

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

The ToxCast LTEA Assay

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Stephen Ferguson

Quintiles

(formerly Expression Analysis)

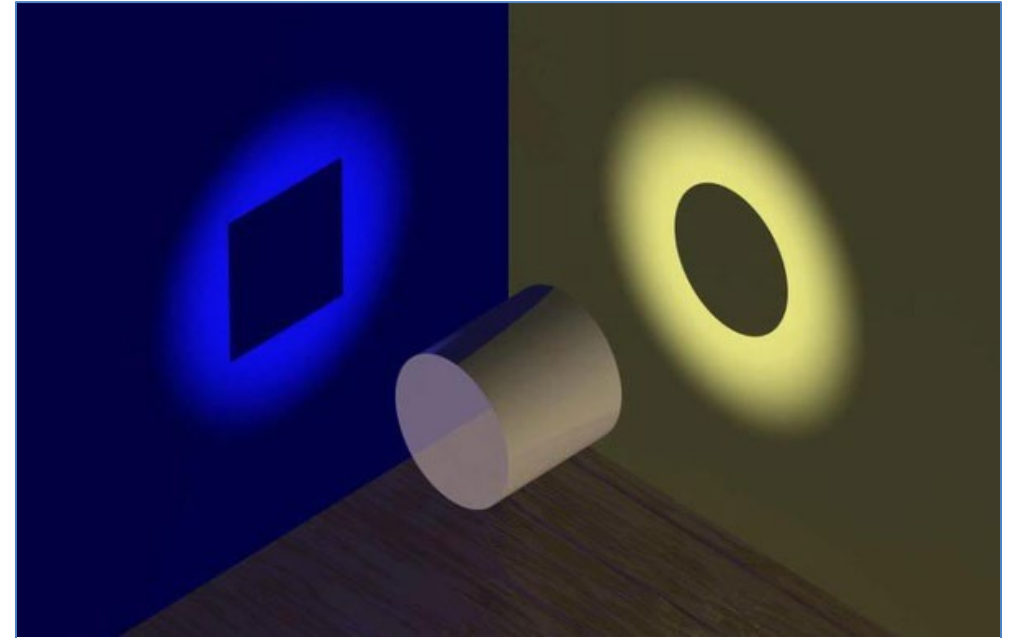
Patrick Hurban

Stephen Siferd

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. Bonzo^{1,2}, John Jack¹, Nancy C. Baker^{1,3}, Parth Kothiyia¹, Rafal P. Witek², Patrick Hurban⁴, Stephen Siferd⁴, Susan Hester^{1,5}, Imran Shah¹, Stephen S. Ferguson^{1,5}, Keith A. Houck¹ and John F. Wambaugh^{1,6}

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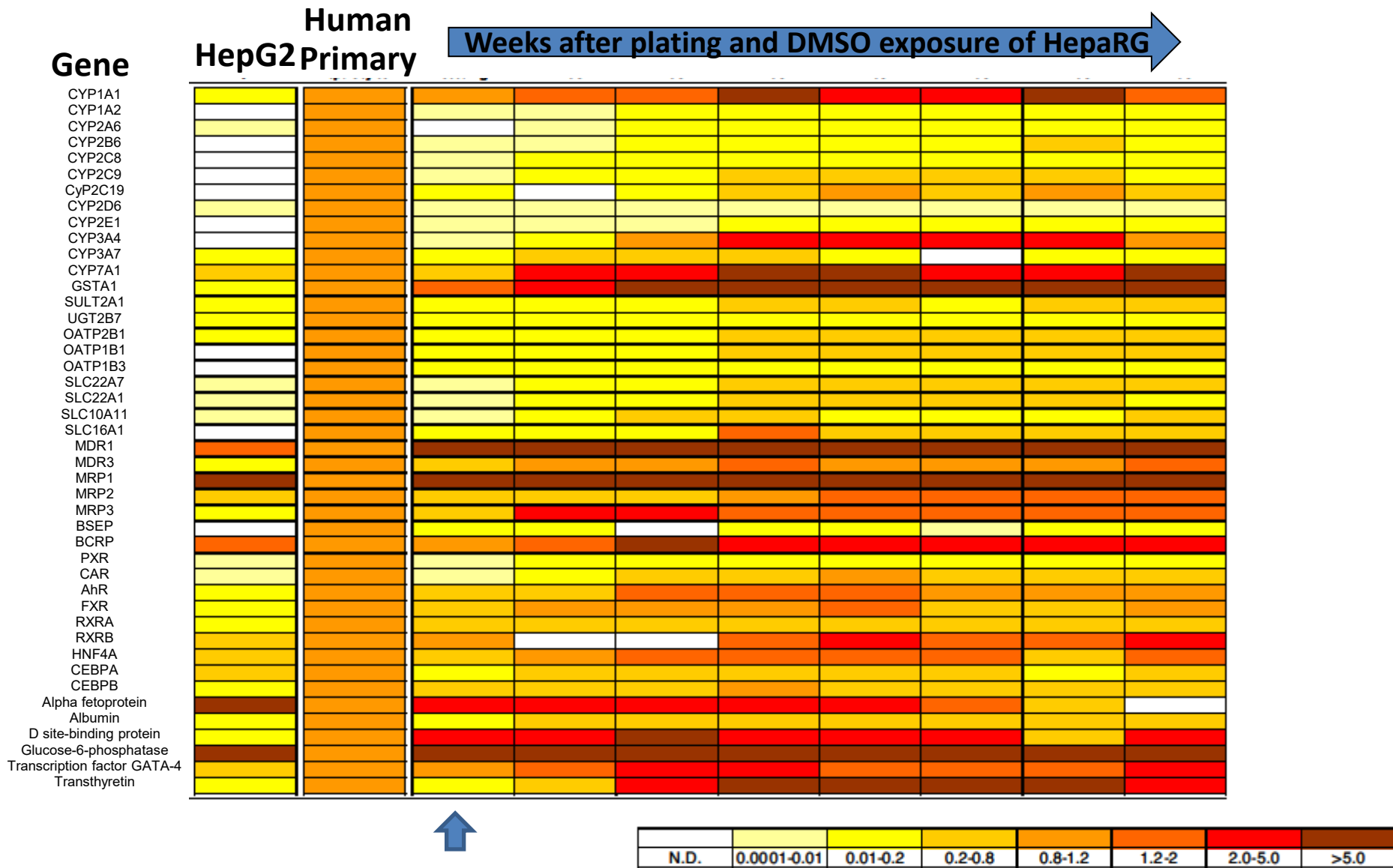
The ToxCast LTEA Assay Endpoints

- 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:

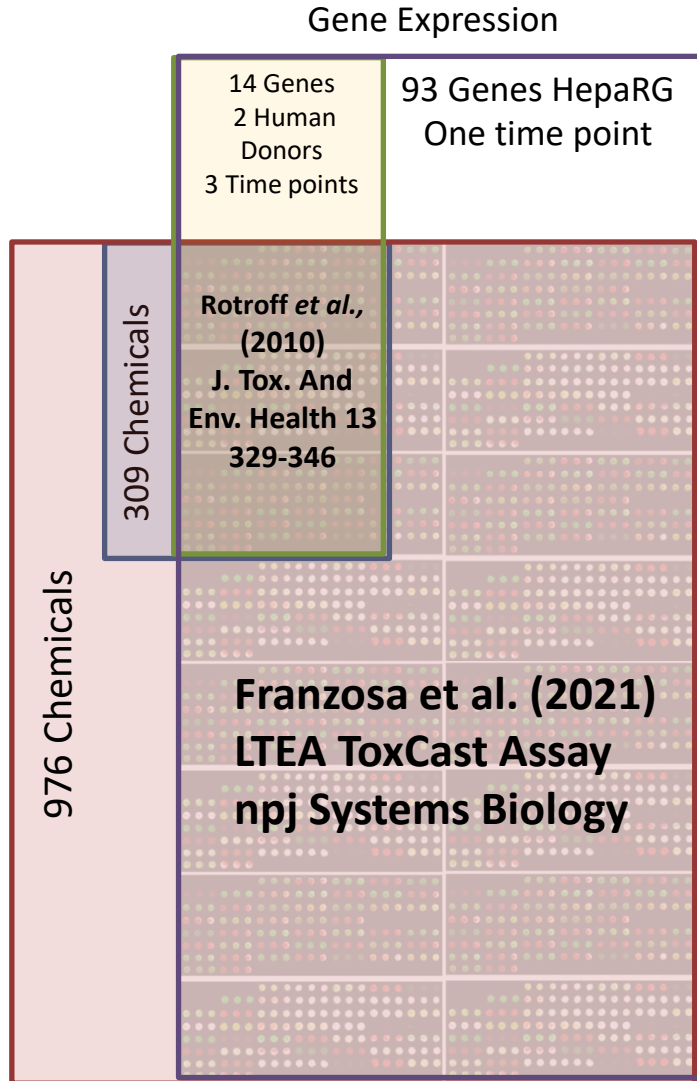
- Cholangiocytes (bile producing)
- Hepatocytes (which are responsible for much of metabolism)



ToxCast HepaRG at 96h

Kanebratt and Anderssen (2008)

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

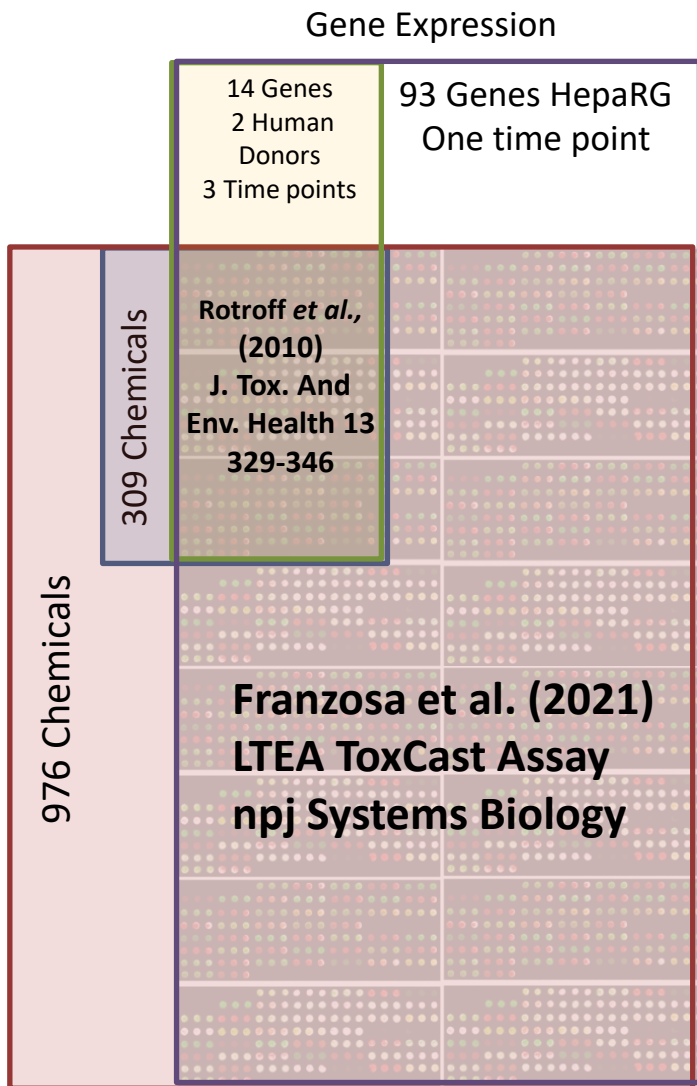
- qRT-PCR using Fluidigm 96.96 microfluidic technology
- $\Delta\Delta Ct$ (fold-change relative to DMSO and 3 housekeeping genes)
- 93 genes covering Phase I & II enzymes, transporters, cell-cycle, and disease states

Enumerating the Problem

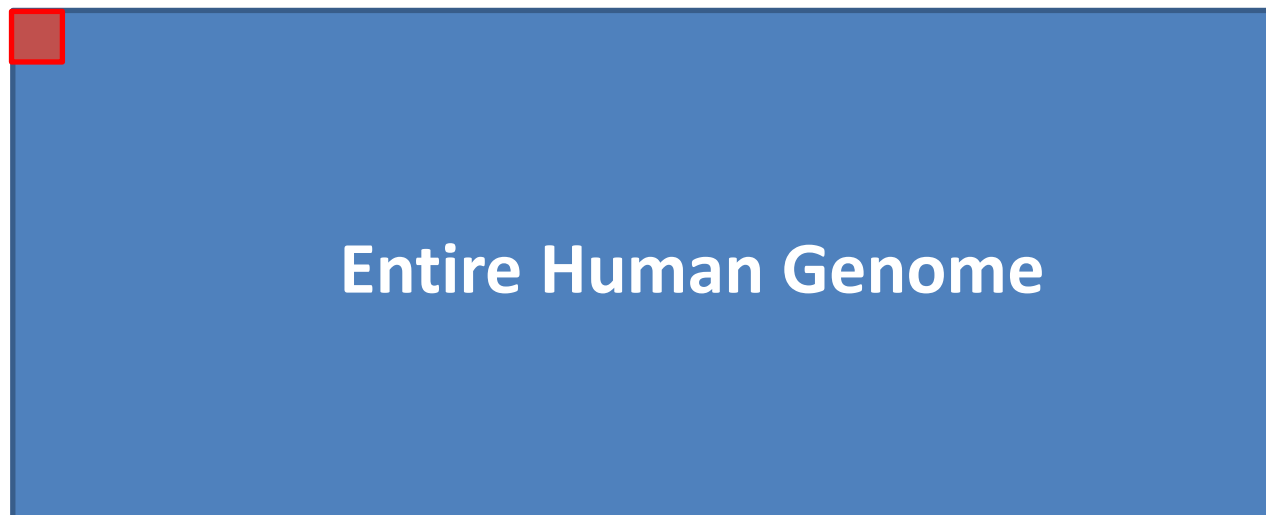
- 93 genes, eight concentrations, two replicates, across 1060 chemicals is 1,577,280 data points (not counting reference plates, control genes, LDH cytotox assay)
 - Rotroff et al. (2010) was 14 genes, eight concentrations, two replicates, by 309 chemicals for 69,216 data points
- Hill-model curve fits per gene per chemical are 4 parameters * 1060 chemicals * 93 genes * two 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

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DOI: 10.1080/10937404.2010.483949



XENOBIOTIC-METABOLIZING ENZYME AND TRANSPORTER GENE EXPRESSION IN PRIMARY CULTURES OF HUMAN HEPATOCTES 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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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.

14 genes, 309 chemicals

93 genes, 976 chemicals

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XENOBIOTIC-METABOLIZING GENE EXPRESSION IN PRIMAR HEPATOCTES MODULATED I

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Primary human hepatocyte culture when cultured under appropriate such as metabolism, transport, and regulate the concentration- and time-expression of genes regulated by quantitative nuclease protection and transporters, 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 of one or more of the 14 genes. Significant relative risk assessment reported for the five major receptors reported into the larger ToxCast pr

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

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The ToxCast in vitro screening program has provided concentration-response bioactivity data across more than a thousand assay endpoints for thousands of chemicals found in our environment and commerce. However, most ToxCast screening assays have evaluated individual biological targets in cancer cell lines lacking integrated physiological functionality (such as receptor signaling, metabolism). We evaluated differentiated HepaRGTM cells, a human liver-derived cell model understood to effectively model physiologically relevant hepatic signaling. Expression of 93 gene transcripts was measured by quantitative polymerase chain reaction using Fluidigm 96.96 dynamic arrays in response to 1060 chemicals tested in eight-point concentration-response. A Bayesian framework quantitatively modeled chemical-induced changes in gene expression via six transcription factors including: aryl hydrocarbon receptor, constitutive androstane receptor, pregnane X receptor, farnesoid X receptor, androgen receptor, and peroxisome proliferator-activated receptor alpha. For these chemicals the network model translates transcriptomic data into Bayesian inferences about molecular targets known to activate toxicological adverse outcome pathways. These data also provide new insights into the molecular signaling network of HepaRGTM cell cultures.

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Toxicogenomic Screening Continues to Evolve

14 genes, 309 chemicals

93 genes, 976 chemicals

>20,000 genes, 42 chemicals

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High-throughput toxicology environment using meta cultures

Jill A. Franzosa¹, Jessica A. Bonzo^{1,2}, John Jack^{1,3},
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The ToxCast in vitro screening program has provided endpoints for thousands of chemicals found in our environment (evaluated individual biological targets in cancer and metabolism). We evaluated differentiated HepaRG cells for physiologically relevant hepatic signaling. Expression analysis using Fluidigm 96.96 dynamic arrays in a Bayesian framework quantitatively modeled chemical activity at the aryl hydrocarbon receptor, constitutive androstano receptor, and peroxisome proliferator-activated receptor alpha. Bayesian inferences about molecular targets known and unknown provided new insights into the molecular signaling network.

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Research Article

High-Throughput Transcriptomics Platform for Screening Environmental Chemicals

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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

14 genes, 309 chemicals

93 genes, 976 chemicals

>20,000 genes, 42 chemicals

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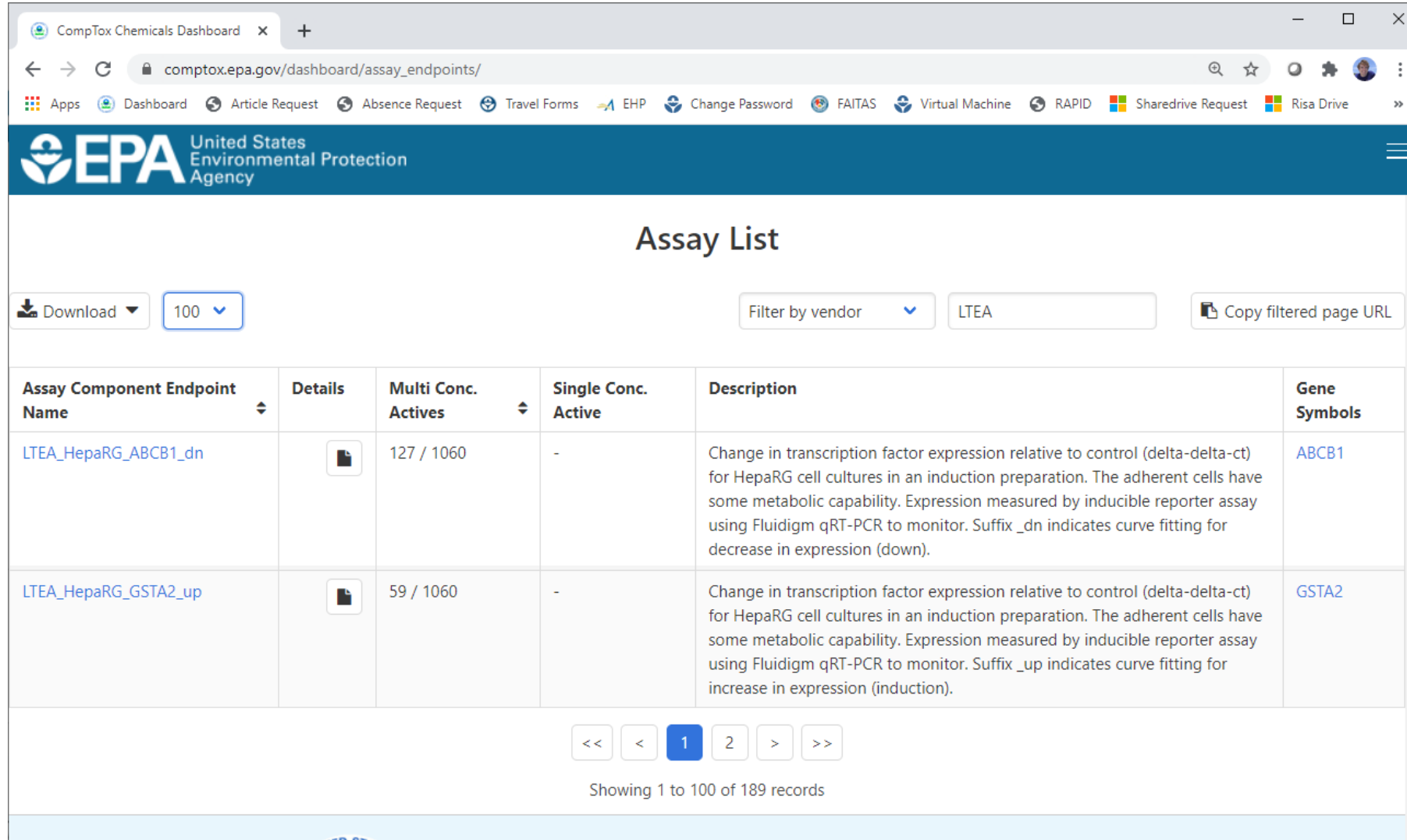
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Downloaded from <https://academic.oup.com/toxsci/advance-article/doi/10.1093/toxsci/ktab0>



The LTEA Data are on the Dashboard

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



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 |

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 |
| 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 |
| H | PB.8 | PB.8 | OMP. 8 | OMP. 8 | FF.8 | FF.8 | CDCA. 8 | CDCA. 8 | AFL.8 | AFL.8 | | EA |

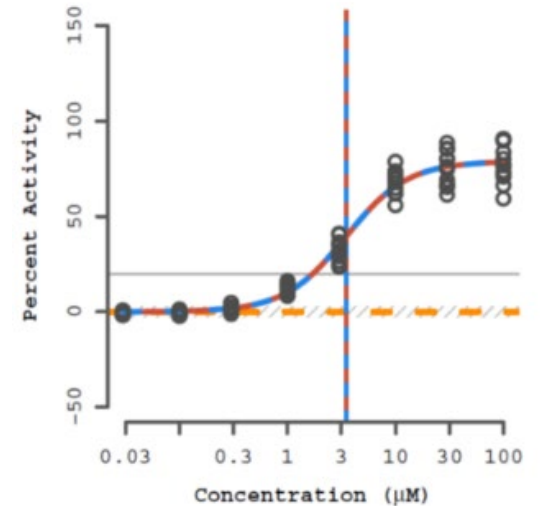
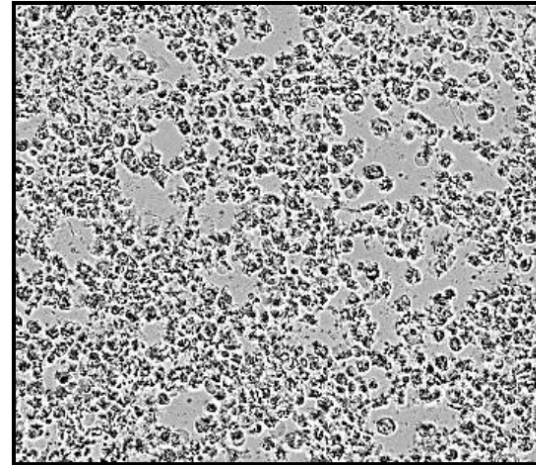
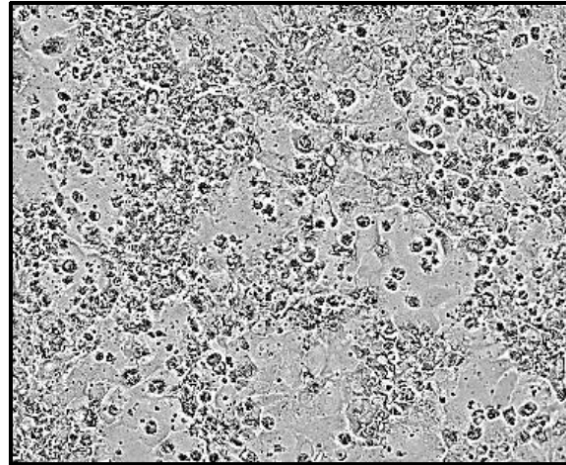
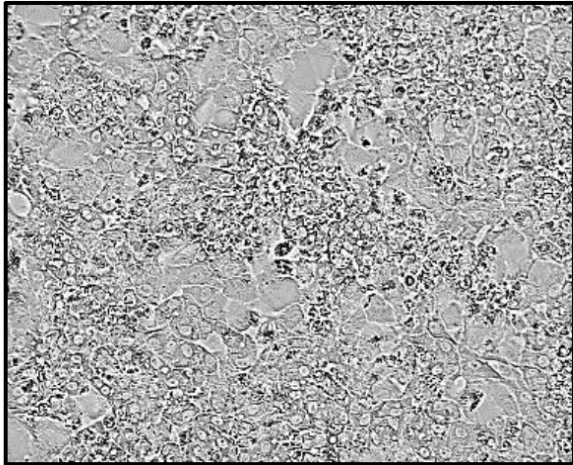
No cells

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

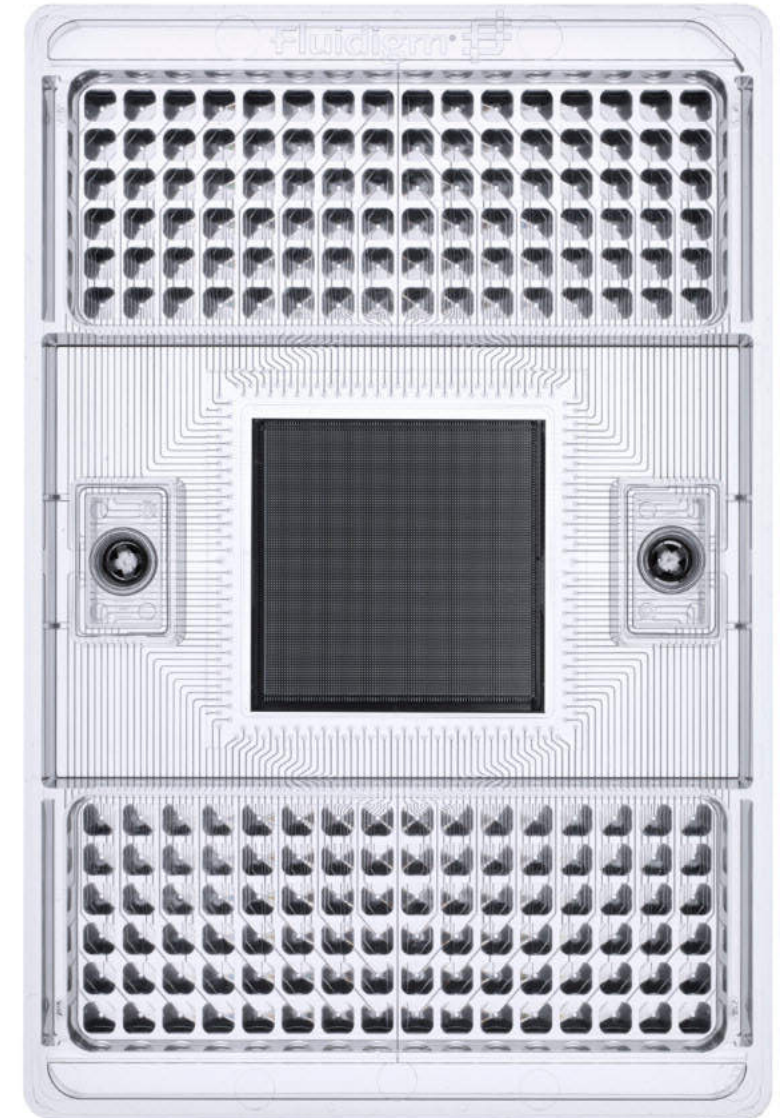
Metabolic Competency

“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

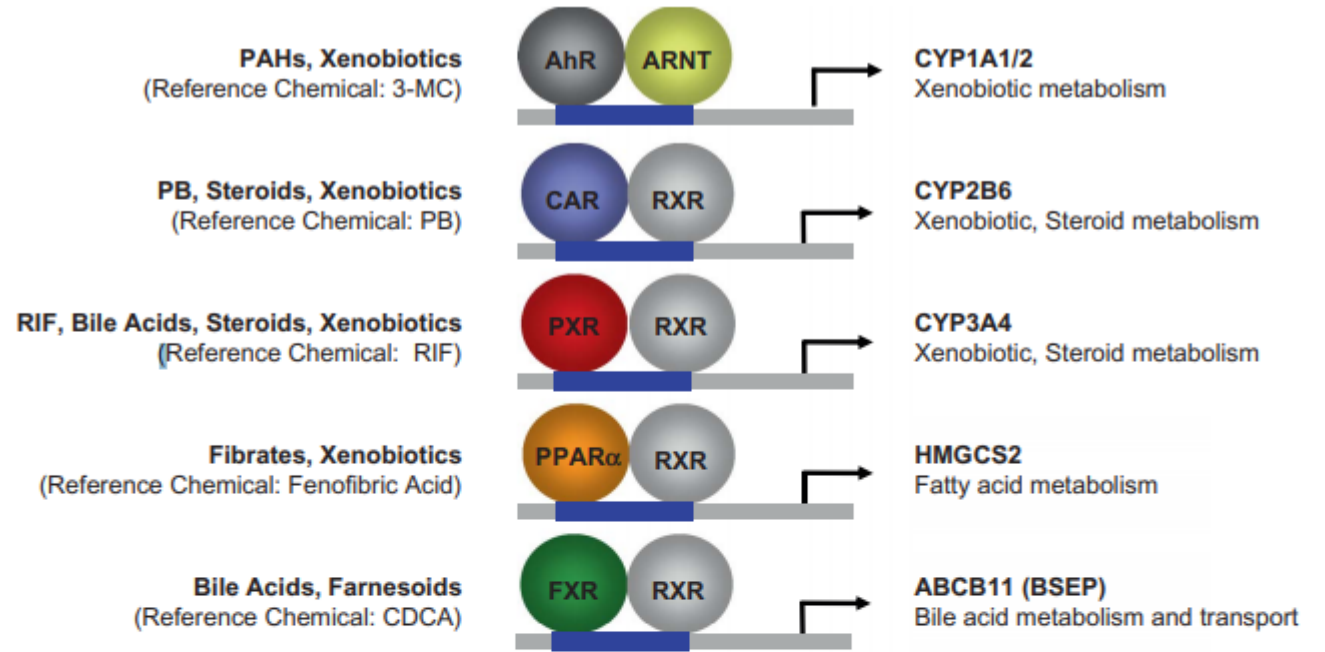
- Patrick Hurban led gene expression analysis at Expression analysis/Quintiles
- Fluidigm's 96.96 Dynamic Array was used for gene expression analyses by quantitative reverse transcription polymerase chain reaction (qRT-PCR)
- Standard TaqMan Assays were used to assess the expression of 93 genes
- Three “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



Target Gene Categories

- CYP450 (6)
- Transporter (4)
- Phase II Metabolism (3)
- Cholesterol Synthesis (1)
- Endogenous control (2)



Rotroff et al. (2010)

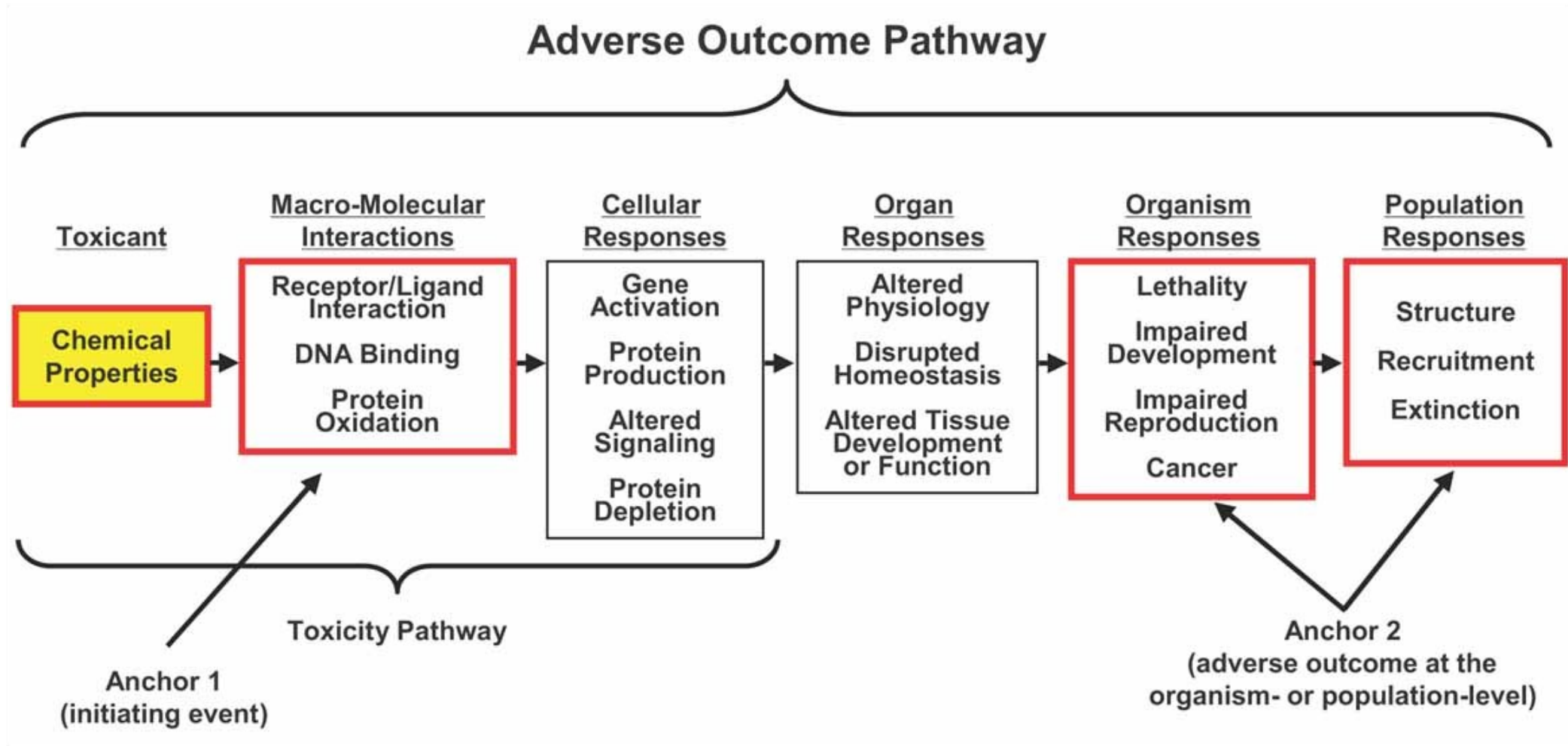
Supplemental Table I Provides Gene Selection Reasoning

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

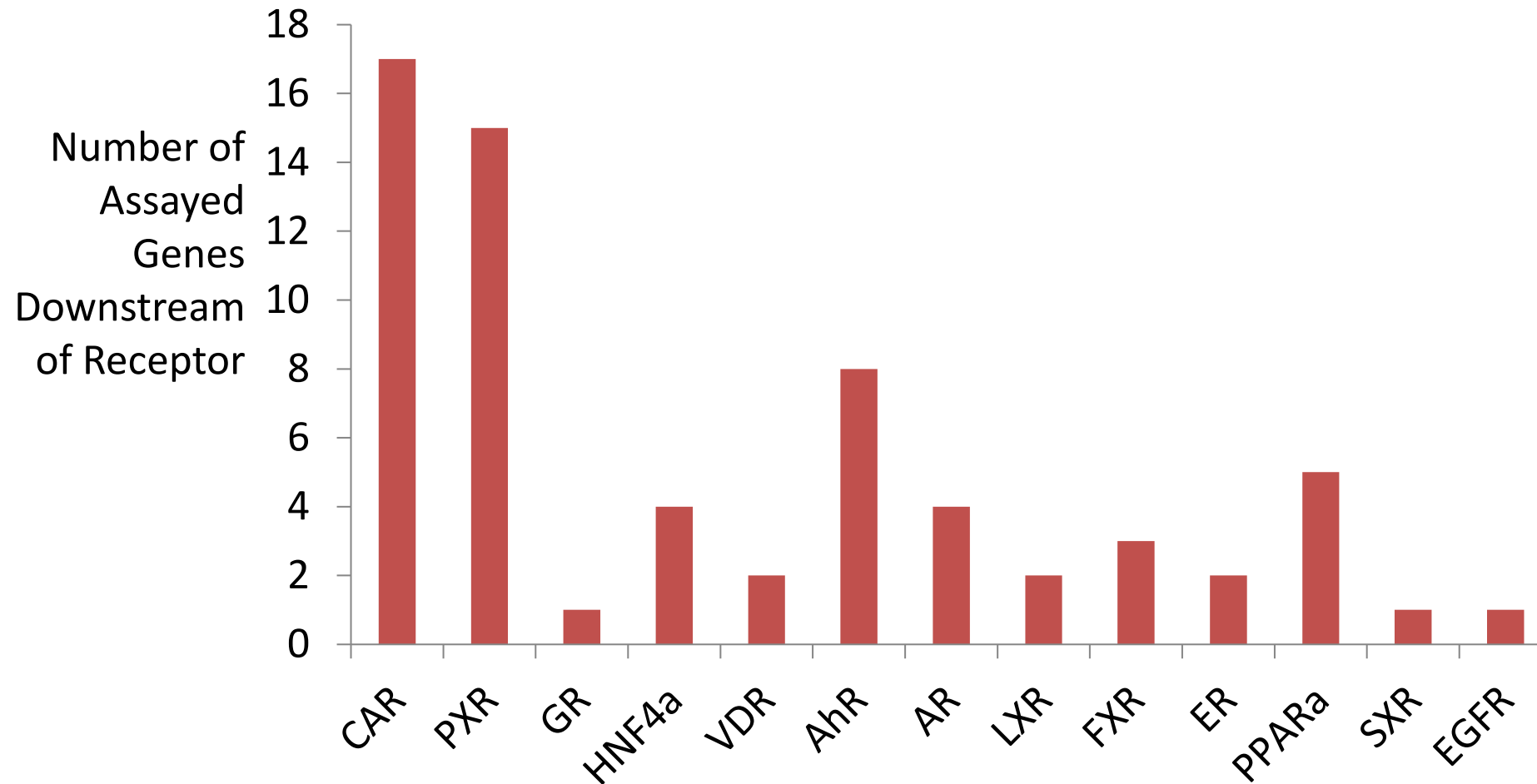
Supplementary Table 1: Genes Assayed as Part of ToxCast Screen

| 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

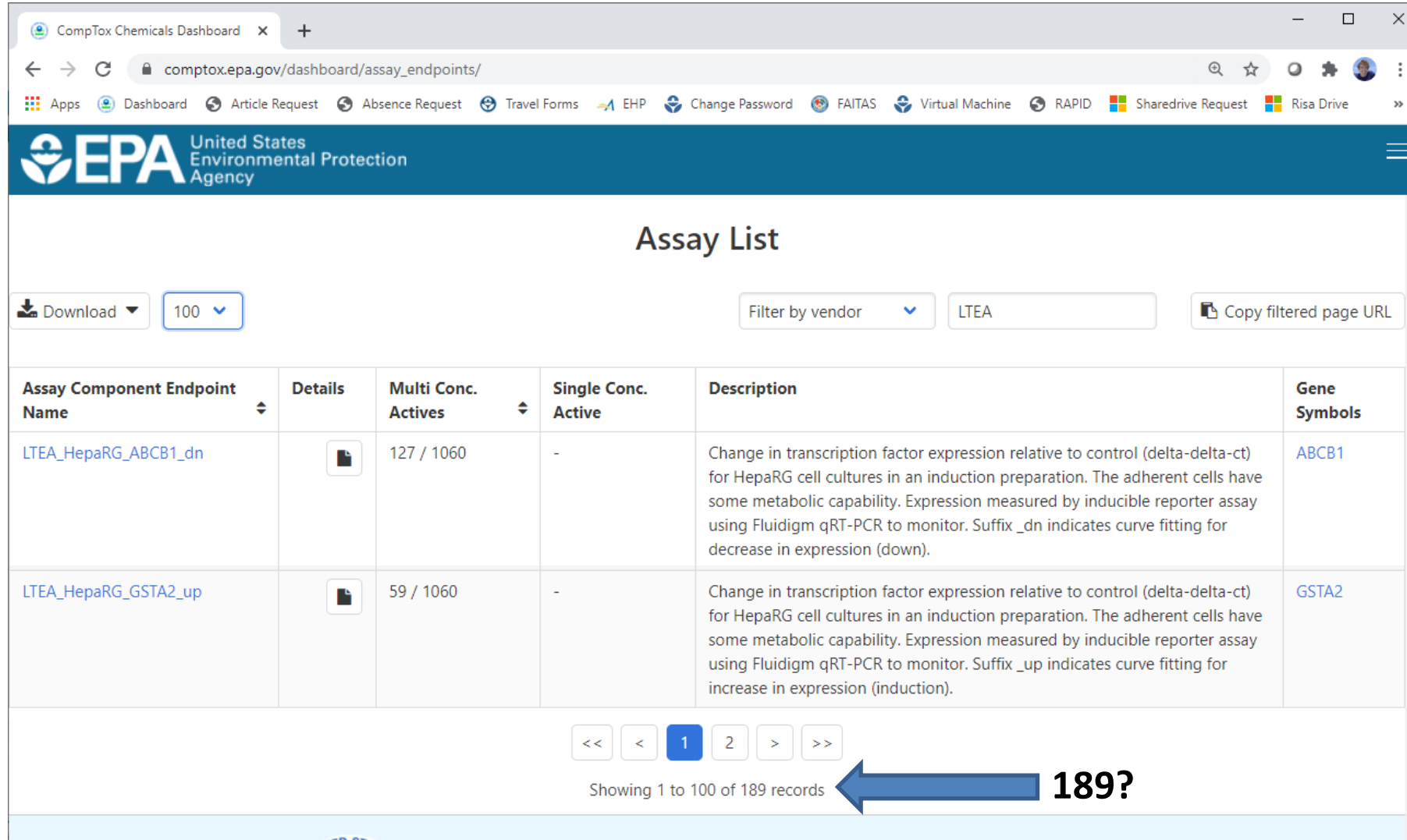


LTEA Coverage of Putative Molecular Initiating Events





The LTEA Data are on the Dashboard

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



Assay List

Download 100 Filter by vendor LTEA Copy filtered page URL

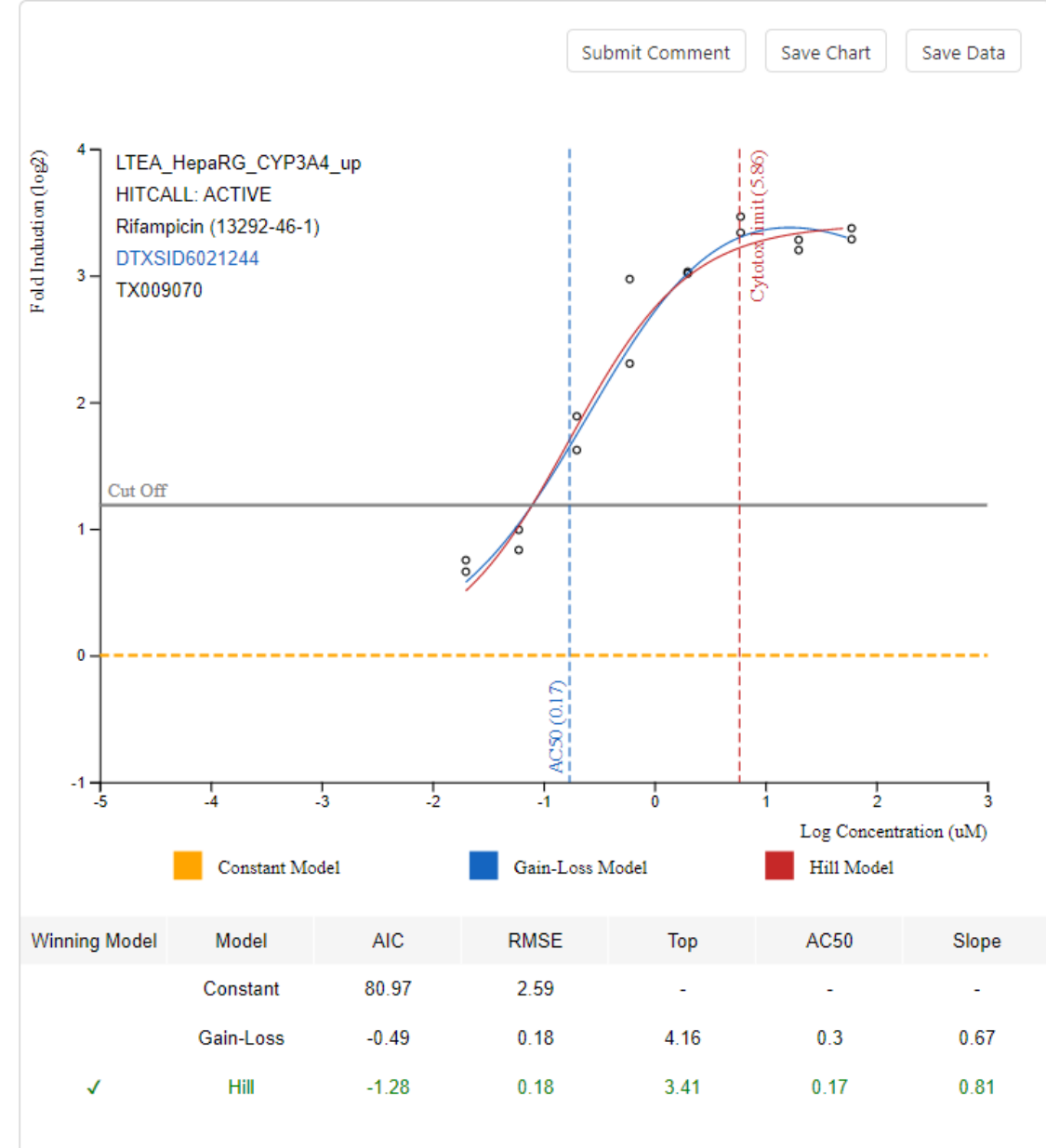
| Assay Component Endpoint Name | Details | Multi Conc. Actives | Single Conc. Active | Description | Gene Symbols |
|--------------------------------------|---|---------------------|---------------------|---|-----------------------|
| LTEA_HepaRG_ABCB1_dn |  | 127 / 1060 | - | Change in transcription 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 |

Showing 1 to 100 of 189 records **189?**

189 LTEA ToxCast Assay Endpoints

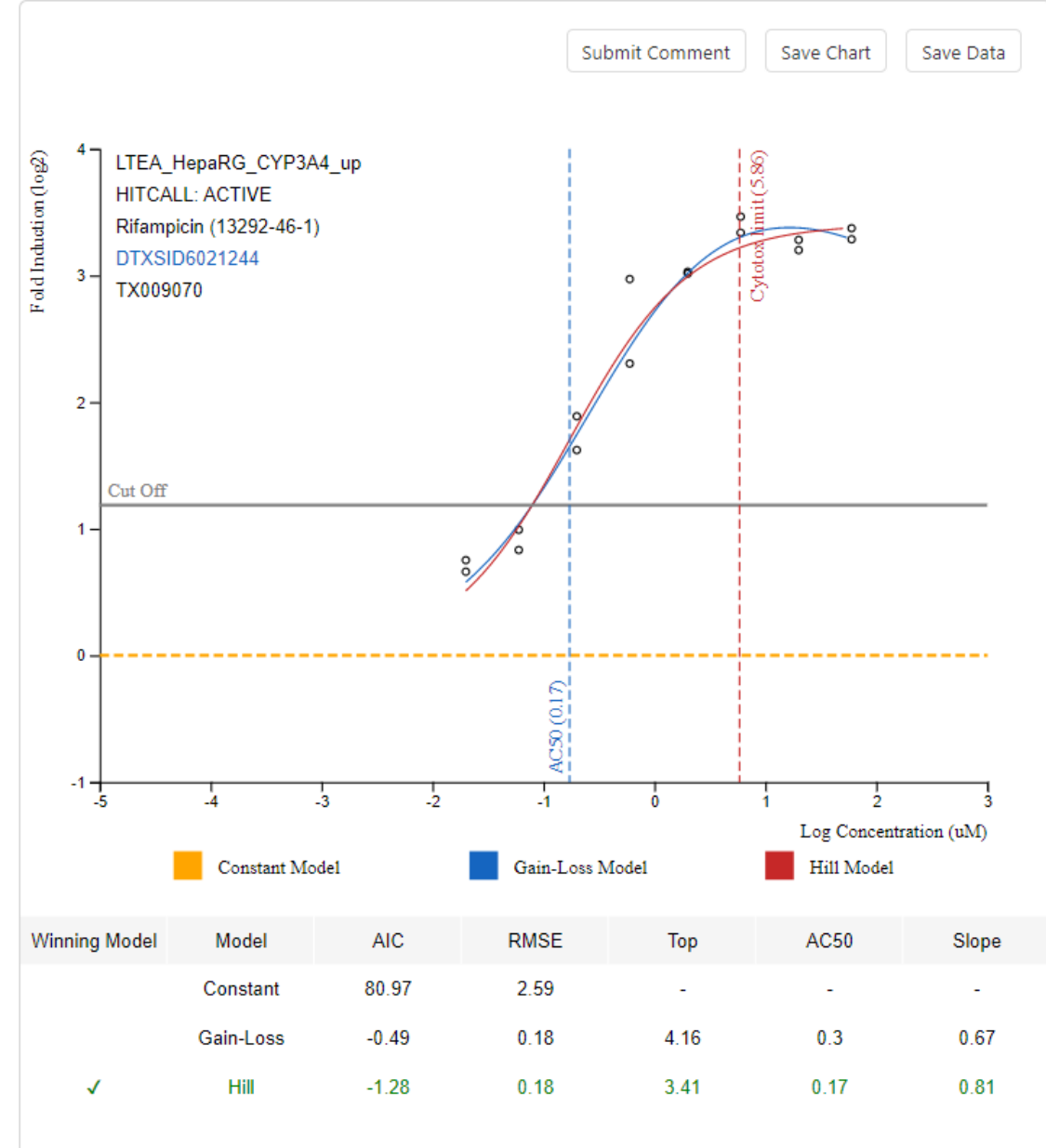
- Curve fitting was performed by Jill Franzosa using R package “tcpl” (the ToxCast Pipeline)
- Each concentration response was fit twice, once for up and once for down
- 90/93 genes has some observed activity ($90 \times 2 = 180$)
- 4 genes were replaced for noisiness after first 96 chemicals ($4 \times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



189 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)

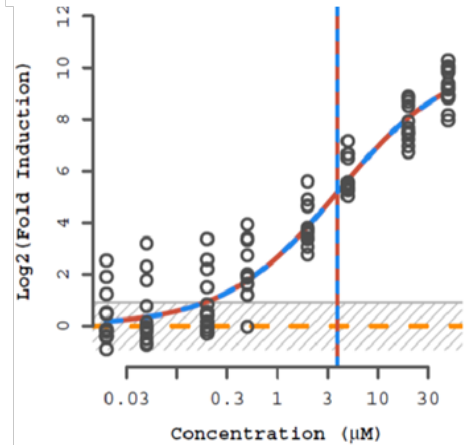


Reference Receptor Activators

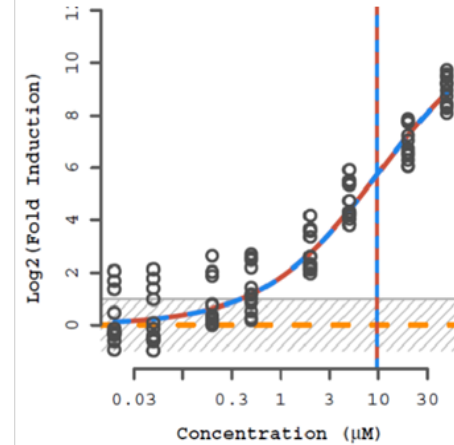
Dose-response curves for reference chemicals and transcriptionally regulated genes. Log₂ (Fold Induction) response profiles of:

- 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

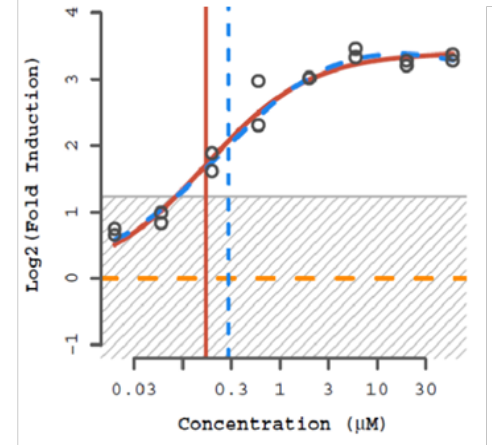
A. AhR: Omeprazole, CYP1A1



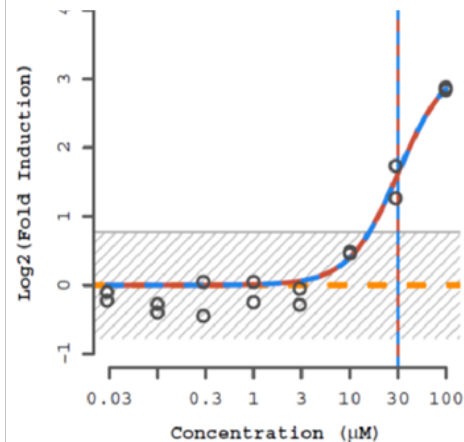
B. AhR: Omeprazole, CYP1A2



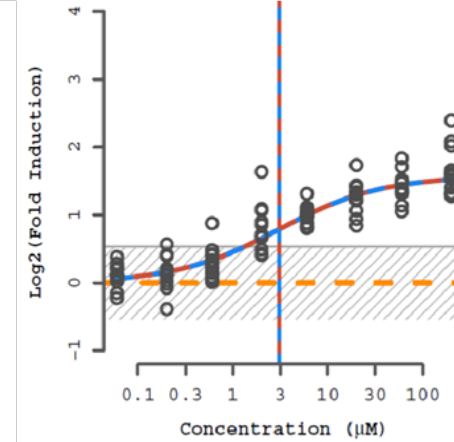
C. PXR: Rifampicin, CYP3A4



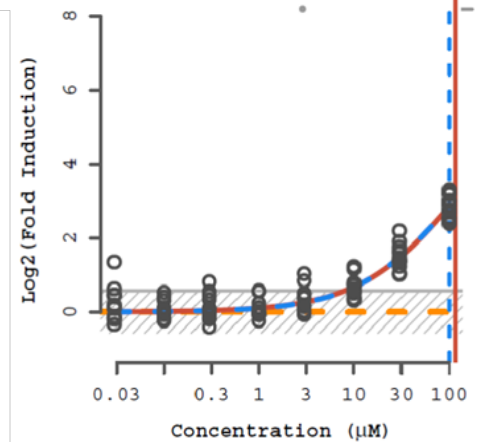
D. CAR: Phenobarbital, CYP2B6



E. PPARα: Fenofibric Acid, HMGCS2



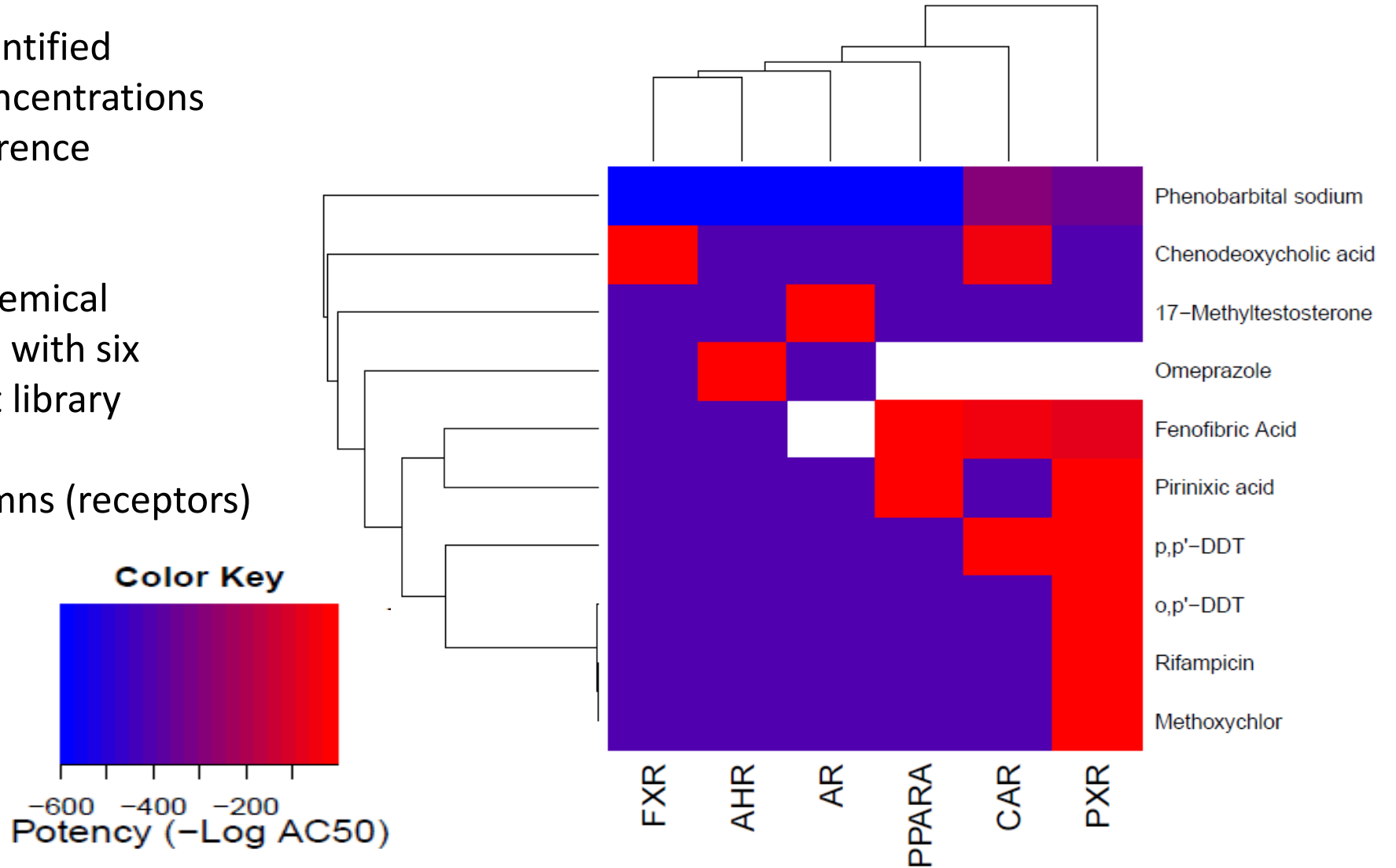
F. FXR: Chenodeoxycholic acid, ABCB11



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.

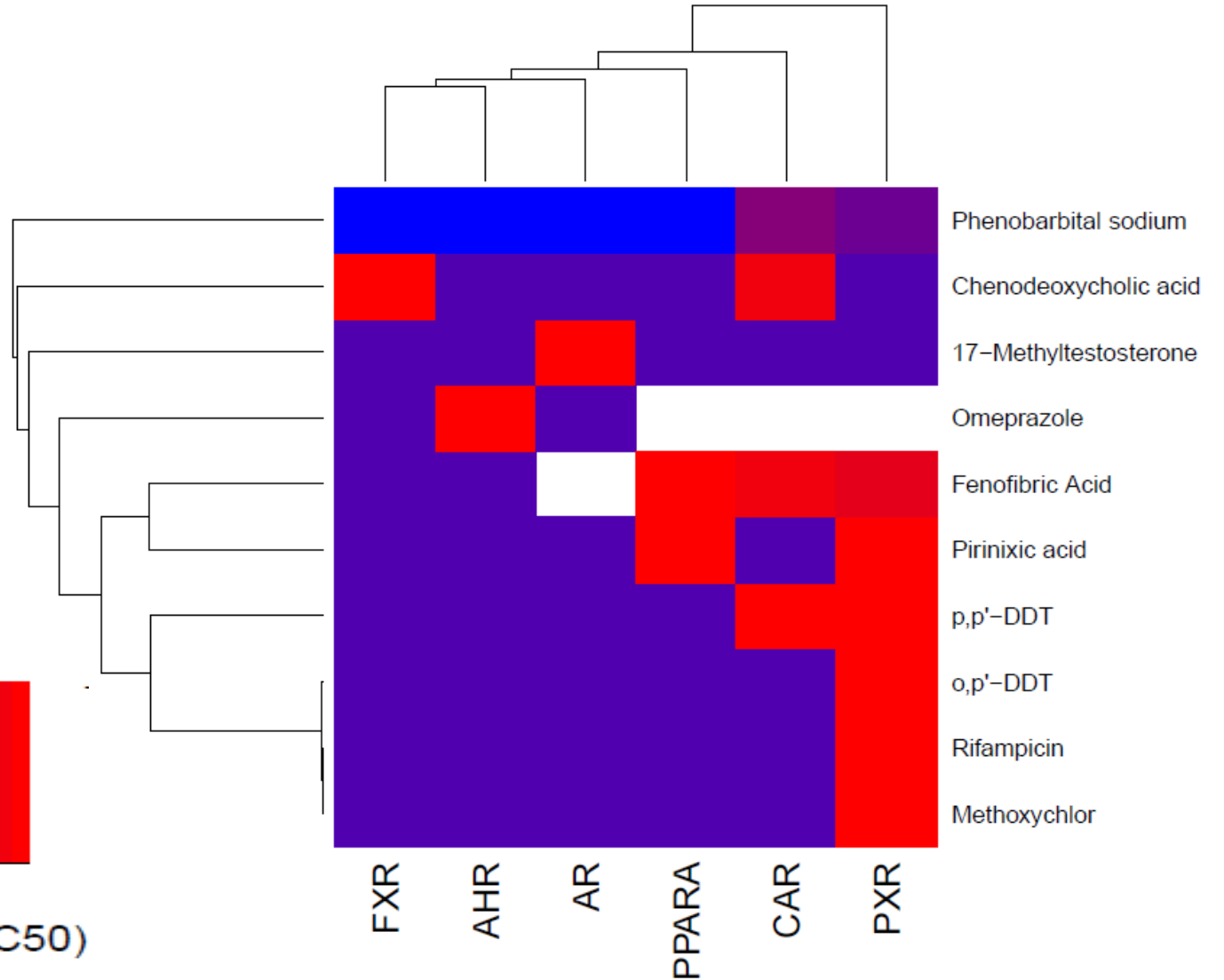
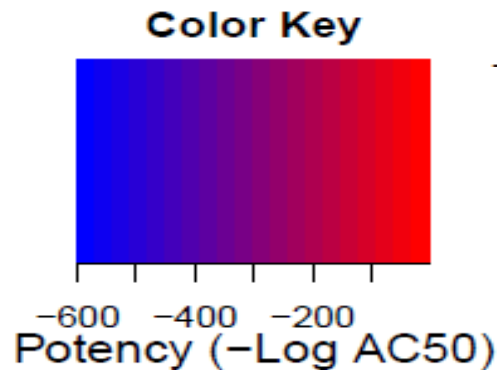
Reference Receptor Activators

- 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.



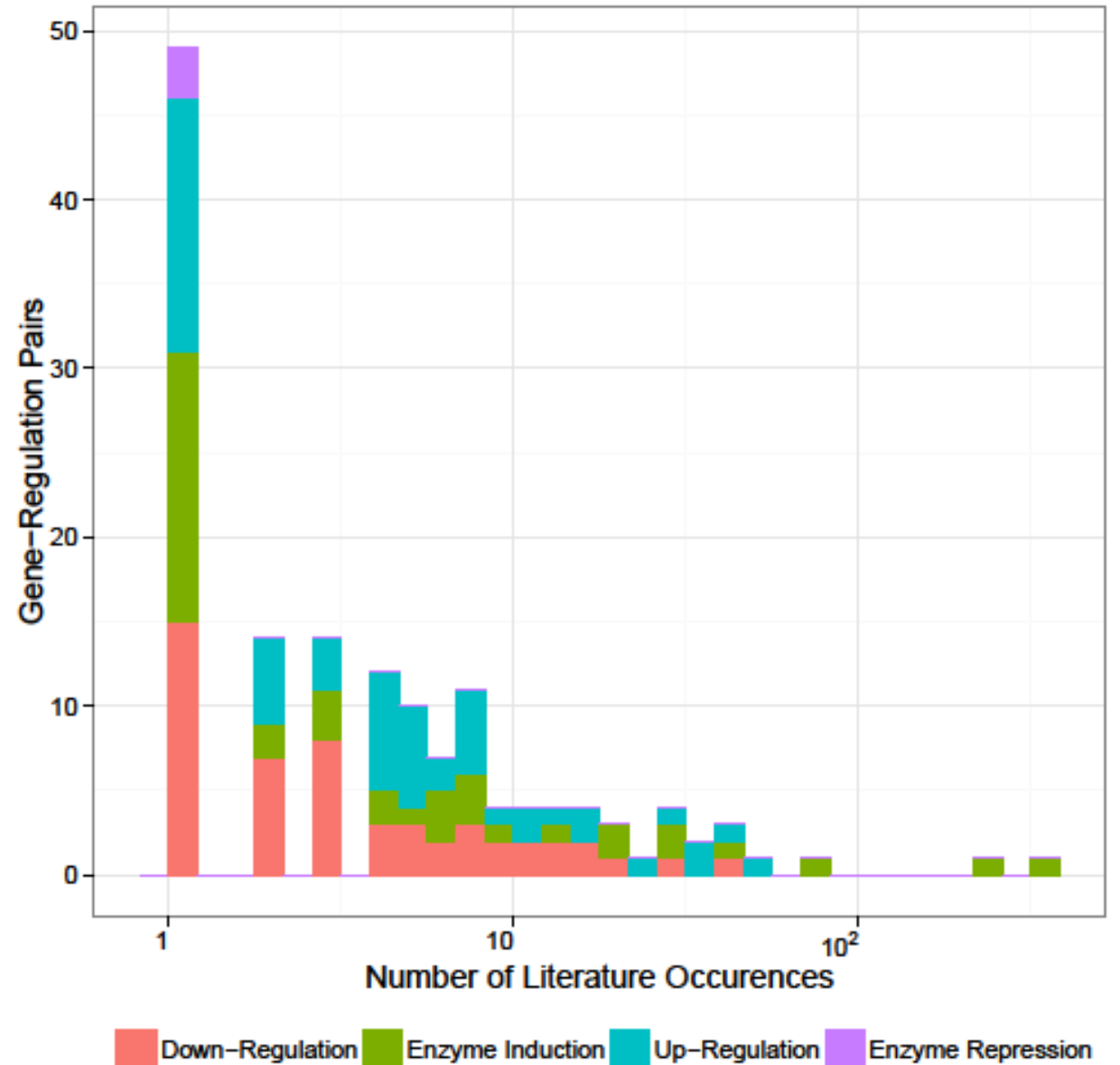
Reference Receptor Activators

- 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.



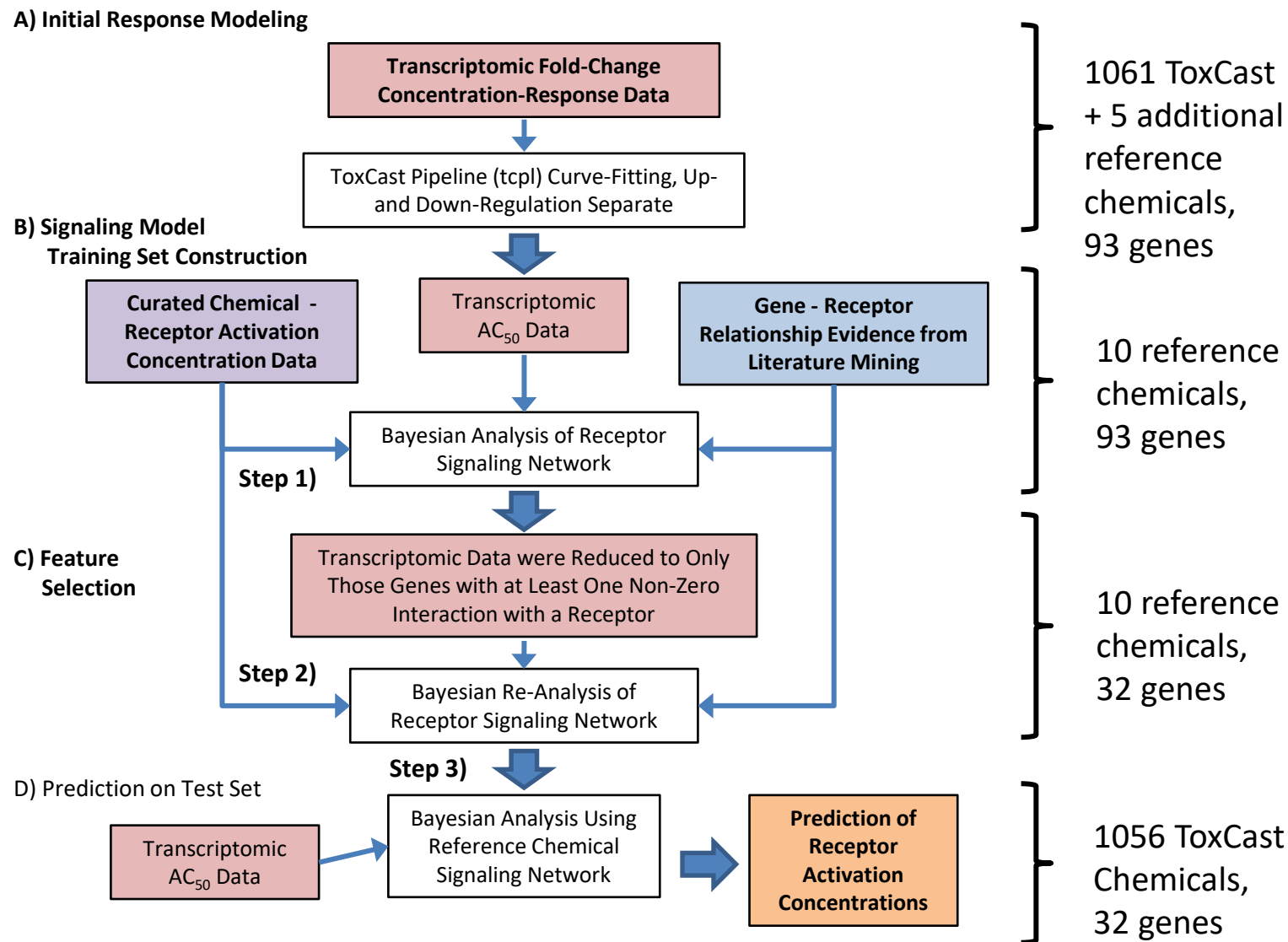
Literature Mining on Receptor-Gene Interactions

- 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

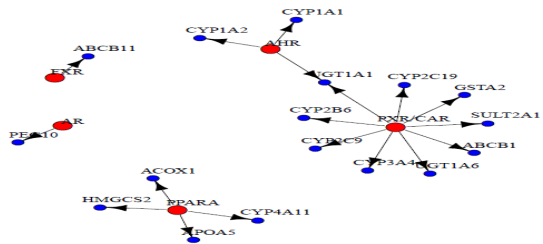


Bayesian Analysis of Gene Expression Data

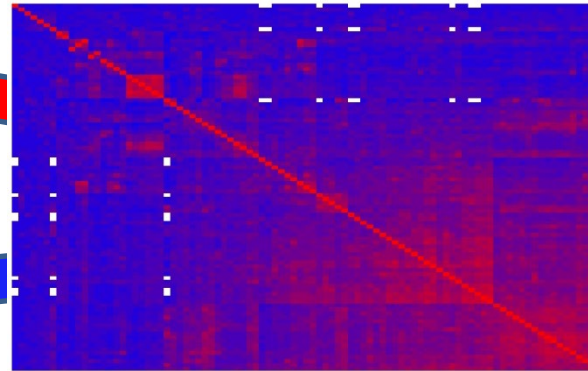
Reference Chemicals

| Compound | CAS | NR | AC50 | Reference Pctg |
|-----------------------|------------|-------|------|-------------------|
| Omeprazole | 73590-58-6 | AHR | 50 | Yes |
| Fenofibric Acid | 42017-89-0 | PPARα | 1 | Yes |
| Chenodeoxycholic Acid | 474-25-9 | FXR | 100 | Yes |
| Phenobarbital | 57-30-7 | CAR | 100 | Yes |
| Rifampicin | 13292-46-1 | PKR | 1 | No |
| Methyl testosterone | 58-18-4 | AR | 1 | No |

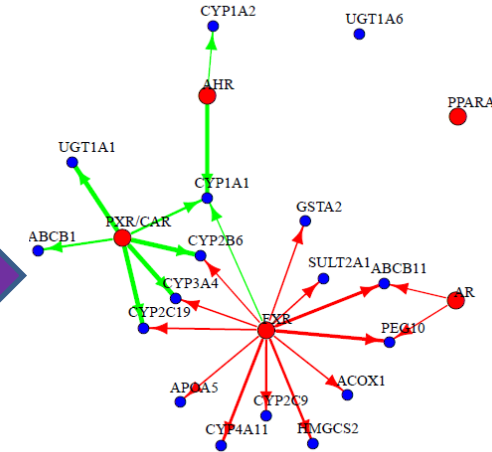
Literature Interactions



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

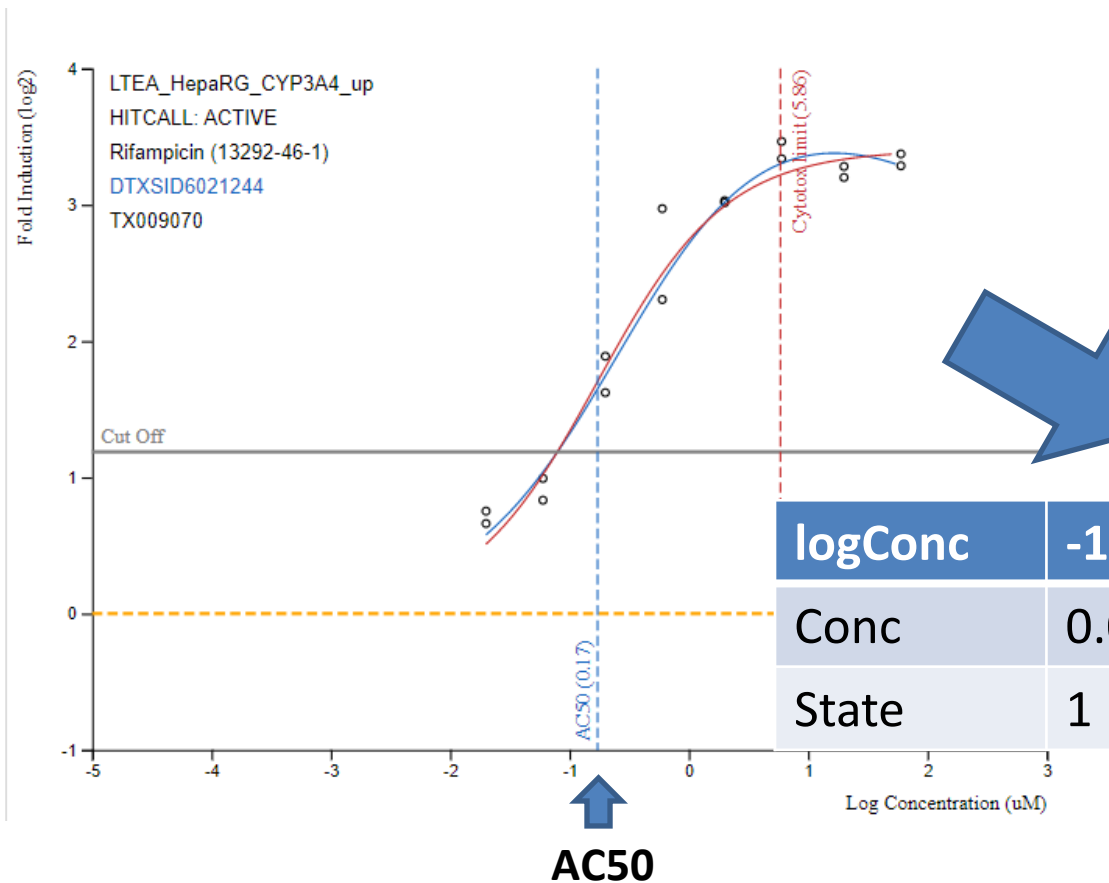
- Model is not deterministic – up-signaling makes up-regulation more likely, down-signaling makes down-regulation more likely
- We simplify each gene expression levels to three states: basal, up-regulated or down-regulated
- For each chemical and nuclear receptor, we estimate an “AC50”
 - Set NR state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

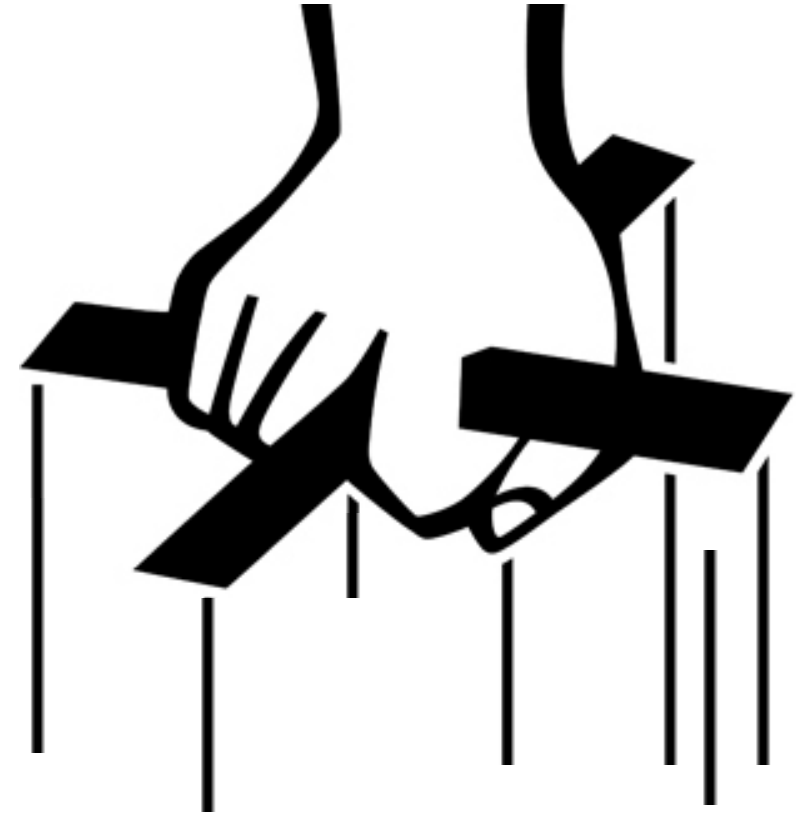
Gene State Model

- 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



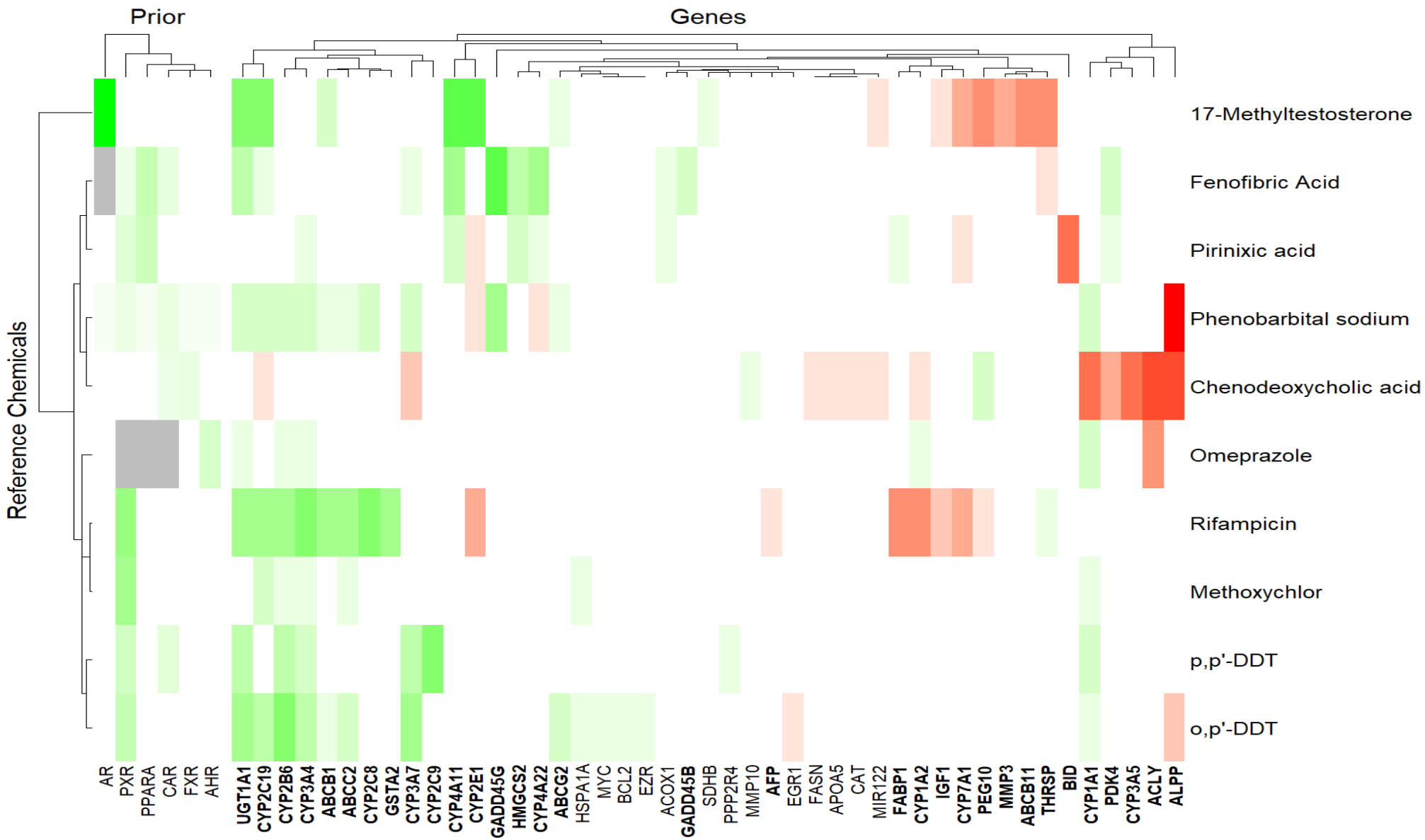
Feature Selection

| 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.

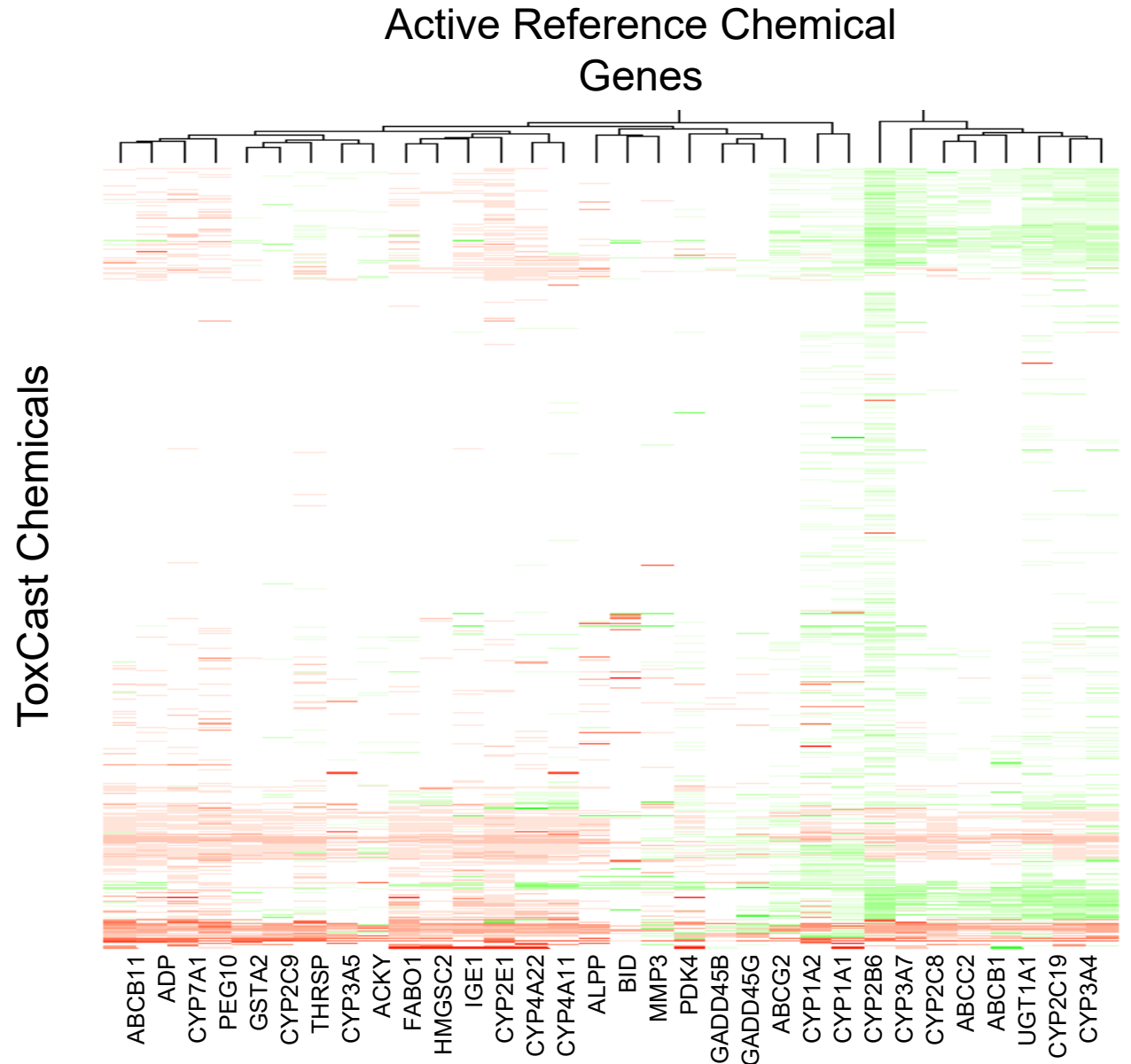
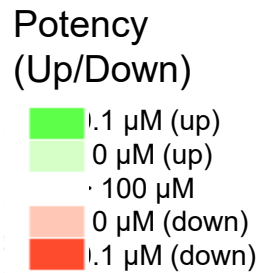
| Gene | CYP7A1 | CYP2C19 | CYP2E1 | UGT1A1 | ABCB1 | ABCB11 | AFP | CYP1A1 | CYP1A2 | CYP3A4 | FABP1 | IGF1 | CYP3A7 | CYP4A11 | CYP4A22 | HMGCS2 | MMP3 | PEG10 | THRSP | ABCC2 | ABCG2 | ACLY | ALPP | CYP2B6 | CYP2C8 | GADD45B | GADD45G | GSTA2 | PDK4 | BID | CYP2C9 | CYP3A5 | |
|----------------------------|--------|---------|--------|--------|-------|--------|-----|--------|--------|--------|-------|------|--------|---------|---------|--------|------|-------|-------|-------|-------|------|------|--------|--------|---------|---------|-------|------|-----|--------|--------|----|
| 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 | - | ++ | -- | ++ | ++ | - | - | + | - | ++ | - | - | + | - | | | | | | | ++ | + | | - | ++ | ++ | | + | | | | | |

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. 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. |

ToxCast LTEA Assay

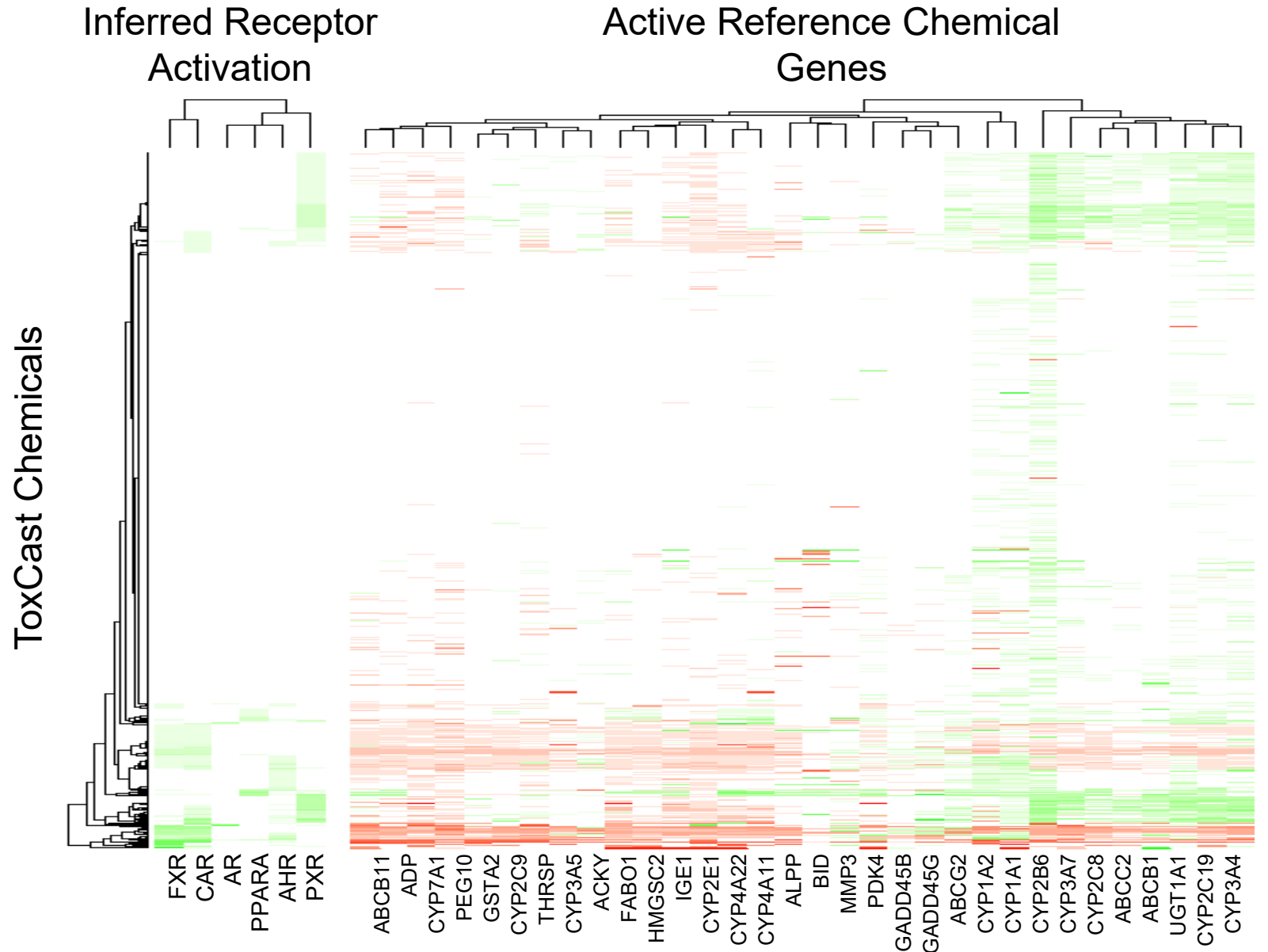
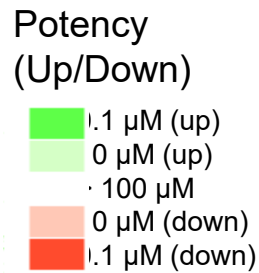
The heatmap at the right presents the observed transcriptional response of transcripts identified as part of the reference chemical signatures



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



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 | | | | | | | |

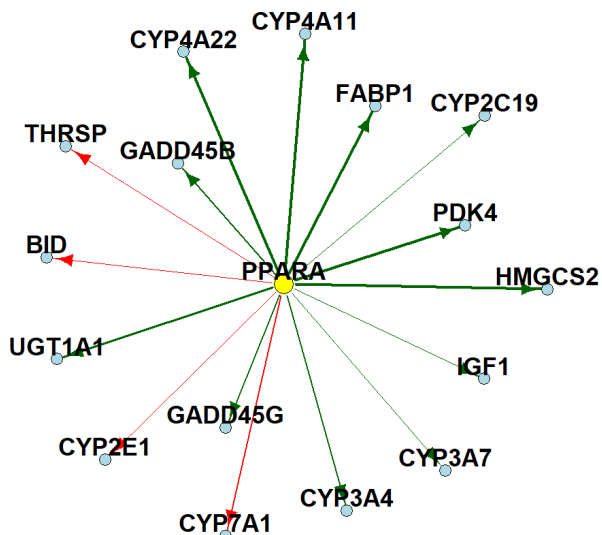
No receptor activity was inferred for 43% of the chemicals and only 37% have any activity inferred below 100 μM

Comparison with Other ToxCast Assays

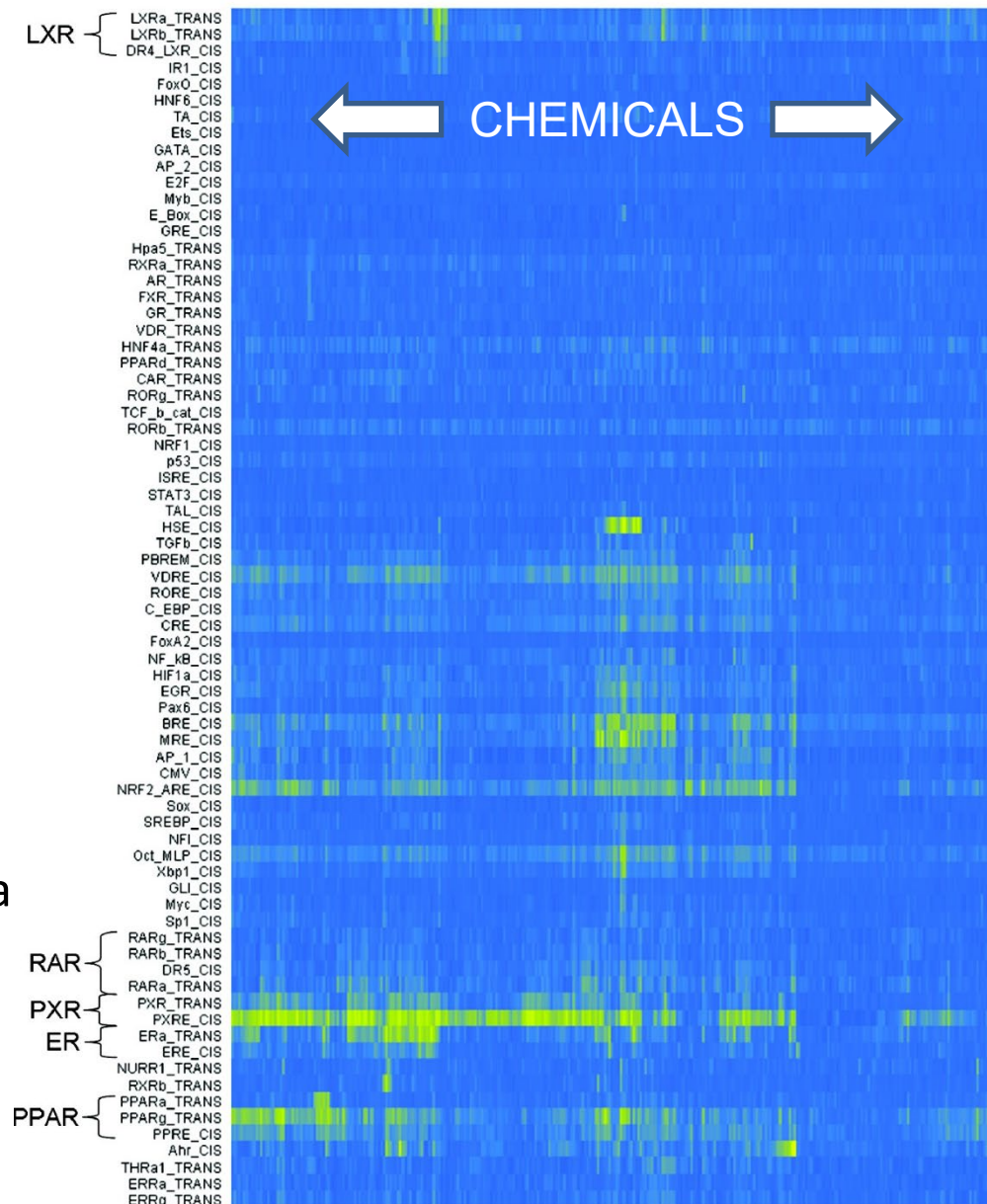
- We did not observe activity for most chemicals.
- While we did not observe as frequent activity as other ToxCast screens, we believe that the synthesis of multiple transcript activities into signatures that must be consistently observed reduces the likelihood of false positives.
- The ToxCast Factorial 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.

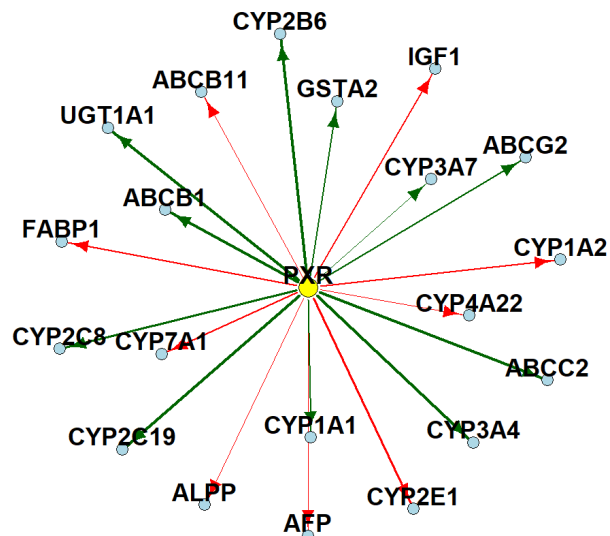


- **PPAR α** - the model identified **28** chemicals that also indicated activity with ToxCast Factorial (mean probability of 61%)
 - identified **45** chemicals not identified by the ToxCast Factorial assay that had an average probability of 32%
 - the Factorial assay found **52** chemicals not identified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

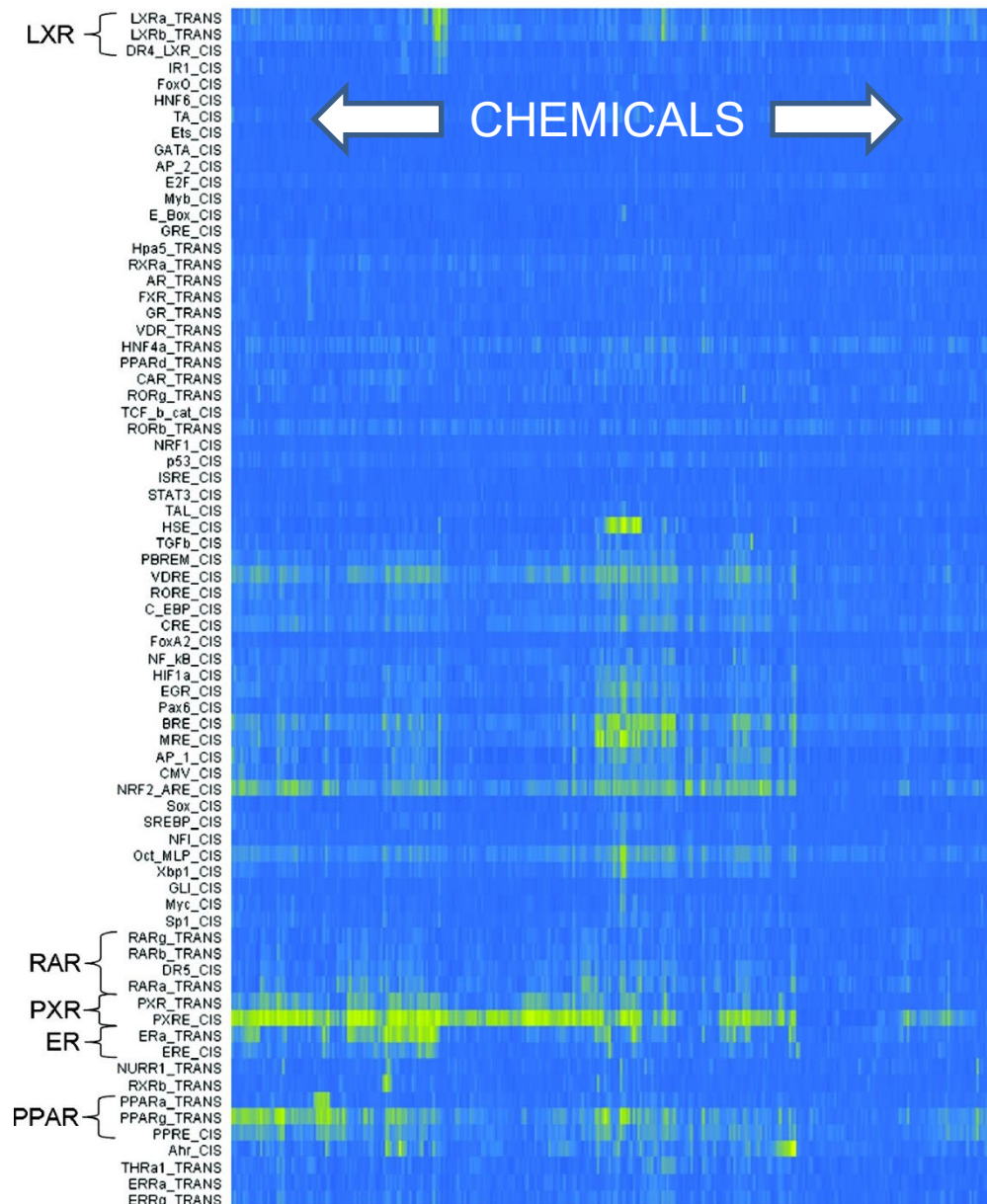


Comparison with ToxCast Factorial (Attagene) Assay

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

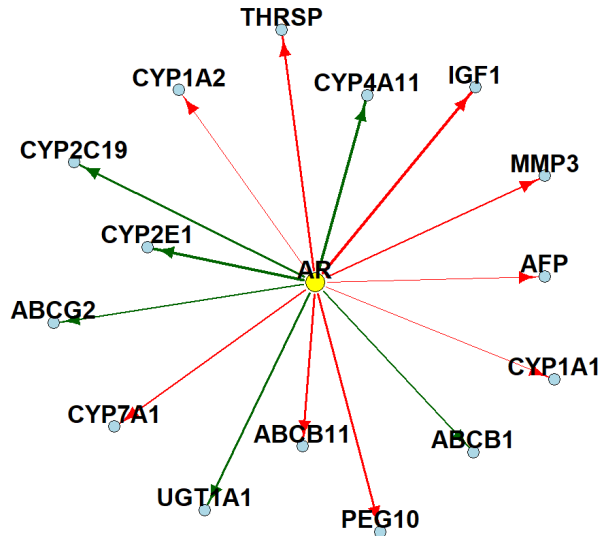


- **PXR** - the model agreed with the Factorial assay on **131** chemicals (mean probability 77%)
 - identified an additional **91** chemicals with a mean probability of 71%
 - assigned 0% probability to **187** chemicals identified with the Factorial assay

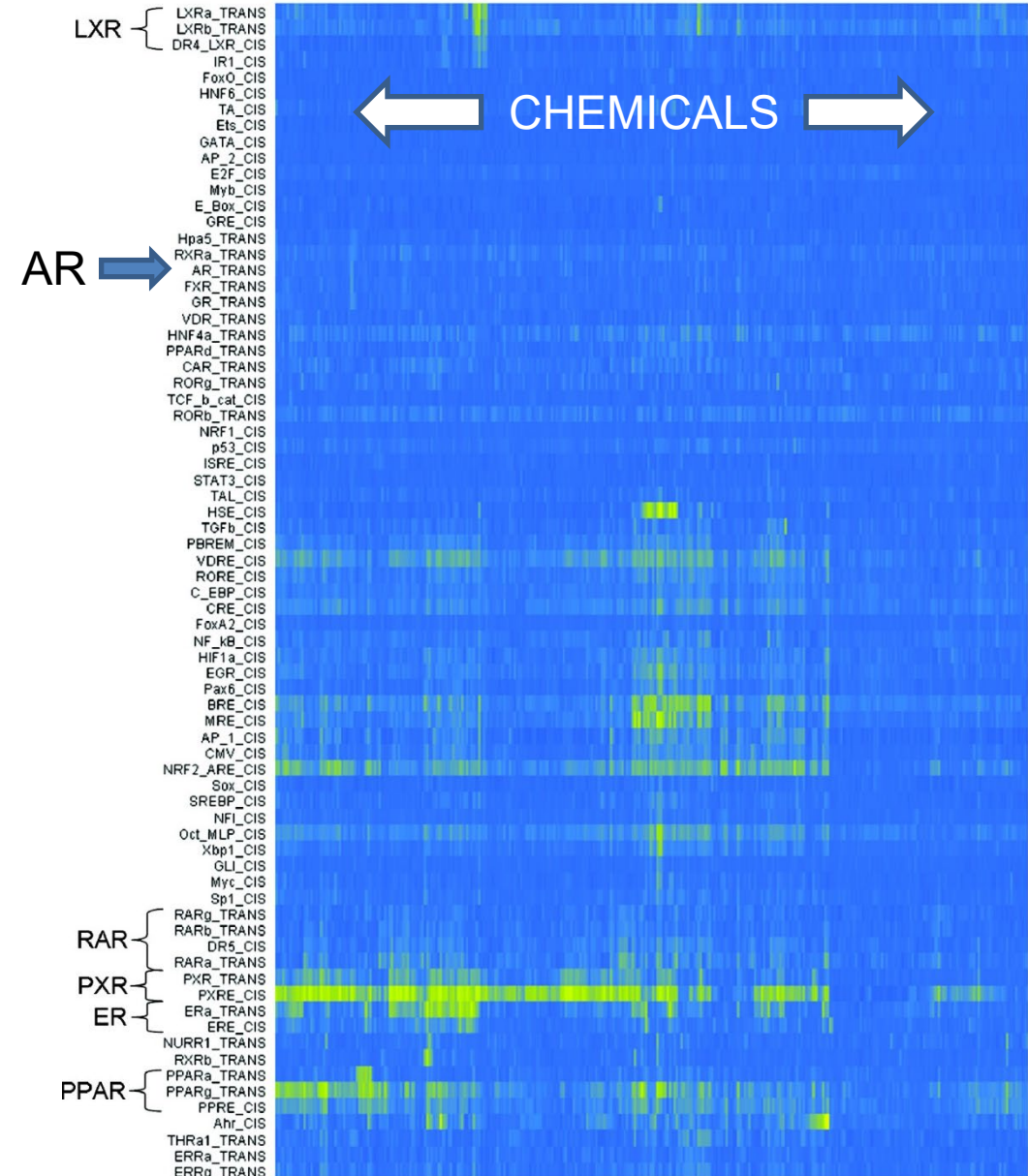


Comparison with ToxCast Factorial (Attagene) Assay

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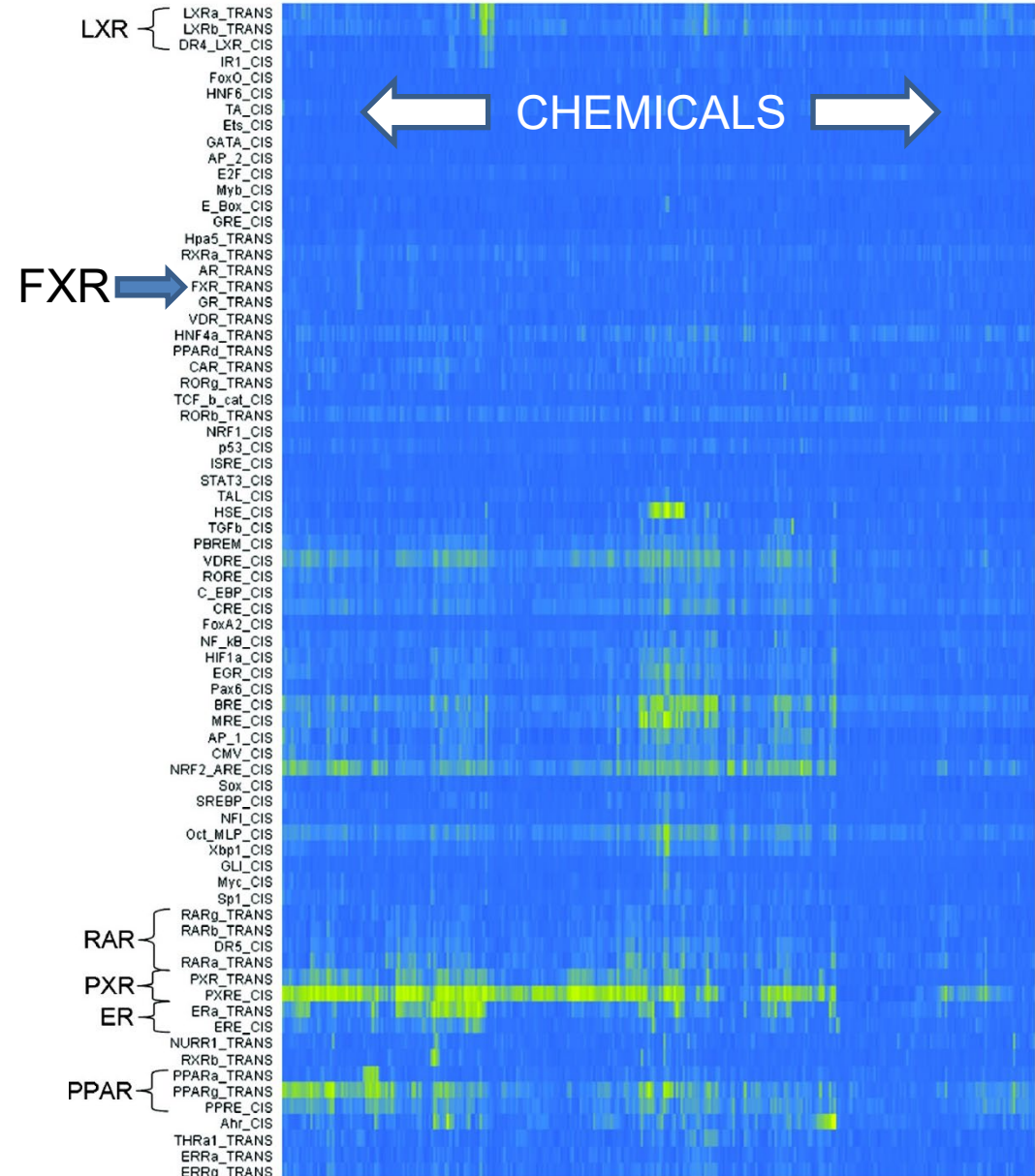
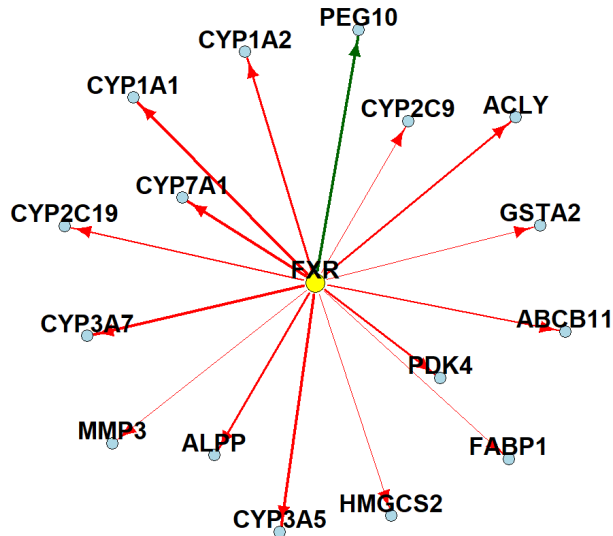


- **AR** - the assays agreed on **8** chemicals and the Bayesian transcriptomics model (mean probability 75%)
 - identified **29** chemicals not found with the ToxCast Factorial assay, but the mean probability was only 8.5%
 - assigned 0% probability to **14** chemicals identified as AR regulators by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



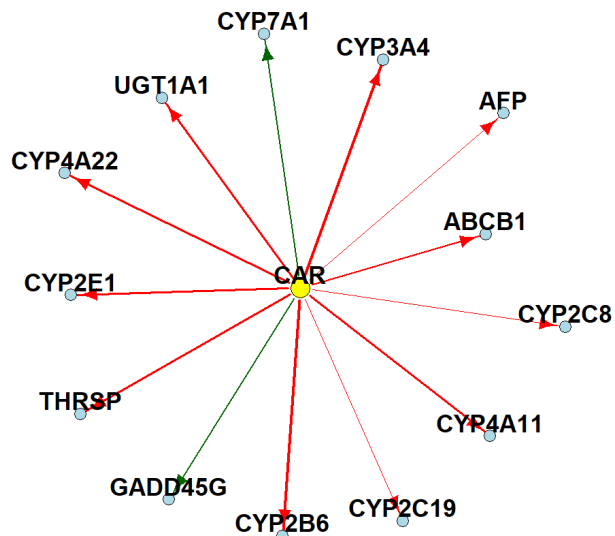
Comparison with ToxCast Factorial (Attagene) Assay

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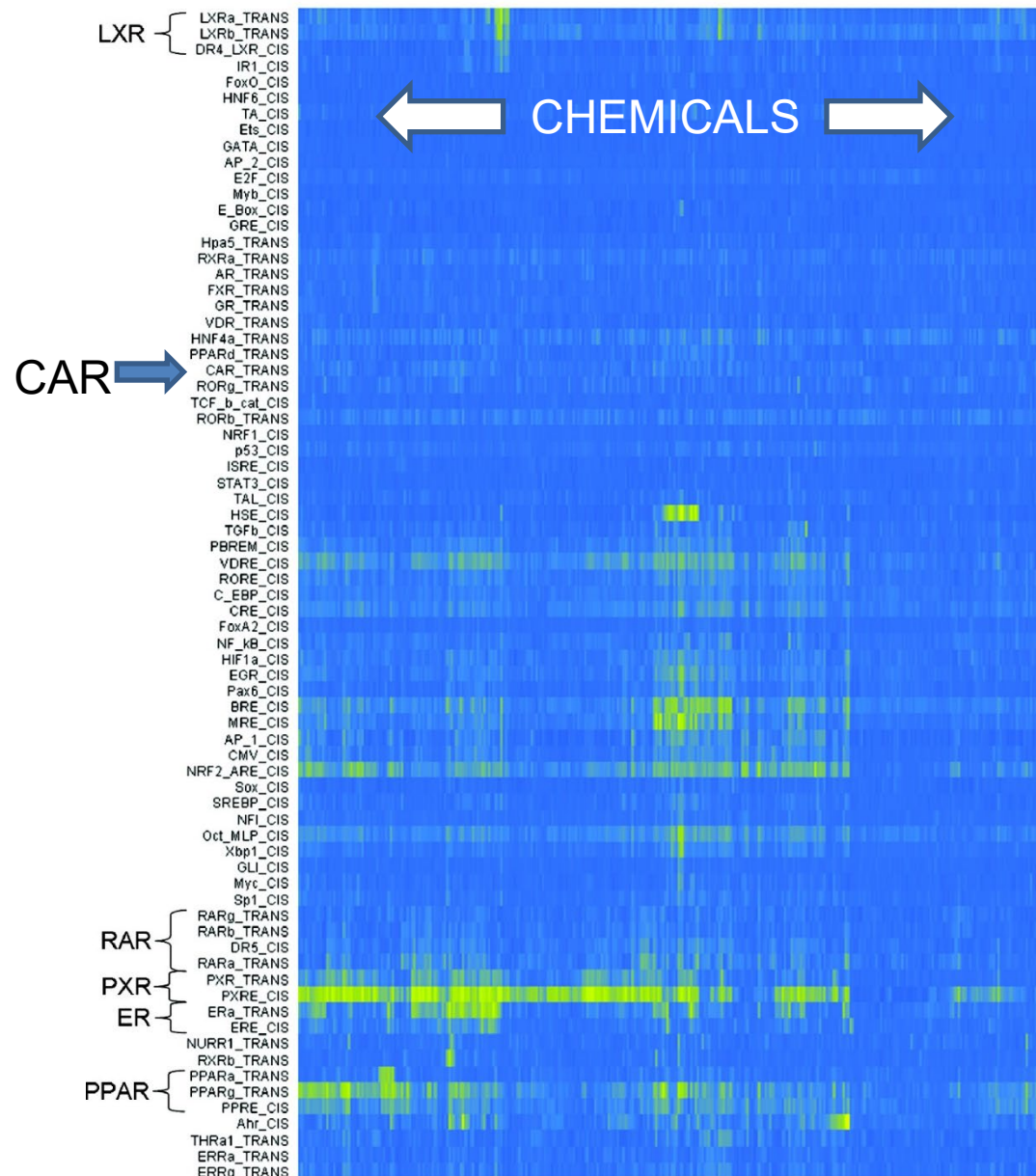


- **FXR** - the two assays agreed on **15** chemicals as potential agonists (mean probability 73%)
 - identified an additional **130** with a mean probability of 70%
 - **40** chemicals identified by the Factorial assay were assigned 0%

Comparison with ToxCast Factorial (Attagene) Assay



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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

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