United States Environmental Protection Agency

High-Throughput Transcriptomics (HTTr) in *In Vitro* Chemical Screening, A Sensitive Tool for Benchmark Dose Assessment

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Office of Research and Development

American Society of Pharmacology and Experimental Therapeutics
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United States Environmental Protection Agency

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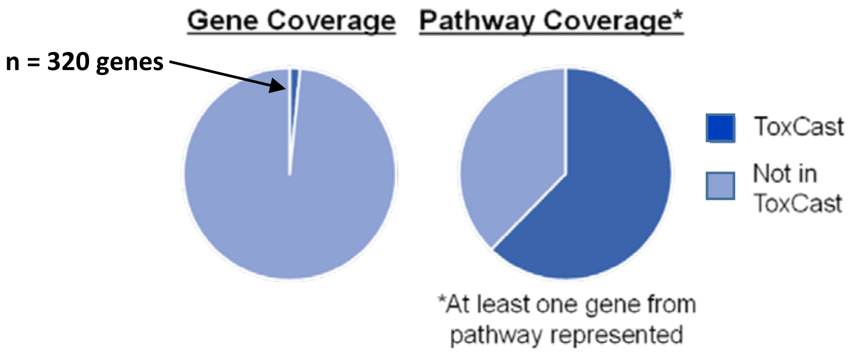
Outline

- **Background**
  - Why high-throughput transcriptomics (HTTr)?
  - TempO-Seq Technology Overveiw
- **Experimental Design & Workflows**
  - Screening format
  - Treatment Randomization
  - Quality Control Samples
- **Technical Reproducibility**
- **Applications**
  - Concentration-Response Modeling
  - Bioactivity Thresholds
  - Comparison of Structurally Related Chemicals
  - Gene Set Analysis
  - Mechanism of Action (MoA) Prediction

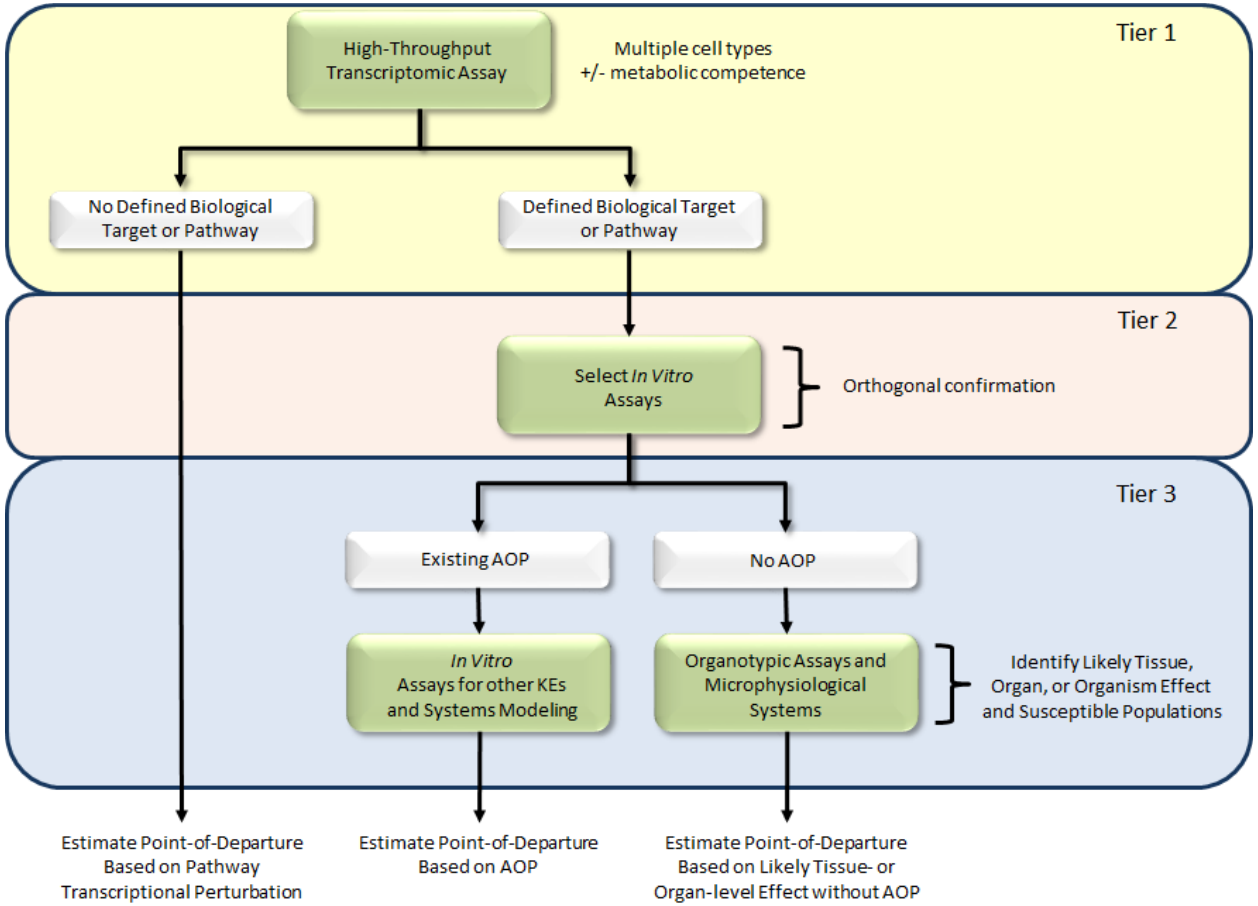
Background

- ToxCast assays cover many genes and pathways, but do not provide complete coverage of biological space.



- **USEPA Strategic Vision and Operational Roadmap:**
  - Tier 1 strategy must cast the broadest net possible for capturing hazards associated with chemical exposure.
  - Global gene expression provides a robust and comprehensive evaluation of chemically induced changes in biological processes.
  - Increasing efficiency and declining cost of generating whole transcriptome profiles has made high-throughput transcriptomics (HTTr) a practical option for determining bioactivity thresholds in *in vitro* models.

A strategic vision and operational road map for computational toxicology at the U.S. Environmental Protection Agency

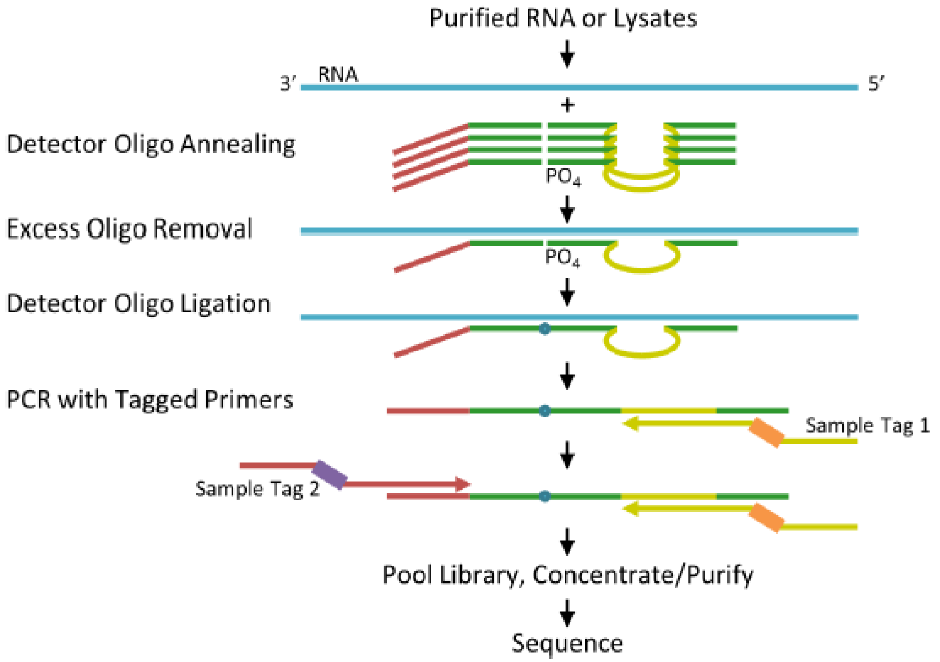


Background

Technology

- The **TempO-Seq** human whole transcriptome assay measures the expression of greater than 20,000 transcripts.
- Requires only picogram amounts of total RNA per sample.
- Compatible with purified RNA samples or **cell lysates**.
- Transcripts in cell lysates generated in 384-well format are barcoded according to well position and combined in a single library for sequencing using industry standard instrumentation.
- Scalable, targeted assay:
  - 1) specifically measures transcripts of interest
  - 2) ~50-bp reads for all genes
  - 3) requires less flow cell capacity than RNA-Seq
- Per sample fastq files are generated and aligned to BioSpyder sequence manifest to generate integer count tables.

TempO-Seq Assay Illustration



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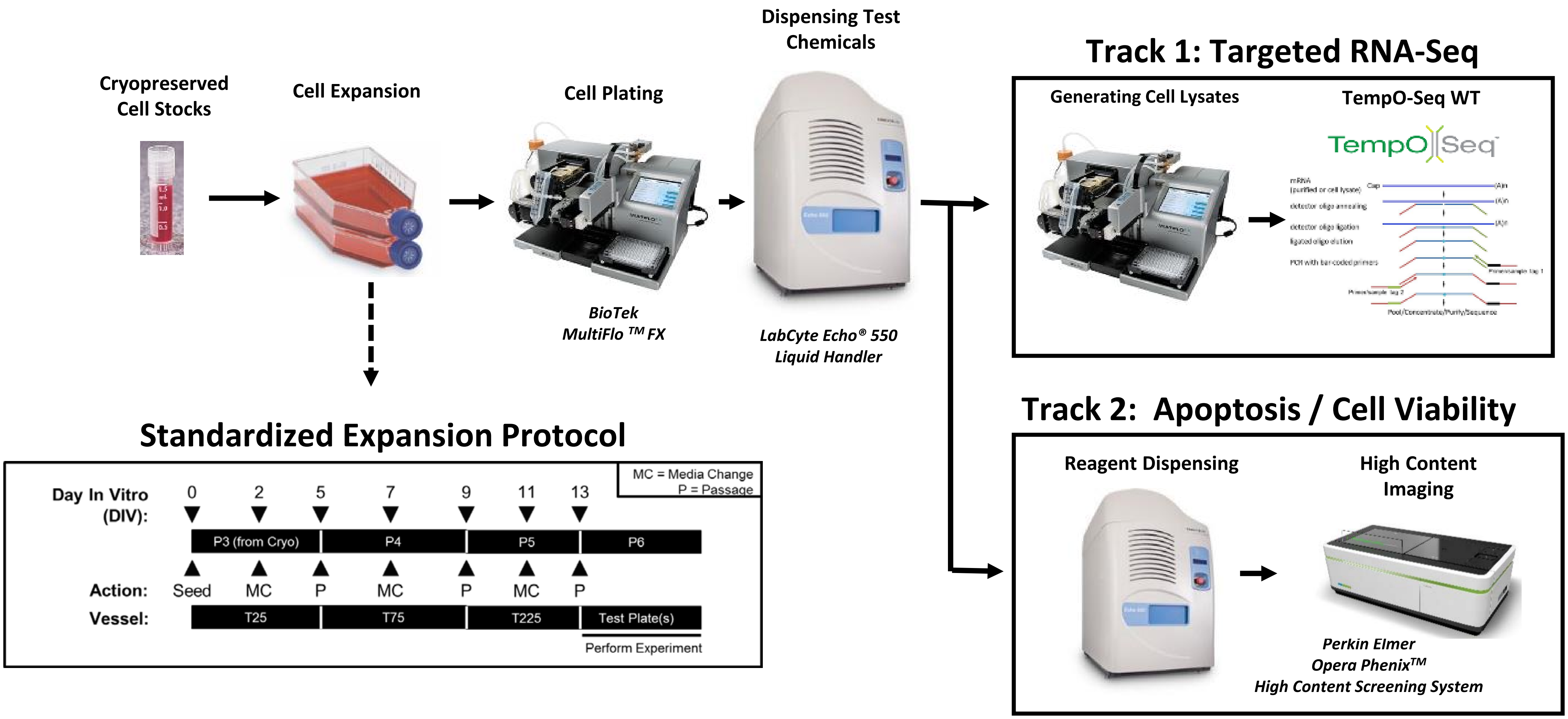
HTTr MCF Screen: Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	MCF7
Culture Condition	1	DMEM + 10% HI-FBS [a]
Chemicals	2,112	ToxCast ph1, ph2 Nominated chemicals from e1k / ph3
Time Points:	1	6 hours
Assay Formats:	2	TempO-Seq HCI Cell Viability & Apoptosis
Concentrations:	8	3.5 $\log_{10}$ units; semi $\log_{10}$ spacing
Biological Replicates:	3	--

- **Total number of samples:** 54,432
- **Total number of endpoint readouts:** 1.15×10^9
- **Total size of fastq files:** 32.5 to 54.4 TB

[a] MCF7 cells cultured in DMEM + 10% HI-FBS was selected as the test system to facilitate comparability to the Broad Institute Connectivity Map (CMAP) database (http://portals.broadinstitute.org/cmap/).

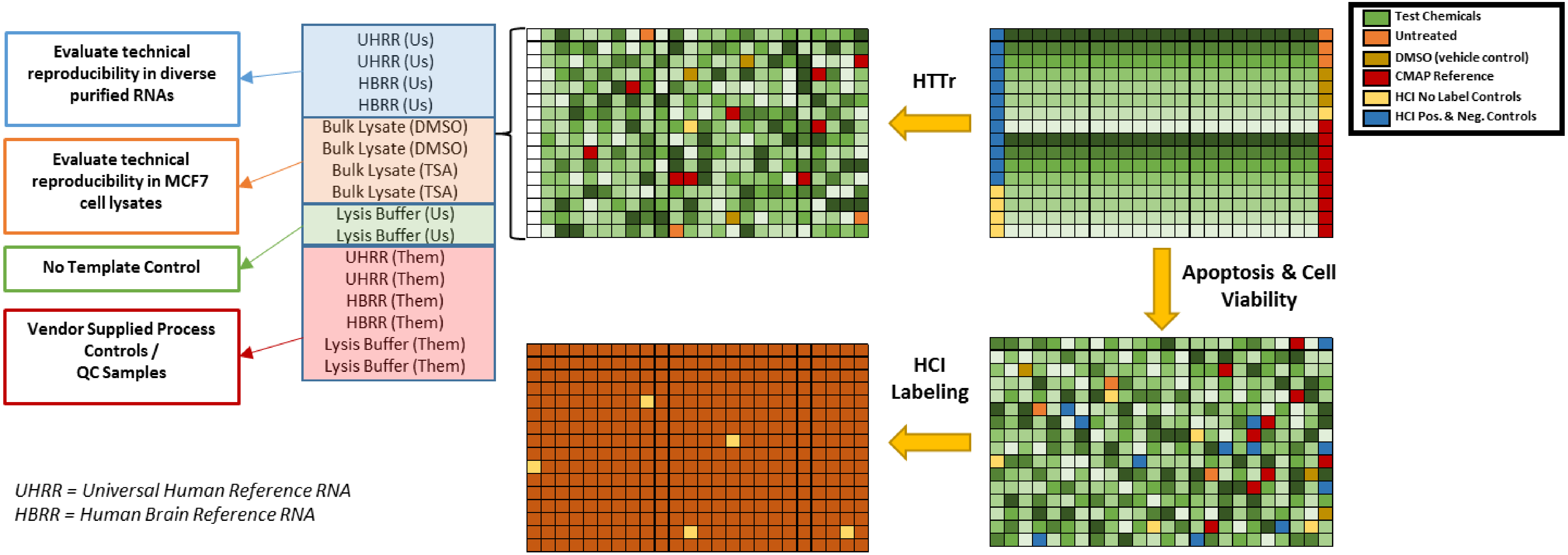
Experimental Workflow



Treatment Randomization & Quality Control Samples

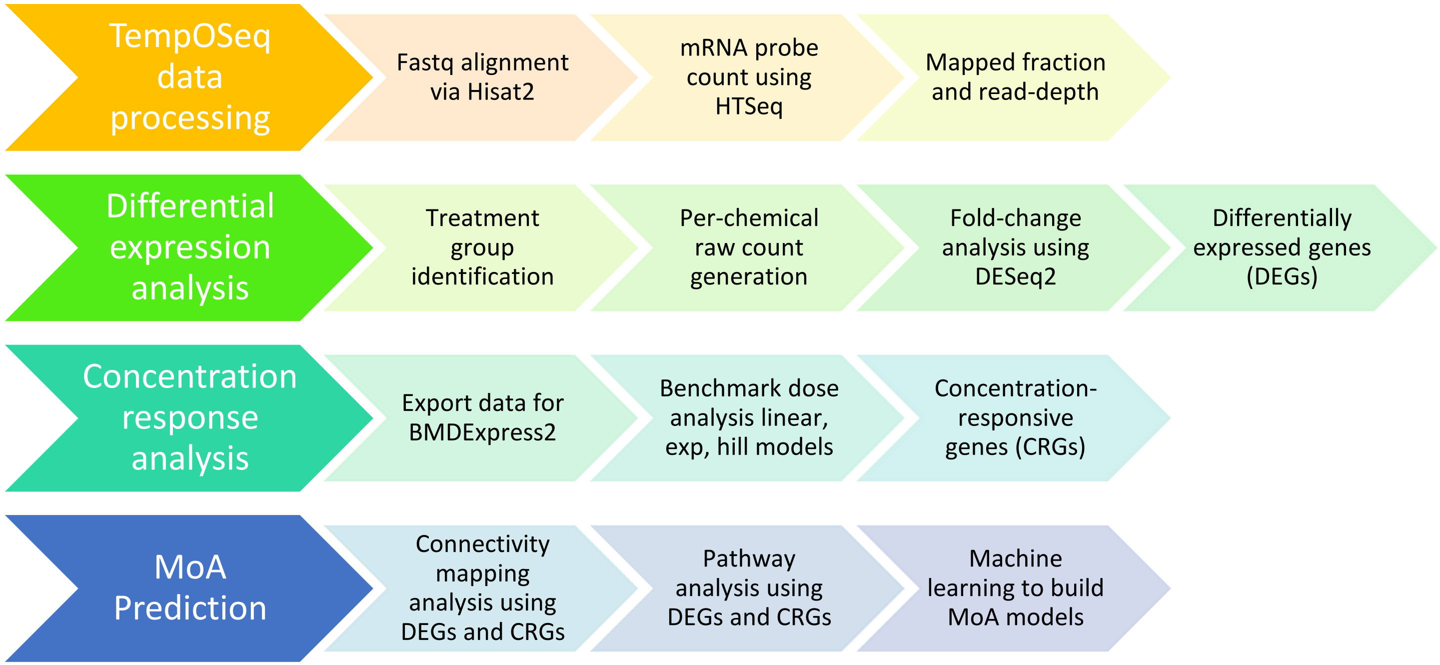
Treatment Randomization: *Each test plate uniquely randomized with respect to treatment.*

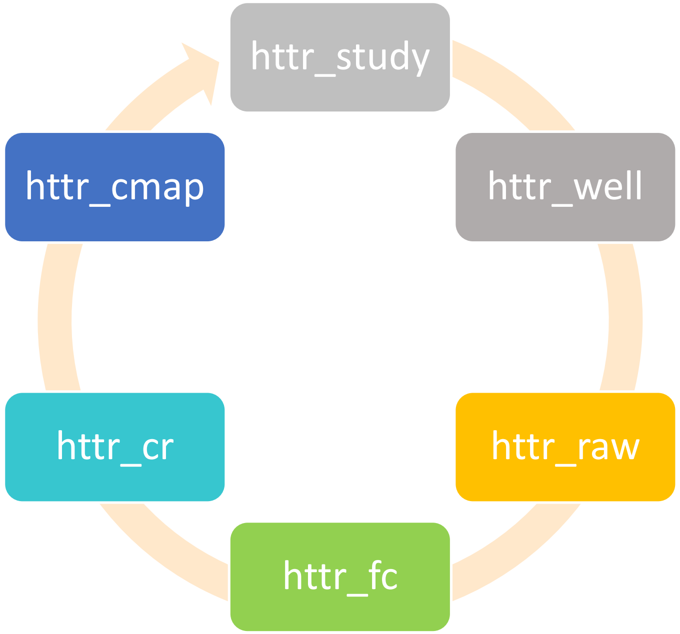
QC Samples: *Quality Control samples included on each plate*



UHRR = Universal Human Reference RNA
HBRR = Human Brain Reference RNA

HTTr Analysis Pipeline & Infrastructure





Python & R analysis pipeline

http://bitbucket.zn.epa.gov/projects/HTTR/repos/httr-wf-dev

Imran Shan
Josh Harrill
Woodrow Setzer
Richard Judson
Derik Haggard

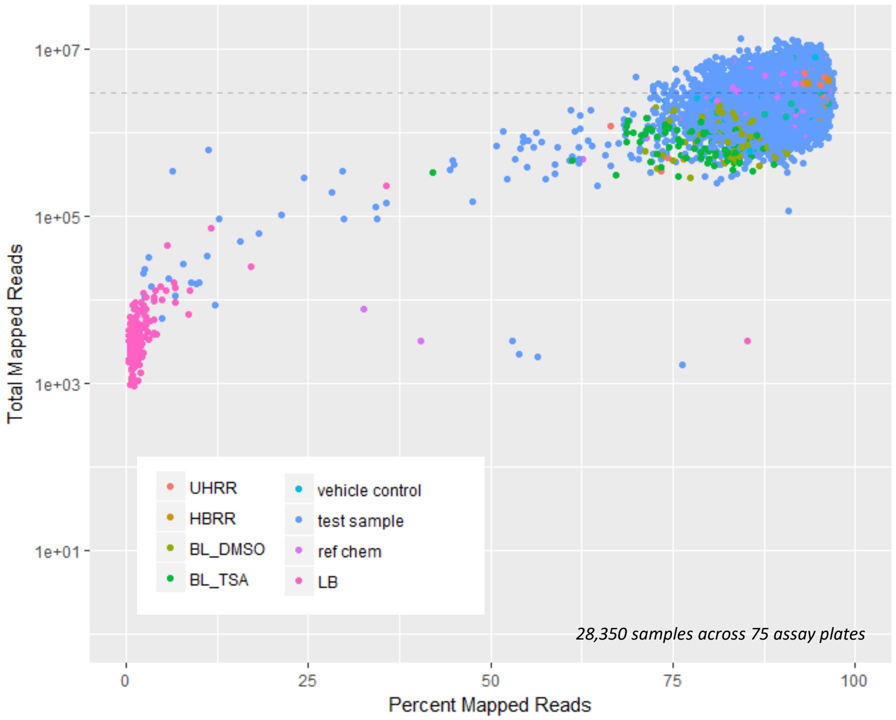
MongoDB

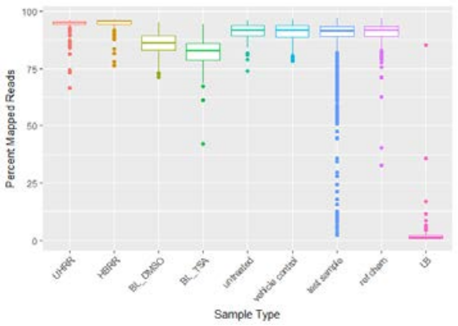
mongodb://pb.epa.gov/httr_v1
mongodb://pb.epa.gov/cmap_v2

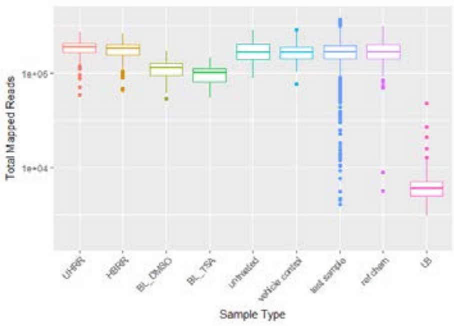
NCCT Investigators
NCCT IT

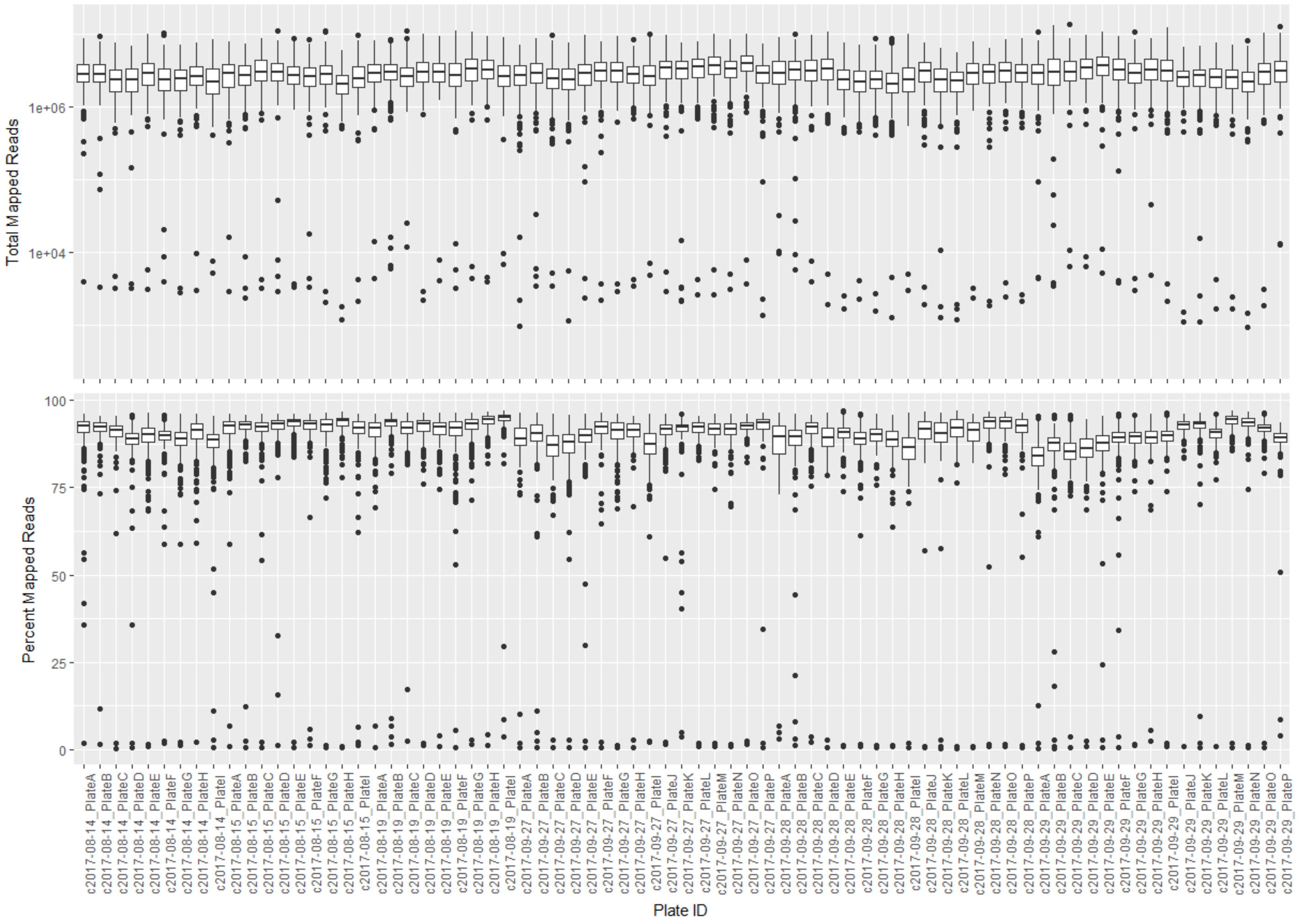
Technical Reproducibility

Reproducibility of Read Depths and Mapping Rates

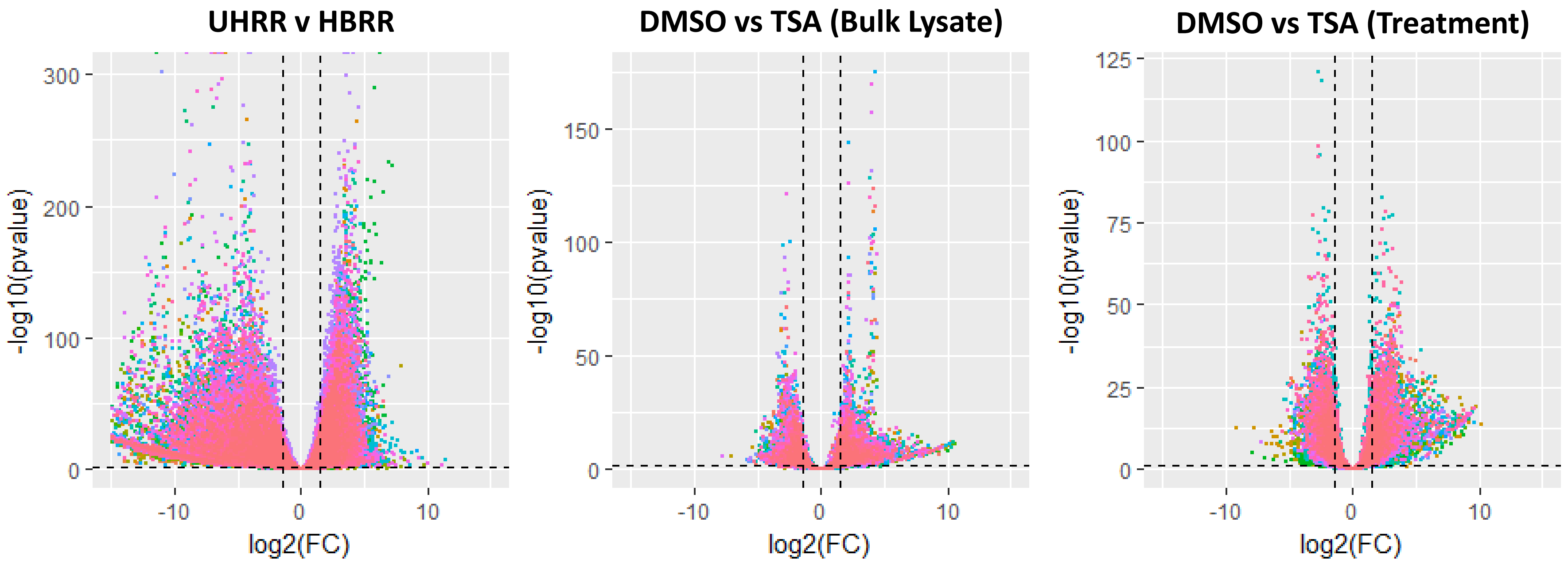


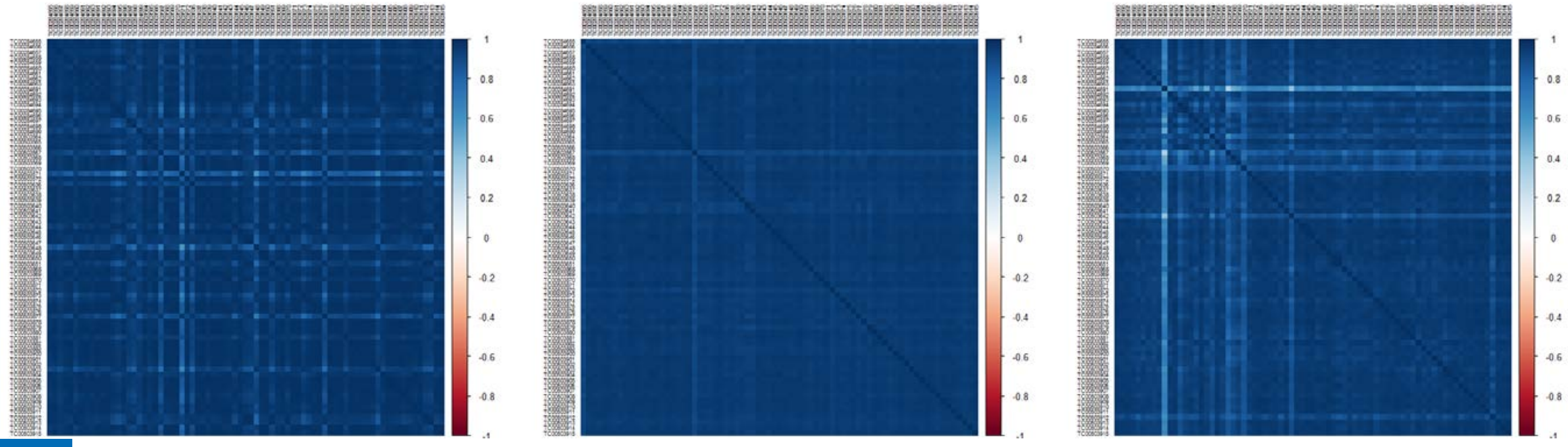


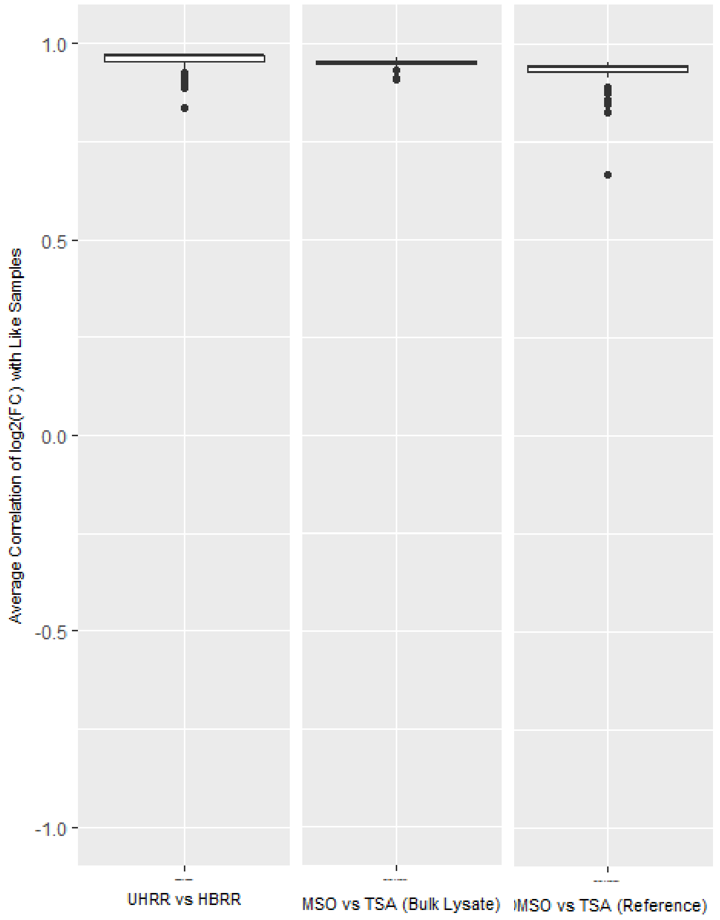




Reproducibility of Log_2(FC) Estimates







Concentration-Response Modeling / Bioactivity Thresholds

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Benchmark Dose Modeling

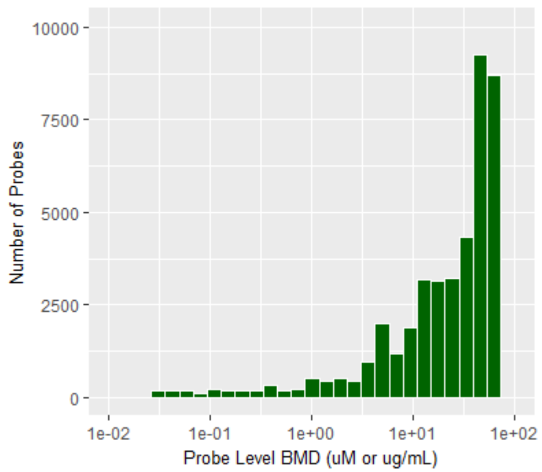
Parameter	Criteria [a]
Pre-filter:	ANOVA ($p_{raw} < 0.05$ & $\lvert FC \rvert \geq 2$)
Models	Hill, Exponential 2, *poly2, power, linear*
BMR Factor:	1.349 (10 %)
Best Model Selection:	Lowest AIC
Hill Model Flagging [b]:	'k' < 1/3 Lowest Positive Dose Retain Flagged Models
Pathway Analysis:	Genes with BMD <= Highest Dose ≥ 3 ≥ 1% Gene Set Coverage
Gene Set Collections [c]:	MSigDB_C2 MSigDB_H Reactome BioPlanet KEGG

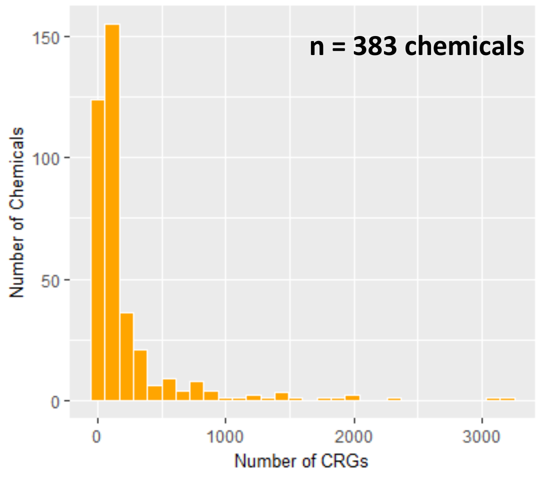
[a] *Exploratory analysis – modeling criteria not finalized*

[c] Gene Set Collections:

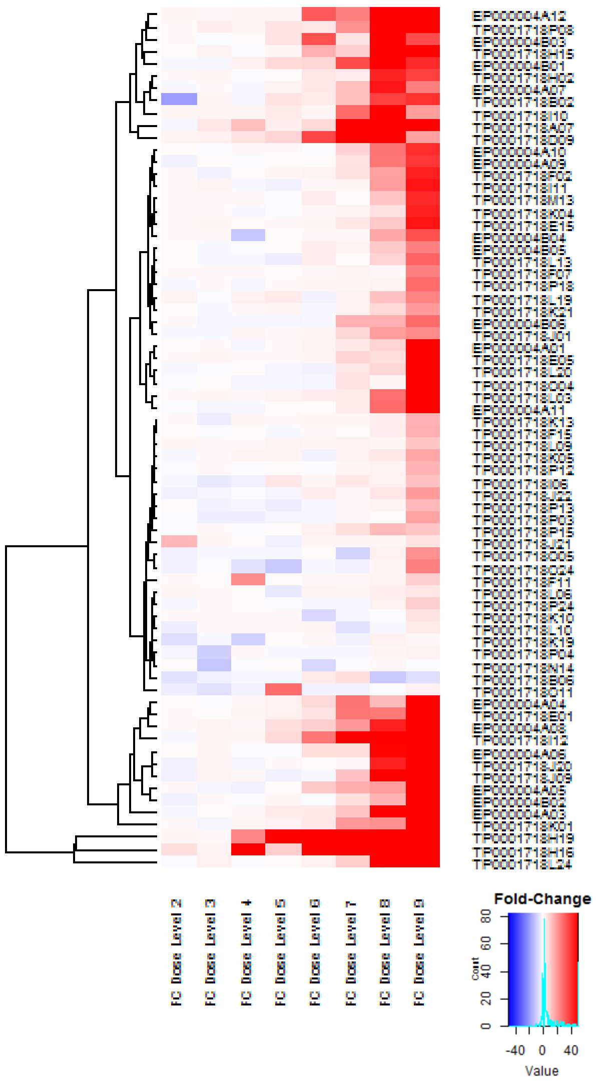
- **MSigDB_C2:** Curated gene sets from online pathway databases, publications and knowledge of domain experts (n = 4738).
- **MSigDB_H:** Coherently expressed signatures derived by aggregating many MSigDB gene sets to represent well-defined biological states or processes (n = 50).
- **Reactome:** Open-source, curated and peer reviewed pathway database with hierarchical pathway relationships in specific domains of biology. (n = 1764). Some pathways included in MSigDB_C2.
- **BioPlanet** (n = 1700): Curated pathway set developed by National Toxicology Program.

Benchmark Dose Modeling Summary

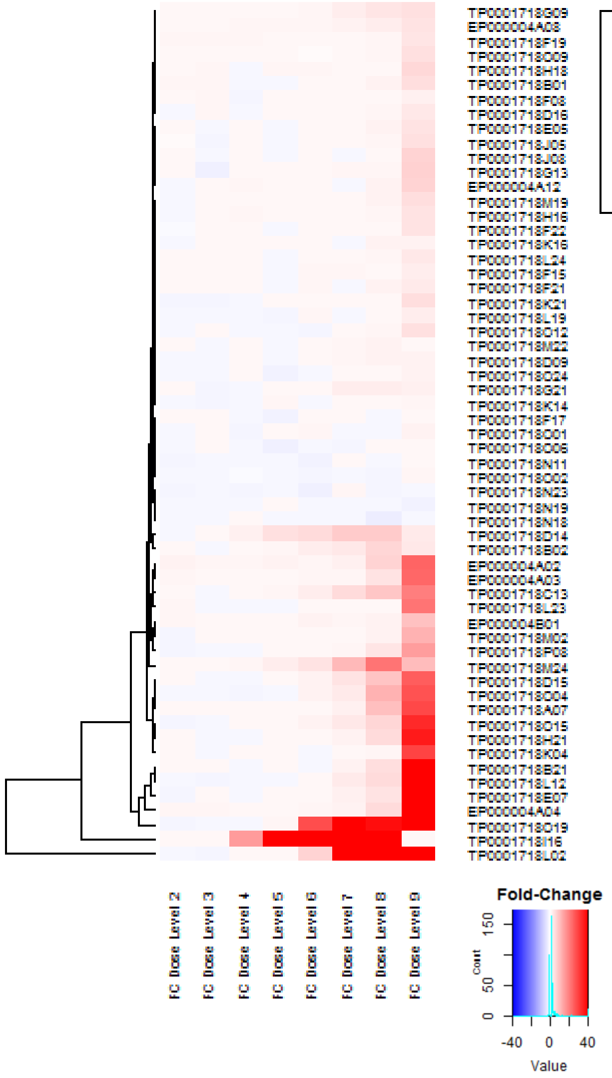




CYP1A1_10775



HMOX1_3041



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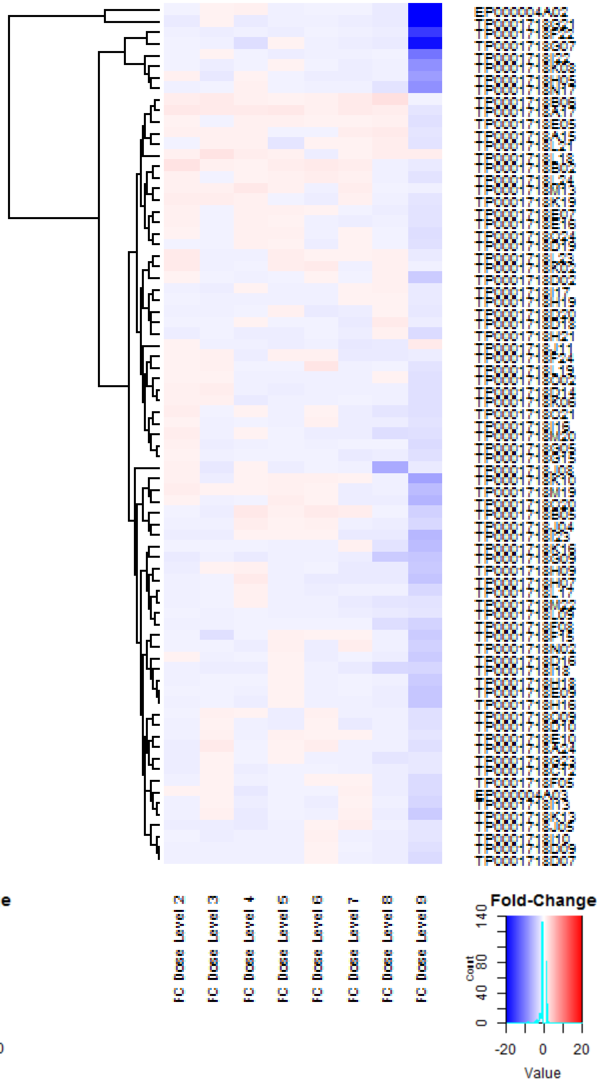

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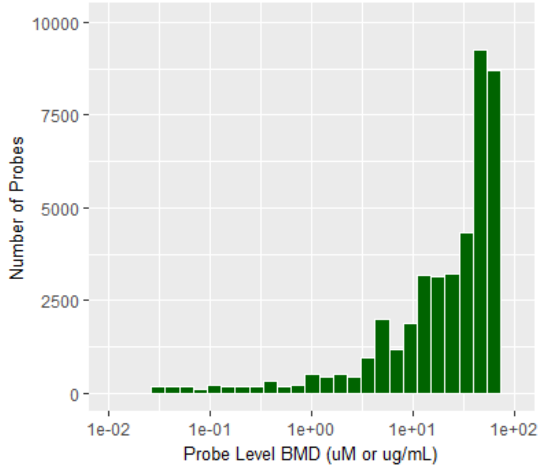
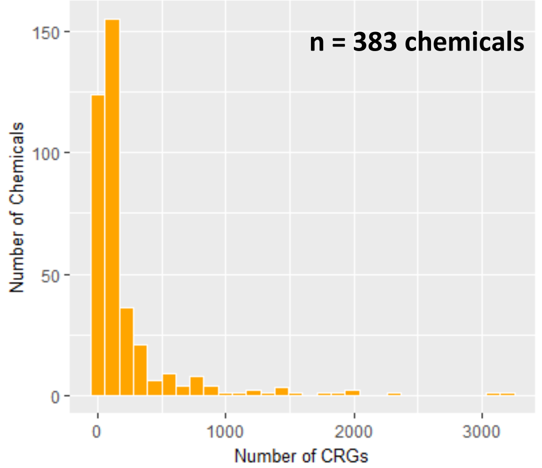
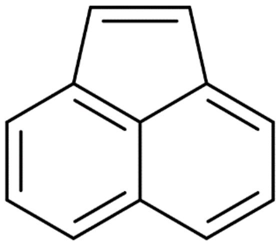
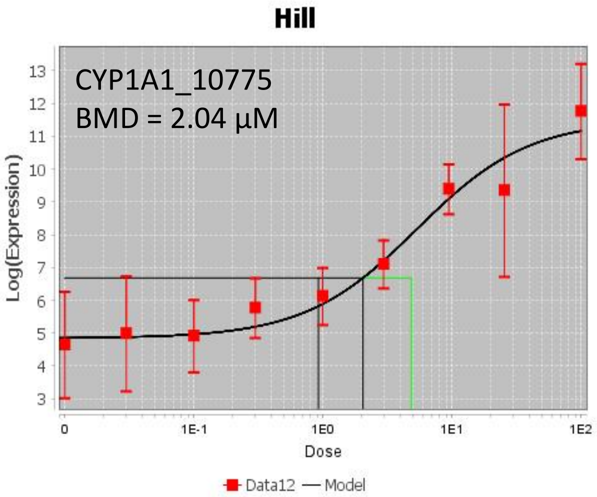
Benchmark Dose Modeling Summary



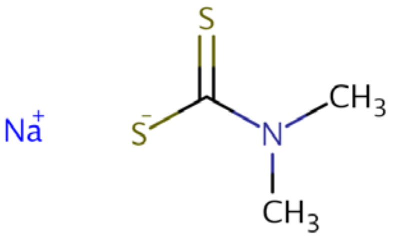


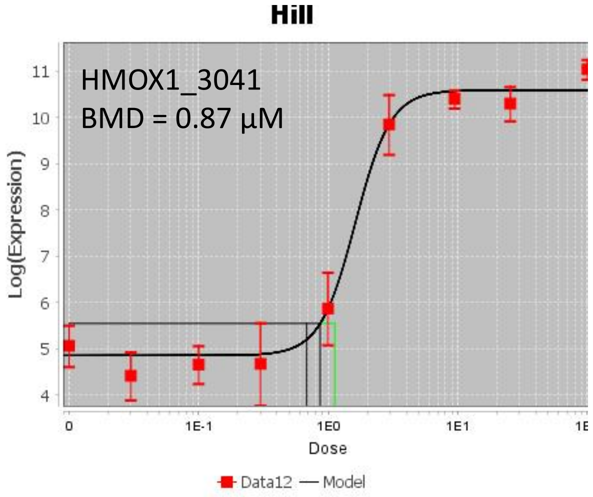
Acenaphthylene
208-96-8 | DTXSID3023845



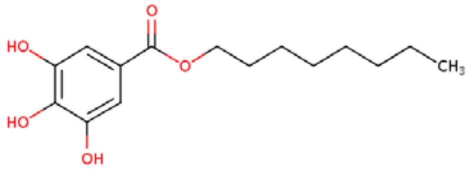


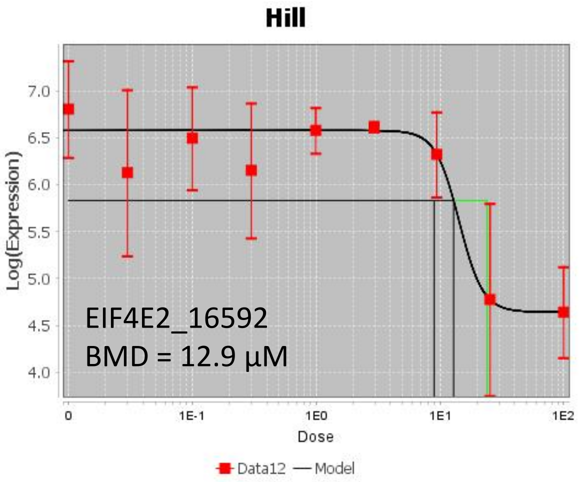
Sodium dimethyldithiocarbamate
128-04-1 | DTXSID6027050



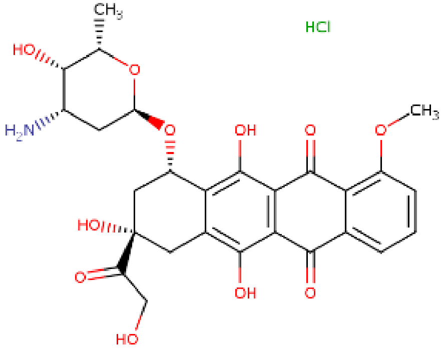


Octyl gallate
1034-01-1 | DTXSID4040713

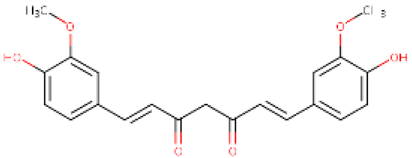




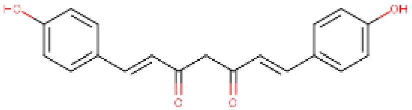
Case Study Chemicals



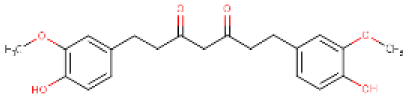
Adriamycin hydrochloride | DTXSID3030636



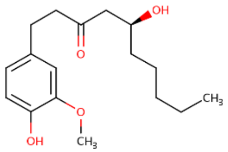
Curcumin | DTXSID8031077



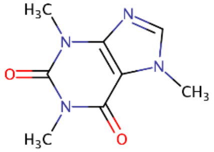
Bisdemethoxycurcumin | DTXSID00872663



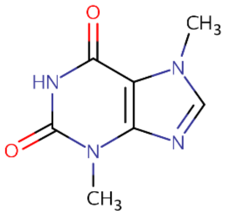
Tetrahydrocurcumin | DTXSID30865801



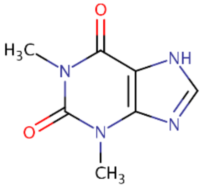
(6)-Gingerol | DTXSID3041035



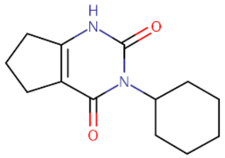
Caffeine | DTXSID0020232



Theobromine | DTXSID9026132

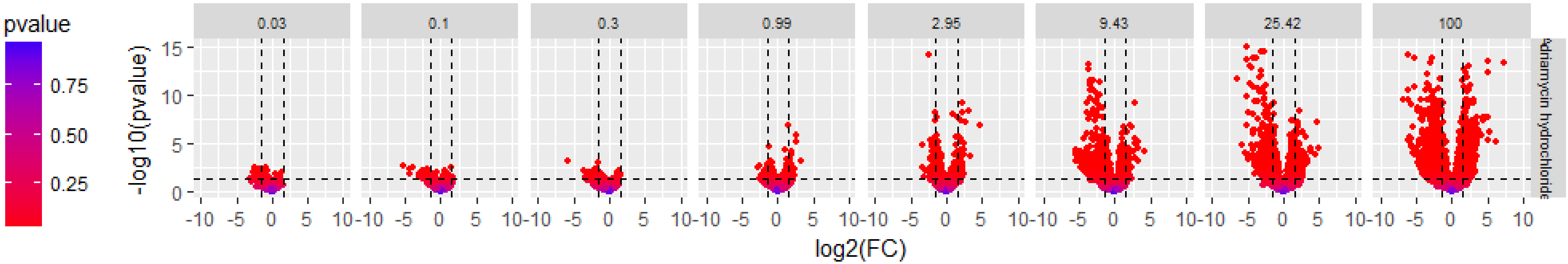


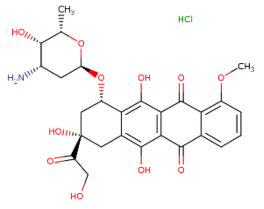
Theophylline | DTXSID5021336



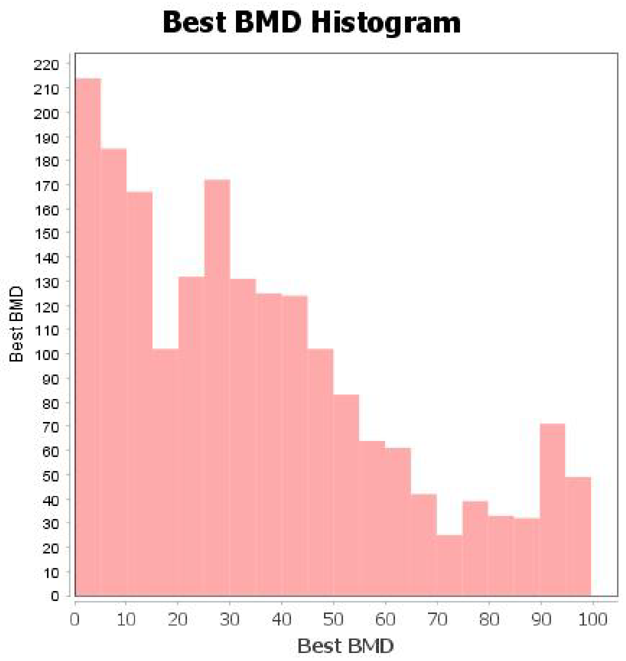
Lenacil | DTXSID9042093

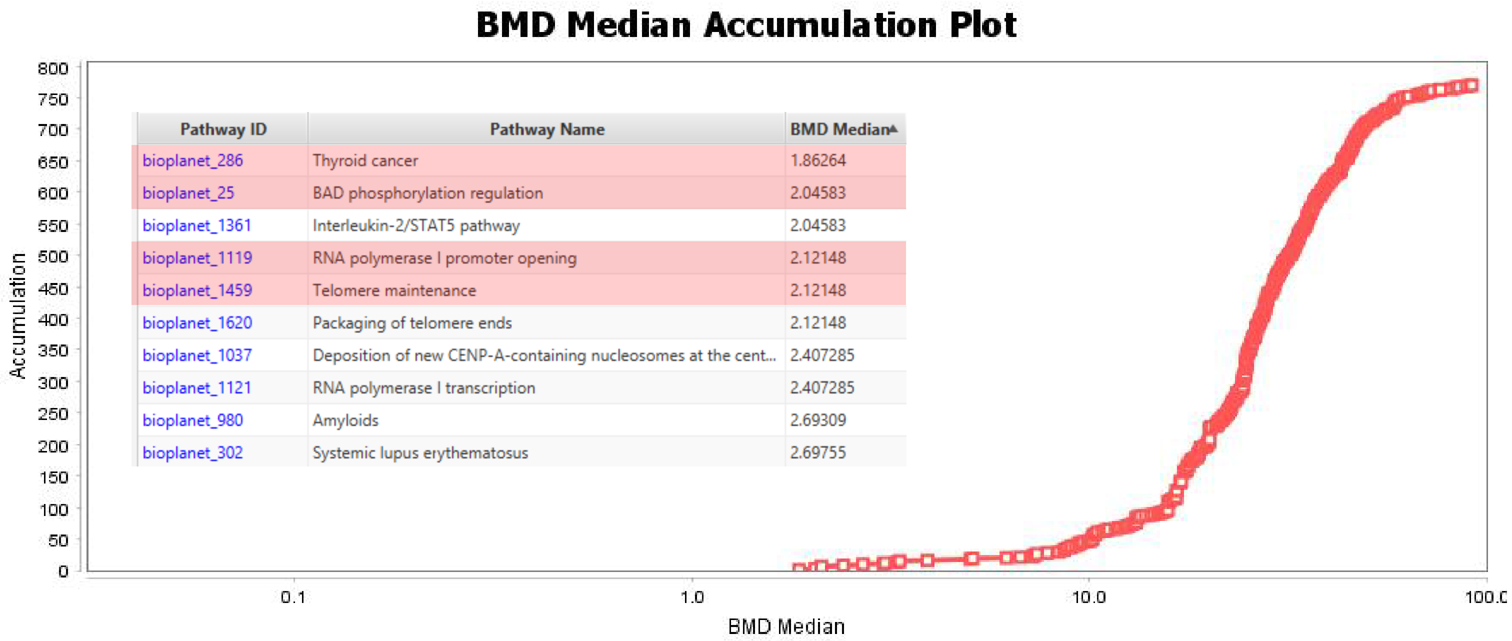
Doxorubicin (aka Adriamycin hydrochloride)





Adriamycin hydrochloride | DTXSID3030636

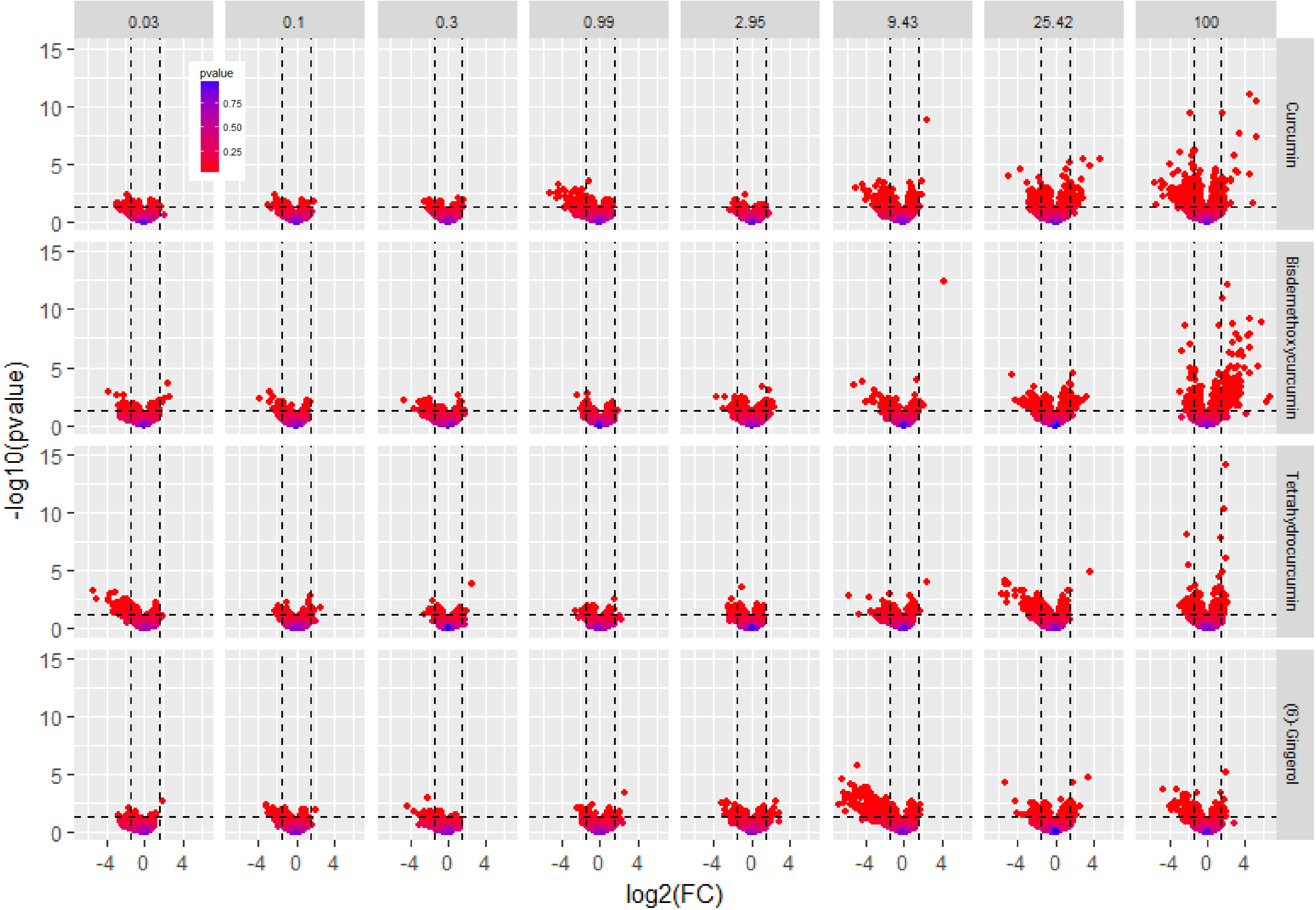


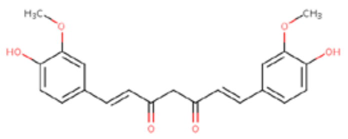


Pathway ID	Pathway Name	BMD Median
bioplanet_286	Thyroid cancer	1.86264
bioplanet_25	BAD phosphorylation regulation	2.04583
bioplanet_1361	Interleukin-2/STAT5 pathway	2.04583
bioplanet_1119	RNA polymerase I promoter opening	2.12148
bioplanet_1459	Telomere maintenance	2.12148
bioplanet_1620	Packaging of telomere ends	2.12148
bioplanet_1037	Deposition of new CENP-A-containing nucleosomes at the cent...	2.407285
bioplanet_1121	RNA polymerase I transcription	2.407285
bioplanet_980	Amyloids	2.69309
bioplanet_302	Systemic lupus erythematosus	2.69755

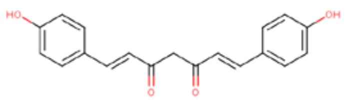
Int J Mol Cell Med. 2015 Spring;4(2):94-102.
Elevation of cAMP Levels Inhibits Doxorubicin-Induced Apoptosis in Pre- B ALL NALM- 6 Cells Through Induction of BAD Phosphorylation and Inhibition of P53 Accumulation.
Fatemi A[1], Kazemi A[1], Kashiri M[1], Safa M[2].

Evaluating Structurally Related Chemicals

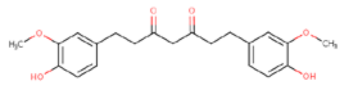




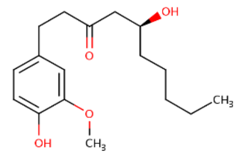
Curcumin | DTXSID8031077



Bisdemethoxycurcumin | DTXSID00872663

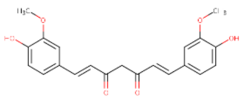


Tetrahydrocurcumin | DTXSID30865801

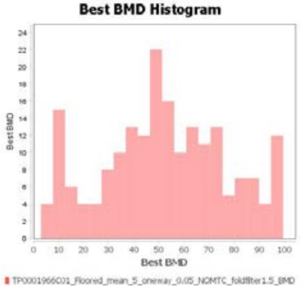


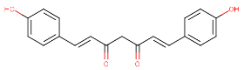
(6)-Gingerol | DTXSID3041035

Gene Set Analysis Using BMD Modeling Results



Curcumin | DTXSID8031077





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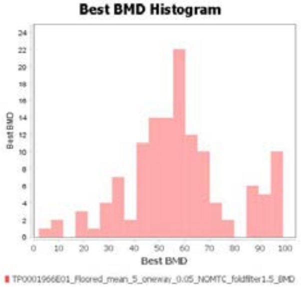
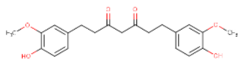
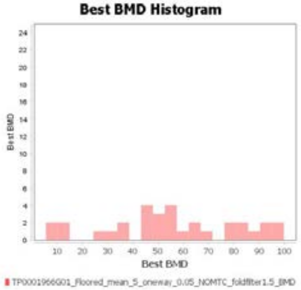
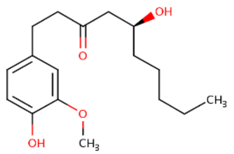


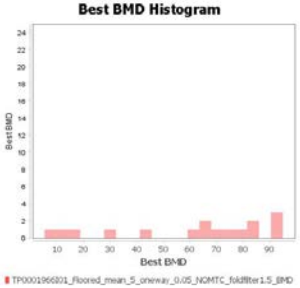


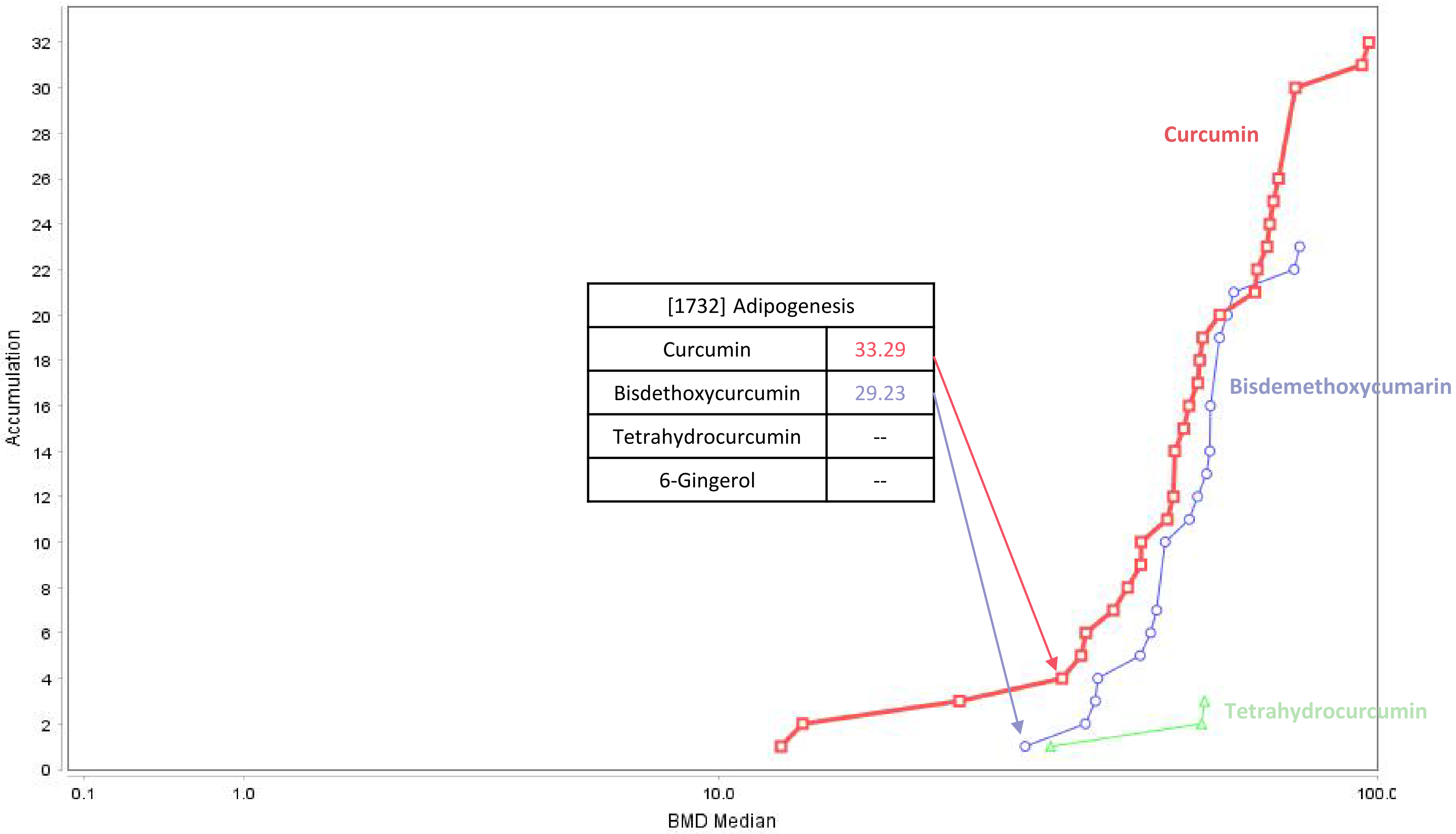
Tetrahydrocurcumin | DTXSID30865801





(6)-Gingerol | DTXSID3041035





[1732] Adipogenesis	
Curcumin	33.29
Bisdethoxycurcumin	29.23
Tetrahydrocurcumin	--
6-Gingerol	--

Toxicol Appl Pharmacol. 2017 Aug 15;329:158-164. doi: 10.1016/j.taap.2017.05.036.
Curcumin inhibits adipogenesis induced by benzyl butyl phthalate in 3T3-L1 cells.
Sakuma S[1], Sumida M[2], Endoh Y[2], Kurita A[2], Yamaguchi A[2], Watanabe T[2], Kohda T[2], Tsukiyama Y[2], Fujimoto Y[3].

J Agric Food Chem. 2016 Feb 3;64(4):821-30. doi: 10.1021/acs.jafc.5b05577. Epub 2016 Jan 25.
Bisdemethoxycurcumin Inhibits Adipogenesis in 3T3-L1 Preadipocytes and Suppresses Obesity in High-Fat Diet-Fed C57BL/6 Mice.
Lai CS[1,2], Chen YY[1], Lee PS[1], Kalyanam N[3], Ho CT[4], Liou WS[5], Yu RC[1], Pan MH[1,6,7,8].

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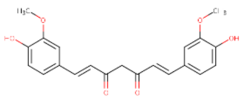
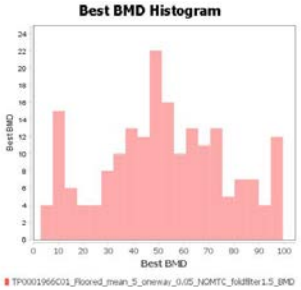
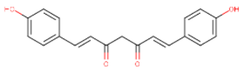
Gene Set Analysis Using BMD Modeling Results

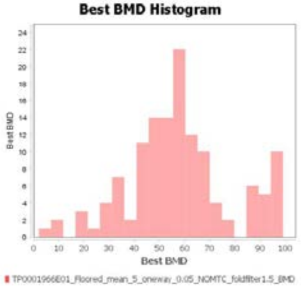


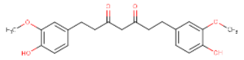
Curcumin | DTXSID8031077



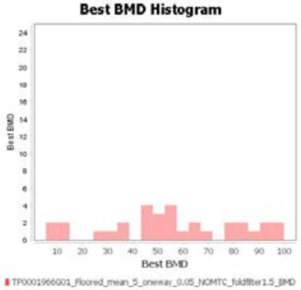


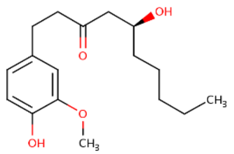
Bisdemethoxycurcumin | DTXSID00872663



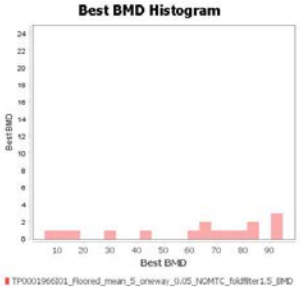


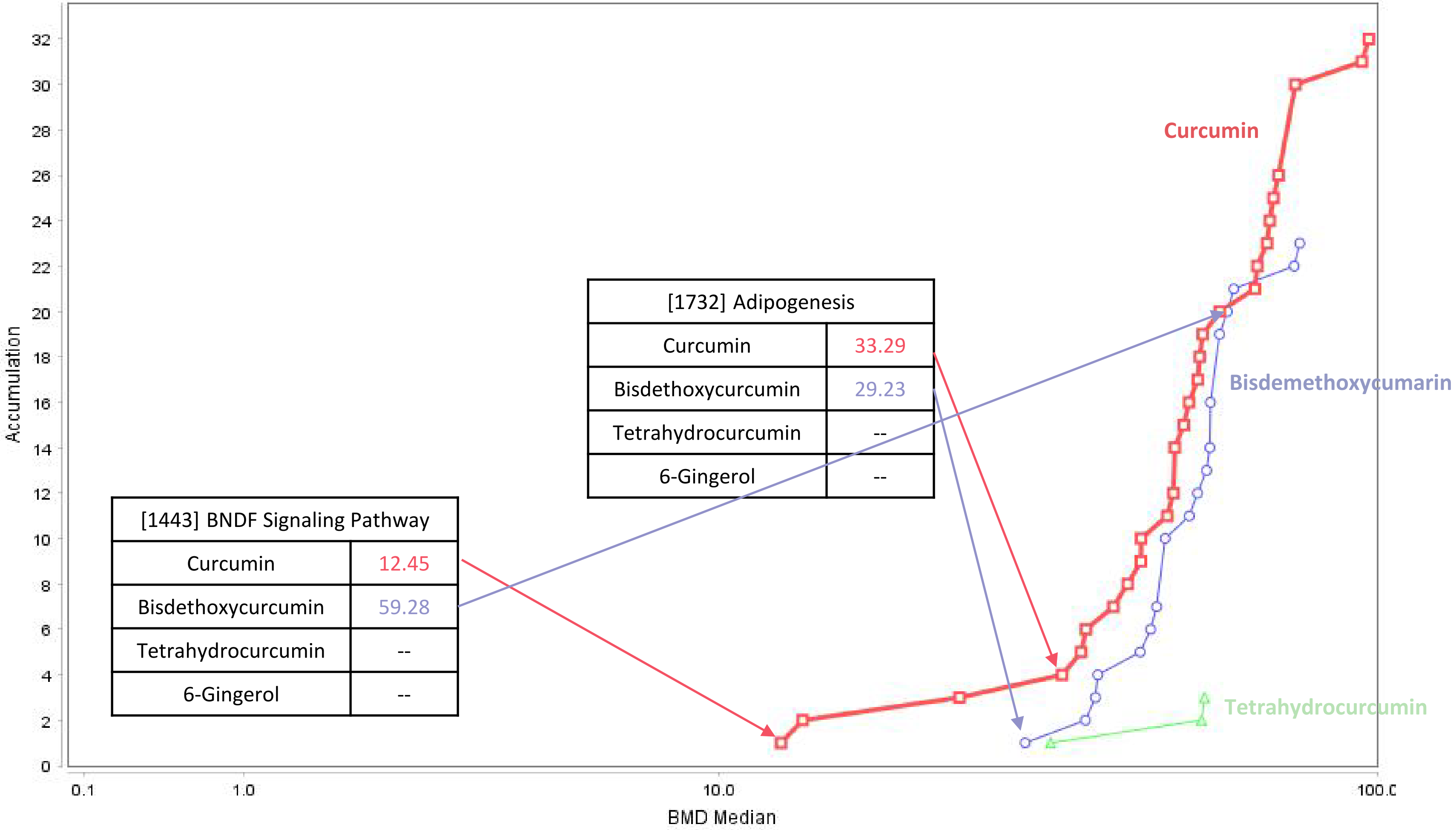
Tetrahydrocurcumin | DTXSID30865801





(6)-Gingerol | DTXSID3041035





[1732] Adipogenesis	
Curcumin	33.29
Bisdethoxycurcumin	29.23
Tetrahydrocurcumin	--
6-Gingerol	--

[1443] BNDF Signaling Pathway	
Curcumin	12.45
Bisdethoxycurcumin	59.28
Tetrahydrocurcumin	--
6-Gingerol	--

Neuropeptides. 2016 Apr;56:25-31. doi: 10.1016/j.npep.2015.11.003. Epub 2015 Nov 11.
Effect of curcumin on serum brain-derived neurotrophic factor levels in women with premenstrual syndrome: A randomized, double-blind, placebo-controlled trial. Fanaei H[1], Khayat S[2], Kasaeian A[3], Javadimehr M[4].

Biomed Pharmacother. 2017 Mar;87:721-740. doi: 10.1016/j.biopha.2016.12.020. Epub 2017 Jan 14.
Curcumin confers neuroprotection against alcohol-induced hippocampal neurodegeneration via CREB-BDNF pathway in rats. Motaghinejad M[1], Motevalian M[2], Fatima S[3], Hashemi H[1], Gholami M[4].

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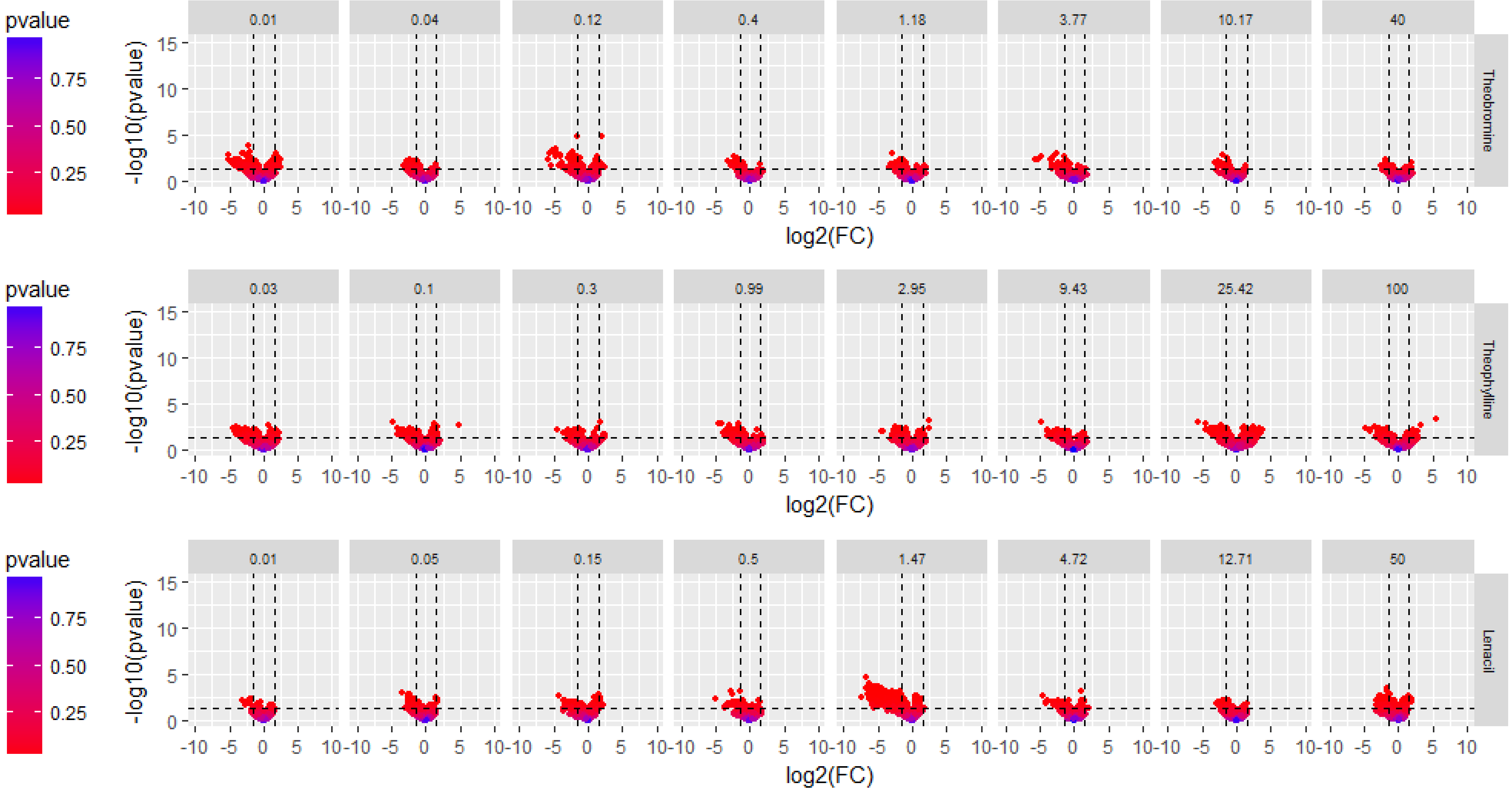
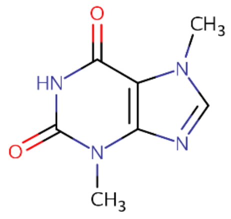
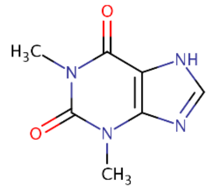
Caffeine Analogs

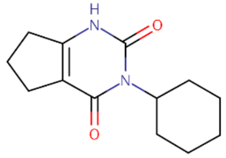




Theobromine | DTXSID9026132



Theophylline | DTXSID5021336



Lenacil | DTXSID9042093

- Very few dose-responsive genes
- No significantly enriched pathways using BMDe standard workflow

Alternative Approach for Gene Set Analysis

Step 1: Calculate Response

- A gene set is a list / bag of genes
- Under one condition (chemical x dose) calculate "gene set response" separately for genes in the set and out of the set:

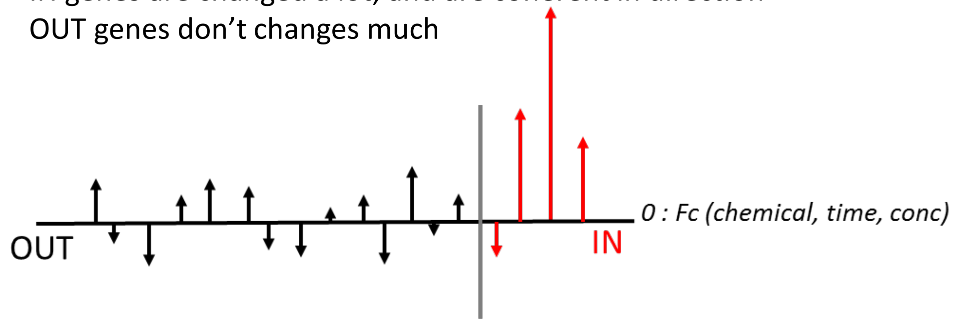
$$M = \sum_{i=1}^{ngene} \frac{\mathrm{fc}_i}{\mathrm{sc}_i^2} \Big/ \sum_{i=1}^{ngene} \frac{1}{\mathrm{sc}_i^2}$$

$$\mathrm{R} = M_{in} - M_{out}$$

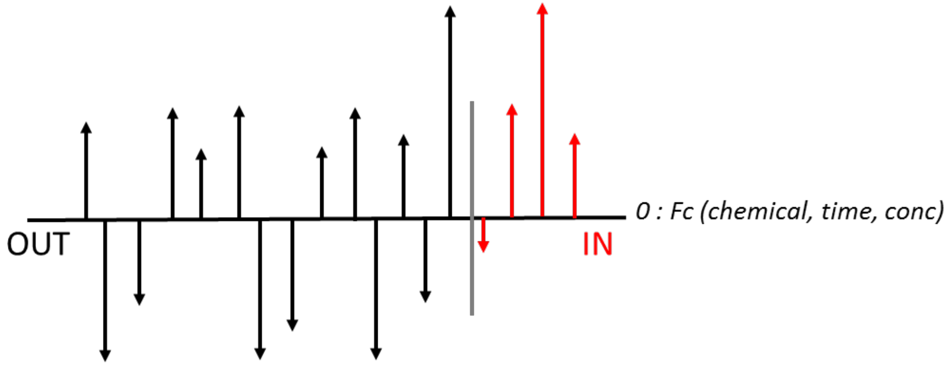
Step 2: CR Modeling

- For each chemical, fit using tcplFit
  - Constant, Hill , Gain-Loss methods
  - BMAD(pathway) = MAD of response for the pathway across the two lowest concentrations across all chemicals and times
- Hitcall:
  - tcplFit calls a hit
  - Top > 3*BMAD

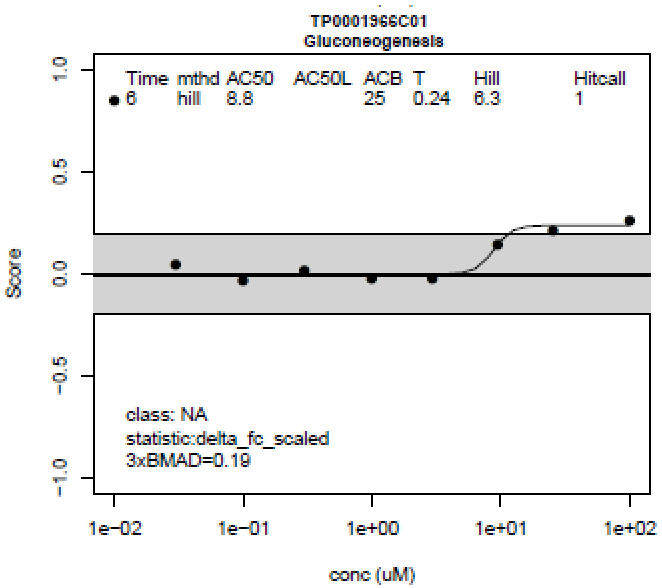
- IN genes are changed a lot, and are coherent in direction
- OUT genes don't changes much

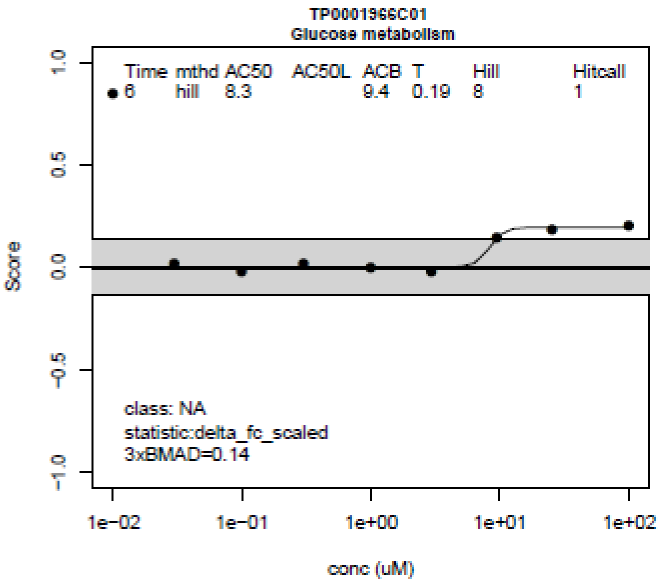


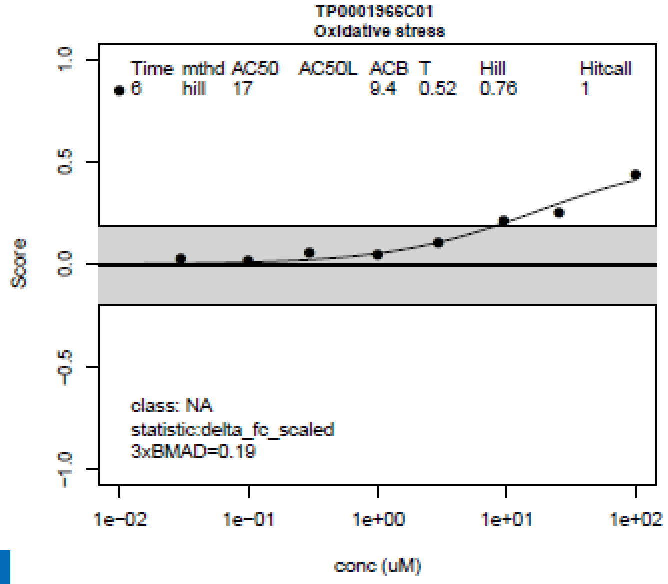
- IN genes are changed a lot, and are coherent in direction
- OUT genes change a lot but are not coherent (mean ~ 0)

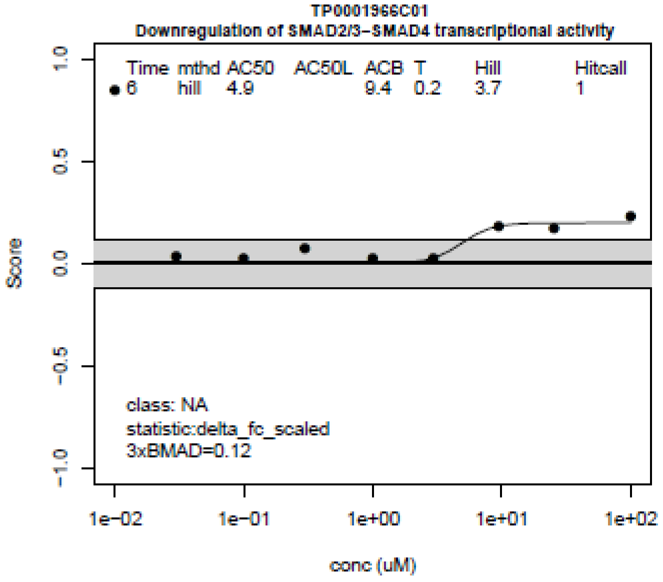


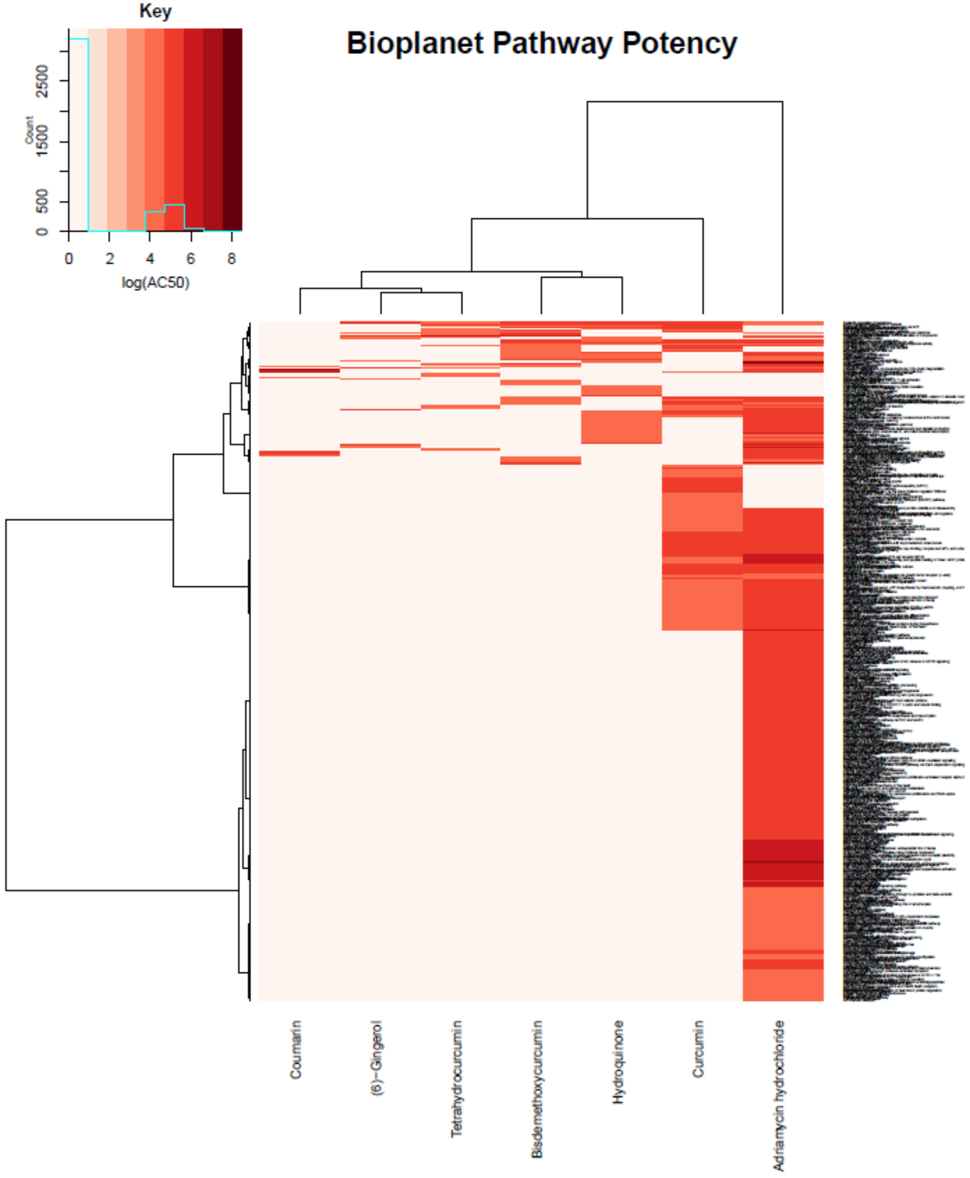
Comparing Activities Across Chemicals











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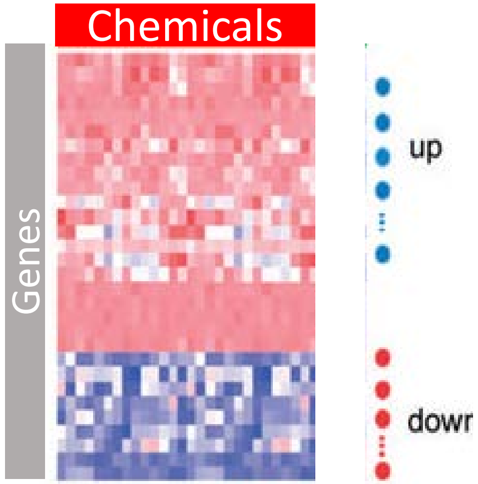
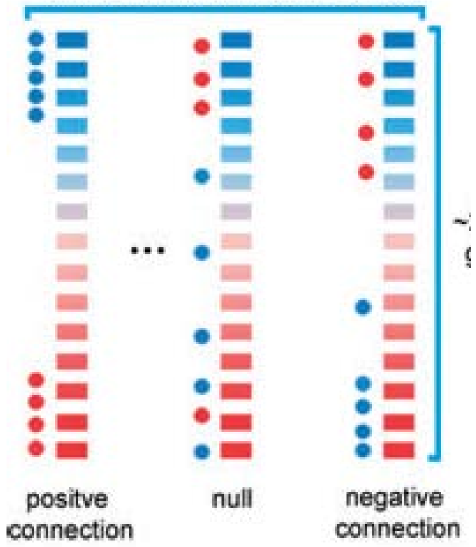
MOA by Connectivity Mapping

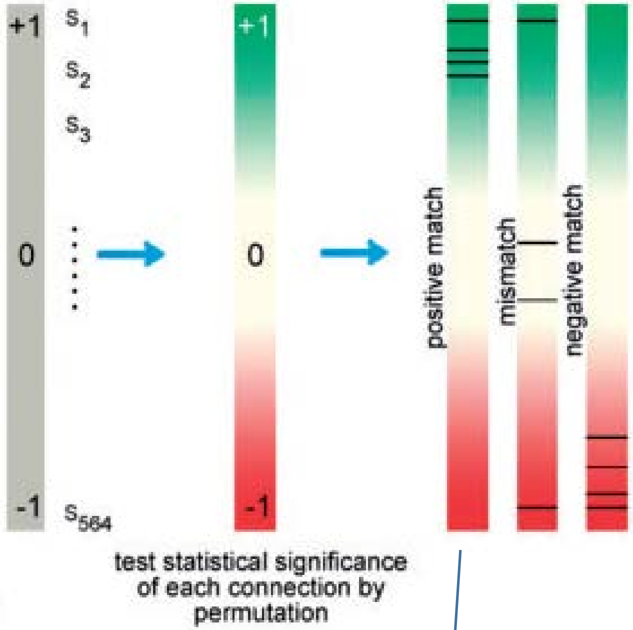
Input Profile



Query Signature DB
CMap or BSP



Find best positive matches



Infer MIE/Target by best match

Issues

- Translating DEG/CRG to signature
- Many measures of similarity
- Only as good as reference chemical MoA annotation
- Highly sensitive but not very specific
- Chemicals that cause global perturbations “hit” all MoAs – how do we distinguish signal from noise ?

Lamb *et al* (2006)
Musa *el al* (2017)

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Various Approaches to Connectivity Mapping

DRG: Differentially Expressed Gene → Based on single-conc. comparison to control.
CRG: Concentration-Responsive Gene → Based on ANOVA or conc.-response modeling.

CMap vs CMap

DEGs
- **Query:** Affymetrix CMap DEGs
- **DB:** Affymetrix CMap

HTTr vs HTTr

DEGs
- **Query:** HTTr DEGs
- **DB:** HTTr DEGs

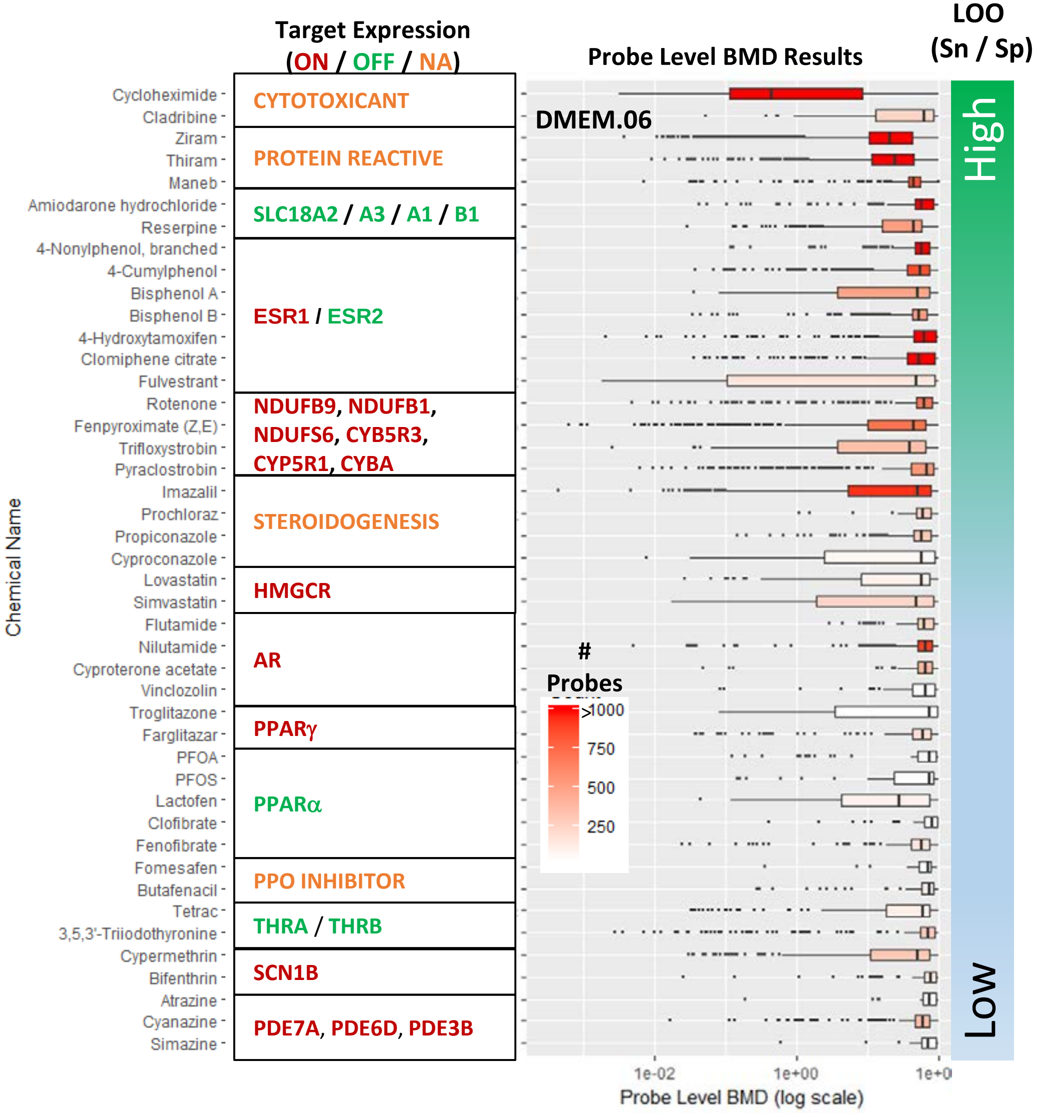
CRGs
- **Query:** HTTr CRGs
- **DB:** HTTr CRGs

HTTr vs CMap

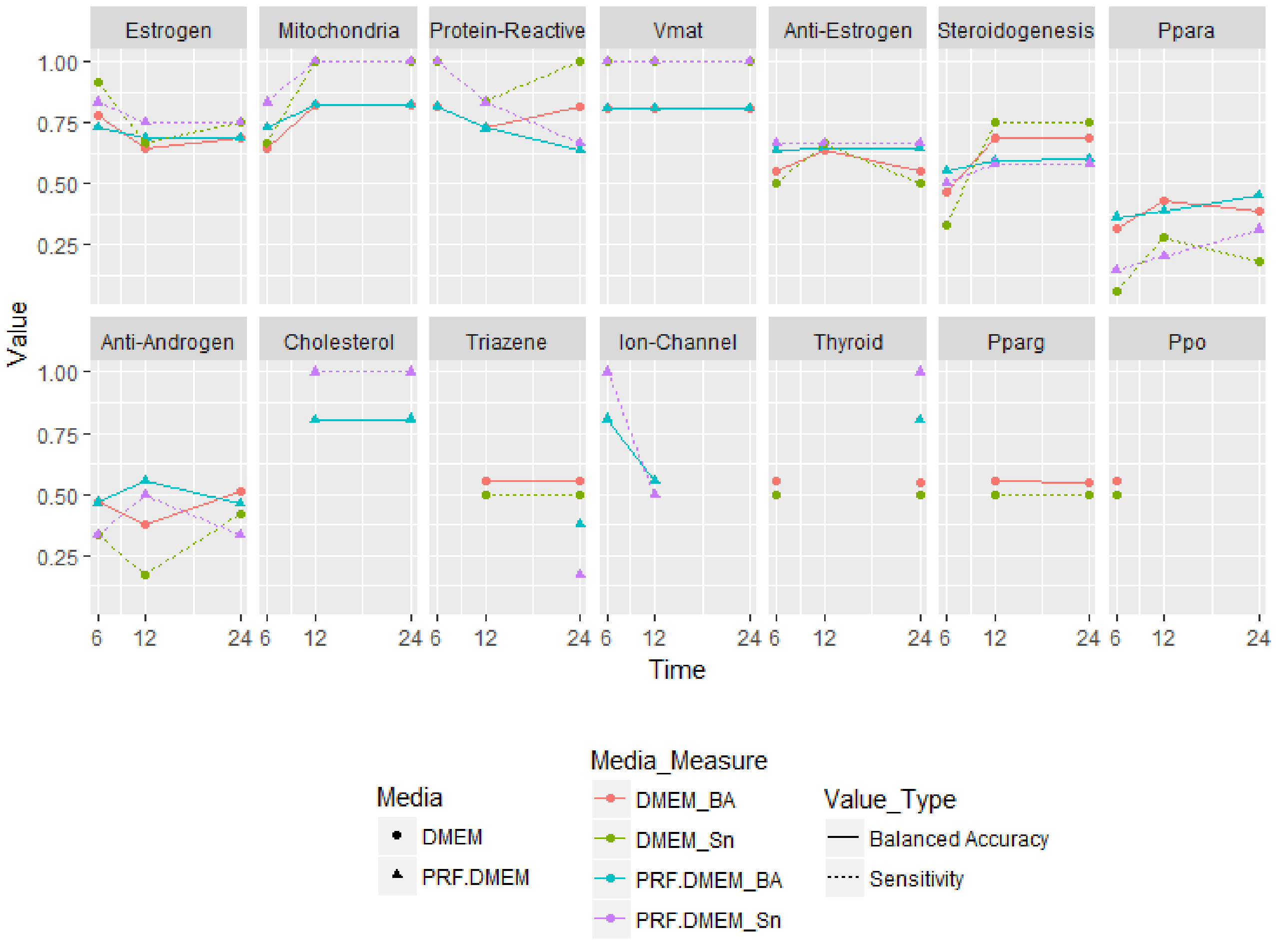
DEGs
- **Query:** TempO-Seq DEGs
- **DB:** Affymetrix CMap

CRGs
- **Query:** TempO-Seq CRGs
- **DB:** Affymetrix CMap

Connectivity Mapping for MoA Prediction



Sensitivity and Balanced Accuracy of MoA Prediction Using CRGs and Leave-One-Out Cross-Validation



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