is a specific statement
a ***forecast*** is a probabilistic statement



- Each ToxCast assay-endpoint has the potential to capture an aspect of chemical biology – more than 1000 to date
- Need many reference chemicals covering diverse mechanisms to establish what different types hazard “look like”

Introduction to ToxCast LTEA Assay

- In Phase I of ToxCast, human primary hepatocytes were incubated with ToxCast chemicals in concentration response to test for changes in regulation of 14 genes (Rotroff et al, 2010)
 - Assay was found to be helpful in many toxicity prediction models (“signatures”), BUT:
 - Confounded by large variability between the two donors
 - Limited supply of primary hepatocytes from any one donor
- This new series of ToxCast HTS makes use of the HepaRG cell line to study chemical-induced gene expression changed in the presence of metabolism (Life Technologies + Expression Analysis = LTEA)
- Presence of metabolism should reduce false positives (detoxication) and false negatives (activation)
- Greater endpoint coverage (more genes) should give more insight into biology

Franzosa et al. (2021)

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High-throughput toxicogenomic screening of chemicals in the environment using metabolically competent hepatic cell cultures

Jill A. Franzosa¹, Jessica A. Bonzo², John Jack¹, Nancy C. Baker³, Parth Kothiya¹, Rafal P. Witek², Patrick Hurban⁴, Stephen Siferd⁴, Susan Hester¹, Imran Shah¹, Stephen S. Ferguson⁵, Keith A. Houck¹ and John F. Wambaugh¹✉

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npj Systems Biology and Applications (2021)7:7 ; <https://doi.org/10.1038/s41540-020-00166-2>

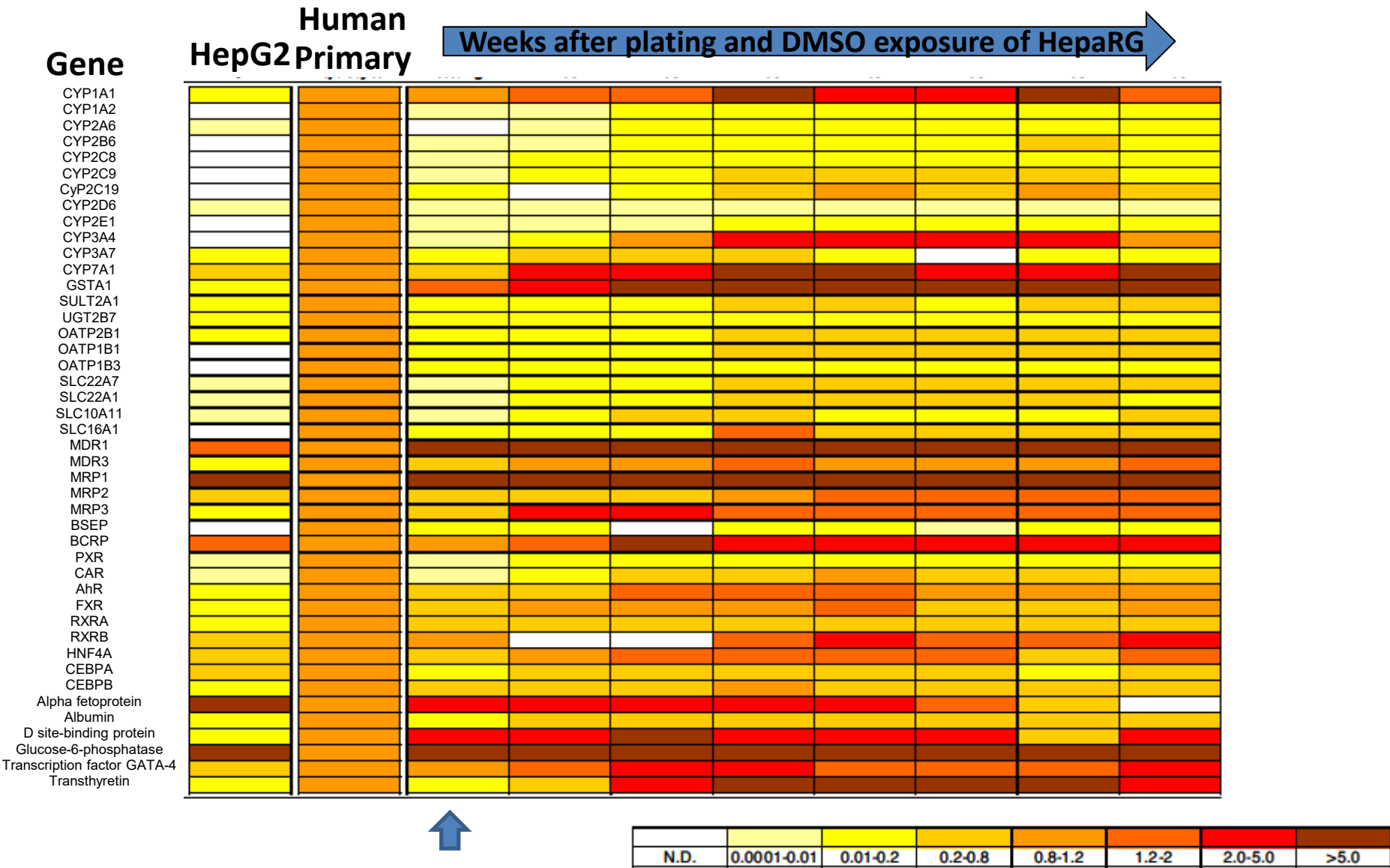
The ToxCast LTEA Assay Endpoints

- 1060 chemicals (ToxCast Phase I and II, with replicates) tested
- 93 transcripts in concentration-response curves fit in up and down mode
- LDH cytotoxicity assay concentration response
- 6 receptor activity inferences in concentration response

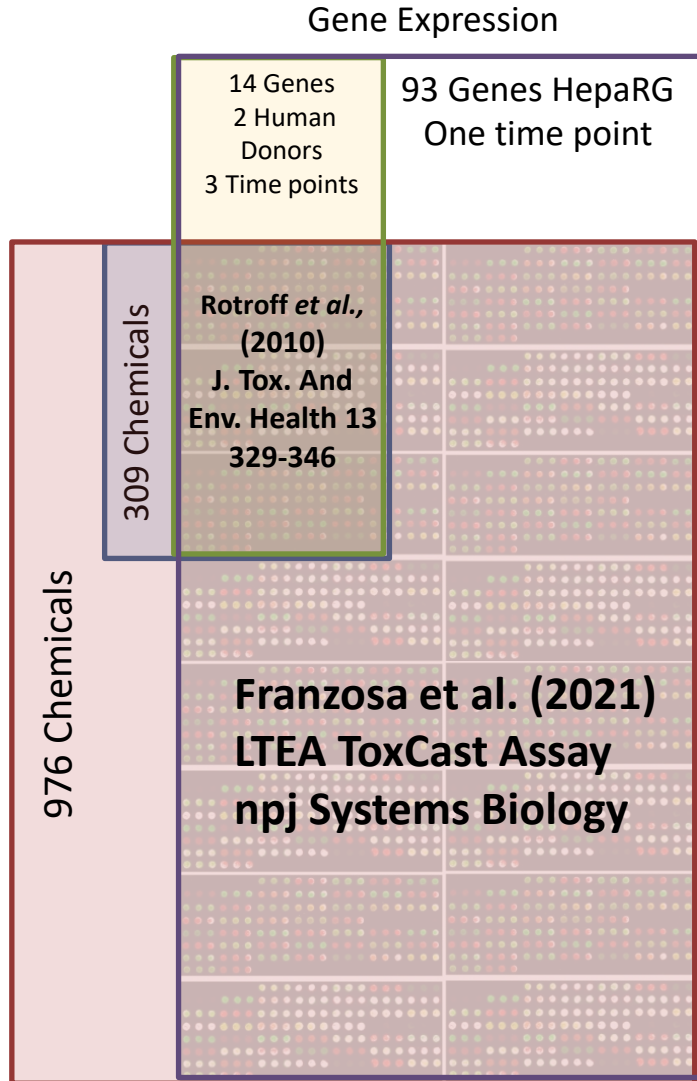
HepaRG Cell Line

HepaRG is a pluripotent cell line that differentiates into a culture of two liver-relevant cell types:

- Cholangiocytes (bile producing)
- Hepatocytes (which are responsible for much of metabolism)



ToxCast LTEA Assay



HepaRG cell cultures treated by ThermoFisher (formerly Life Technologies, CellzDirect)

- 1060 chemicals
- 8 pt concentration response with two replicates
- LDH activity assay (cytotoxicity)
 - Cell morphology images
- One time point (48 hours)
- Positive control plates
- Metabolically-activated cytotoxicity agent (Aflatoxin B1) on each plate

Gene expression conducted by Expression Analysis

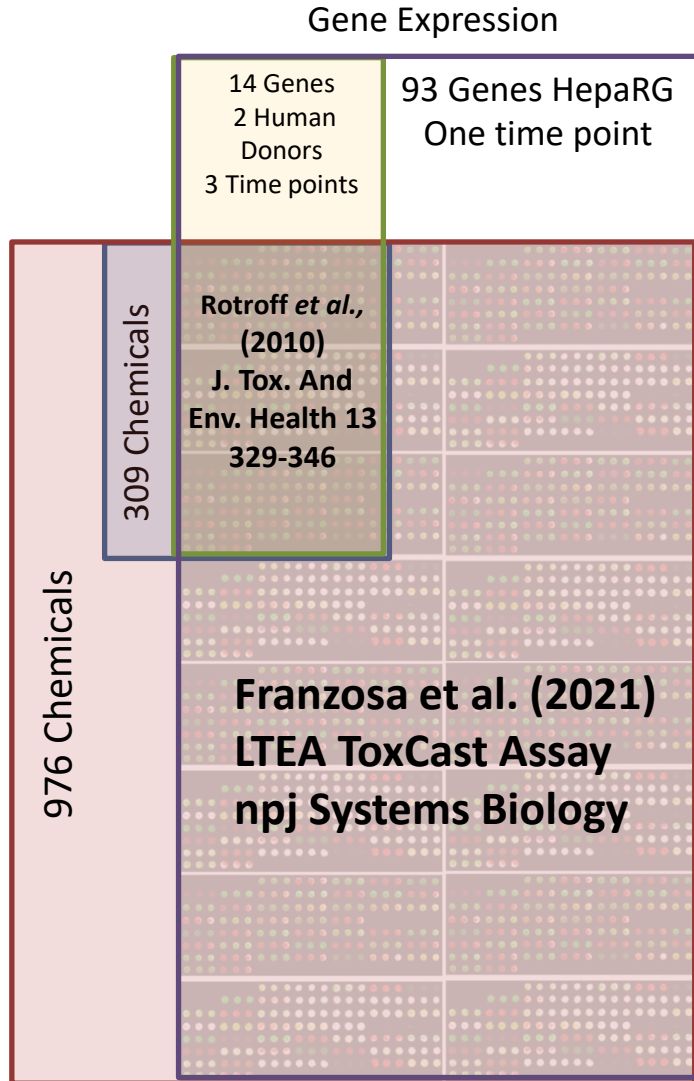
- qRT-PCR using Fluidigm 96.96 microfluidic technology
- $\Delta\Delta C_t$ (fold-change relative to DMSO and 3 housekeeping genes)
- 93 genes covering Phase I & II enzymes, transporters, cell-cycle, and disease states

Enumerating the Problem

- 93 genes, eight concentrations, two replicates, across 1060 chemicals is 1,577,280 data points (not counting reference plates, control genes, LDH cytotox assay)
 - Rotroff et al. (2010) was 14 genes, eight concentrations, two replicates, by 309 chemicals for 69,216 data points
- Hill-model curve fits per gene per chemical are $4 \text{ parameters} * 1060 \text{ chemicals} * 93 \text{ genes} * 2 \text{ directions}$ for 788,640 parameters (smaller with some constraints on Hill curves)
- Big data analysis problem, but we fit 4 parameters at a time
- We fit the data with the ToxCast pipeline (tcpl) but how do we interpret what we see?

LTEA vs. Whole Genome

$$14 < 93 < 23,000$$



LTEA Gene Coverage



We spent ~5 years figuring out how to analyze the data – we hope some of the lessons learned will inform the ToxCast whole transcriptome efforts

14 genes, 309 chemicals

Journal of Toxicology and Environmental Health, Part B, 13:329–346, 2010
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DOI: 10.1080/10937404.2010.483949



XENOBIOTIC-METABOLIZING ENZYME AND TRANSPORTER GENE EXPRESSION IN PRIMARY CULTURES OF HUMAN HEPATOCYTES MODULATED BY TOXCAST CHEMICALS

Daniel M. Rotroff^{1,2}, Andrew L. Beam³, David J. Dix¹, Adam Farmer³, Kimberly M. Freeman³,
Keith A. Houck¹, Richard S. Judson¹, Edward L. LeCluyse³, Matthew T. Martin¹,
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²Department of Environmental Sciences and Engineering, University of North Carolina at Chapel
Hill, Chapel Hill, North Carolina

³CellzDirect/Invitrogen Corporation (a part of Life Technologies), Durham, North Carolina, USA

Primary human hepatocyte cultures are useful *in vitro* model systems of human liver because when cultured under appropriate conditions the hepatocytes retain liver-like functionality such as metabolism, transport, and cell signaling. This model system was used to characterize the concentration- and time-response of the 320 ToxCast chemicals for changes in expression of genes regulated by nuclear receptors. Fourteen gene targets were monitored in quantitative nuclease protection assays: six representative cytochromes P-450, four hepatic transporters, three Phase II conjugating enzymes, and one endogenous metabolism gene involved in cholesterol synthesis. These gene targets are sentinels of five major signaling pathways: AhR, CAR, PXR, FXR, and PPAR α . Besides gene expression, the relative potency and efficacy for these chemicals to modulate cellular health and enzymatic activity were assessed. Results demonstrated that the culture system was an effective model of chemical-induced responses by prototypical inducers such as phenobarbital and rifampicin. Gene expression results identified various ToxCast chemicals that were potent or efficacious inducers of one or more of the 14 genes, and by inference the 5 nuclear receptor signaling pathways. Significant relative risk associations with rodent *in vivo* chronic toxicity effects are reported for the five major receptor pathways. These gene expression data are being incorporated into the larger ToxCast predictive modeling effort.

Toxicogenomic Screening Continues to Evolve

14 genes, 309 chemicals

93 genes, 976 chemicals

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XENOBIOTIC-METABOLIZING GENE EXPRESSION IN PRIMAR HEPATOCYTES MODULATED I

Daniel M. Rotroff^{1,2}, Andrew L. Be
Keith A. Houck¹, Richard S. Judson¹,
David M. Reif¹, Stephen S. Ferguson¹

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University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

³CellDirect/Invitrogen Corporation

Primary human hepatocyte culture when cultured under appropriate conditions such as metabolism, transport, and gene expression. We evaluated the concentration- and time-dependent expression of genes regulated by a quantitative nuclease protection assay. We evaluated the expression of three Phase II conjugation enzymes involved in cholesterol synthesis, three Phase II conjugation pathways: AhR, CAR, PXR, FXR, and efficacy for these chemicals assessed. Results demonstrated that induced responses by prototypic expression results identified various effects of one or more of the 14 genes. Significant relative risk assessment reported for the five major receptors incorporated into the larger ToxCast program.

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High-throughput toxicogenomic screening of chemicals in the environment using metabolically competent hepatic cell cultures

Jill A. Franzosa¹, Jessica A. Bonzo^{1,2}, John Jack^{1,3}, Nancy C. Baker^{1,3}, Parth Kothiyal¹, Rafal P. Witek², Patrick Hurban⁴, Stephen Siferd⁴, Susan Hester^{1,5}, Imran Shah^{1,5}, Stephen S. Ferguson^{1,5}, Keith A. Houck^{1,5} and John F. Wambaugh^{1,5}✉

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Toxicogenomic Screening Continues to Evolve

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93 genes, 976 chemicals

>20,000 genes, 42 chemicals

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Research Article

High-Throughput Transcriptomics Platform for Screening Environmental Chemicals

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Thomas Sheffield,¹ Joseph L. Bundy,^{*} Clinton M. Willis,¹
Russell S. Thomas¹, Imran Shah¹,^{*} and Richard S. Judson¹

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Disclaimer: The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental
Protection Agency.

ABSTRACT

New approach methodologies (NAMs) that efficiently provide information about chemical hazard without using whole

Toxicogenomic Screening Continues to Evolve

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93 genes, 976 chemicals

>20,000 genes, 42 chemicals

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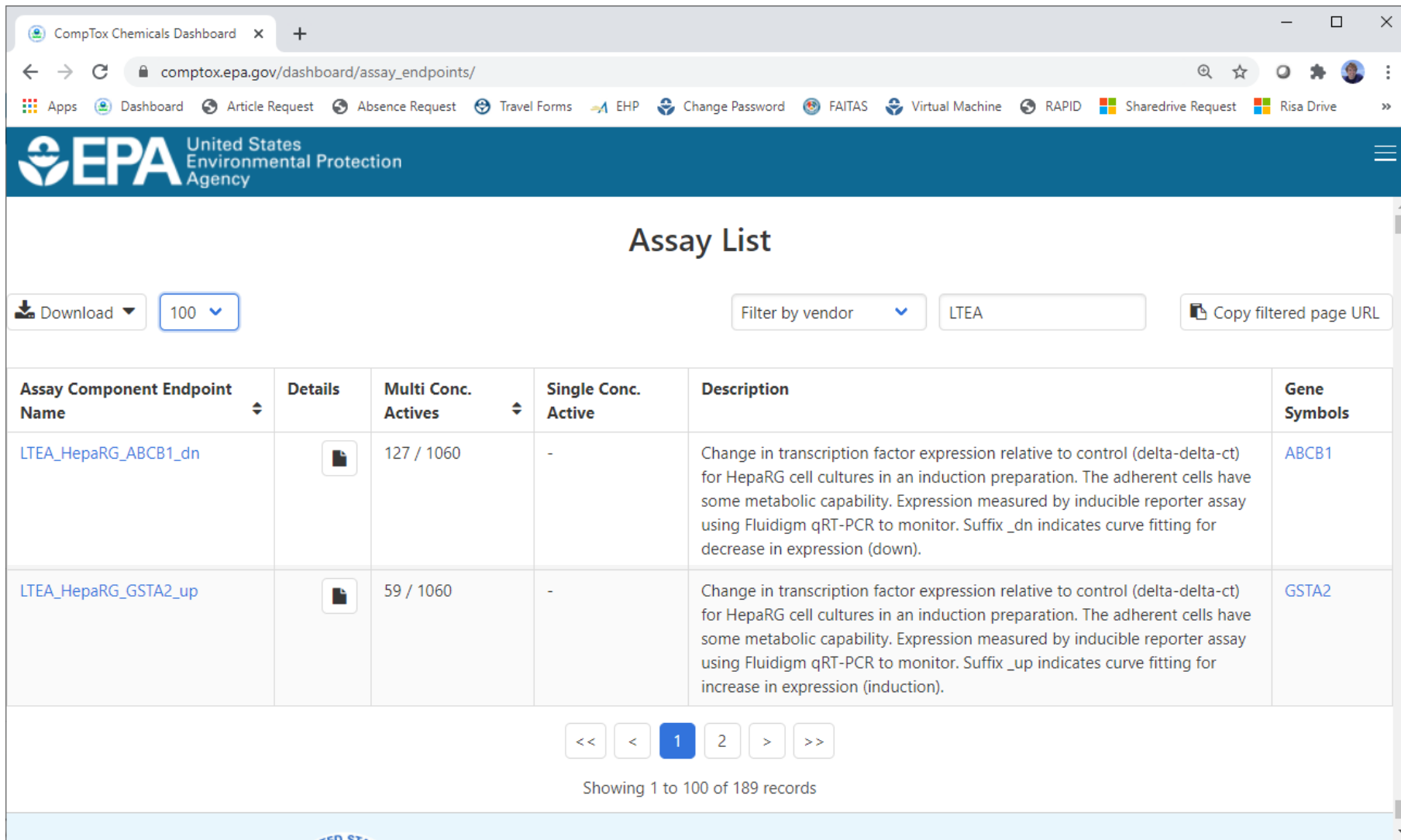
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Downloaded from <https://academic.oup.com/toxsci/advance-article/doi/10.1093/toxsci/kfab0>

The LTEA Data are on the Dashboard



https://comptox.epa.gov/dashboard/assay_endpoints/?link=&vendorFilter=LTEA



The screenshot shows the EPA ComTox Chemicals Dashboard. The top navigation bar includes links to Apps, Dashboard, Article Request, Absence Request, Travel Forms, EHP, Change Password, FAITAS, Virtual Machine, RAPID, Sharedrive Request, and Risa Drive. The main header displays the EPA logo and the text "United States Environmental Protection Agency".

Assay List

Download 100 Filter by vendor LTEA Copy filtered page URL

Assay Component Endpoint Name	Details	Multi Conc. Actives	Single Conc. Active	Description	Gene Symbols
LTEA_HepaRG_ABCB1_dn		127 / 1060	-	Change in transcription factor expression relative to control (delta-delta-ct) for HepaRG cell cultures in an induction preparation. The adherent cells have some metabolic capability. Expression measured by inducible reporter assay using Fluidigm qRT-PCR to monitor. Suffix _dn indicates curve fitting for decrease in expression (down).	ABCB1
LTEA_HepaRG_GSTA2_up		59 / 1060	-	Change in transcription factor expression relative to control (delta-delta-ct) for HepaRG cell cultures in an induction preparation. The adherent cells have some metabolic capability. Expression measured by inducible reporter assay using Fluidigm qRT-PCR to monitor. Suffix _up indicates curve fitting for increase in expression (induction).	GSTA2

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Showing 1 to 100 of 189 records

HepaRG Preparation and Treatment

- Jessica Bonzo (now of FDA) led the cell culture and treatment at Life Technologies/ThermoFisher
- A single lot of human HepaRG™ cells was used for the cell culture experiments
- Cryopreserved HepaRG™ cells were thawed, plated at a density of 100,000 cells/well and incubated for 48 h
- Forty-eight hours after plating the culture medium was changed to serum-free “induction media” and cells were exposed with each test chemical in duplicate. Acoustic Liquid Handling Technology used – likely reducing cross-contamination. Plates were returned to incubators and maintained for 48 h
- 50 µL of spent culture media from each plate was removed for the LDH assay.
- CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used to measure the LDH leakage activity as a measure of membrane integrity and cytotoxicity in the cells.

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- Side note:
 - CellzDirect (Rotroff 2010 contractor) was acquired by Invitrogen (2008)
 - Also, in 2008 Invitrogen renamed itself “Life Technologies”
 - ThermoFisher Acquired Life Technologies in 2014

Study Design: Test Plates

Each test chemical plate contained duplicate eight-point dilutions of five ToxCast compounds.

Experiments took >200 test plates

	1	2	3	4	5	6	7	8	9	10	11	12
A	PB.1	1.1	2.1	3.1	4.1	5.1	1.1	2.1	3.1	4.1	5.1	DMSO
B	PB.2	1.2	2.2	3.2	4.2	5.2	1.2	2.2	3.2	4.2	5.2	DMSO
C	PB.3	1.3	2.3	3.3	4.3	5.3	1.3	2.3	3.3	4.3	5.3	Total lysate
D	AFL.1	1.4	2.4	3.4	4.4	5.4	1.4	2.4	3.4	4.4	5.4	Total lysate
E	AFL.2	1.5	2.5	3.5	4.5	5.5	1.5	2.5	3.5	4.5	5.5	EA
F	AFL.3	1.6	2.6	3.6	4.6	5.6	1.6	2.6	3.6	4.6	5.6	EA
G	DMSO	1.7	2.7	3.7	4.7	5.7	1.7	2.7	3.7	4.7	5.7	EA
H	DMSO	1.8	2.8	3.8	4.8	5.8	1.8	2.8	3.8	4.8	5.8	EA

No cells

- 1.1, 2.1, 3.1, ..., 5.8 ToxCast compound N, dilution 1, 2, 3,...
- PB Phenobarbital
- DMSO Vehicle control
- Total Lysate LDH assay control
- . AFL Aflatoxin B1
- EA Empty wells for EA: one no template control, one no enzyme control, and two for universal human reference RNA

Study Design: Reference Plates

Seven reference chemical plates were interspersed throughout the experimental process.

Reference chemical plates contained both reference chemicals for metabolic activity (aflatoxin b1) and reference receptor activators.

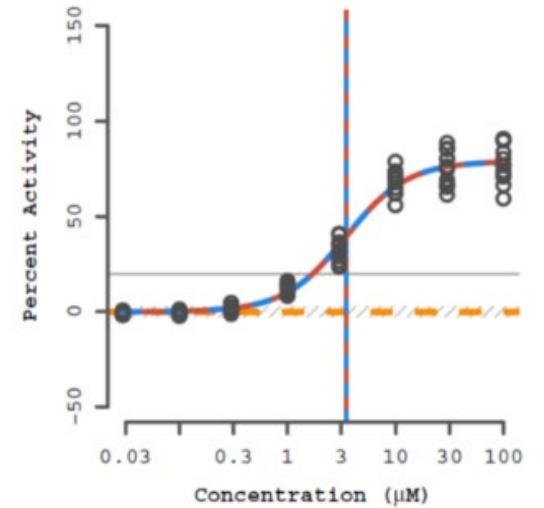
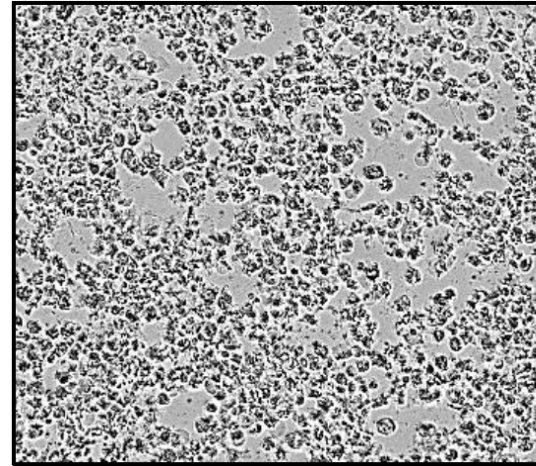
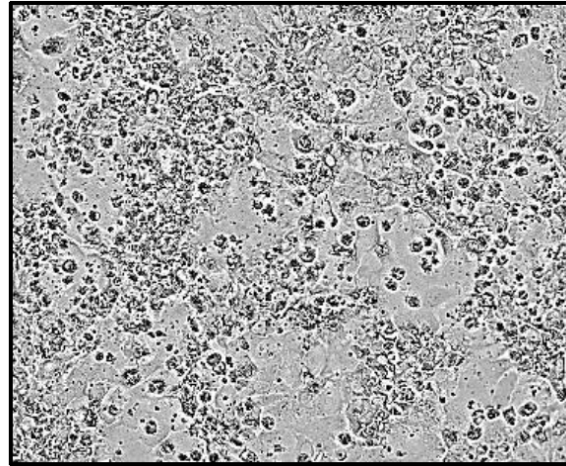
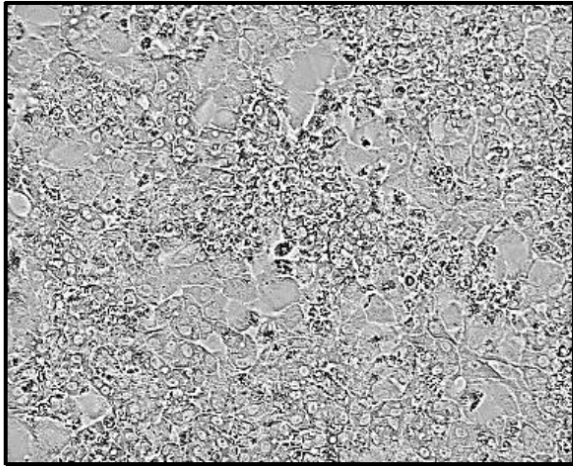
	1	2	3	4	5	6	7	8	9	10	11	12
A	PB.1	PB.1	OMP. 1	OMP. 1	FF.1	FF.1	CDCA. 1	CDCA. 1	AFL.1	AFL.1	DMSO	DMSO
B	PB.2	PB.2	OMP. 2	OMP. 2	FF.2	FF.2	CDCA. 2	CDCA. 2	AFL.2	AFL.2	DMSO	DMSO
C	PB.3	PB.3	OMP. 3	OMP. 3	FF.3	FF.3	CDCA. 3	CDCA. 3	AFL.3	AFL.3		Total lysate
D	PB.4	PB.4	OMP. 4	OMP. 4	FF.4	FF.4	CDCA. 4	CDCA. 4	AFL.4	AFL.4		Total lysate
E	PB.5	PB.5	OMP. 5	OMP. 5	FF.5	FF.5	CDCA. 5	CDCA. 5	AFL.5	AFL.5	EA	No cells
F	PB.6	PB.6	OMP. 6	OMP. 6	FF.6	FF.6	CDCA. 6	CDCA. 6	AFL.6	AFL.6	EA	
G	PB.7	PB.7	OMP. 7	OMP. 7	FF.7	FF.7	CDCA. 7	CDCA. 7	AFL.7	AFL.7	EA	
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CAR/PXR PB: Phenobarbital
 AhR OMP: Omeprazole
 PPAR α FF: Fenofibric Acid
 FXR CDCA: Chenodeoxycholic Acid
 Toxicity AFL: Aflatoxin B1

DMSO: Vehicle control
 Total Lysate: LDH assay control
 EA: Empty wells for EA to add universal human reference RNA

Metabolic Competency

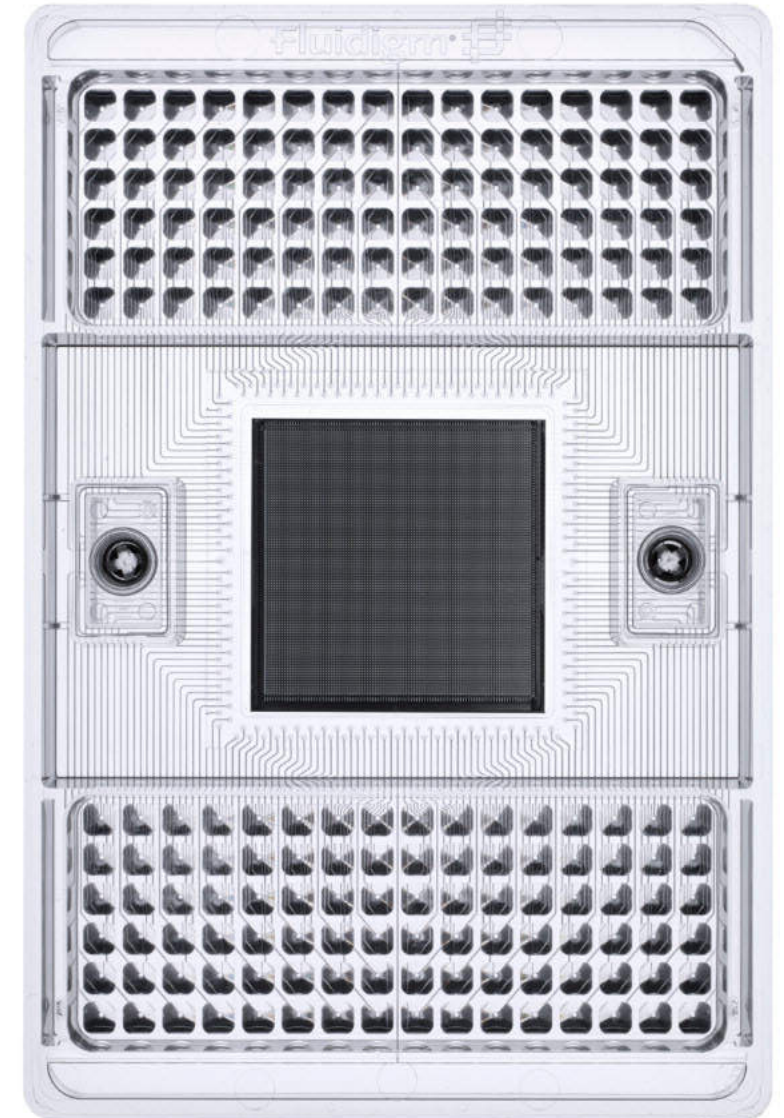
“Induction media” (0.5% DMSO) was used instead of 2% DMSO from other experiments, somewhat reducing metabolic capacity, but **metabolism-mediated cytotoxicity of aflatoxin B1 (AFL)** was still observed



Visual assessment of AFL cytotoxicity in cells. Images of cells treated for 48 h with (A) 0.5% DMSO vehicle control, (B) 3.16 μM AFL (~ EC10) or (C) 100 μM AFL. (D) LDH assay dose-response curve for AFL treated cells

Gene expression

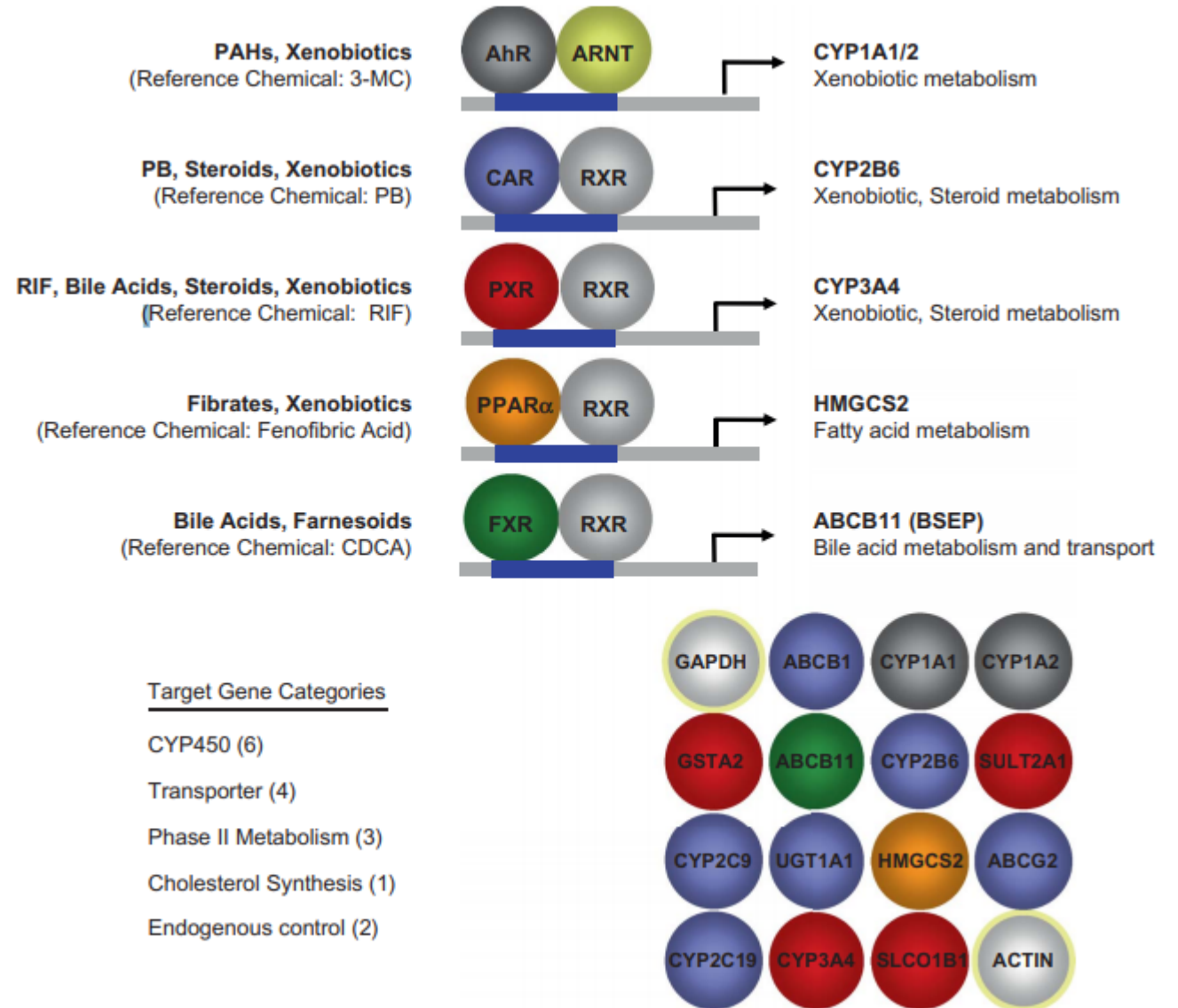
- Patrick Hurban led gene expression analysis at Expression analysis/Quintiles
- Fluidigm's 96.96 Dynamic Array was used for gene expression analyses by quantitative reverse transcription polymerase chain reaction (qRT-PCR)
- Standard TaqMan Assays were used to assess the expression of 93 genes
- Three “housekeeping” endogenous control genes were also included and used for normalization:
 - Actin β (ACTB)
 - Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
 - RNA Polymerase II Subunit A (POLR2A).



Fluidigm 96.96 microfluidic plate

- Genes were selected based on their selectivity and sensitivity to important hepatic receptors known to be modulated by environmental chemicals; importance in human hepatocyte functionality; and hepatotoxicity.
- Jill Franzosa, John Jack, Patrick Hurban, Steve Siferd, Susan Hester, Steve Ferguson, Keith Houck helped create the list
- Steve Siferd and Patrick Hurban identified TaqMan Assays and helped identify more responsive genes
- Franzosa et al. (2021) Supplemental Table 1 Provides Gene Selection Reasoning

Target Gene List



Rotroff et al. (2010)

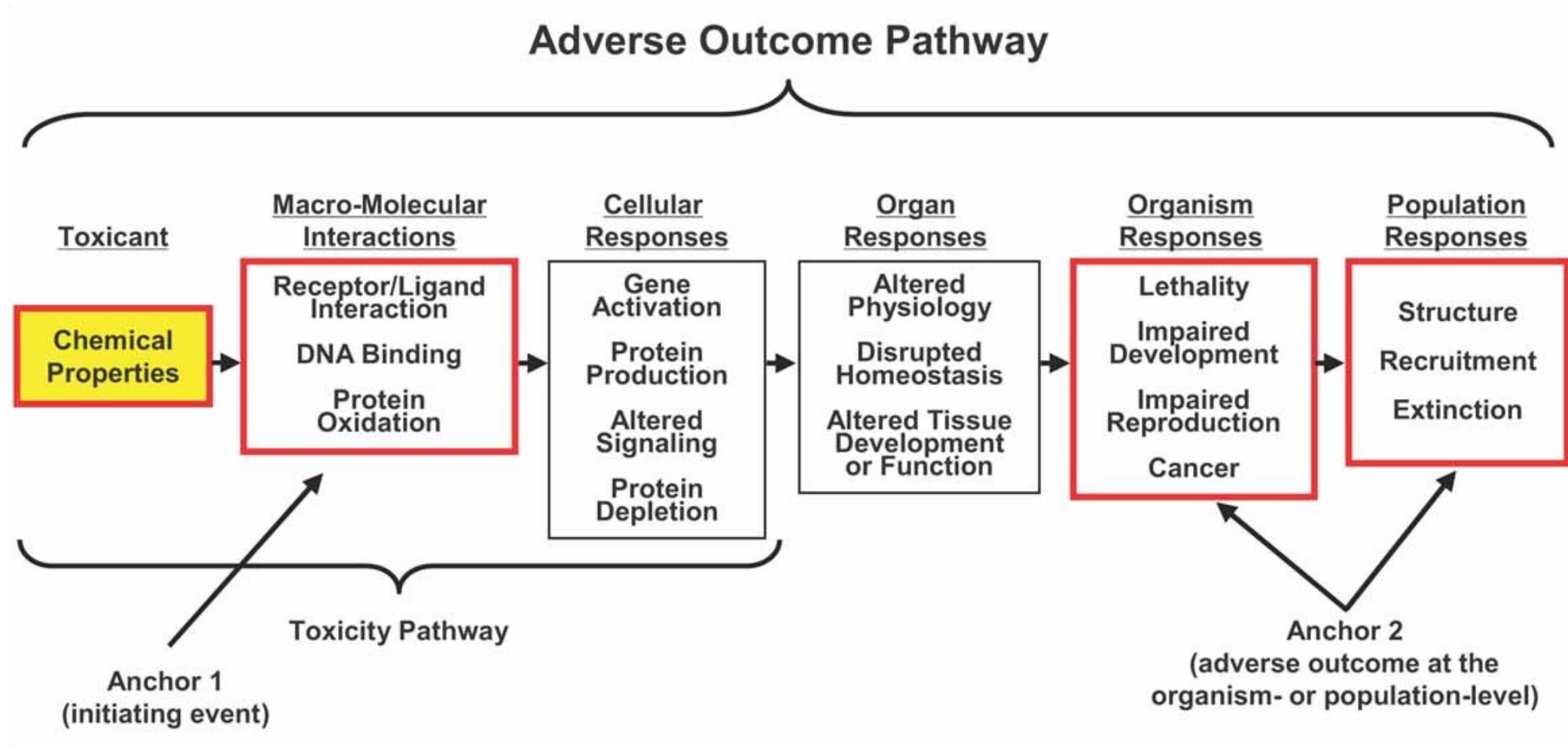
Supplemental Table I Provides Gene Selection Reasoning

Supplementary Material for “High-Throughput Toxicogenomic Screening of Chemicals in the Environment Using Metabolically Competent Hepatic Cell Cultures”

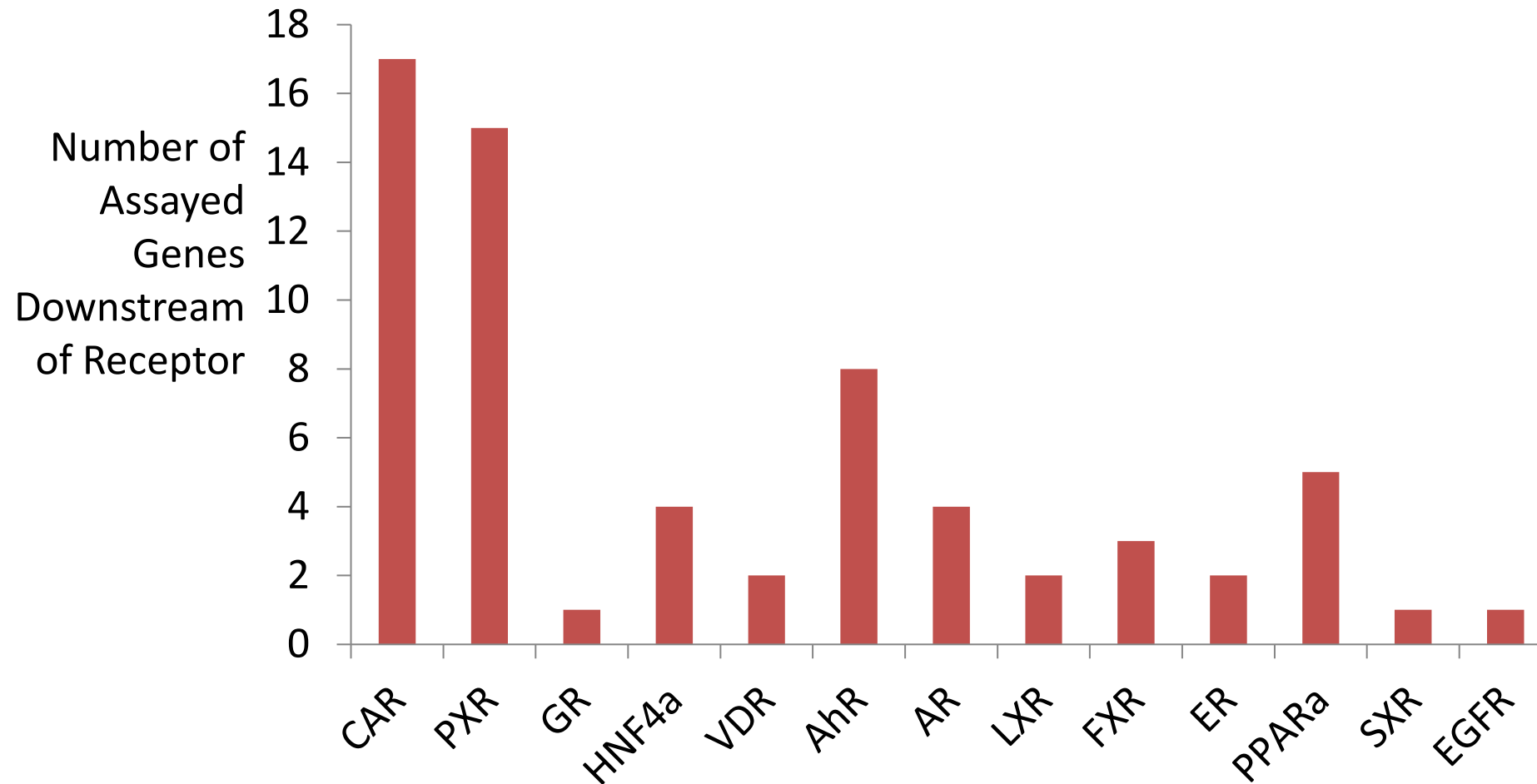
Supplementary Table 1: Genes Assayed as Part of ToxCast Screen

EntrezID	TaqMan Assay	Gene ID	Feature	Receptors	Rotroff et al. (2010)
5243	Hs00184500_m1	ABCB1	Steatosis/NR mediated transport	CAR/PXR/VDR/AHR	Y
8647	Hs00184824_m1	ABCB11	Steatosis/NR mediated transport	FXR/LXR	Y
1244	Hs00166123_m1	ABCC2	Steatosis/NR mediated transport	FXR/CAR/PXR	
8714	Hs00978473_m1	ABCC3	NR mediated transport	AHR/PXR/CAR	
9429	Hs01053790_m1	ABCG2	Steatosis/NR mediated transport	EGFR/CAR/PXR/AHR	Y
47	Hs00982738_m1	ACLY	Steatosis		
51	Hs01074241_m1	ACOX1	Steatosis	PPARA	
132	Hs00417073_m1	ADK	Steatosis		
174	Hs00173490_m1	AFP	Undifferentiated hepatocyte		
250	Hs01654626_s1	ALPP	Cell proliferation, survival, death		
116519	Hs00983449_g1	APOA5	Steatosis	PPARA	
572	Hs00188930_m1	BAD	Cell proliferation, survival, death		
581	Hs00180269_m1	BAX	Cell proliferation, survival, death		
596	Hs00608023_m1	BCL2	Cell proliferation, survival, death		
10018	Hs00708019_s1	BCL2L11	Cell proliferation, survival, death		
637	Hs00609632_m1	BID	Cell proliferation, survival, death		

Molecular Initiating Events and Toxicology

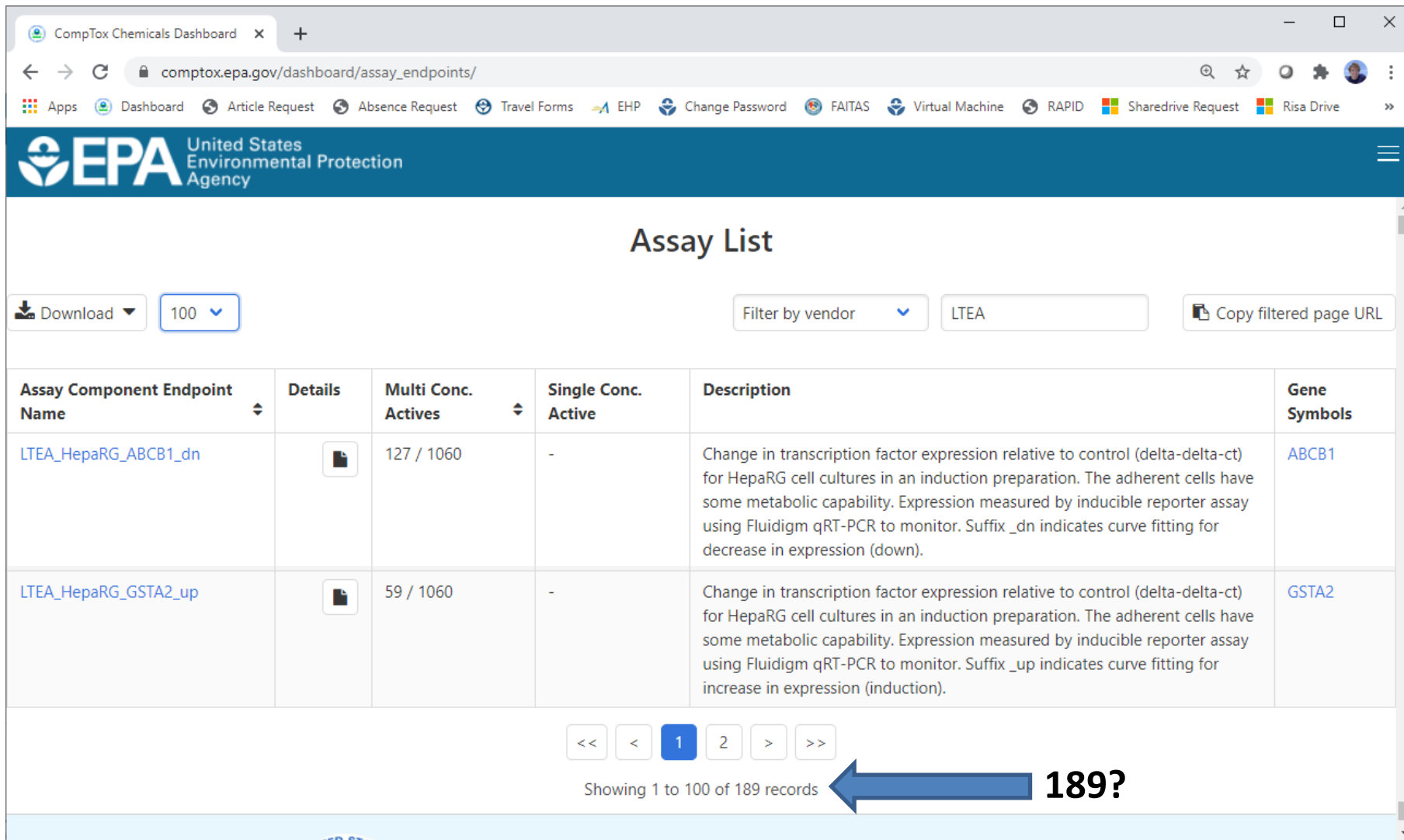


LTEA Coverage of Putative Molecular Initiating Events





The LTEA Data are on the Dashboard

https://comptox.epa.gov/dashboard/assay_endpoints/?link=&vendorFilter=LTEA



The screenshot shows the EPA Comptox Chemicals Dashboard. The page title is "Assay List". There are filters for "Download" (100), "Filter by vendor" (LTEA), and a "Copy filtered page URL" button. The table below lists assay data:

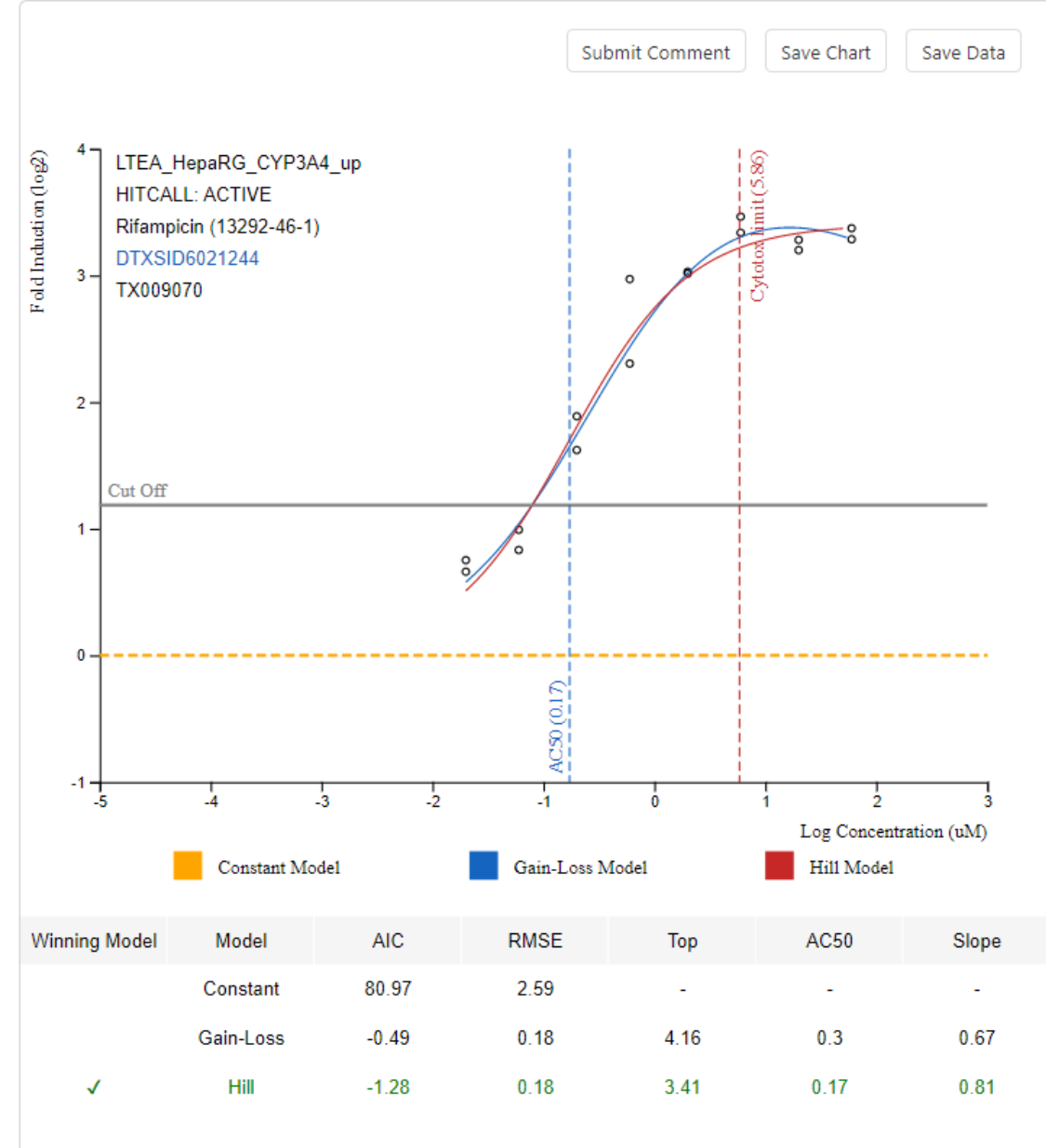
Assay Component Endpoint Name	Details	Multi Conc. Actives	Single Conc. Active	Description	Gene Symbols
LTEA_HepaRG_ABCB1_dn		127 / 1060	-	Change in transcription factor expression relative to control (delta-delta-ct) for HepaRG cell cultures in an induction preparation. The adherent cells have some metabolic capability. Expression measured by inducible reporter assay using Fluidigm qRT-PCR to monitor. Suffix _dn indicates curve fitting for decrease in expression (down).	ABCB1
LTEA_HepaRG_GSTA2_up		59 / 1060	-	Change in transcription factor expression relative to control (delta-delta-ct) for HepaRG cell cultures in an induction preparation. The adherent cells have some metabolic capability. Expression measured by inducible reporter assay using Fluidigm qRT-PCR to monitor. Suffix _up indicates curve fitting for increase in expression (induction).	GSTA2

At the bottom, there is a pagination bar showing "Showing 1 to 100 of 189 records". A blue arrow points to the number "189?" in the pagination bar.

I 89 LTEA ToxCast Assay Endpoints

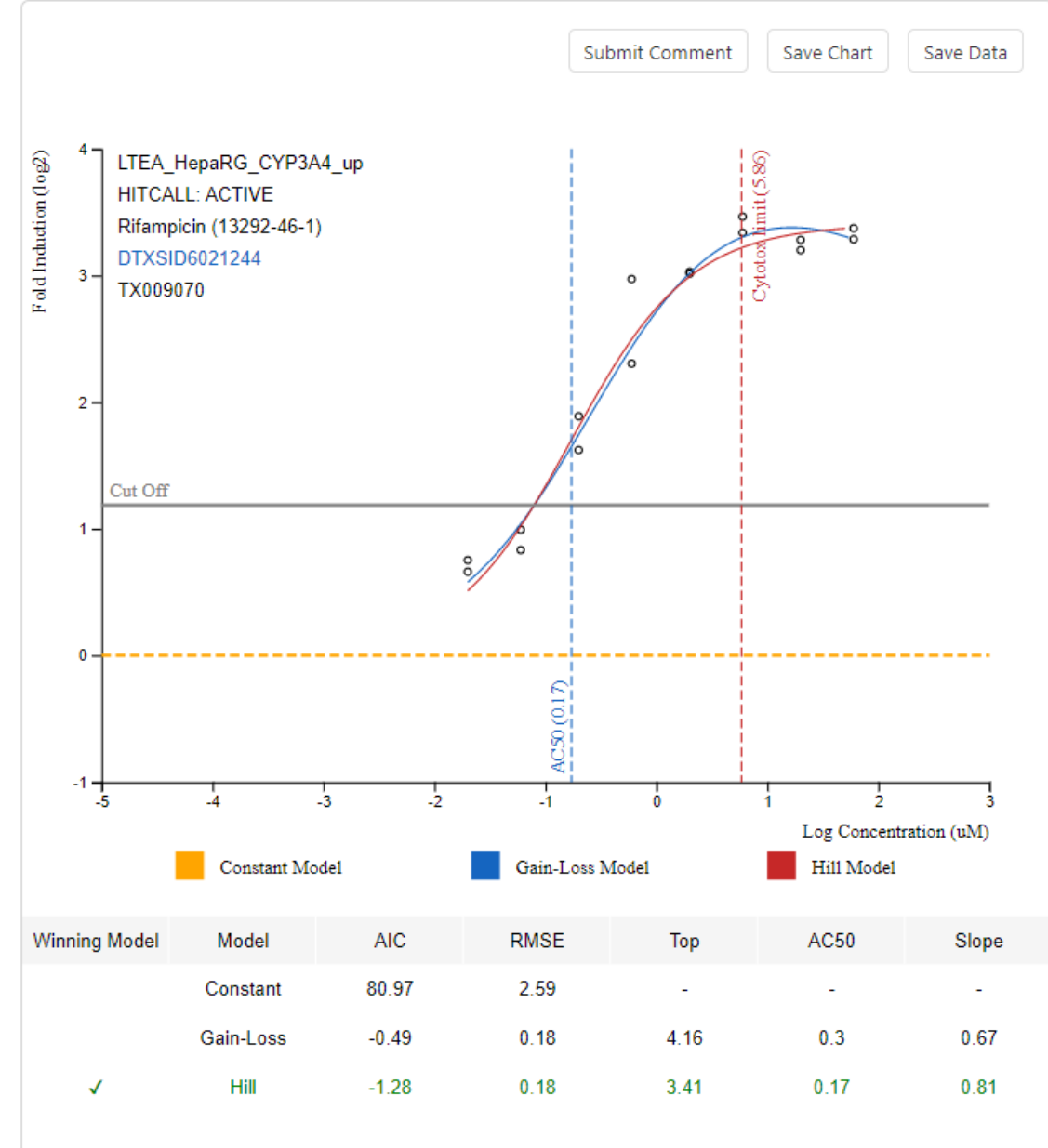
- Curve fitting was performed by Jill Franzosa using R package “tcpl” (the ToxCast Pipeline)
- Each concentration response was fit twice, once for up and once for down
- 90/93 genes has some observed activity ($90 \times 2 = 180$)
- 4 genes were replaced for noisiness after first 96 chemicals ($4 \times 2 = 8$)
- LDH cytotoxicity assay
- 189 total endpoints

Each gene expression concentration response that varied systematically with concentration can be characterized by an 50% activity concentration (AC50)



I 89 LTEA ToxCast Assay Endpoints

- Out of 1060 chemical samples (there were replicates), 1037 chemicals (98%) had at least one systematic relationship between concentration and transcriptional response.
- If all relationships with curve-fit warning flags are omitted only 718 (68%) of chemicals had a clear systematic response.
- Among the 718 chemicals, the median number of responding transcripts was 6, with a maximum of 90 (for the chemical mancozeb)
- The most commonly occurring responses were:
 - Upregulation of CYP1A1 (360 chemicals)
 - Upregulation of CYP2B6 (352)
 - Downregulation of CYP2E1 (323)

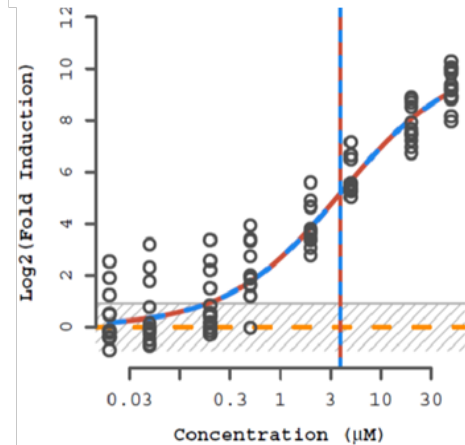


Reference Receptor Activators

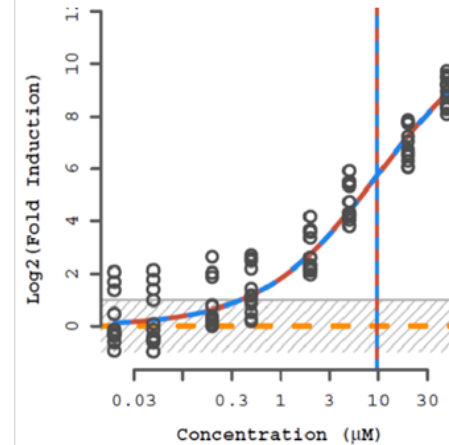
Dose-response curves for reference chemicals and transcriptionally regulated genes. Log2 (Fold Induction) response profiles of:

- A. CYP1A1 and B. CYP1A2 upon exposure to AhR positive control omeprazole
- C. CYP3A4 with rifampicin
- D. CYP2B6 with phenobarbital
- E. HMGCS2 after fenofibric acid
- F. ABCB11 with chenodeoxycholic acid

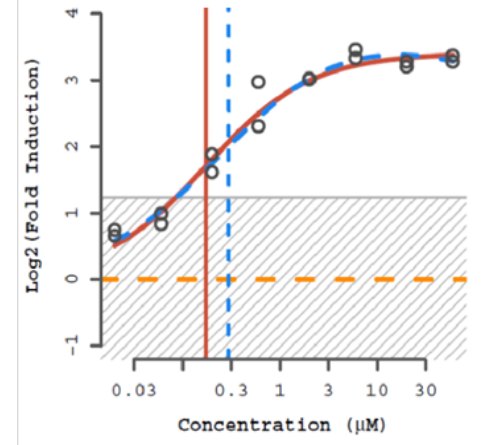
A. AhR: Omeprazole, CYP1A1



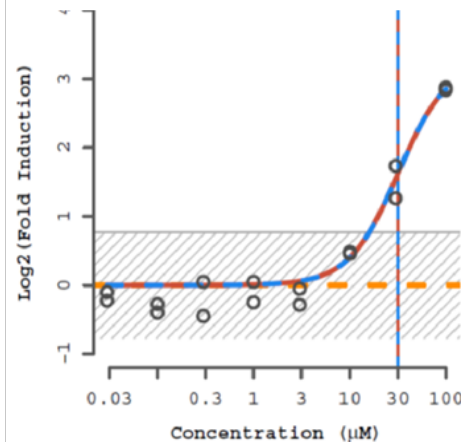
B. AhR: Omeprazole, CYP1A2



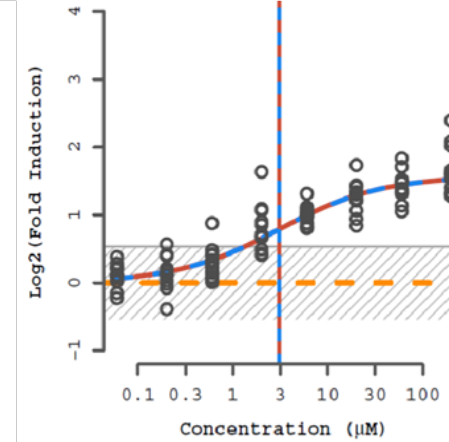
C. PXR: Rifampicin, CYP3A4



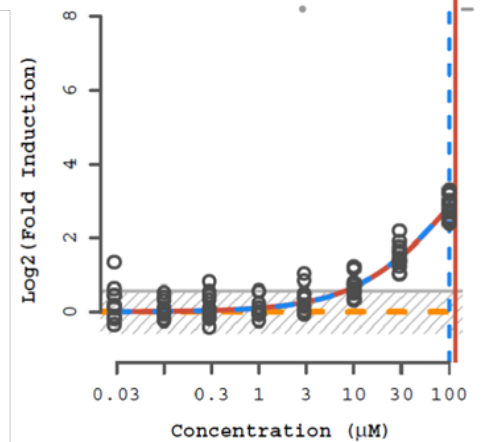
D. CAR: Phenobarbital, CYP2B6



E. PPARα: Fenofibric Acid, HMGCS2



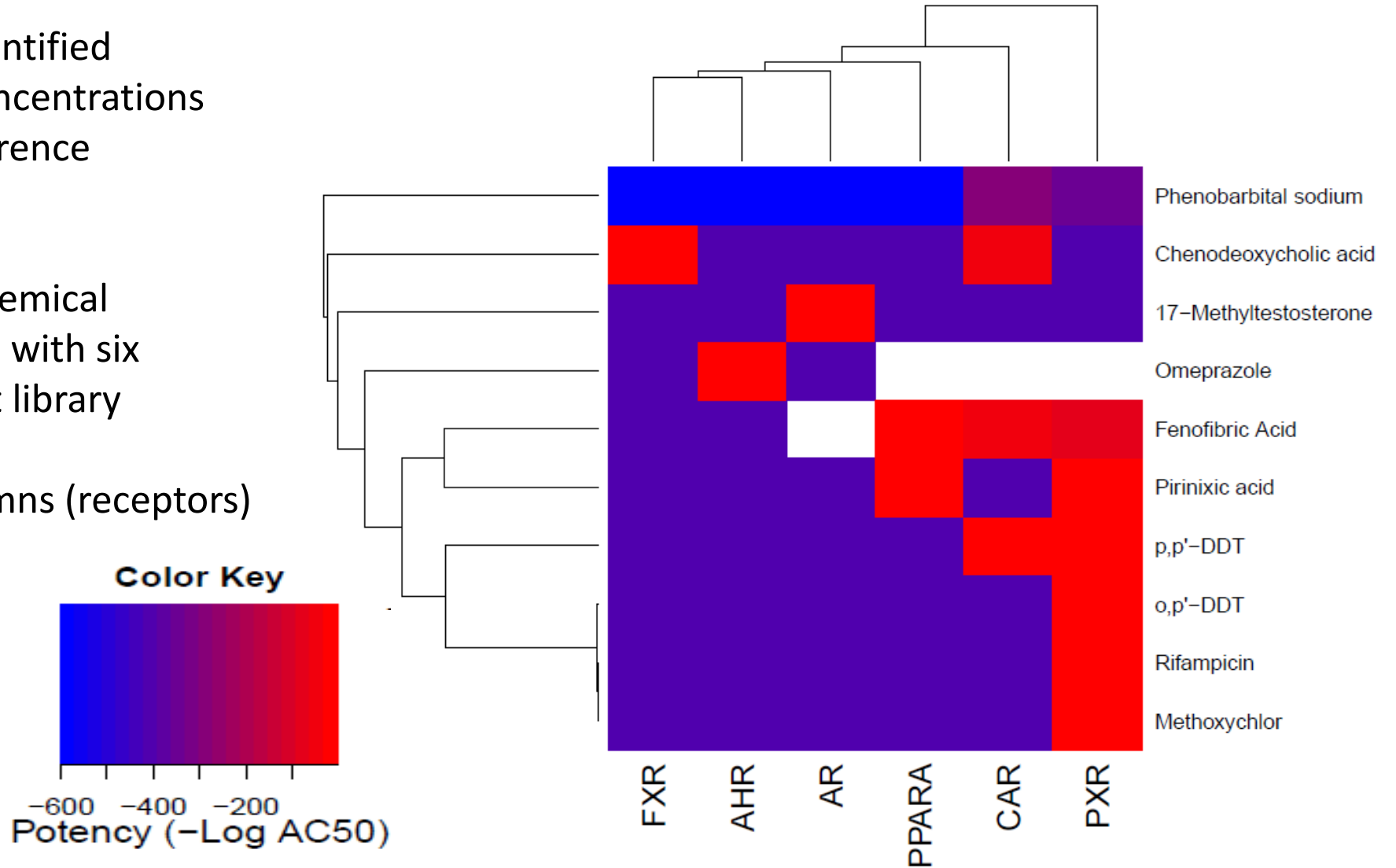
F. FXR: Chenodeoxycholic acid, ABCB11



Three dose-response relationships are indicated in each plot, the first (no response) is a horizontal long-dashed line, while the Hill function (short-dashed line) and gain-loss (solid line) response models change with the points. The vertical lines indicate the estimate 50% activation concentration (AC50) for the two response models. The grey shaded region indicates estimated background.

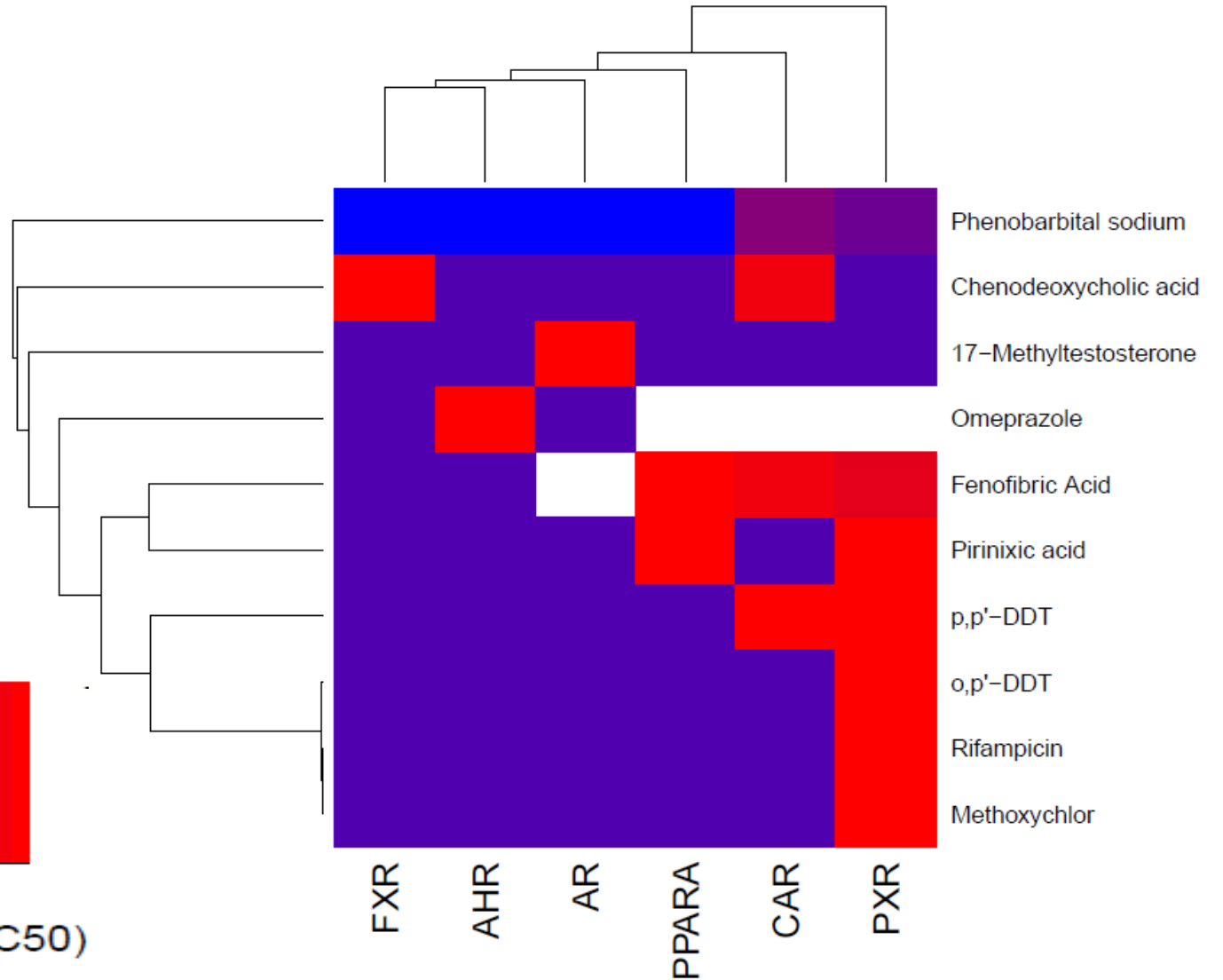
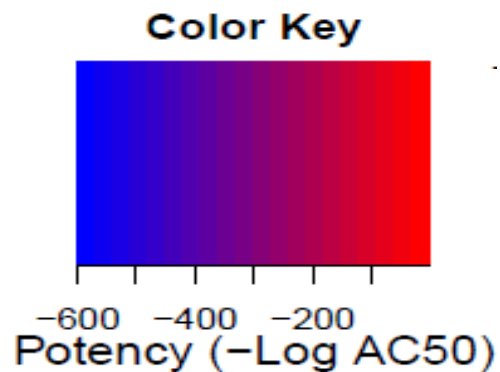
Reference Receptor Activators

- Curation of *in vitro* data identified receptor 50% activation concentrations (potencies) for the ten reference chemicals.
- The four reference plate chemical activators were augmented with six selections from the ToxCast library
- Rows (chemicals) and columns (receptors) are hierarchically clustered
- Color in the heatmap indicates the potency, with white indicating instances where no data were available.



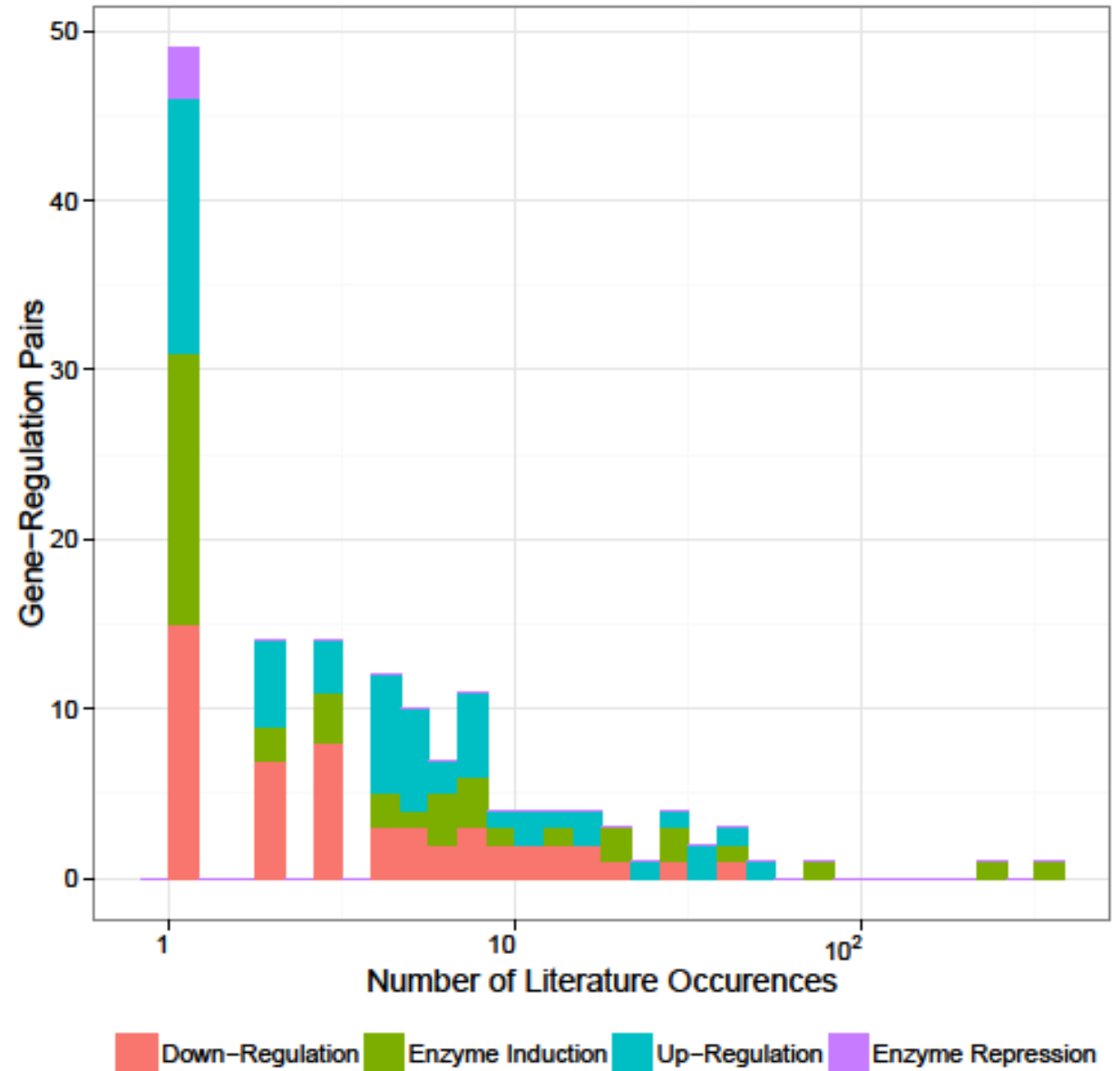
Reference Receptor Activators

- It's just as important to know if a reference chemical does NOT activate a certain receptor
- Unfortunately, many “reference” chemicals have activity in multiple receptors, albeit with different potencies
- Prior information pulled together from ToxCast/Tox21 assays and Rotroff et al. (2010)
- Color in the heatmap indicates the potency, with white indicating instances where no data were available.



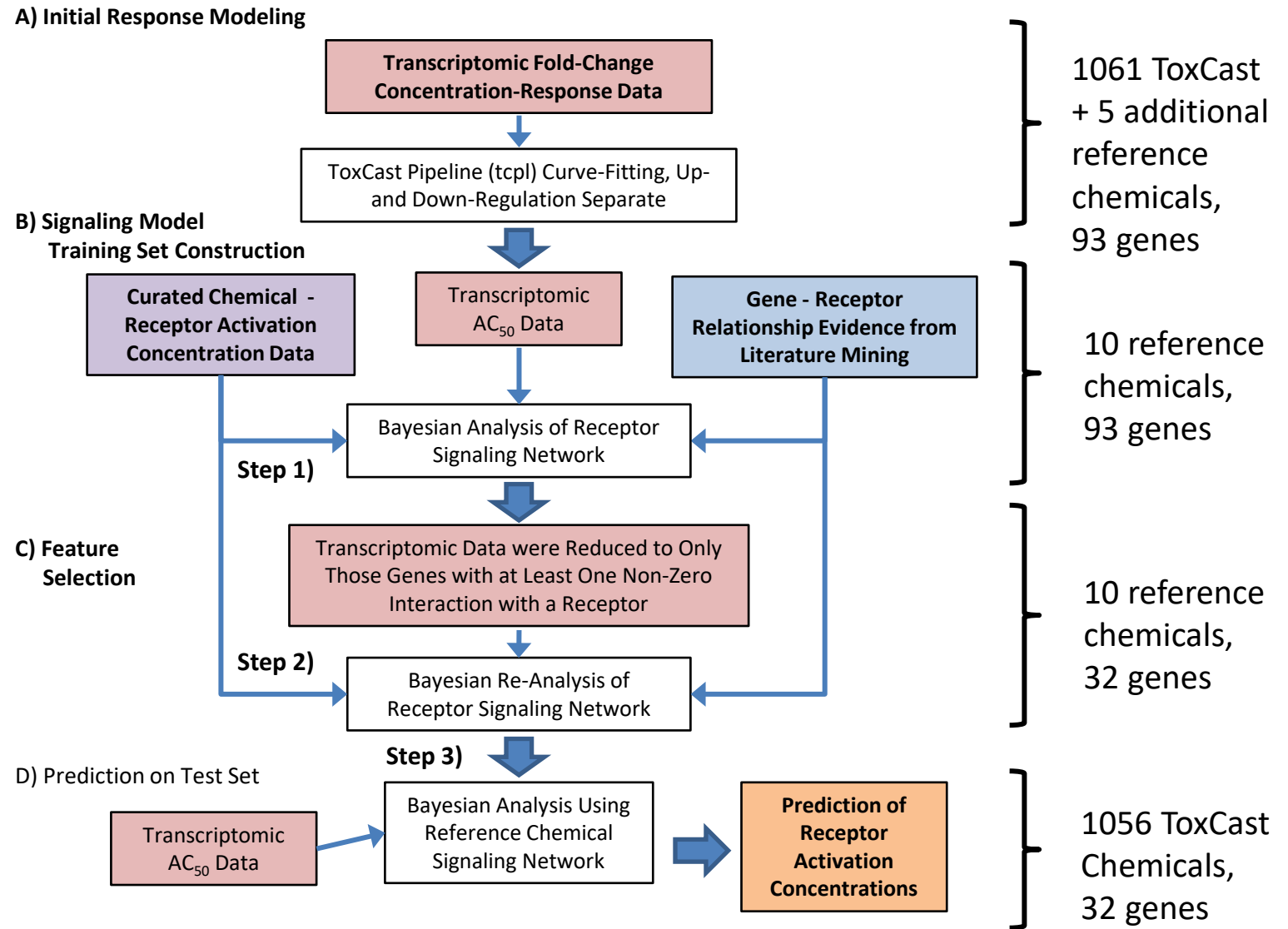
Literature Mining on Receptor-Gene Interactions

- Literature mining was performed by Nancy Baker
- Co-occurring mentions of receptors and transcripts were curated from the published literature
- The histogram shows that most occurrences were of a single instance of a receptor and transcript being mentioned, while in some cases there were several hundred occurrences
- Occurrence of receptor-gene and direction was turned into a prior for Bayesian analysis



Bayesian Analysis of LTEA Data

- We have “prior” information:
 - Chemical-Receptor interactions
 - Receptor-Gene Interactions
- Plus, we have new data to interpret
- Bayesian methods using a statistical model to combine prior information and new data to determine probabilities
- This is a full bayesian analysis as apposed to approximate Bayesian network methods common in genomics

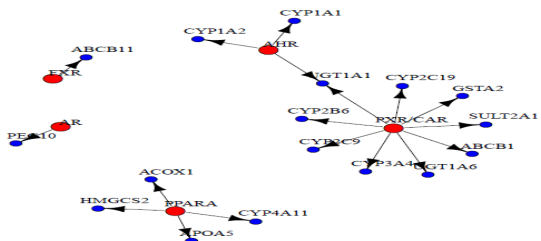


Bayesian Analysis of Gene Expression Data

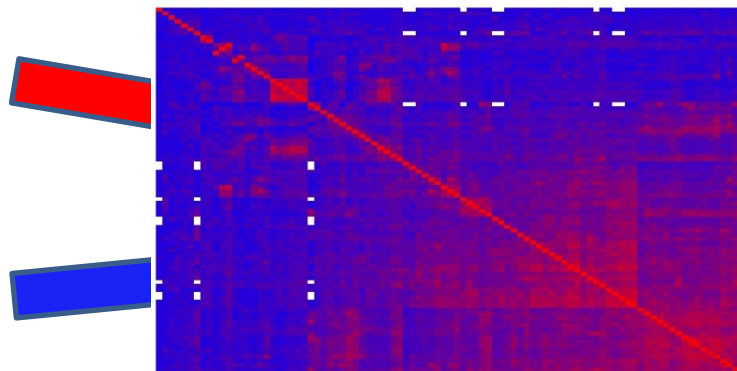
Reference Chemicals

Compound	CAS	NR	AC50	Reference Peto
Omeprazole	73590-58-6	AHR	50	Yes
Perofibric Acid	42017-89-0	PPARα	1	Yes
Chenodeoxycholic Acid	474-25-9	FXR	100	Yes
Phenobarbital	57-30-7	CAR	100	Yes
Rifampicin	11292-46-1	FXR	1	No
Methyl testosterone	58-18-4	AR	1	No

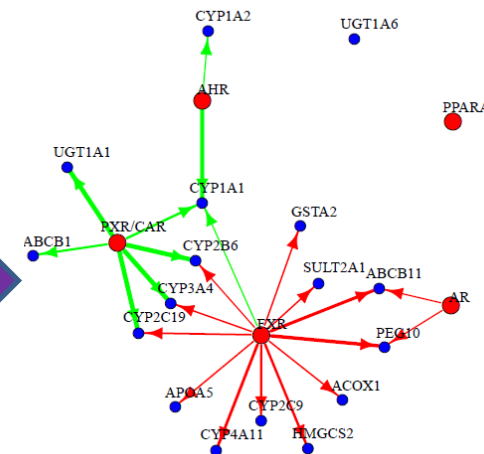
Literature Interactions



AC50 Data for ToxCast Library



Inferred HepaRG Network



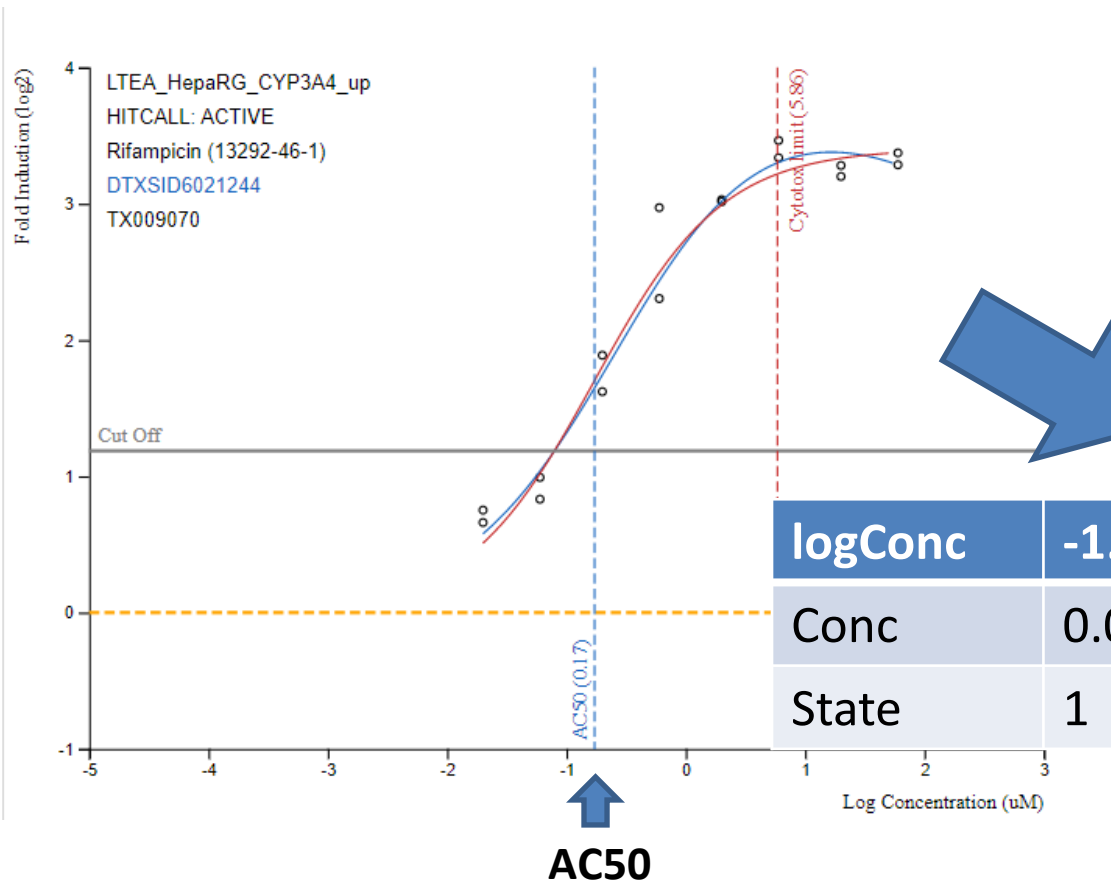
- Assay expression changes were modeled as being driven by a NR-gene interaction network
- Bayesian analysis combined prior information (literature interaction network and reference chemicals) with the observed gene expression changes in order to estimate:
 - Weights of NR-gene interactions (0 = no interaction)
 - Chemical-specific AC50's for each NR
- Analysis was performed using JAGS via the R package "runjags"

Gene State Model

- Model is not deterministic – up-signaling makes up-regulation more likely, down-signaling makes down-regulation more likely
- We simplify each gene expression levels to three states: basal, up-regulated or down-regulated
- For each chemical and nuclear receptor, we estimate an “AC50”
 - Set NR state to 0 if concentration is below the AC50 and 1 if above

State	Probability	State Calculation	
Basal	$P_i^1 = \frac{S_i^1}{S_i^1 + S_i^2 + S_i^3}$	Fixed contribution for a given gene (labelled <i>i</i>). This term includes on measurement noise.	S_i^1
Up-Regulated	$P_i^2 = \frac{S_i^2}{S_i^1 + S_i^2 + S_i^3}$	Chemical conc. and gene dependent	$S_i^2(Conc.) = \sum_{j=1}^6 UP_{i,j} * NR^j(Conc.)$
Down-Regulated	$P_i^3 = \frac{S_i^3}{S_i^1 + S_i^2 + S_i^3}$	Chemical conc. and gene dependent	$S_i^3(Conc.)_i = \sum_{j=1}^6 DOWN_{i,j} * NR^j(Conc.)$

Gene State Model



The dose-response curve is distilled to a vector of states (1/basal, 2/up-regulated, 3/down-regulated)

Anything below the AC50 is basal, anything above is up/down as appropriate

logConc	-1.5	-1	-0.5	0	0.5	1	1.5	2.0
Conc	0.032	0.1	0.32	1	3.2	10	32	100
State	1	1	1	2	2	2	2	2

We include every gene for every chemical – if there is no hit then we have a vector of all 1’s

We include higher concentrations for Life Tech chemicals where appropriate

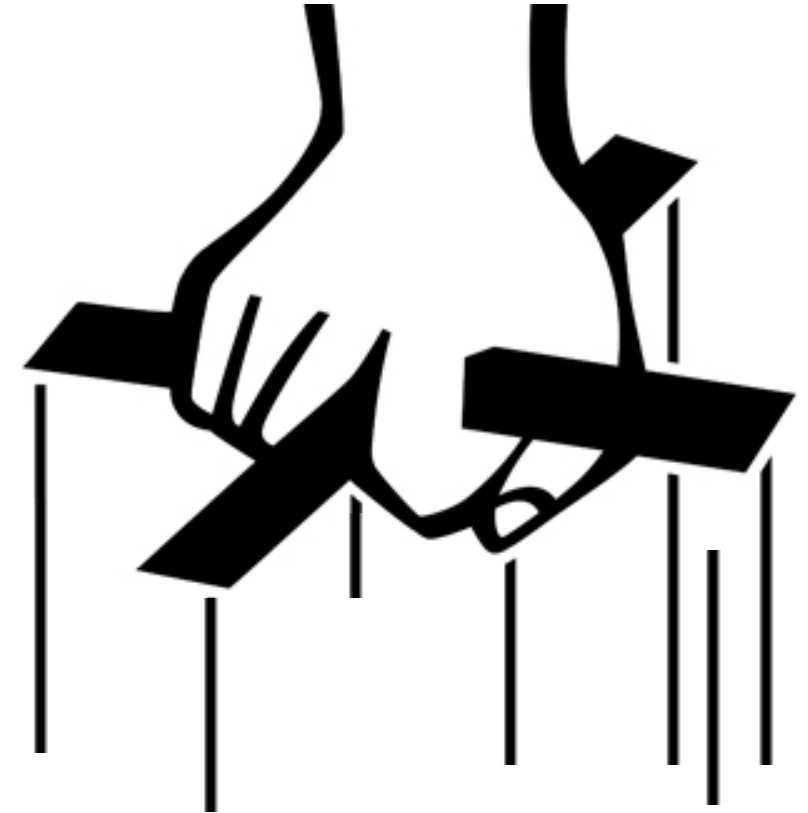
Gene State Model

- For each chemical and nuclear receptor, we estimate an “AC50”
 - Set NR state to 0 if concentration is below the AC50 and 1 if above

Conc.	NR1	NR2	NR3	NR4	NR5	NR6
0.032	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.32	0	0	0	0	0	0
1	0	0	1	0	0	0
3.2	0	0	1	0	0	0
10	0	0	1	0	0	0
32	0	0	1	0	0	0
100	0	0	1	1	0	0

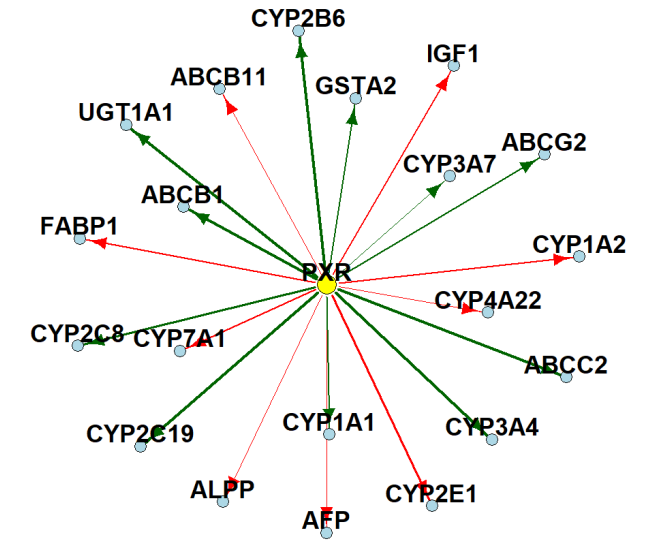
Simplifying the Problem

- Each chemical has six receptor parameters (6 different AC50's)
 - 6360 parameters
 - AC50 may be above the tested concentrations, in which case they have no effect for that gene
- 6 NRs interact with 87 genes (limited to those that were active for reference chemicals)
 - This is a 6x87 matrix with 522 parameters
- One extra parameter for each gene representing stiffness of response (S1)
- By simplifying the problem to basal/up/down with each NR either contributing to up or down regulating we have already reduced the problem from 788,640 to 6882
- Start by looking at the reference chemicals only – 651 parameters



Feature Selection

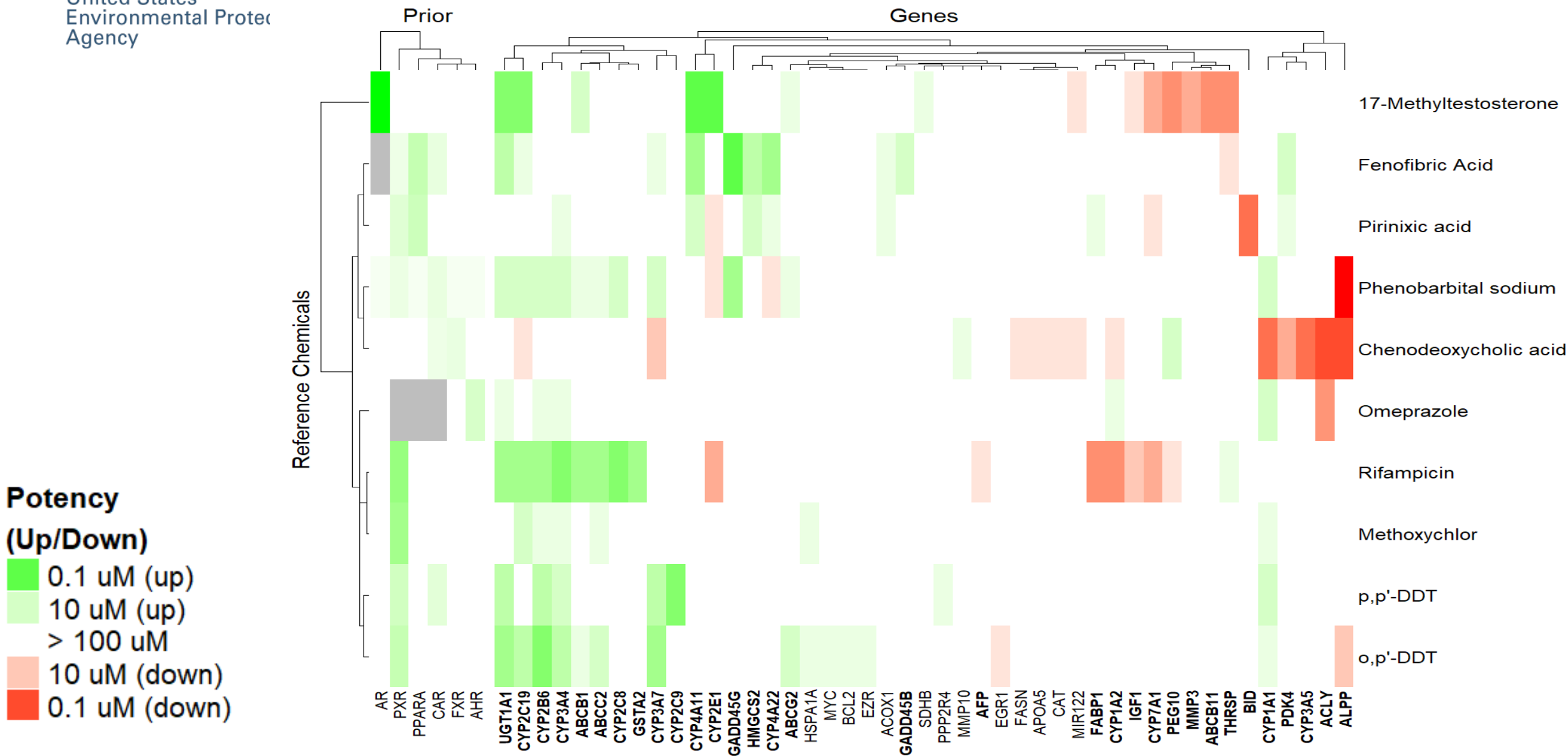
Bayesian Analysis	Description	Data	Prior	Posterior
Step One	Univariate (one receptor at a time) analysis of reference chemicals	Full reference chemical concentration-response data for all reference chemicals and only those transcripts where a change was observed.	Literature associations between reference chemicals and transcripts under investigation. Texting mining of MeSH term co-occurrence for receptors and transcripts.	Estimates of strength of interaction for every receptor and all transcripts where the reference chemicals displayed activity.
Step Two	Multivariate analysis of reference chemicals	Reference chemicals but only those transcripts where there was a 50% or greater chance of interaction in Step One.	Same as above	Estimates of strength of interaction for every receptor and every transcript identified as likely to be associated with a receptor in Step One.
Step Three	Multivariate analysis of test chemicals	Full concentration response data for all test chemicals for the same transcripts as Step Two.	The posterior from Step Two: a correlated, multivariate normal distribution of receptor-transcript interactions.	Estimates of the probability and potency of receptor activation for all test chemicals.



Feature Down-Selection

Bayesian Analysis	Description	Data	Prior	Posterior
Step One	Univariate (one receptor at a time) analysis of reference chemicals	Full reference chemical concentration-response data for all reference chemicals and only those transcripts where a change was observed.	Literature associations between reference chemicals and transcripts under investigation. Texting mining of MeSH term co-occurrence for receptors and transcripts.	Estimates of strength of interaction for every receptor and all transcripts where the reference chemicals displayed activity.
Step Two	Multivariate analysis of reference chemicals	Reference chemicals but only those transcripts where there was a 50% or greater chance of interaction in Step One.	Same as above	Estimates of strength of interaction for every receptor and every transcript identified as likely to be associated with a receptor in Step One.
Step Three	Multivariate analysis of test chemicals	Full concentration response data for all test chemicals for the same transcripts as Step Two.	The posterior from Step Two: a correlated, multivariate normal distribution of receptor-transcript interactions.	Estimates of the probability and potency of receptor activation for all test chemicals.

Reference Receptor Activators

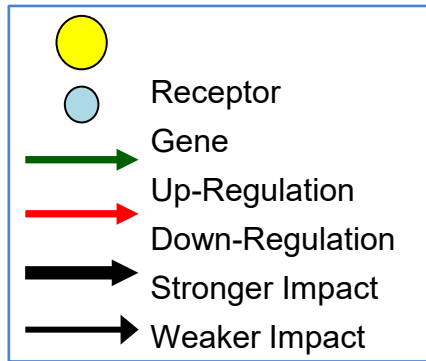


HepaRG Signaling

The list of genes used to identify receptor activity based on analysis of reference chemicals. “+” indicates up-regulation, “-” indicates down-regulation. “++” and “--” indicate above median receptor-gene strength of interaction, while “+” and “-” indicate below median interaction strength.

Gene	CYP7A1	CYP2C19	CYP2E1	UGT1A1	ABCB1	ABCB11	AFP	CYP1A1	CYP1A2	CYP3A4	FABP1	IGF1	CYP3A7	CYP4A11	CYP4A22	HMGCS2	MMP3	PEG10	THRSP	ABCC2	ABCG2	ACLY	ALPP	CYP2B6	CYP2C8	GADD45B	GADD45G	GSTA2	PKK4	BID	CYP2C9	CYP3A5	
Figure Label on Next Slide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19														
AHR	-		--	++	-	--	-	++	++	+	-	--				--	--	-		-		--				--							
AR	-	++	++	++	+	--	-	-	-			--		++			-	--	--		+												
CAR	+	-	--	--	-		-			--				--	--				--					--	-		+						
FXR	--	-				-		--	--		-		--			-	-	++				--	--						-	--		-	--
PPARA	-	+	-	++						+	++	+	+	++	++	++			-							+	+		++	-			
PXR	-	++	--	++	++	-	-	+	-	++	-	-	+		-					++	+		-	++	++			+					

Genes regulated by three or more receptors are labeled numerically, as described in the previous table.



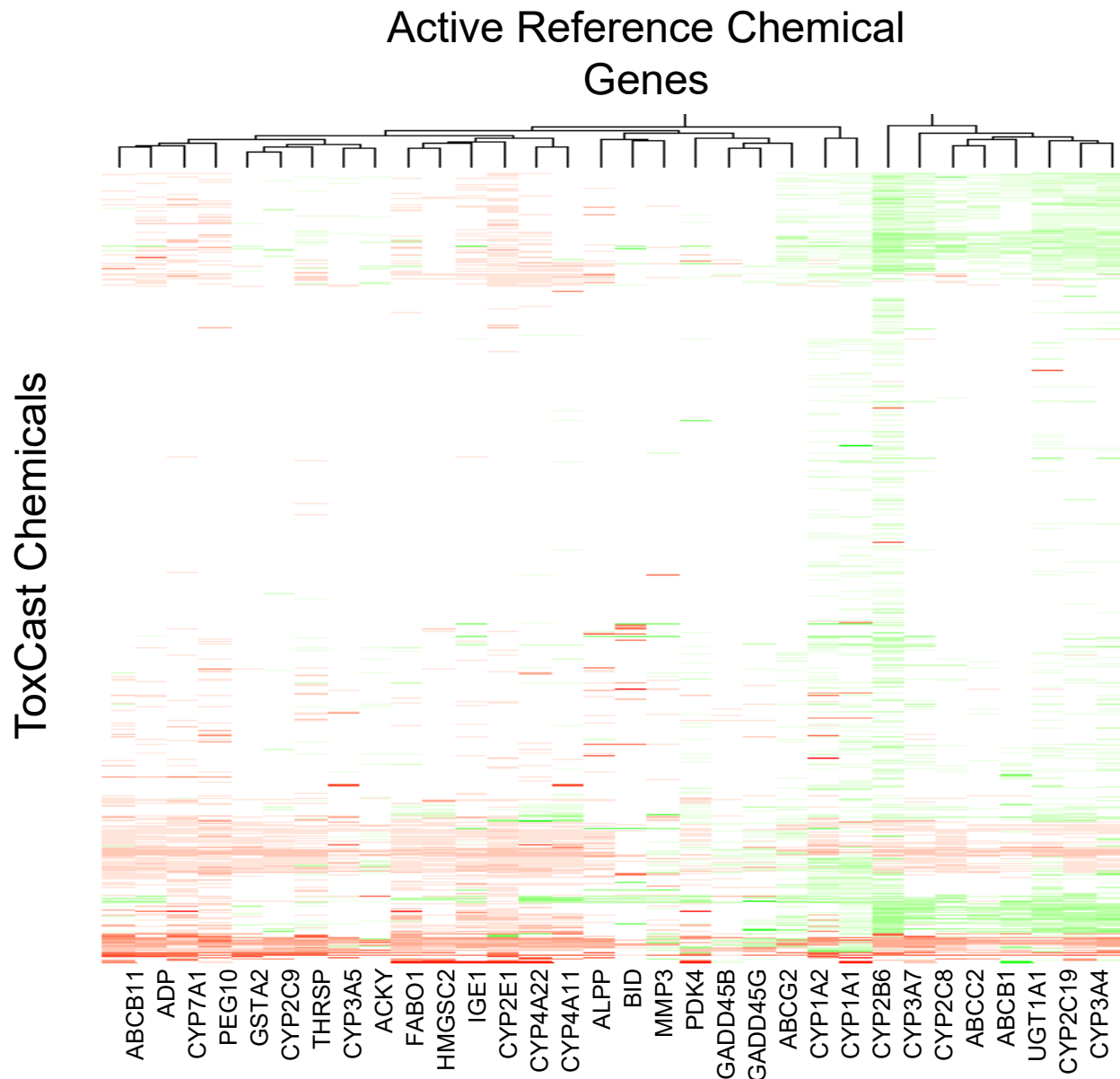
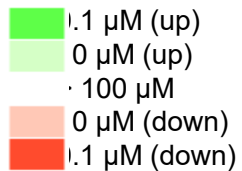
ToxCast Screening

Bayesian Analysis	Description	Data	Prior	Posterior
Step One	Univariate (one receptor at a time) analysis of reference chemicals	Full reference chemical concentration-response data for all reference chemicals and only those transcripts where a change was observed.	Literature associations between reference chemicals and transcripts under investigation. Text mining of MeSH term co-occurrence for receptors and transcripts.	Estimates of strength of interaction for every receptor and all transcripts where the reference chemicals displayed activity.
Step Two	Multivariate analysis of reference chemicals	Reference chemicals but only those transcripts where there was a 50% or greater chance of interaction in Step One.	Same as above	Estimates of strength of interaction for every receptor and every transcript identified as likely to be associated with a receptor in Step One.
Step Three	Multivariate analysis of test chemicals	Full concentration response data for all test chemicals for the same transcripts as Step Two.	The posterior from Step Two: a correlated, multivariate normal distribution of receptor-transcript interactions.	Estimates of the probability and potency of receptor activation for all test chemicals.

ToxCast LTEA Assay

The heatmap at the right presents the observed transcriptional response of transcripts identified as part of the reference chemical signatures

Potency
(Up/Down)

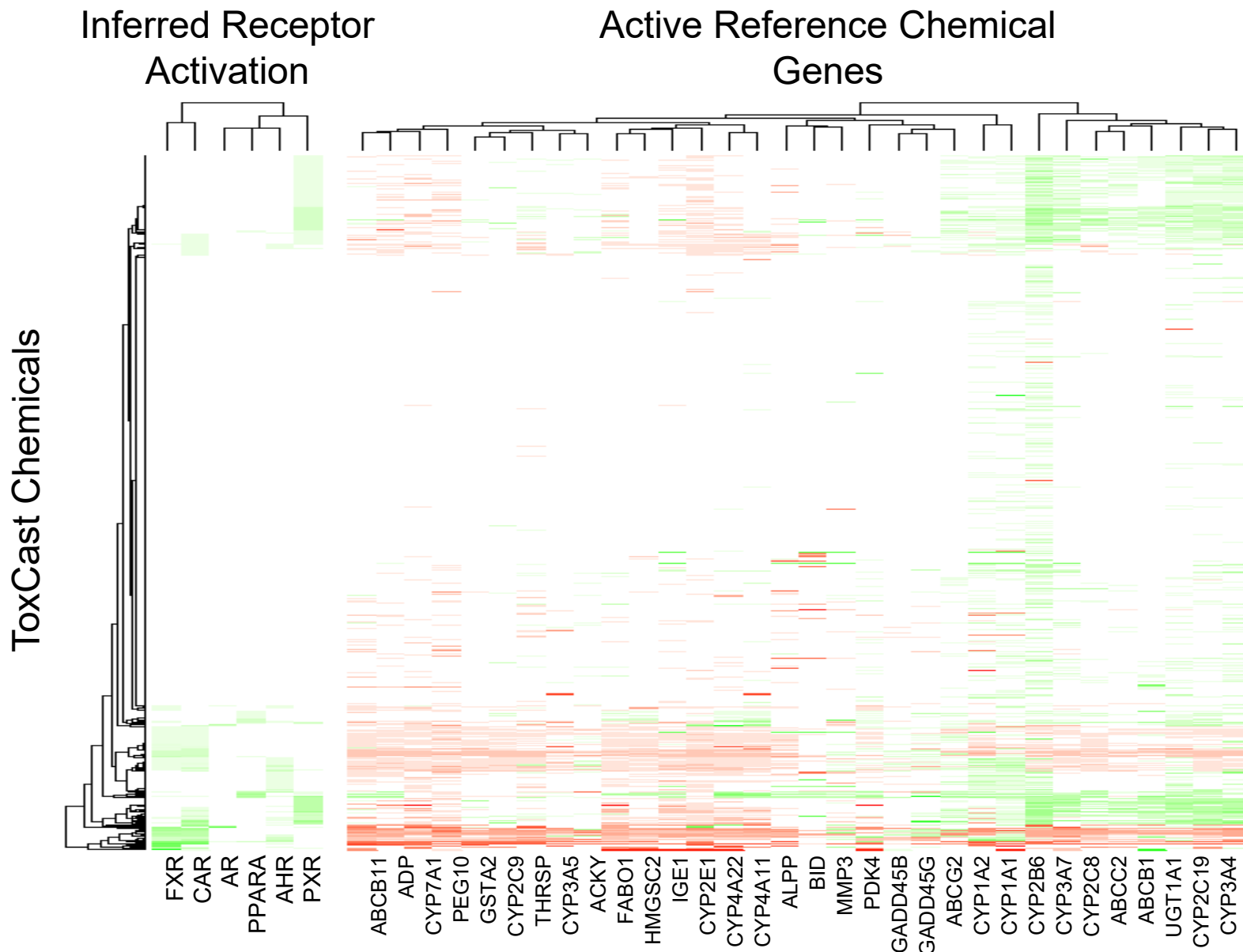
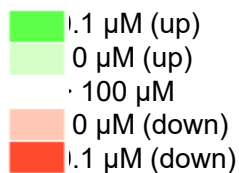


Putative MIE's from ToxCast LTEA Assay

The heatmap at the right presents the observed transcriptional response of transcripts identified as part of the reference chemical signatures

The left-hand heatmap indicates the relative potency inferred for the six receptors

Potency
(Up/Down)



MIE Inferences

Supplementary Table 5: ToxCast Chemical Receptor Potency Inference (μM)

	AHR	AR	CAR	FXR	PPARA	PXR	<200 μM
Aflatoxin B1	1000000	1000000	63.09573	316.2278	1000000	1000000	Yes
Triflumizole	3.162278	1000000	3.162278	3.162278	1000000	1000000	Yes
4,4'-Sulfonylbis[2-(prop-2-en-1-yl)phenol]	1000000	1000000	1000000	1000000	1000000	1000000	
Tamoxifen	1000000	1000000	3.162278	1000000	1000000	1000000	Yes
FR167356	3.162278	1000000	1000000	1000000	1000000	1000000	Yes
Niclosamide	12.58925	1000000	12.58925	10	1000000	1000000	Yes
Phenylmercuric acetate	1000000	1000000	15.84893	10	1000000	1000000	Yes
Benzo(b)fluoranthene	10	1000000	10	3.162278	1000000	1000000	Yes
Fabesetron hydrochloride	3.162278	1000000	3.162278	1000000	1000000	1000000	Yes
Abamectin	1000000	1000000	3.162278	3.162278	1000000	1000000	Yes
PFOSA	1000000	1000000	10	10	1000000	1000000	Yes
Haloperidol	1000000	1000000	1000000	1000000	1000000	1000000	

“1000000” means no activity inferred

MIE Inferences

Supplementary Table 5: ToxCast Chemical Receptor Potency Inference (μM)

	AHR	AR	CAR	FXR	PPARA	PXR	<200 μM
Aflatoxin B1			63.09573	316.2278			Yes
Triflumizole	3.162278		3.162278	3.162278			Yes
4,4'-Sulfonylbis[2-(prop-2-en-1-yl)phenol]							
Tamoxifen			3.162278				Yes
FR167356	3.162278						Yes
Niclosamide	12.58925		12.58925	10			Yes
Phenylmercuric acetate			15.84893	10			Yes
Benzo(b)fluoranthene	10		10	3.162278			Yes
Fabesetron hydrochloride	3.162278		3.162278				Yes
Abamectin			3.162278	3.162278			Yes
PFOSA			10	10			Yes
Haloperidol							

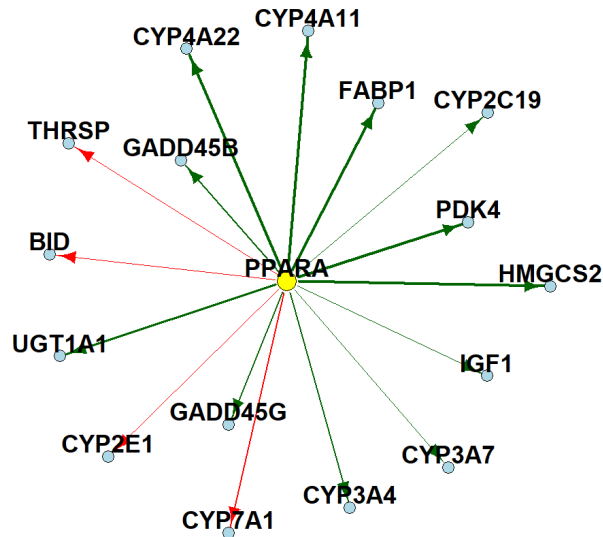
No receptor activity was inferred for 43% of the chemicals and only 37% have any activity inferred below 100 μM

Comparison with Other ToxCast Assays

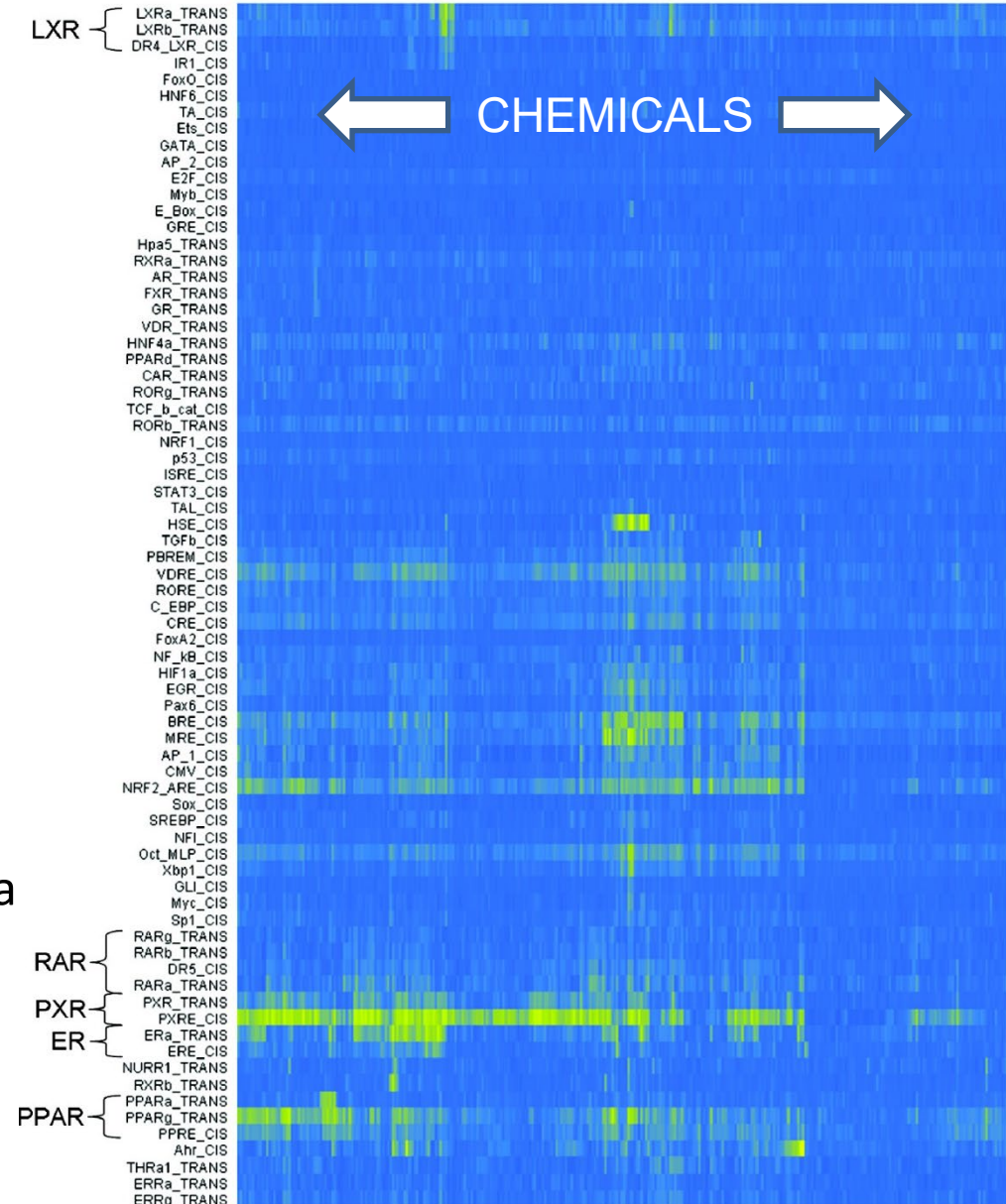
- We did not observe activity for most chemicals.
- While we did not observe as frequent activity as other ToxCast screens, we believe that the synthesis of multiple transcript activities into signatures that must be consistently observed reduces the likelihood of false positives.
- The ToxCast Factorial assay (Attagene) uses modified HepG2 cells to identify chemical perturbations of many transcription factors, including CAR, PXR, PPAR α , FXR, and AR

Comparison with ToxCast Factorial (Attagene) Assay

Steve Ferguson:
If each transcript is a note,
then each receptor plays a chord.



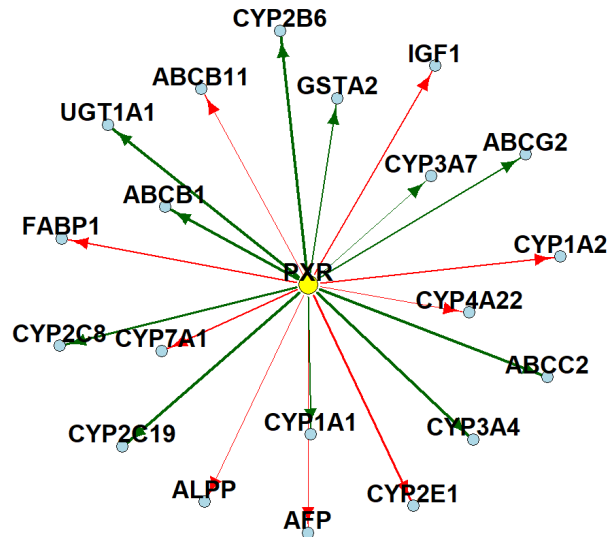
- **PPAR α** - the model identified **28** chemicals that also indicated activity with ToxCast Factorial (mean probability of 61%)
 - identified **45** chemicals not identified by the ToxCast Factorial assay that had an average probability of 32%
 - the Factorial assay found **52** chemicals not identified with transcriptomics



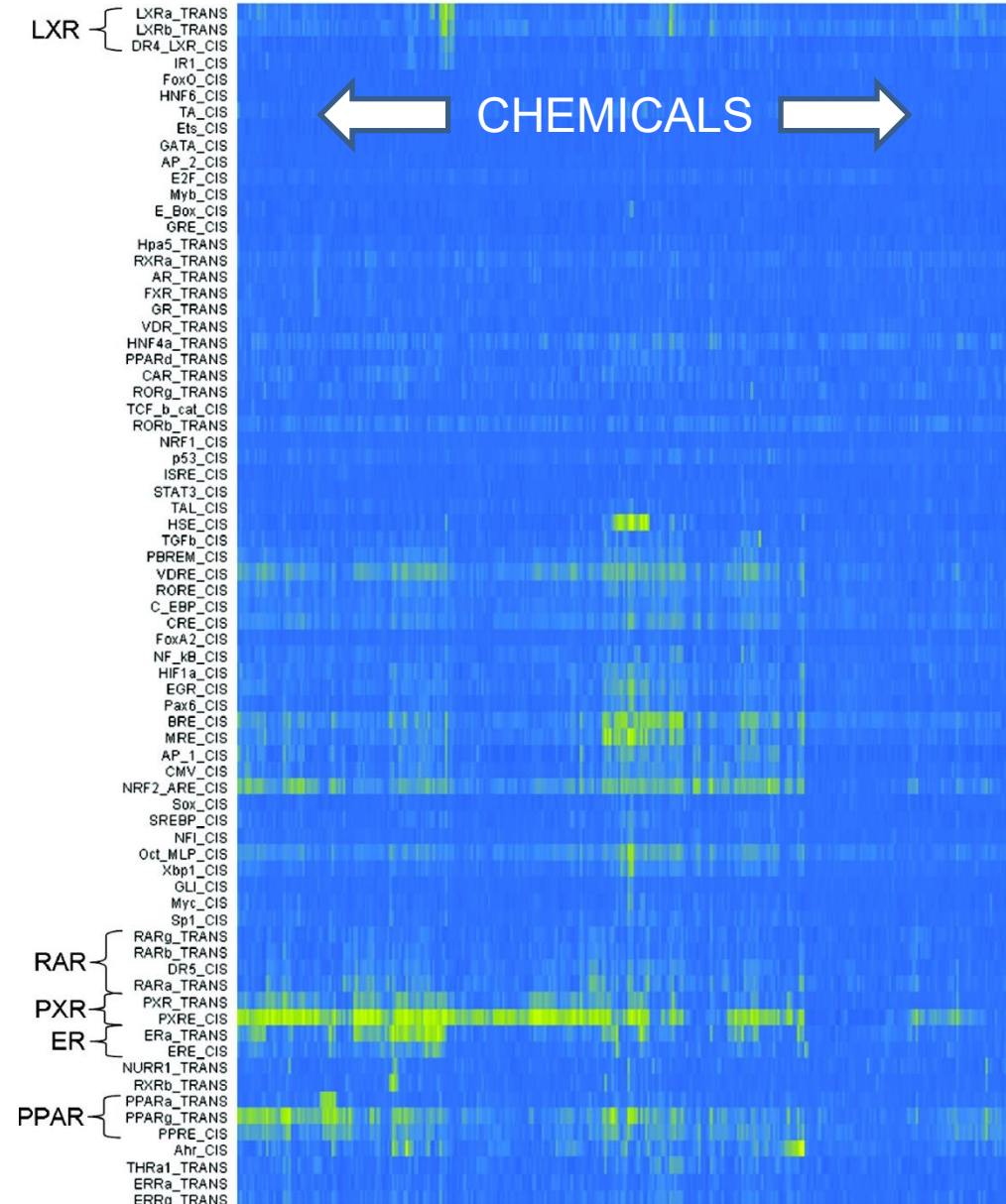
Martin et al. (2010)

Comparison with ToxCast Factorial (Attagene) Assay

Steve Ferguson:
If each transcript is a note,
then each receptor plays a chord.



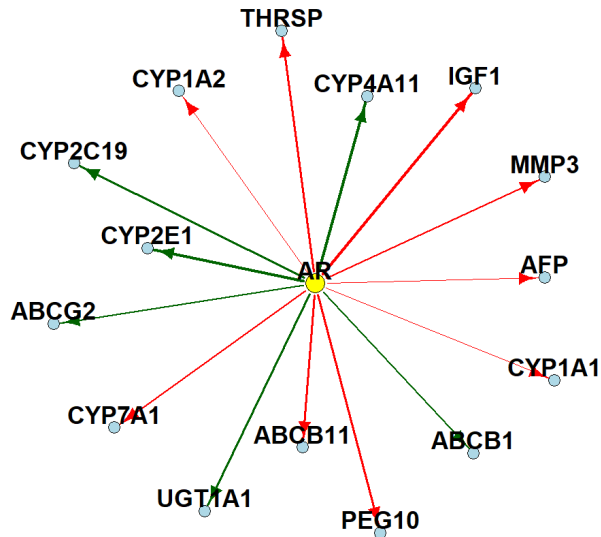
- **PXR** - the model agreed with the Factorial assay on **131** chemicals (mean probability 77%)
 - identified an additional **91** chemicals with a mean probability of 71%
 - assigned 0% probability to **187** chemicals identified with the Factorial assay



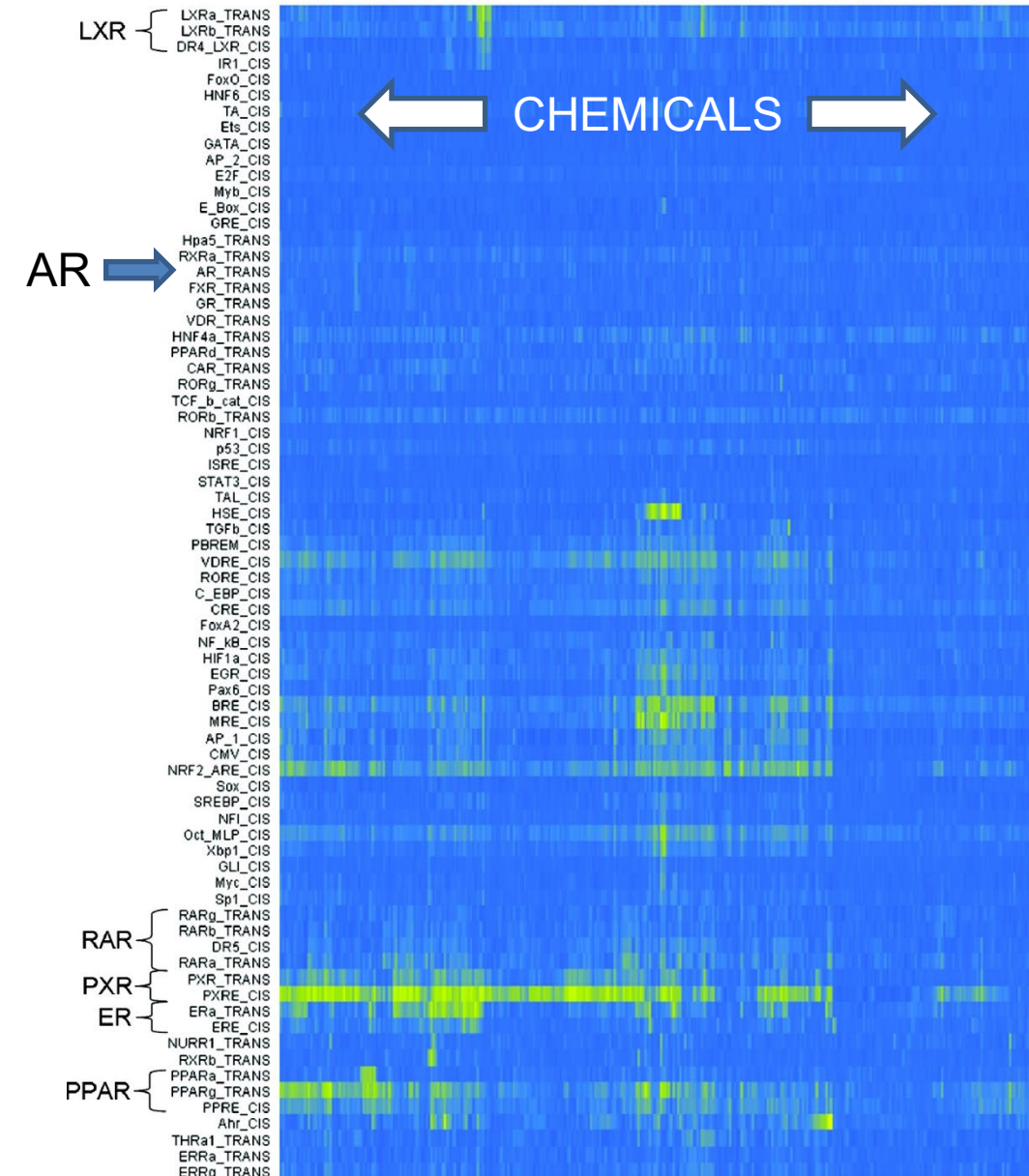
Martin et al. (2010)

Comparison with ToxCast Factorial (Attagene) Assay

Steve Ferguson:
If each transcript is a note,
then each receptor plays a chord.



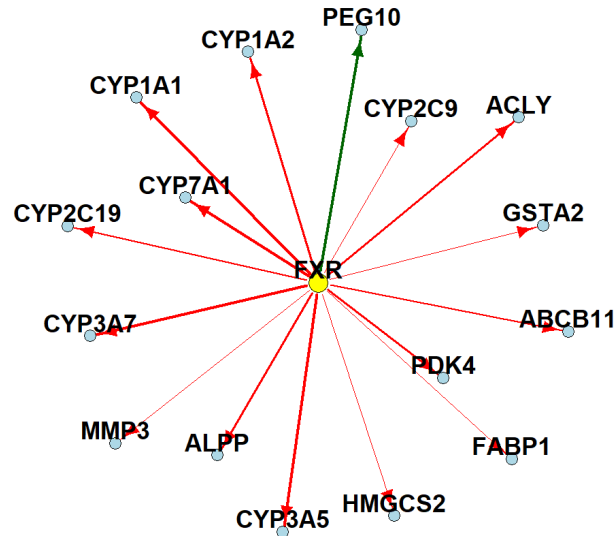
- **AR** - the assays agreed on **8** chemicals and the Bayesian transcriptomics model (mean probability 75%)
 - identified **29** chemicals not found with the ToxCast Factorial assay, but the mean probability was only 8.5%
 - assigned 0% probability to **14** chemicals identified as AR regulators by the ToxCast Factorial assay



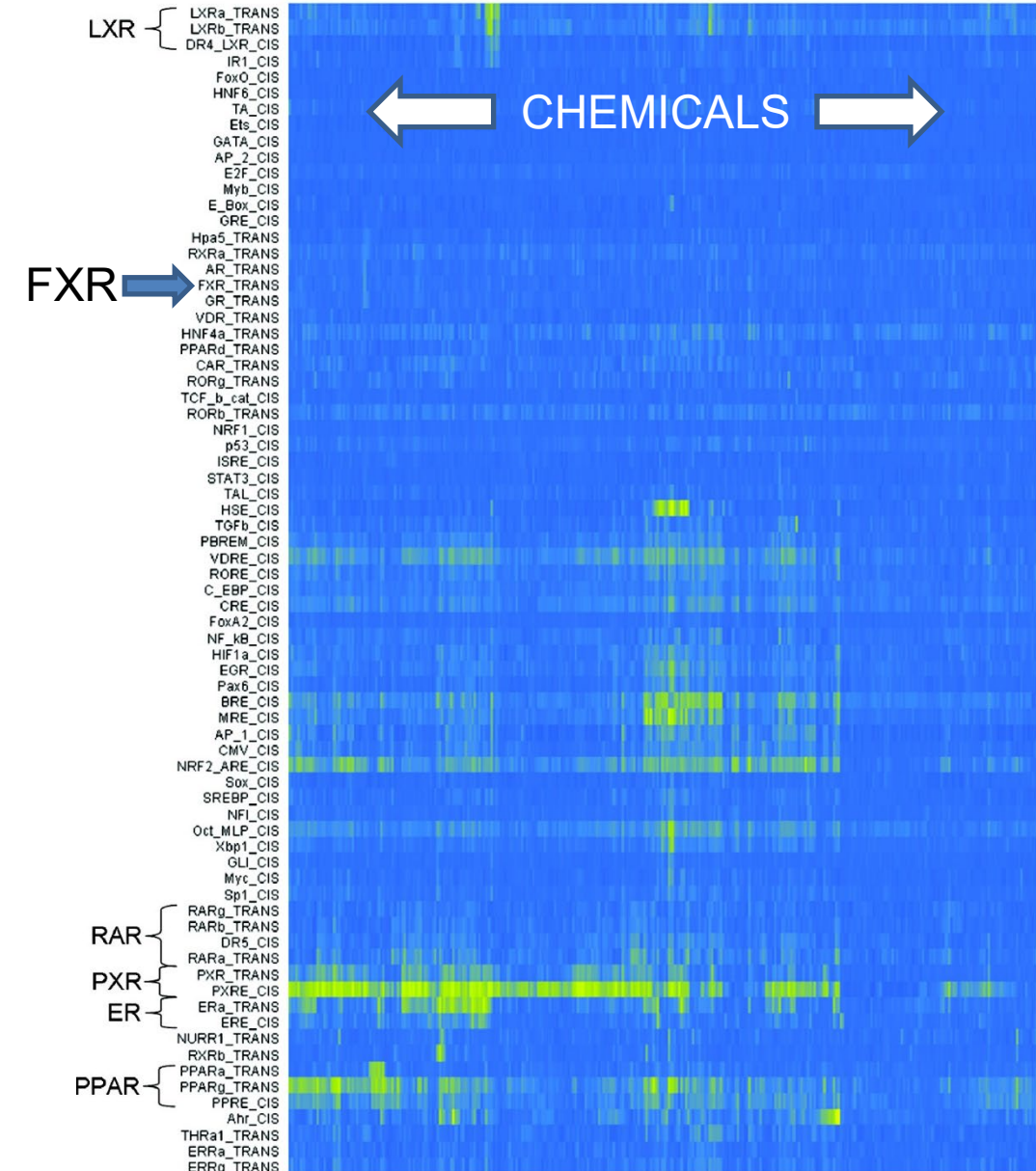
Martin et al. (2010)

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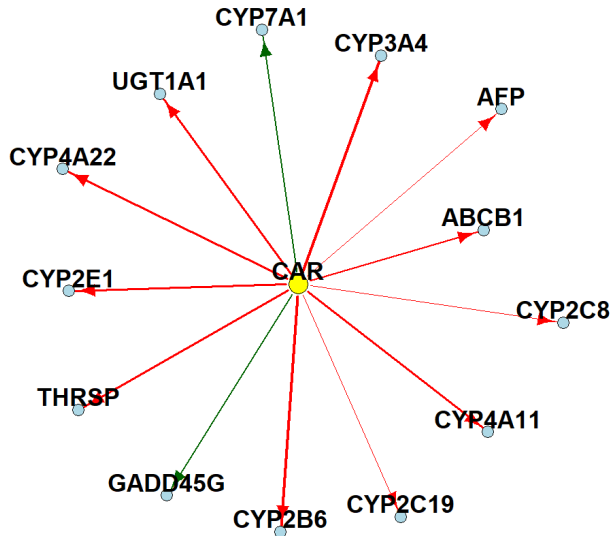
- **FXR** - the two assays agreed on **15** chemicals as potential agonists (mean probability 73%)
 - identified an additional **130** with a mean probability of 70%
 - **40** chemicals identified by the Factorial assay were assigned 0%



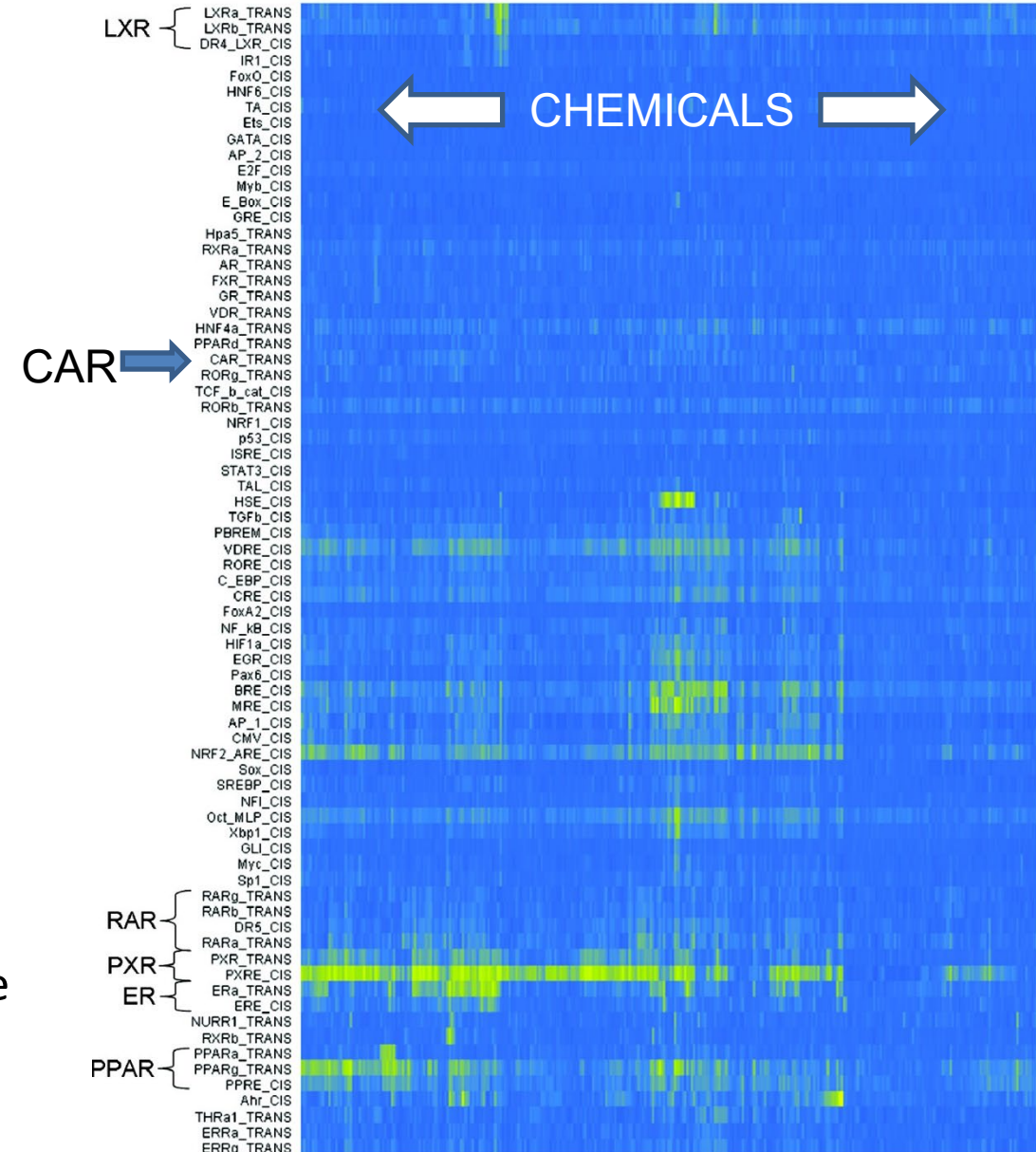
Martin et al. (2010)

Comparison with ToxCast Factorial (Attagene) Assay

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If each transcript is a note,
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- **CAR** - the Factorial assay agreed on only **4** chemicals (mean probability 1.3%)
 - identified an additional **330** chemicals with a mean probability 44%
 - assigned 0% probability to **15** chemicals identified by the Factorial assay



Martin et al. (2010)

Conclusions

- Transcriptomics with metabolically-competent *in vitro* models presents an opportunity for more thorough, accurate screening
- LTEA data characterizes perturbations on sentinel targets of cellular signaling pathways for 1,060 chemicals
- We analyzed these data to identify patterns of transcription that are indicative of six different molecular initiating events and assess the probability of those events occurring as a function of concentrations for all the chemicals
 - Receptor activity inference method complexity grows with number of receptors, so not too many more than six at once
- We can identify putative MIEs for receptors as a steppingstone toward more quantitative AOP-based toxicological research.
 - See Supplemental Table 5