EPA United States Environmental Protection Agency

High Throughput Transcriptomics (HTTr) Concentration-Response Screening in MCF7 Cells

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Conflict of Interest Statement

- No conflict of interest declared.
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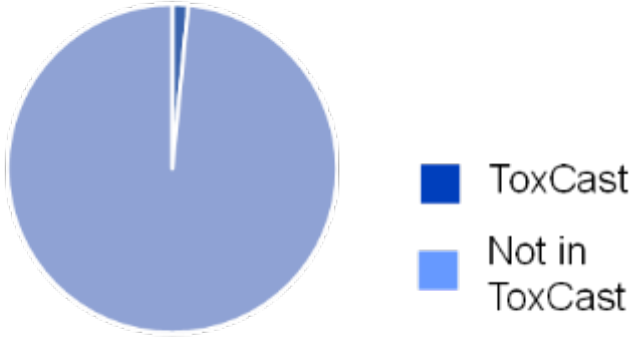
Outline

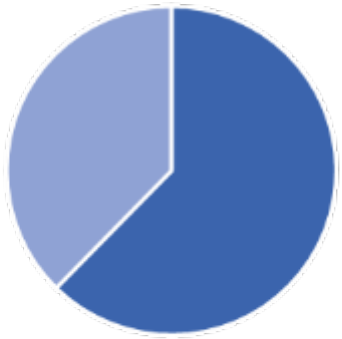
- Background & Objectives
- HTTr Pilot Experiment
    - Optimization Steps
    - Attenuation
    - Experimental Layout
- Results
    - Assay Performance Metrics
    - Concentration-Response Modeling
- Current Activities & Future Directions

Background

Gene Coverage



Pathway Coverage*

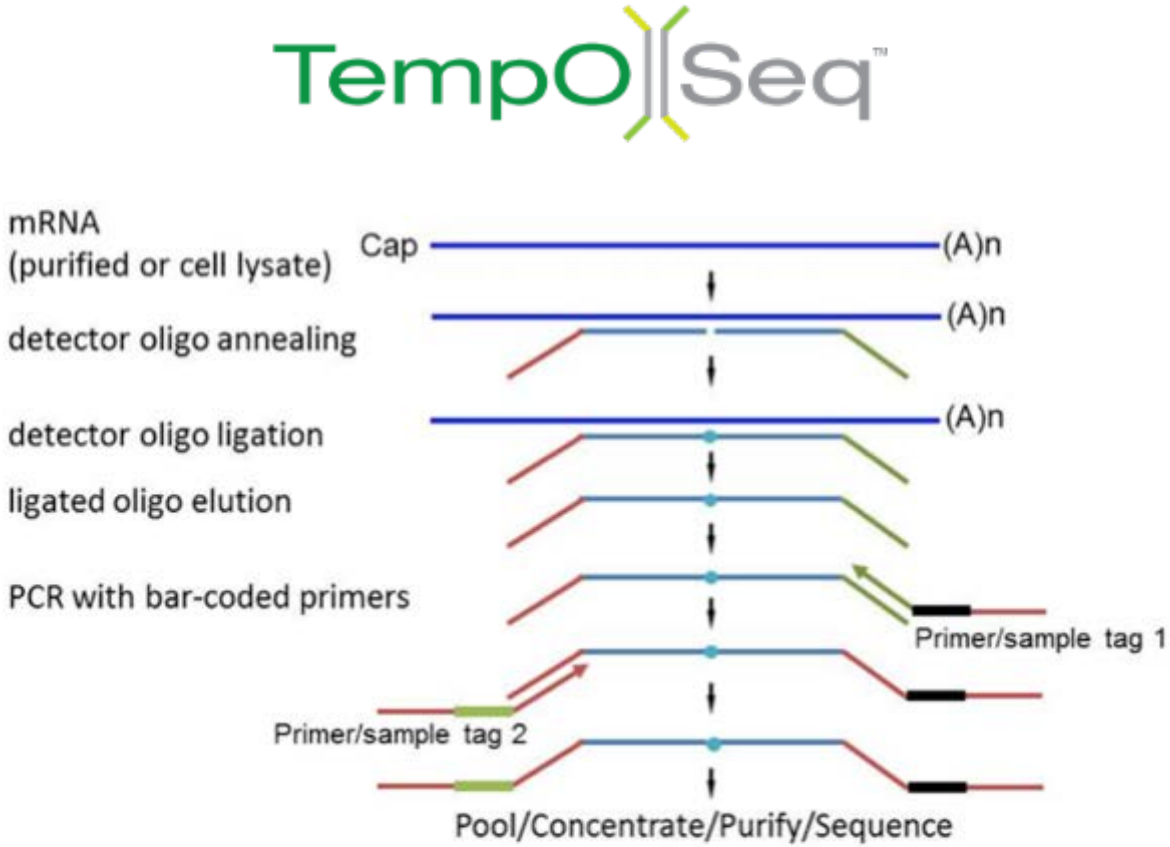


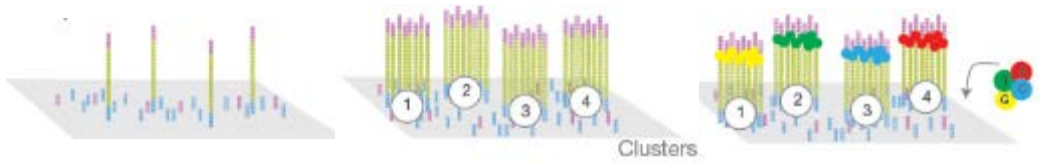
*At least one gene from pathway represented

- ToxCast assays cover about 320 genes.
- Pathway coverage is higher but still leaves large gaps
- Recent technological advances in transcriptomics are very promising for rapid and cost-effective whole transcriptome screening.
- Increase biological coverage by using high throughput transcriptomics (HTTr) as broad-based Tier 0 bioactivity screen.

BioSpyder TempO-Seq

- Targeted RNA-Seq technology
- Whole transcriptome assay provides output on > 20,000 transcripts.
- Requires very low input (< 10 pg total RNA).
- Performed on “standard” PCR and Next Gen Sequencers.
- Compatible with purified RNA or cell lysates.





Objectives

- Optimize culture and assay conditions for HTTr screening in MCF7 cells using the TempO-Seq human whole transcriptome assay.

- Perform a pilot experiment with a limited number of chemicals (n=44) in order to:

    1) Evaluate TempO-Seq assay performance.
    2) Determine the ability of the TempO-Seq assay to detect known biological signatures following chemical perbations
    3) Guide experimental design of larger screening studies.

HTTr Pilot: Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	MCF7
Culture Condition	2	DMEM + 10% HI-FBS PRF-DMEM + 10% CS-HI-FBS
Chemicals	44	see subsequent slides
Time Points:	3	6, 12, 24 hours
Assay Formats:	3	TempO-Seq HCI-Apoptosis HCI-Cytotoxicity
Concentrations:	8	3.5 $\log_{10}$ units; ½ $\log_{10}$ spacing
Biological Replicates:	4	3 TempO-Seq; 1 Reserve

[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/).

[a] Dulbecoo's Modified Eagle's Media (MediaTech 10-013) + Heat-Inactivated FBS (Sigma-Aldrich F4135)
[B] Phenol Red Free Dulbecco's Modified Eagle's Media (MediaTech 17-205) + Charcoal-Stripped Heat-Inactivated FBS (Sigma-Aldrich 6765)

HTTr Pilot: Workflow

Generate Cryopreserved Cell Stocks

Cell Expansion

Cell Plating

BioTek MultiFlo FX

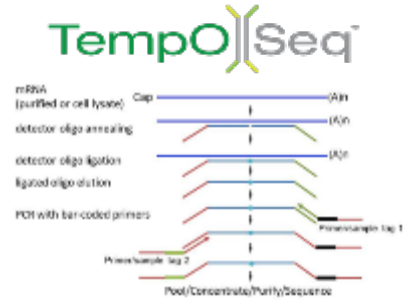
Cell Dosing

LabCyte Echo® 550 Liquid Handler

Track 1: Targented RNA-Seq

Generating Cell Lysates

TempO-Seq WT



Track 2: Cytotoxicity / Apoptosis

Cell Labeling

High Content Imaging

Thermo Cellomics ArrayScan® VTI HCS Reader

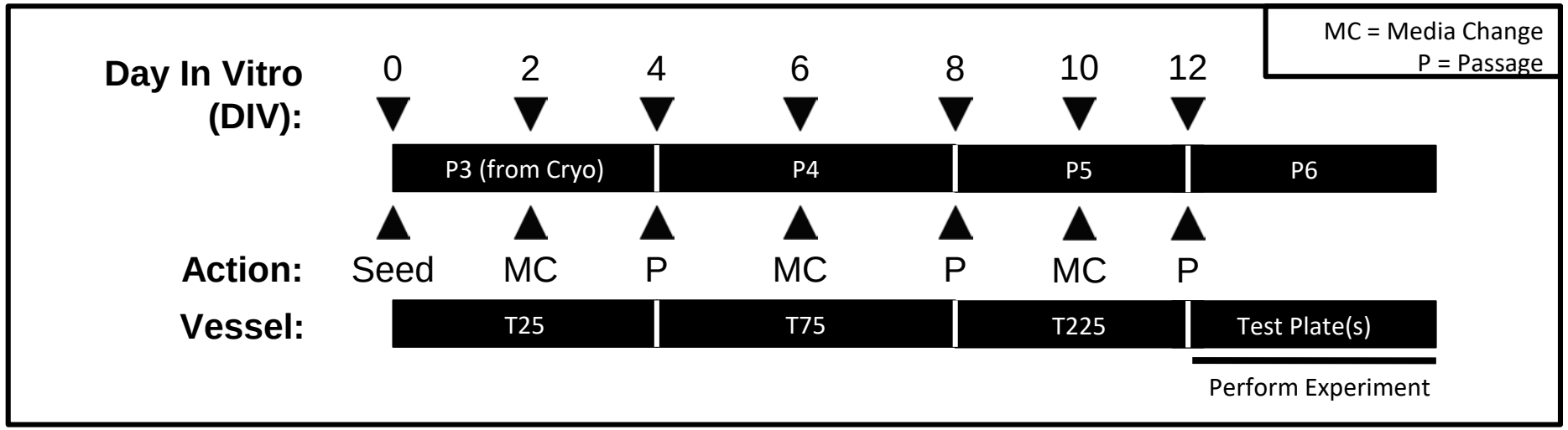
Assay Optimization

- **MCF7 Cell Culture**
  - Authentication
  - Expansion Protocol
  - Media Formulation
  - Seeding Density

- **TempO-Seq Assay**
  - Lysis Conditions
  - Attenuation of Highly Expressed Genes

- **Chemical Treatments**
  - Concentration Range
  - Plate Map Design
  - Exposure Duration

MCF7 Expansion Protocol



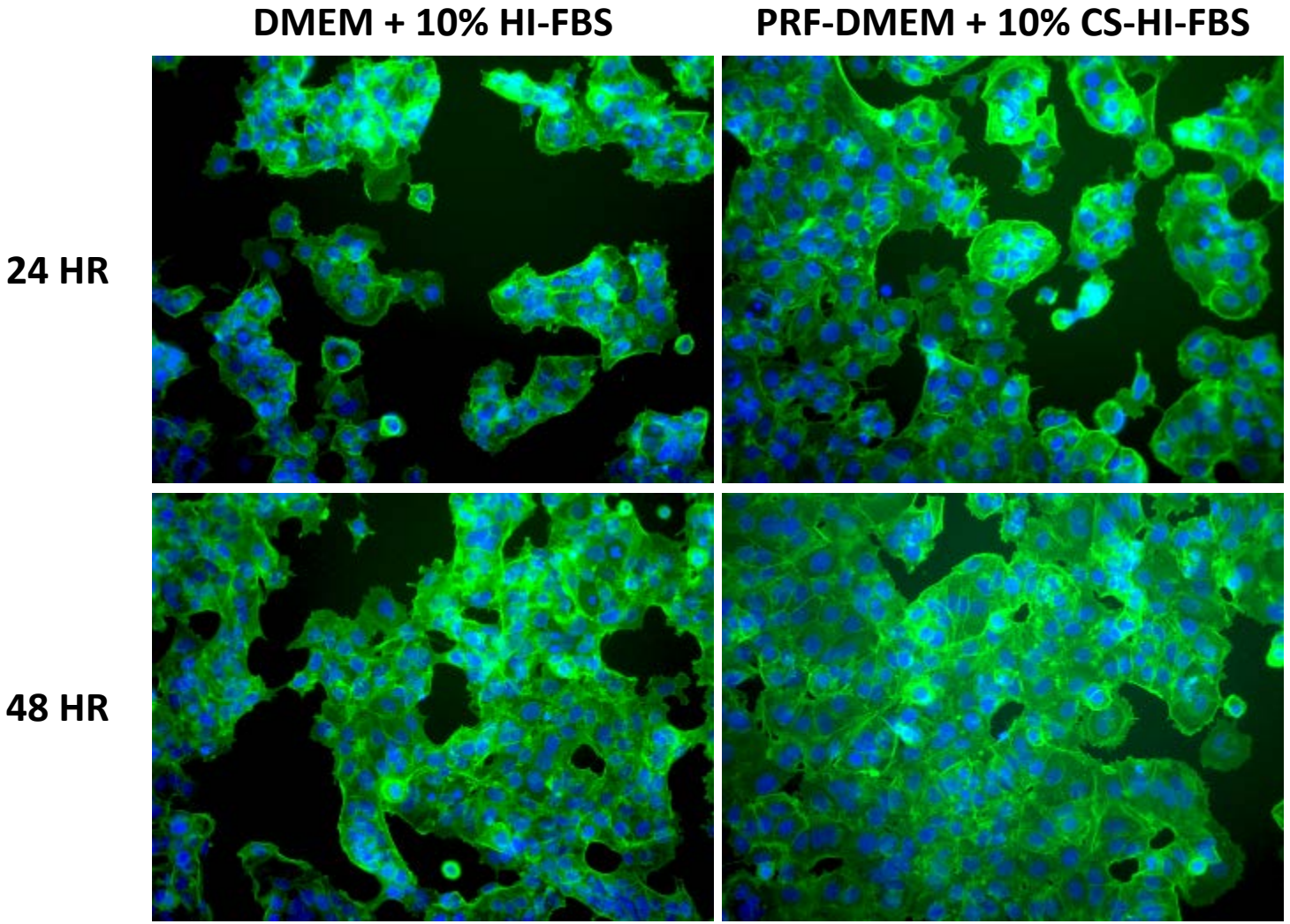
Stage	Culture Vessel	Average Cell Yield [a]	Number of Treatment Wells [b]	Number of Test Plates [c]
Initial Seeding	NA	$1.28x10^7$	182	0.47
P (3→4)	T25	$2.43x10^7$	346	0.90
P (4→5)	T75	$5.86x10^7$	837	2.18
P(5→6)	T225	$1.47x10^8$	2100	5.47

[a] Median values from c2017-08-14, c2017-08-15, c2017-08-19, c2017-08-20
[b] Assumes 384 well plate, 10,000 cells / well.
[c] For experimental needs > 5 plates / experiment, expand multiple cryopreserved MCF7 cell aliquots in parallel. Pool at each passaging stage.

- MCF7 Cells authenticated by STR Profiling and karyotyping prior to use in screening studies.

Media Effects on MCF7 Growth

- DMEM + 10% HI-FBS contains phenol red and an unknown compliment of serum factors which may stimulate ER activation.
- Phenol red-free media with charcoal-stripped FBS reduces endogenous estrogen receptor activation.



Qualitative Observations

- More cell attachment and cell spreading with PRF-DMEM + 10% CS-HI-FBS.
- Greater increase in cell confluency over time in PRF-DMEM + 10% CS-HI-FBS.
- More proliferation over time in DMEM + 10% HI-FBS.

Attenuation

- A method used with BioSpyder TempO-Seq assay to prevent highly expressed genes from occupying a disproportionate amount of available read space and increase the ability to quantify low abundance transcripts.

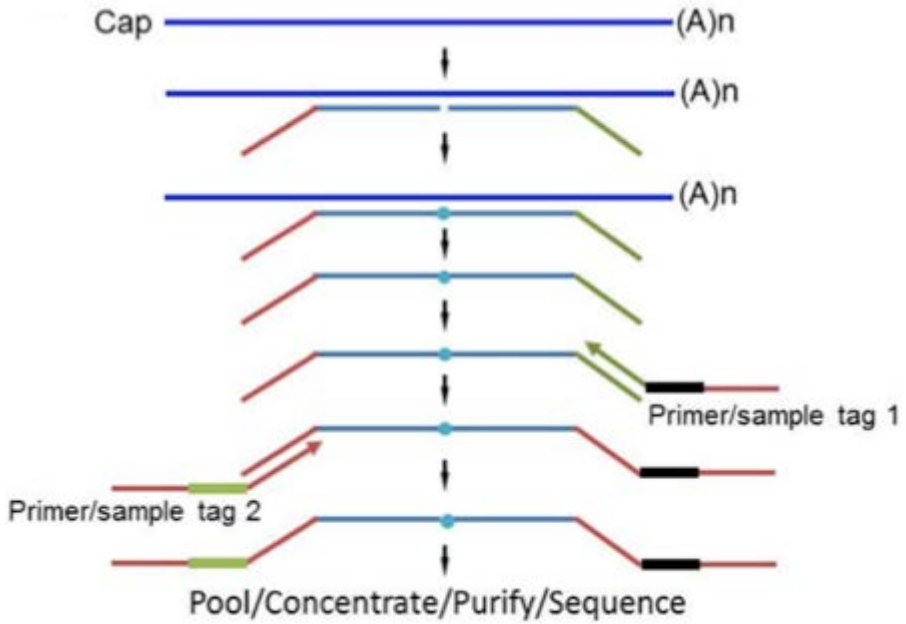
- Attenuation is accomplished by adding “cold probes” which do not have the PCR amplification tags at the 5’ and 3’ ends of the ligated detector oligos.

- The attenuation probe will bind to the same site as the detector oligos, thus decreasing the amount of the target RNA species available for PCR ampliciation.

- A “standard” attenuation for ribosomal RNAs is applied to TempO-Seq whole transcriptome assays.

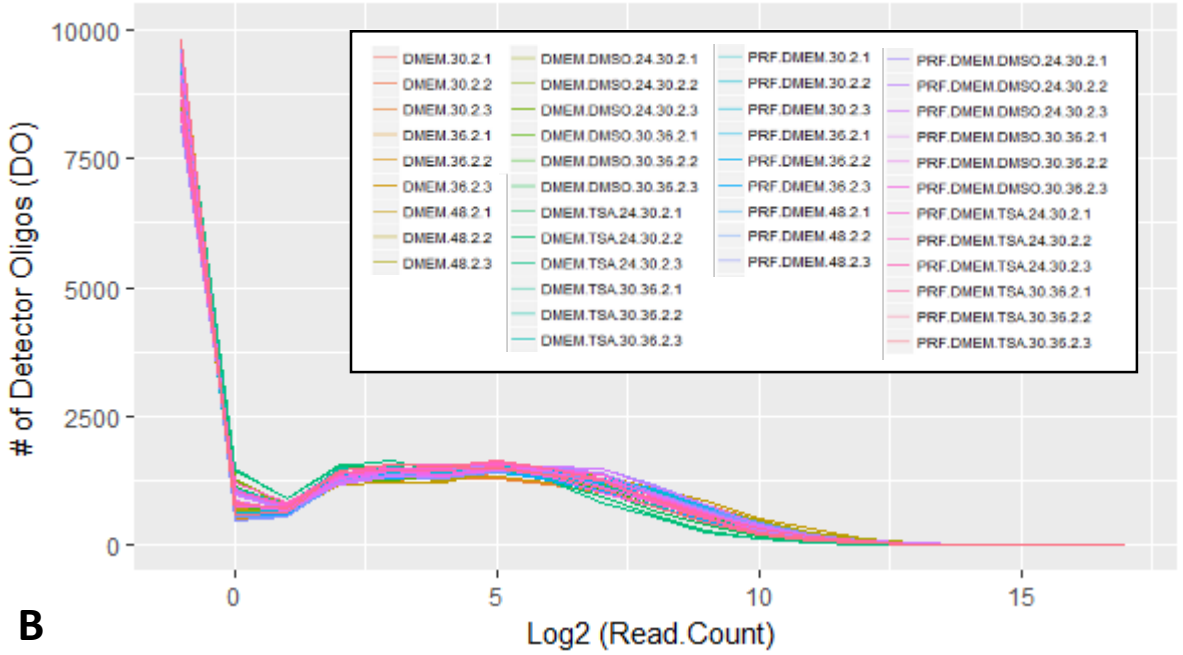
- For additional attenuation, the end user must define:
  - The set of genes to be attenuated, and…
  - What degree of attenuation is appropriate

- **Question(s):**
  - Is additional attenuation needed in the MCF7 cell model?
  - If so, how is the attenuation set defined?



Distribution of Read Counts

A



B

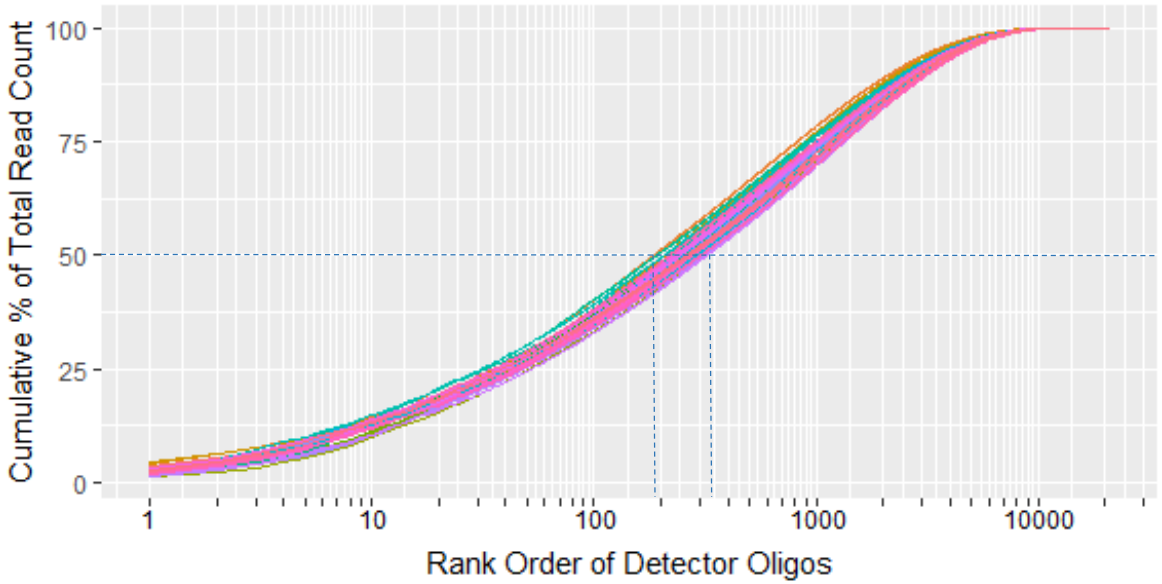


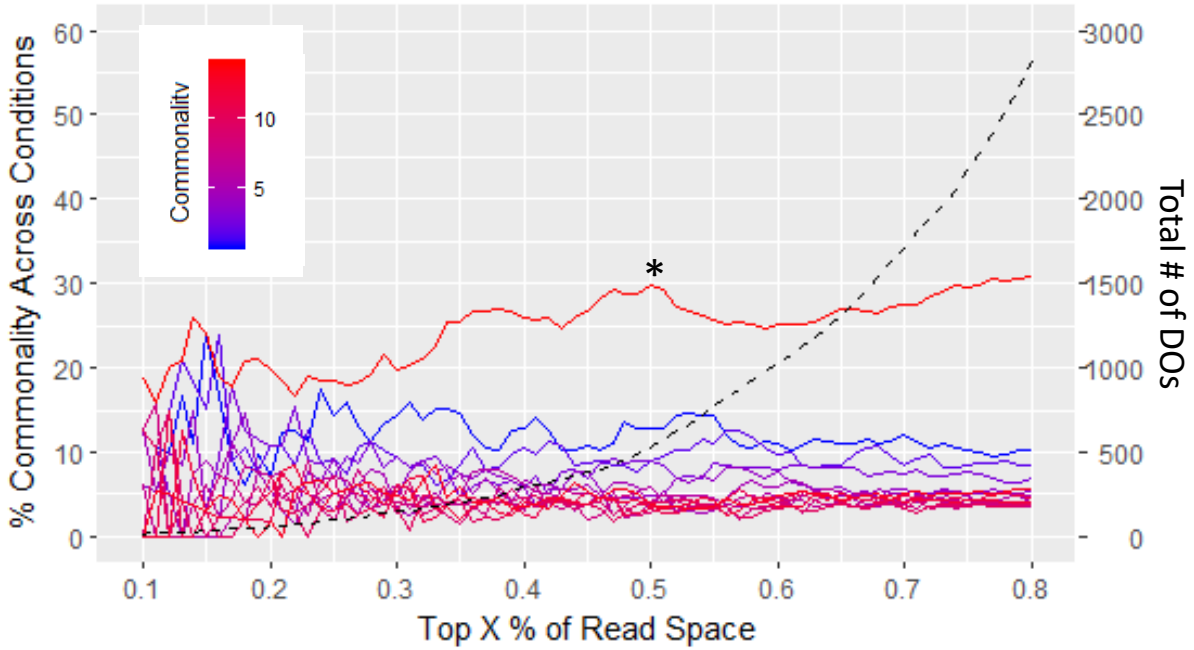
Table 1. Number of DOs Accounting for 50% of Total Read Space, Per Sample Basis

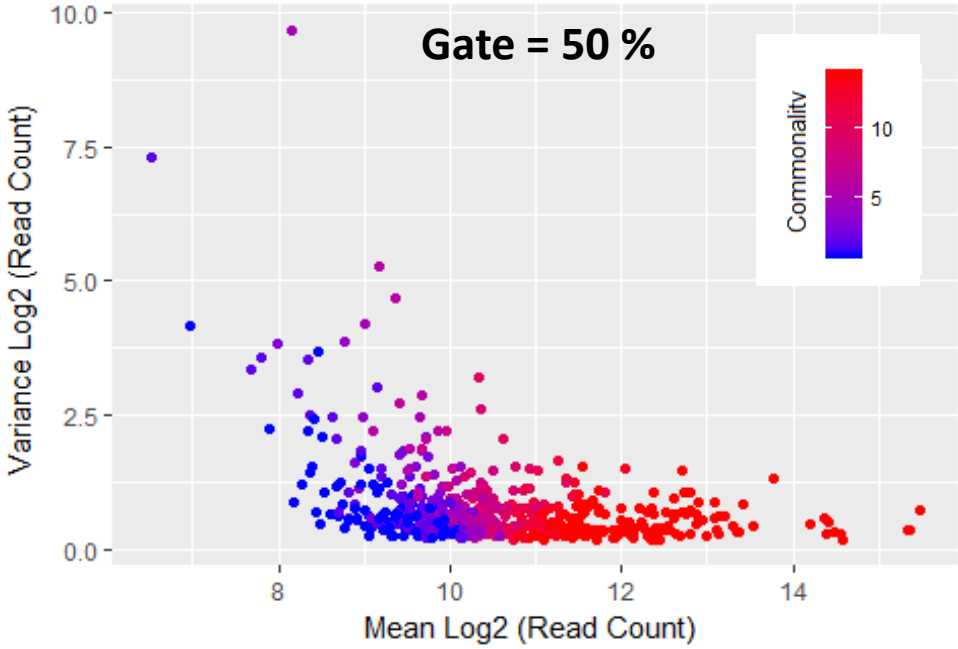
	Media Type	Treatment Type	Treatment Time, h	Sample Time, h	Replicate Number		
					1	2	3
Time Course	DMEM	--	--	30	242	246	186
Time Course	DMEM	--	--	36	273	220	208
Time Course	DMEM	--	--	48	238	249	239
Time Course	PRF.DMEM	--	--	30	276	288	289
Time Course	PRF.DMEM	--	--	36	268	248	244
Time Course	PRF.DMEM	--	--	48	240	240	262
C.Resp.1	DMEM	DMSO	24	30	308	259	269
C.Resp.1	DMEM	TSA, 1 µM	24	30	231	248	253
C.Resp.1	PRF.DMEM	DMSO	24	30	307	303	322
C.Resp.1	PRF.DMEM	TSA, 1 µM	24	30	273	278	303
C.Resp.2	DMEM	DMSO	30	36	242	233	249
C.Resp.2	DMEM	TSA, 1 µM	30	36	192	222	208
C.Resp.2	PRF.DMEM	DMSO	30	36	245	242	232
C.Resp.2	PRF.DMEM	TSA, 1 µM	30	36	220	273	263
				Range of DO Counts:	**186 - 322**		

Results

- Read count distributions similar across samples.
- Broad range of read counts within each sample (0 - ~32K).
- Within each sample, ~50-60% of DOs with non-zero read counts.
- Between 186 - 322 DOs account for 50% of the available read space (varies with sample).

Evaluating Commonality of Highly Expressed Genes Across Test Conditions





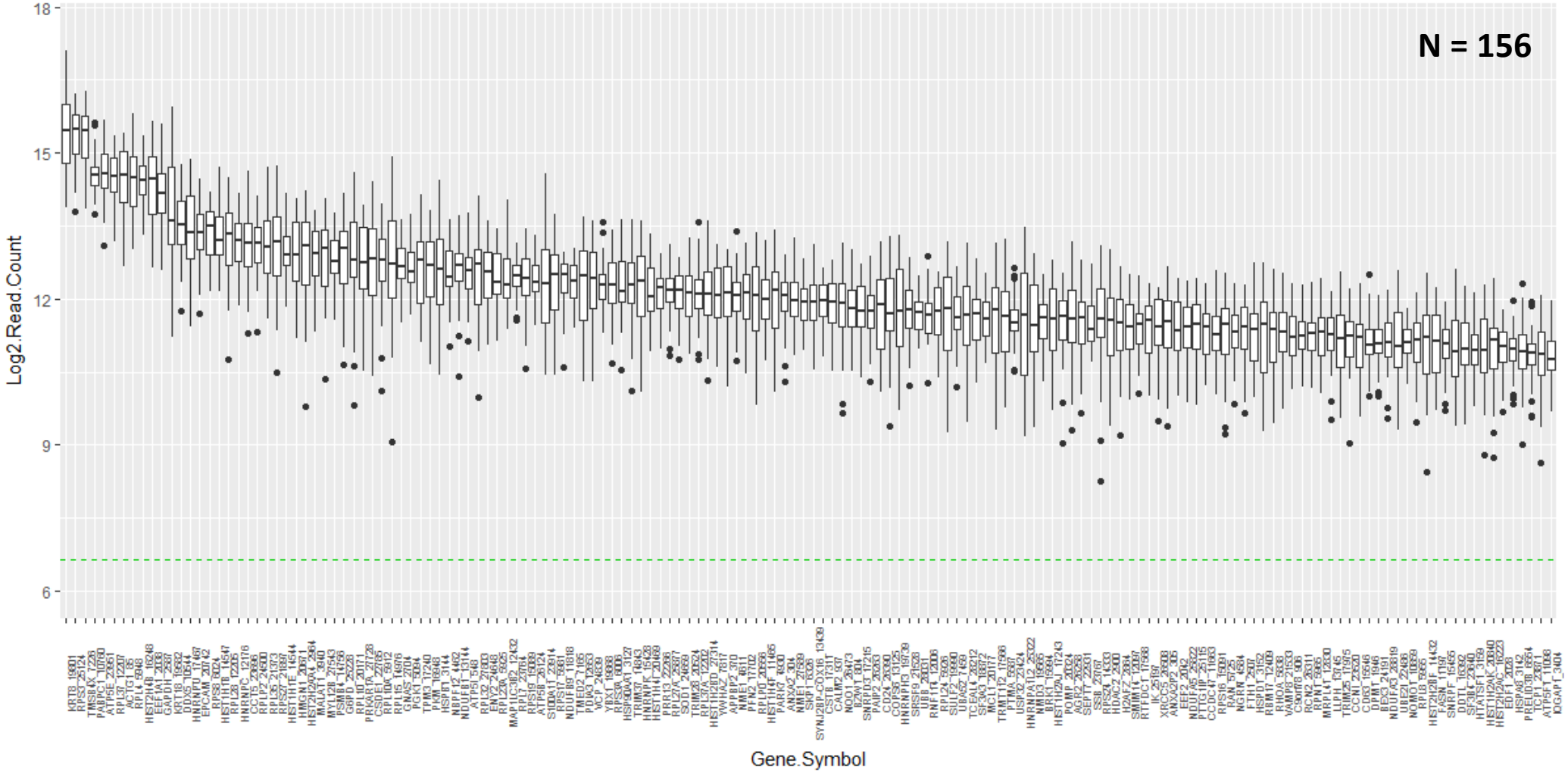
Using a Gate of 50 % of the total read space (*):

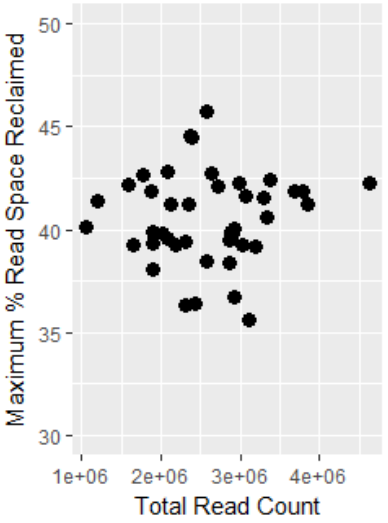
- ***Commonality Score = 14:*** ~ 30% of the DOs are identified as "highly-expressed" in all 14 test conditions (red).
- ***Commonality Score = 1:*** ~12.5% are identified as "highly-expressed" in only 1 test condition (blue).
- ***Commonality Score = 2 – 13:*** Varying number of DOs (< 10%) identified as "highly-expressed" in 2 to 13 test conditions.
- ***Variance:*** Tended to increase in DOs with lower commonality scores.

Conclusions:

- At Gate = 50 %, DOs with Commonality Scores of 14 are consistently identified as "highly-expressed" across all test conditions and have relatively lower variance and higher read counts across all test conditions.
- **N = 156** DOs identified as candidates for attenuation.

Candidate “Highly Expressed Genes” for Attenuation





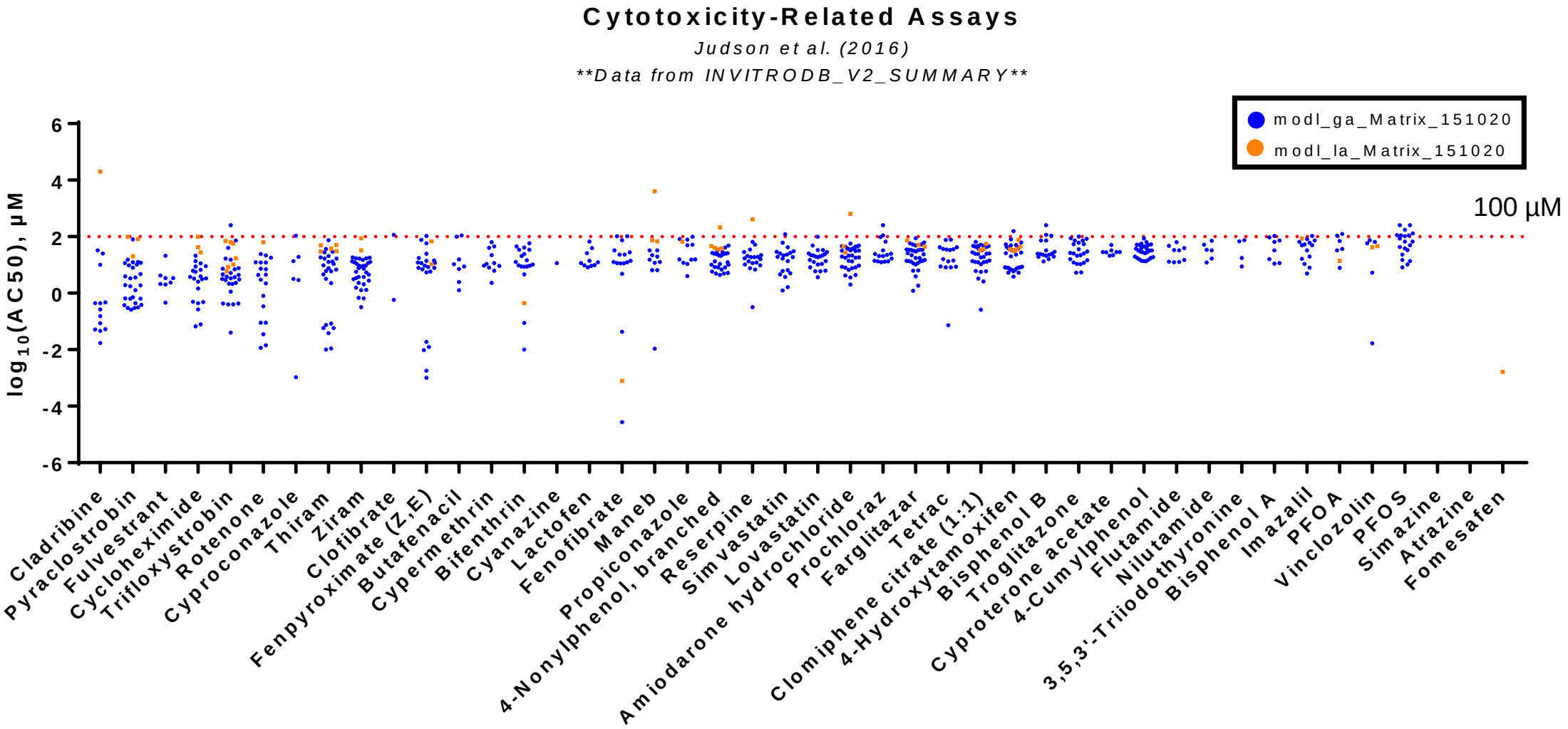
- Rank ordered on x-axis by average read count across all test conditions.
- Green line → Raw read count = 100.
- The most highly expressed genes in the attenuation set are “housekeeping” genes.

HTTr Pilot: Chemical Test Set

Chemical Name	MIE Family	Chemical Name	MIE Family
Flutamide	ANTIANDROGEN	Rotenone	MITOCHONDRIA (COMPLEX I)
Nilutamide		Fenpyroximate (Z,E)	
Cyproterone acetate		Trifloxystrobin	MITOCHONDRIA (COMPLEX II)
Vinclozolin		Pyraclostrobin	
4-Hydroxytamoxifen	ANTIESTROGEN	PFOS	PPAR
Clomiphene citrate (1:1)		PFOA	
Fulvestrant		Troglitazone	
Atrazine	cAMP INDUCERS / PDE INHIBITORS	Farglitazar	
Cyanazine		Lactofen	PPO INHIBITOR / PPAR
Cladribine	CYTOTOXICANTS	Fomesafen	PPO INHIBITOR
Cycloheximide		Butafenacil	
Bisphenol A	ESTROGENS	Maneb	SH REACTIVE
4-Nonylphenol, branched		Thiram	
Bisphenol B		Ziram	
4-Cumylphenol		Imazalil	STEROIDOGENESIS
Clofibrate	FIBRATES	Prochloraz	
Fenofibrate		Cyproconazole	
Lovastatin	HMGCR	Propiconazole	
Simvastatin		Tetrac	THR
Bifenthrin	NA+ CHANNEL	3,5,3'-Triiodothyronine	
Cypermethrin		Reserpine	VMAT
Simazine	PHOTOSYSTEM II INHIBITOR	Amiodarone hydrochloride	

- Chemical set covers broad range of mechanistic diversity with redundancy within mechanistic class.

Dose Range Selection

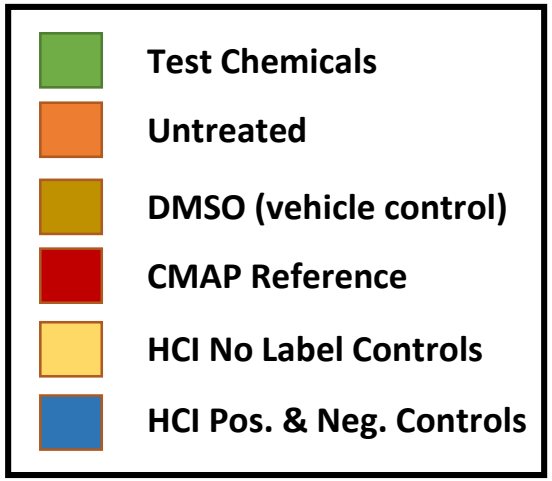


- Upper bound in testing range set at 100 µM based on upper limit of cytotoxicity range for most chemicals.
- **Final dose range:** 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µM

Dosing Plate Layout

DOSING PLATE MAP																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	A	Ionomycin (30 µM)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	non-treated
2	B	Ionomycin (30 µM)	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	non-treated
3	C	Ionomycin (30 µM)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	non-treated
4	D	Staurosporine (1 µM)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	DMSO
5	E	Staurosporine (1 µM)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	DMSO
6	F	Staurosporine (1 µM)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	DMSO
7	G	Saccharin (100 µM)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	DMSO [No Label]
8	H	Saccharin (100 µM)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	Trichostatin (1 µM)
9	I	Saccharin (100 µM)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	Trichostatin (1 µM)
10	J	Sorbitol (100 µM)	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	Trichostatin (1 µM)
11	K	Sorbitol (100 µM)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	Genistein (10 µM)
12	L	Sorbitol (100 µM)	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	Genistein (10 µM)
13	M	Ionomycin (30 µM) [No Label]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Genistein (10 µM)
14	N	Staurosporine (1 µM) [No Label]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	Sirolimus (0.1 µM)
15	O	Saccharin (100 µM) [No Label]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	Sirolimus (0.1 µM)
16	P	Sorbitol (100 µM) [No Label]	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	Sirolimus (0.1 µM)

- 44 chemicals in 8-point concentration-response → all on one plate
- Non-treated (n=3) and DMSO (n=3) control wells.
- Three “CMAP” Reference Compounds, single point, in triplicate
- First column reserved for addition of RNA QC samples by NCCT (pre-shipment) and BioSpyder (post-shipment).

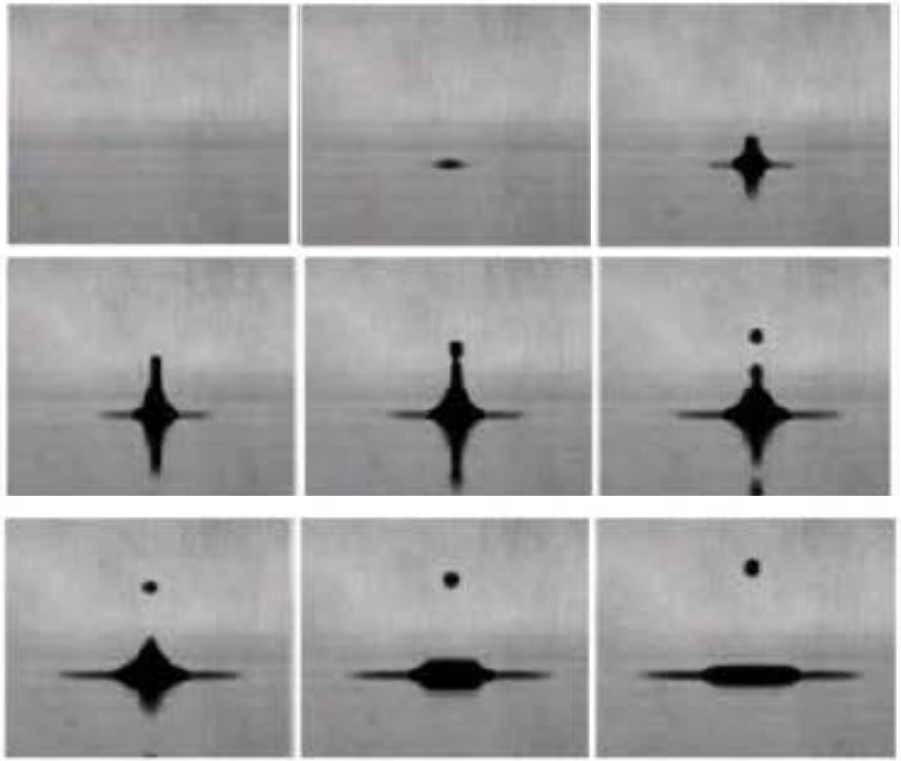


Dose Randomization using Echo 550

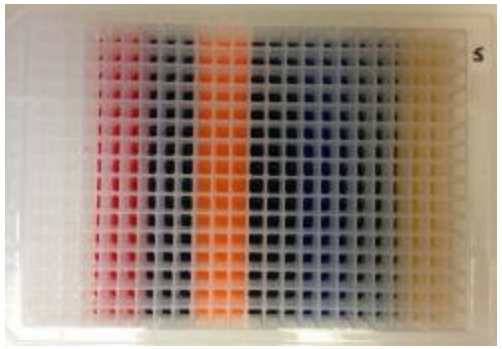
Acoustic dispensing technology:

- Uses soundwaves to precisely transfer small quantities of liquid (nL) from source plate to test plate.
- Allows for randomization of test wells → mitigate potential edge effects without "losing real estate."

LabCyte Echo® 550
Liquid Handler

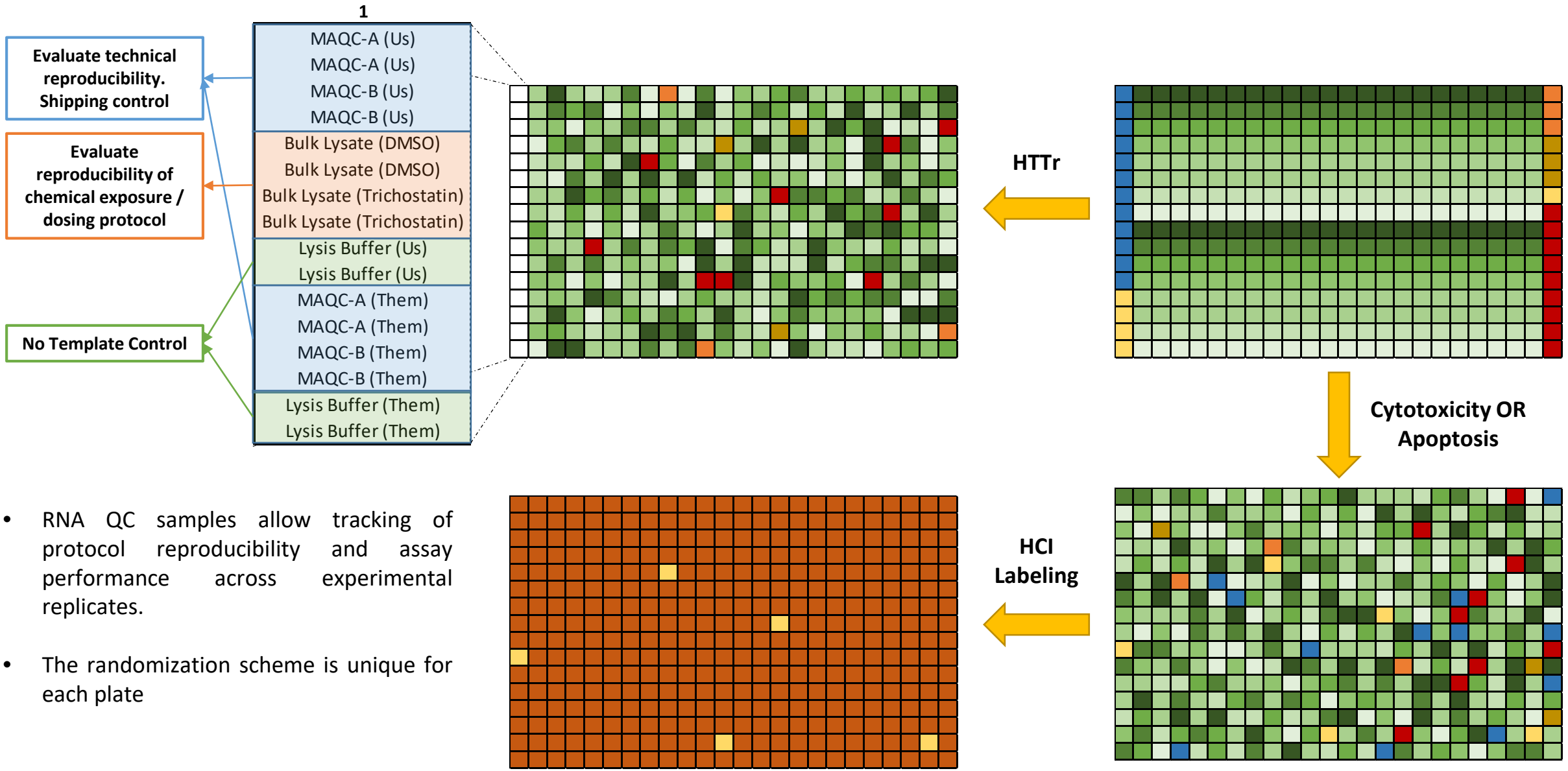


Source Plate



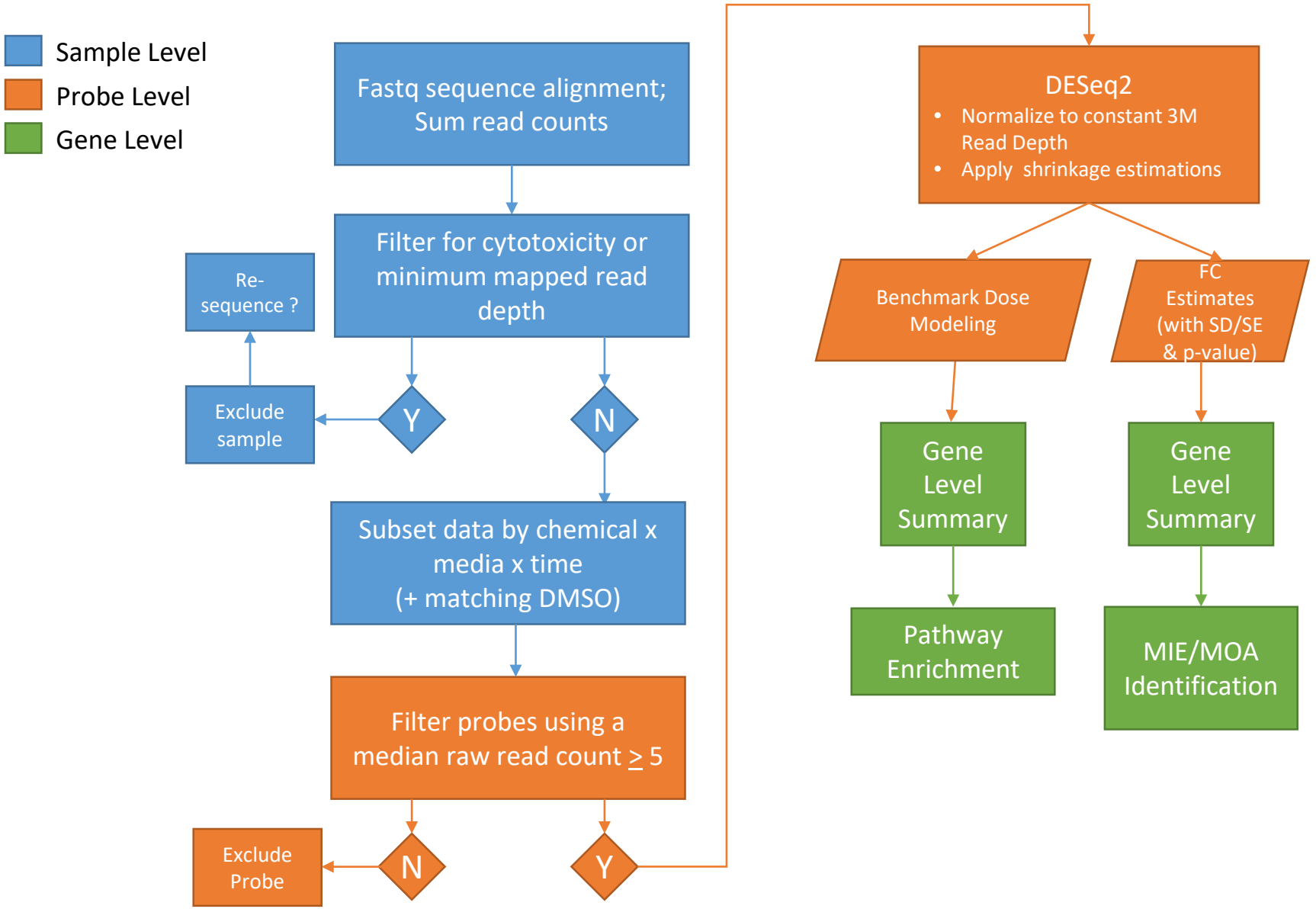
Test Plate

Echo Dispensing



- RNA QC samples allow tracking of protocol reproducibility and assay performance across experimental replicates.
- The randomization scheme is unique for each plate

Data Analysis Pipeline

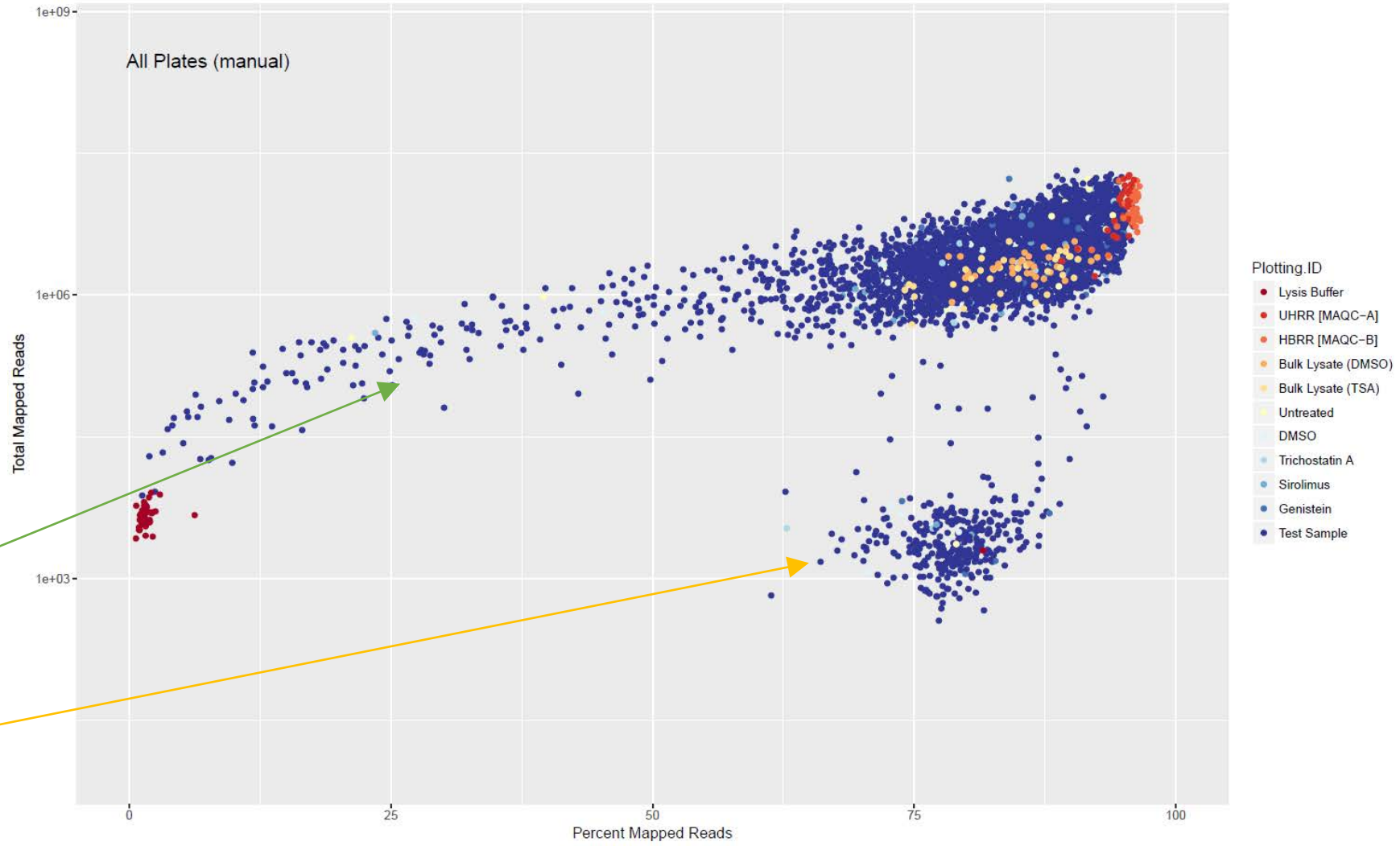


Assay Performance Metrics

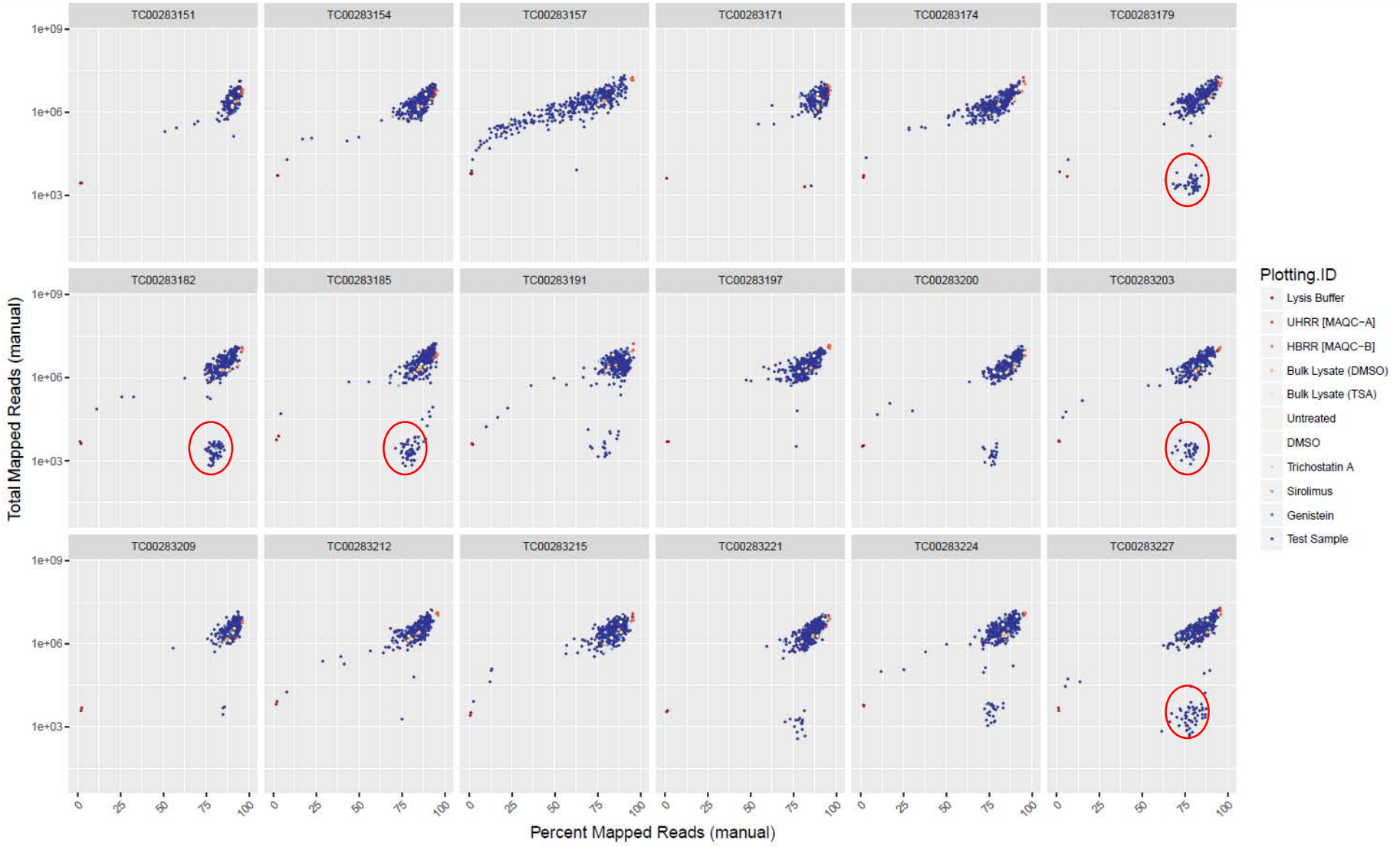
- **Total Mapped Reads vs. Percent Mapped Reads**
- **Correlation and Variation in Technical Replicates [within plate]**
- **Correlation and Variation in Biological Replicates [across plates]**
- **Detection of Biological Signal**
  - Transcriptional Biomarkers
  - Connectivity Mapping

Total Mapped Reads vs. Percent Mapped Reads [All Plates]

- Average total mapped reads of test samples ~ $3.0x10^6$
- Average mapped read count per gene ~150
- Percent mapped reads > 75%
- Lysis Buffer blanks have low total reads, but not zero.
- Purified RNAs clustered at upper left.
- Comet tail ?
- Off-set cluster ?

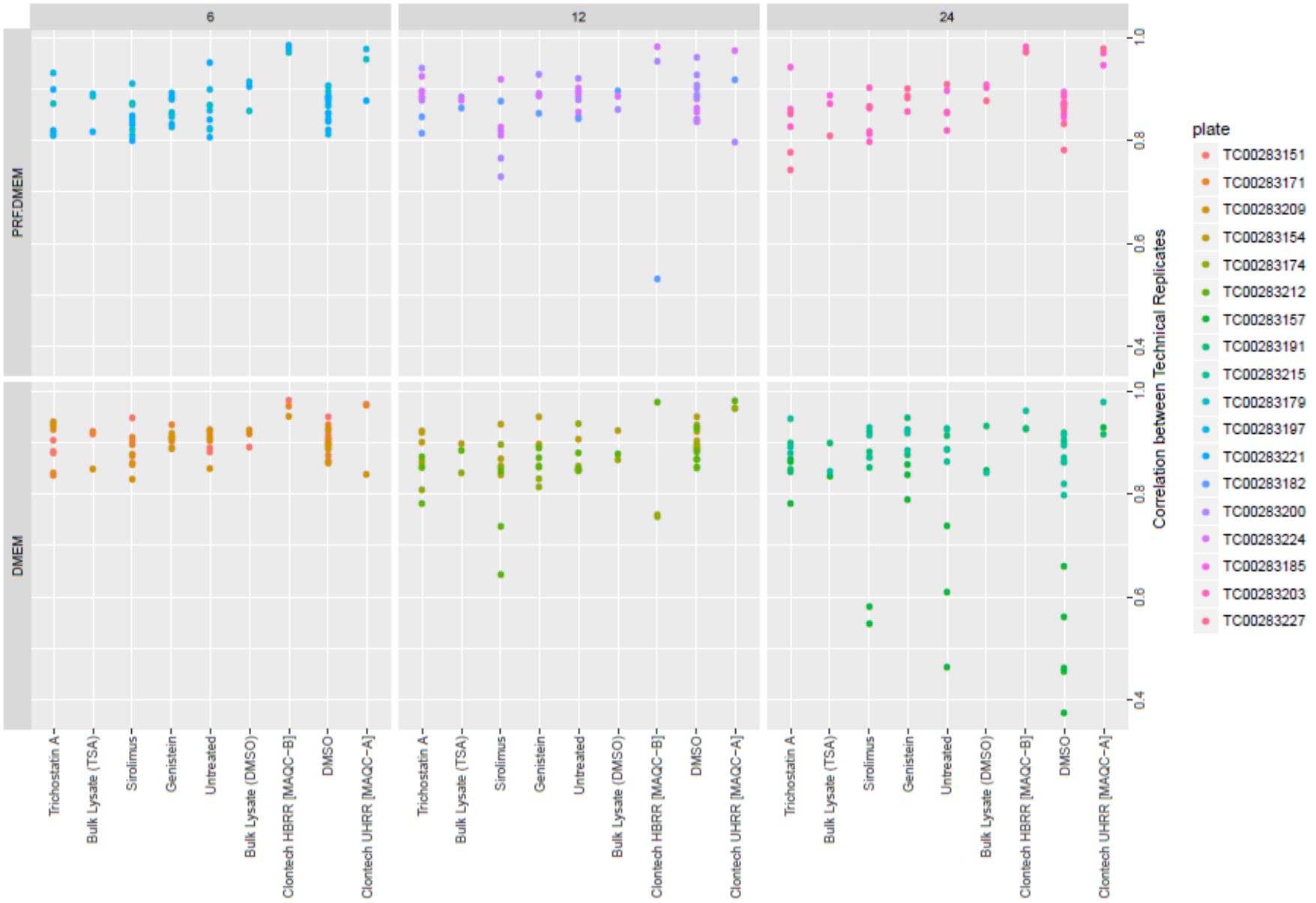


Total Mapped Reads vs. Percent Mapped Reads [By Plates]



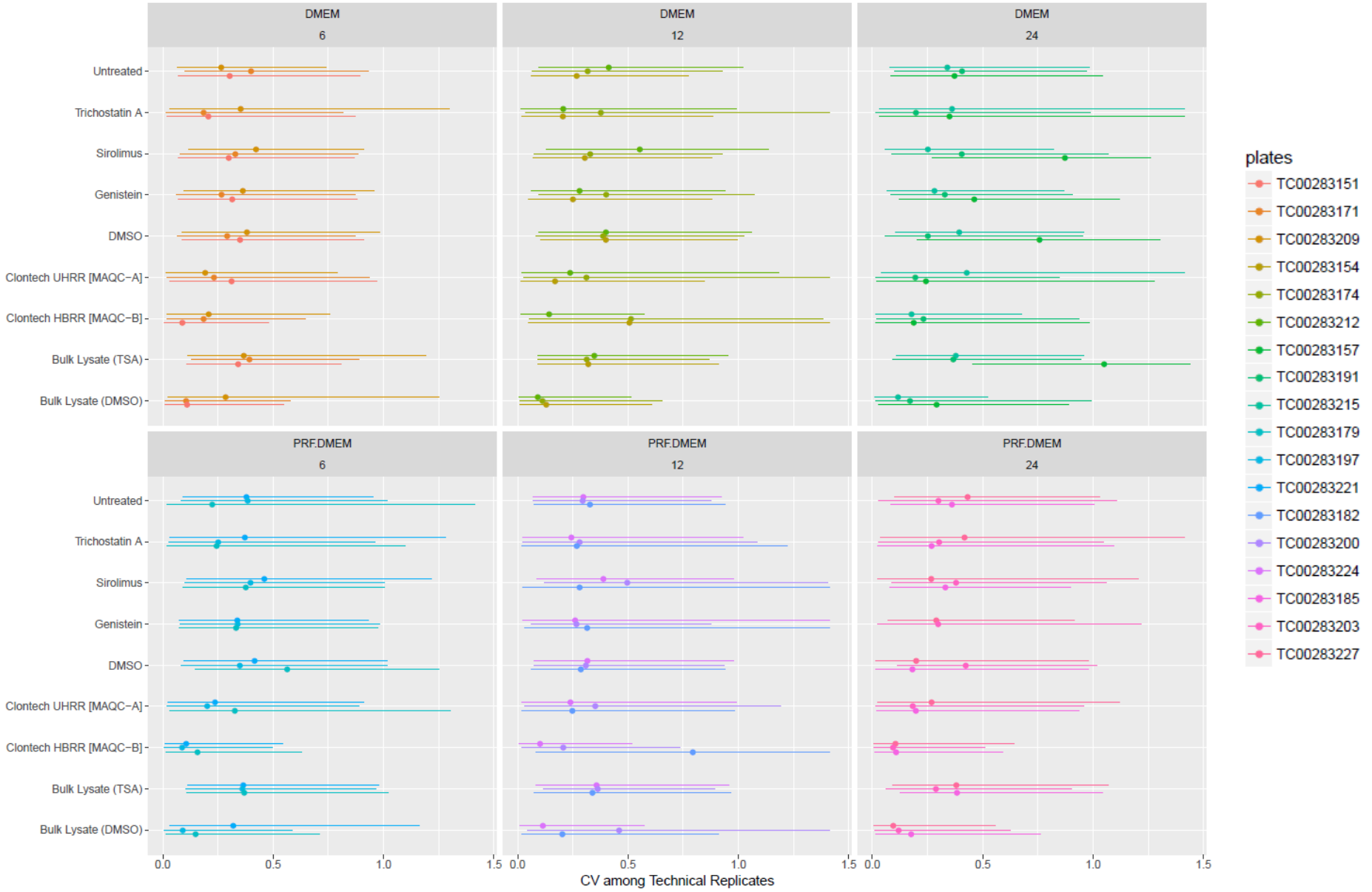
- Comet tail → Due to one “poor performing” plate
- Offset cluster → Low read count samples across many plates (red circles) → Candidates for resequencing.

Correlation Among Technical Replicates



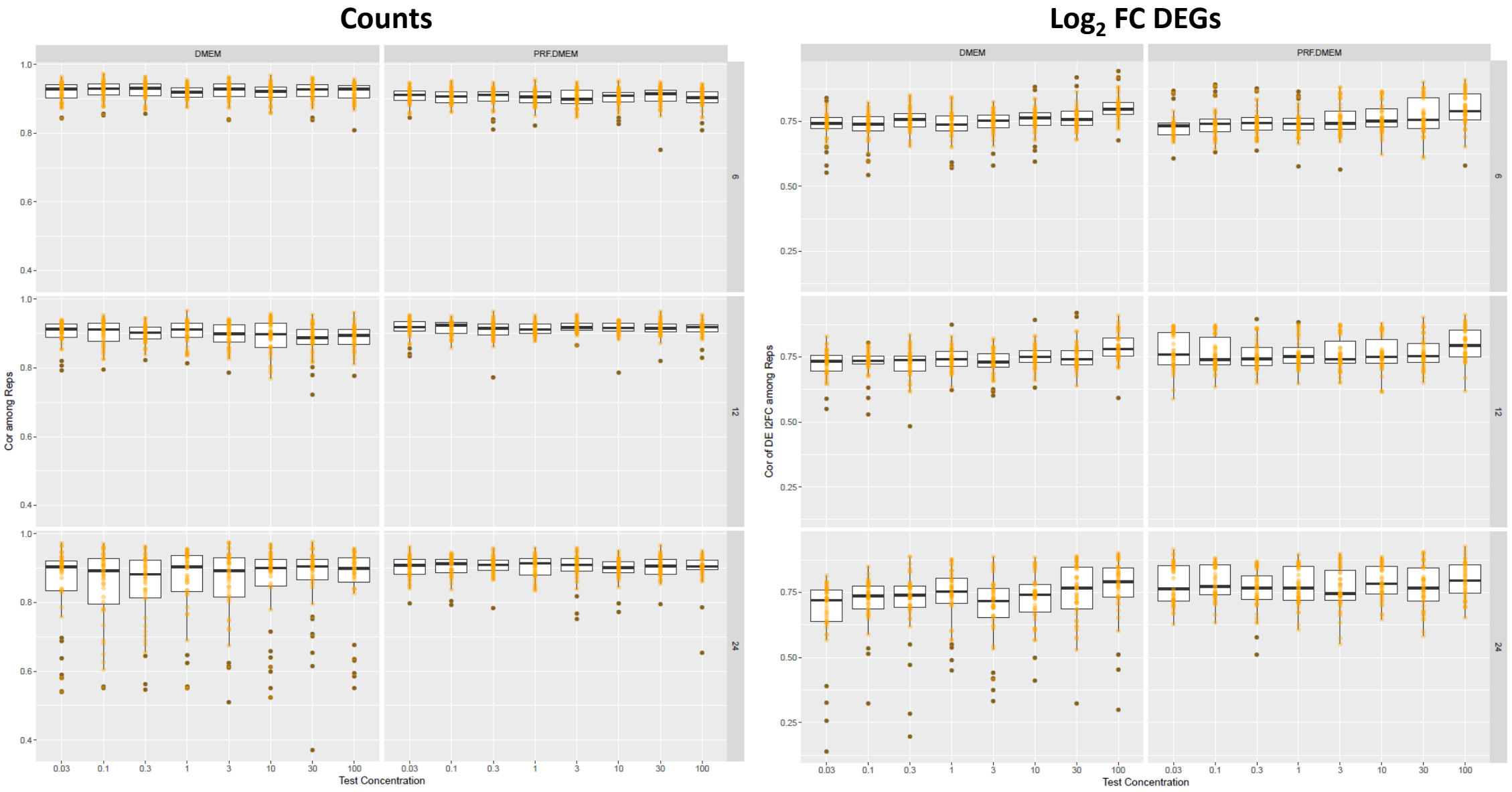
- Correlation among technical replicates is high (> 0.85 %).

Coefficient of Variation (CV) Among Technical Replicates



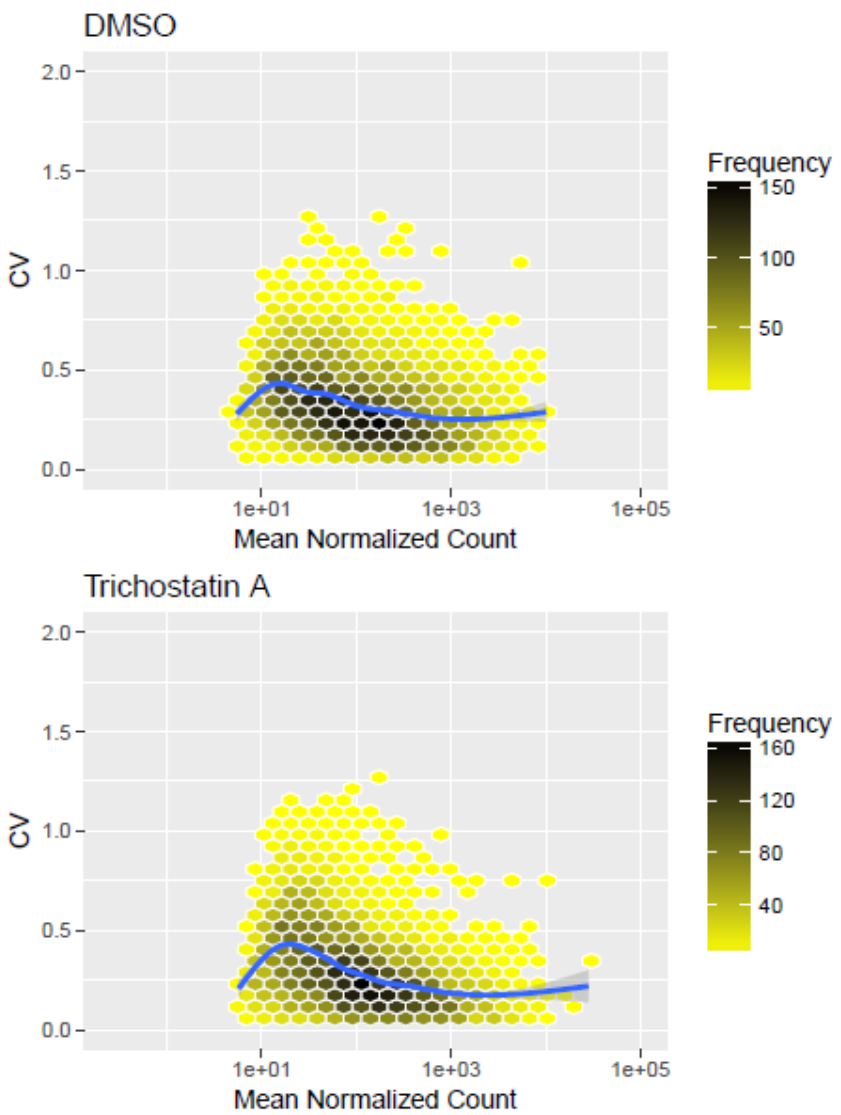
- Coefficient of variation in gene expression values is low (median ~30 %).

Correlations in Biological Replicates, Stratified by Expression Level



- Correlations of raw counts and $\log_2$ FC of DEGs is high ($\geq$ 0.85) for most conditions.

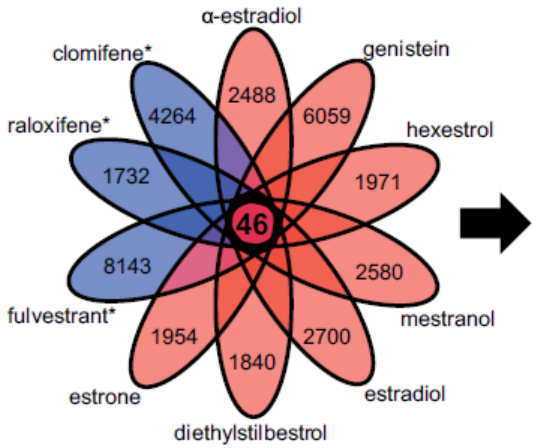
Coefficient of Variation (CV) Stratified by Expression Level

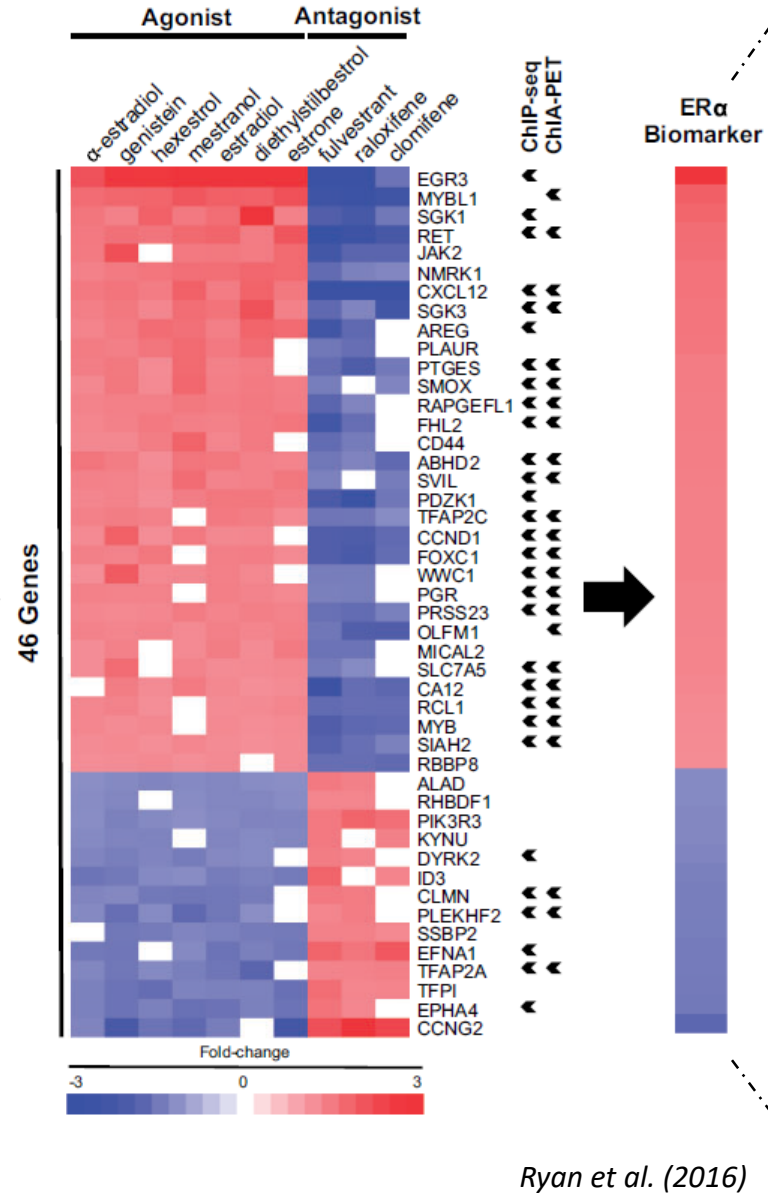


- CVs decrease as a function of mean expression level.

ERα Biomarker Signature

- Biomarker signature determined by treating MCF7 cells with various ERα agonists and antagonists.
- Can we use this to detect biologically meaningful signal in the BioSpyder data?

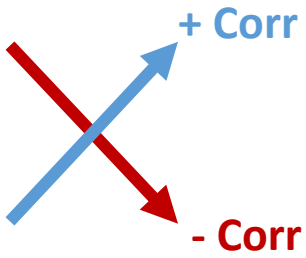


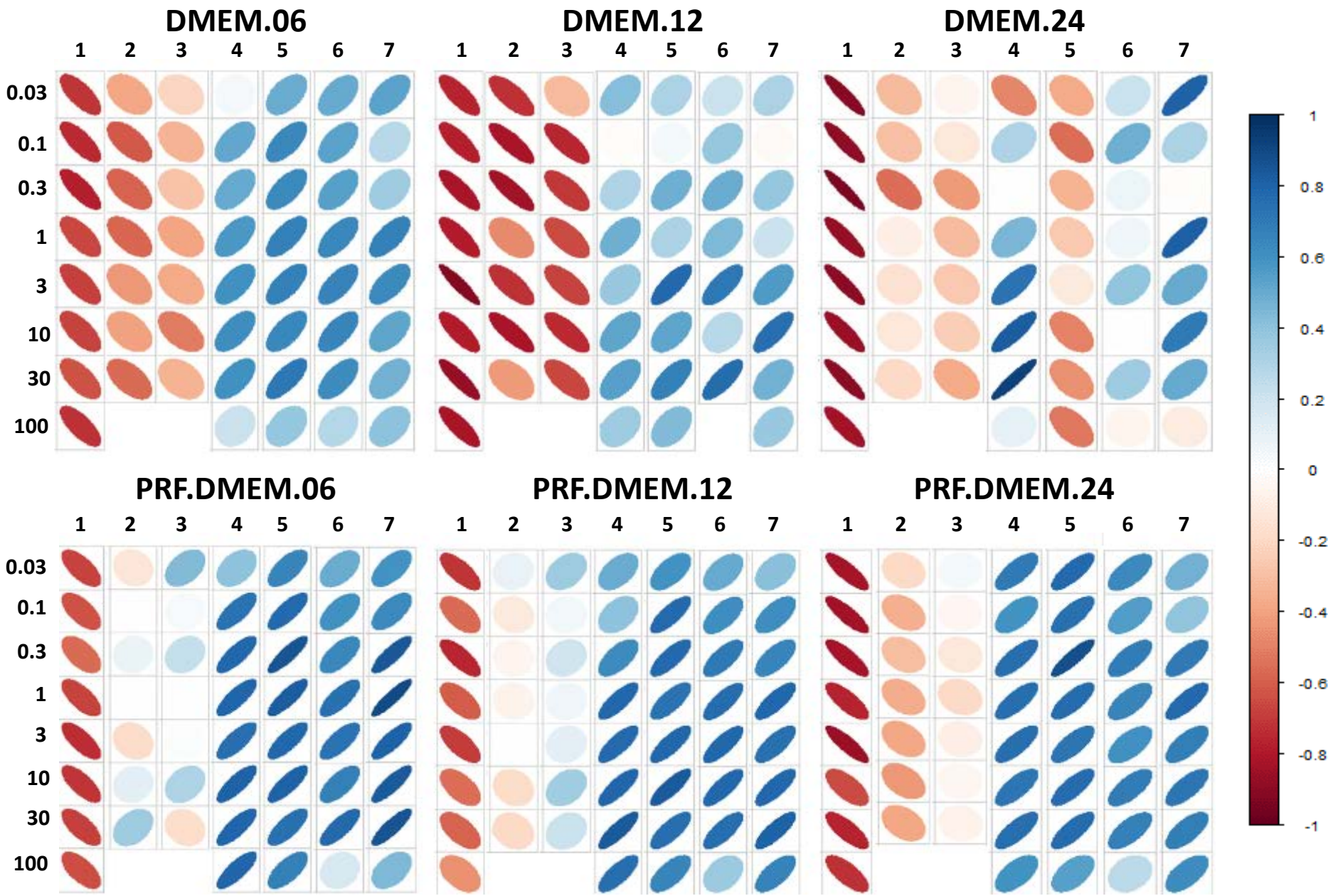


Ryan et al. (2016)

Correlation with ERα Transcriptional Biomarker

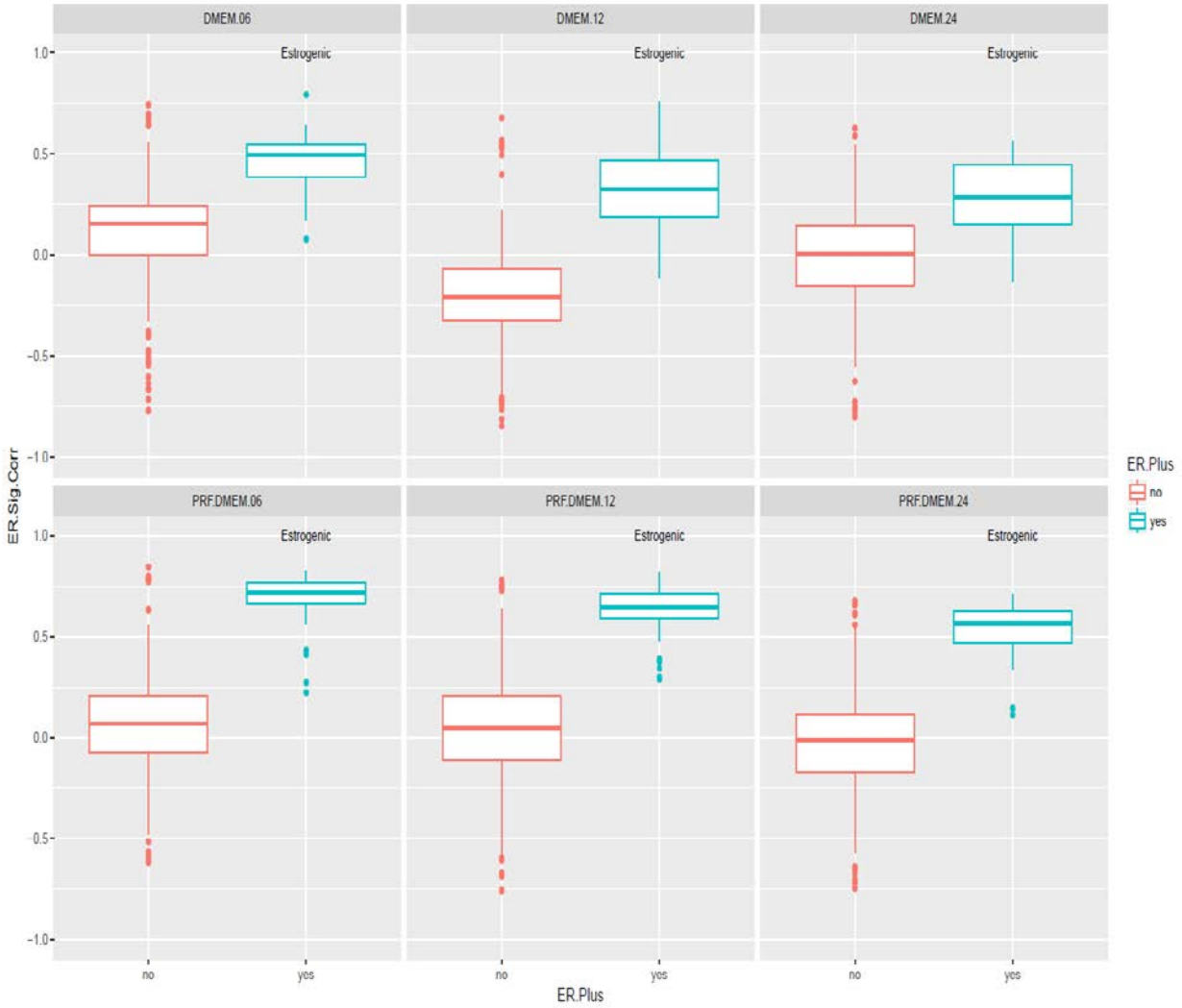
	Chemical	MOA
1	Fulvestrant	Antiestrogen (SERD)
2	4-Hydroxytamoxifen	Antiestrogen (SERM)
3	Clomiphene Citrate	
4	Bisphenol A	Estrogenic
5	Bisphenol B	
6	4-Nonylphenol, branched	
7	4-Cumylphenol	



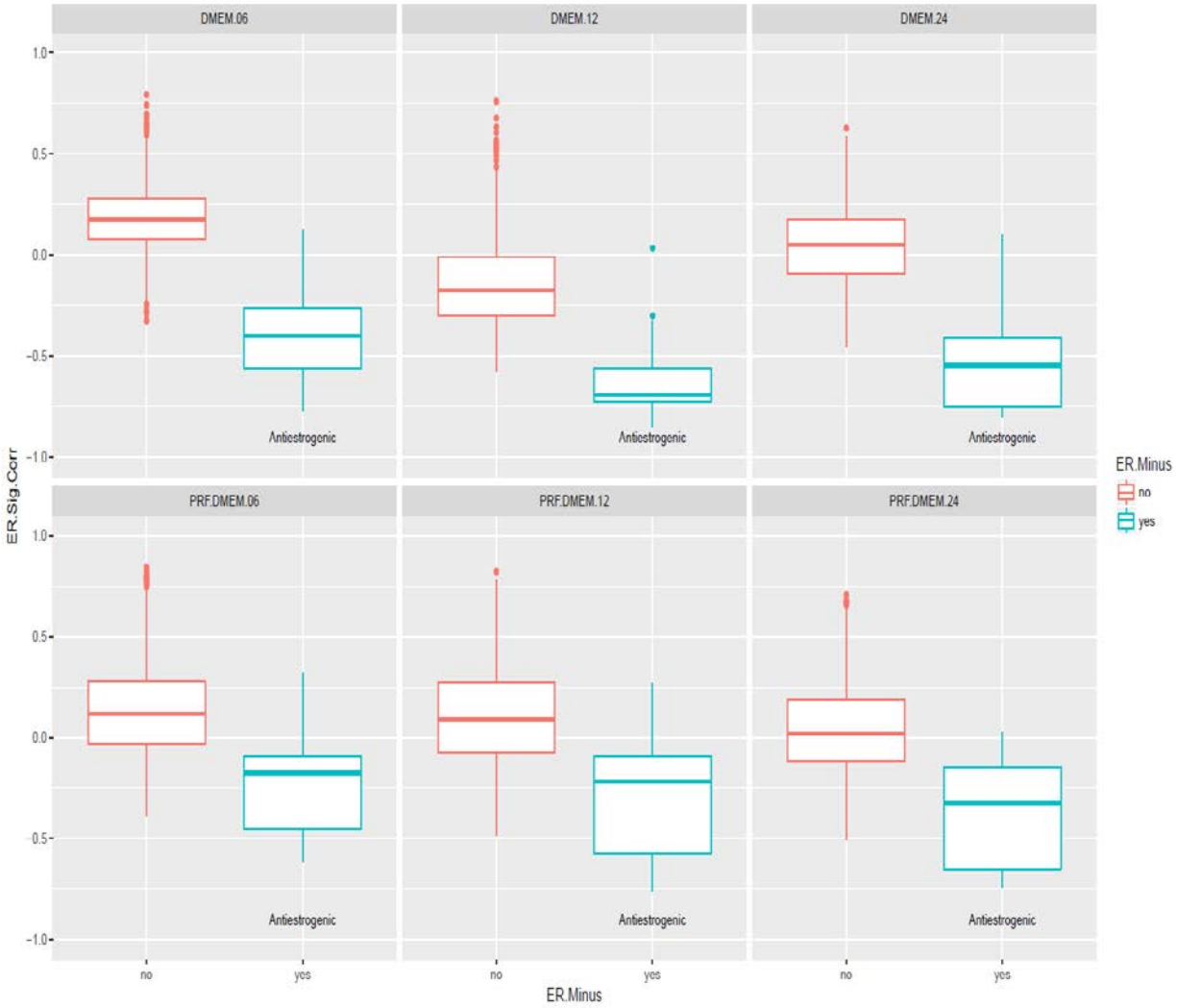


Correlation with ERα Transcriptional Biomarker - Antagonists

Agonists

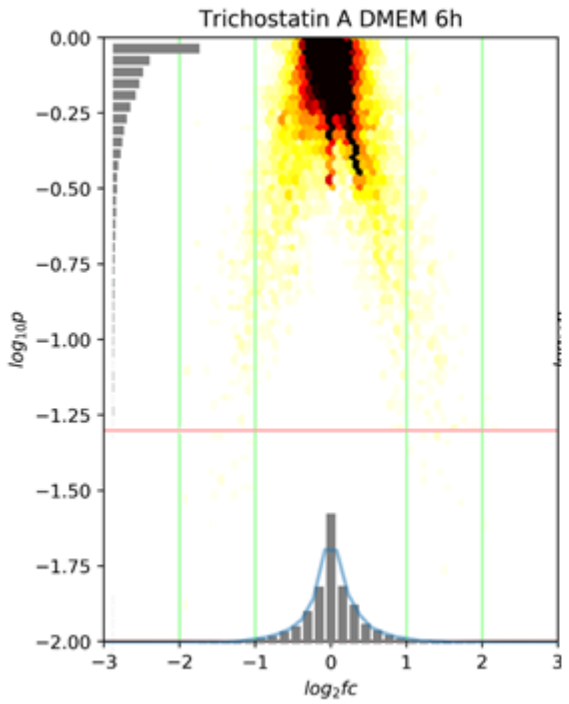


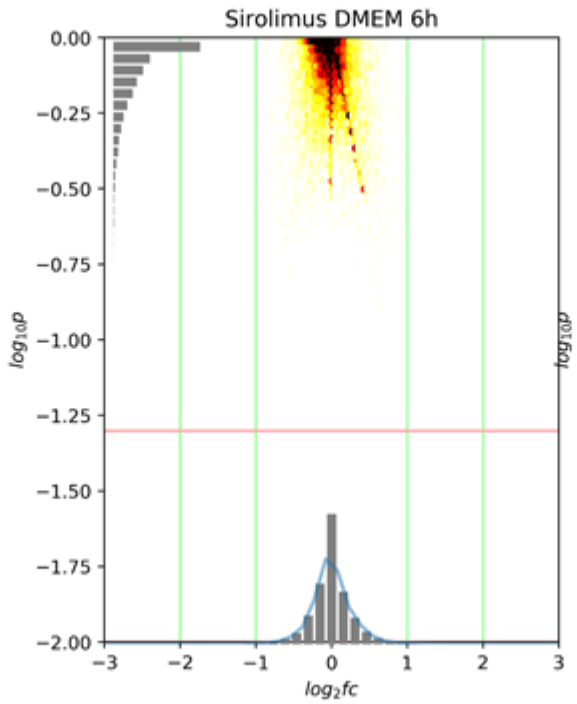
Antagonists

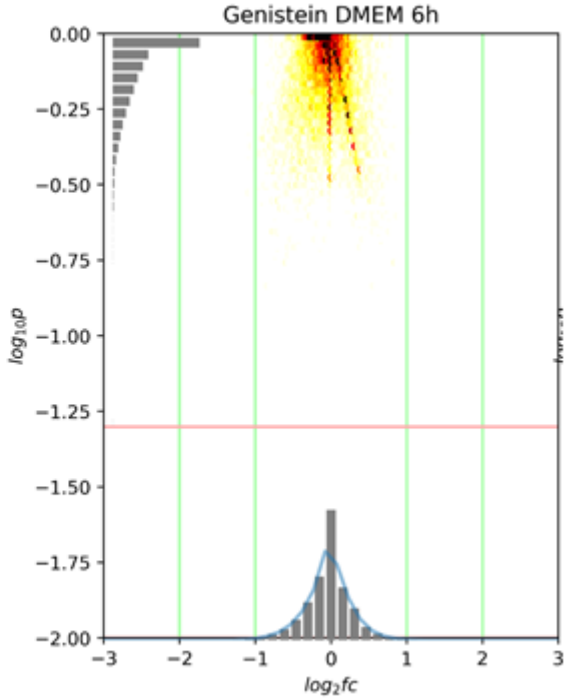


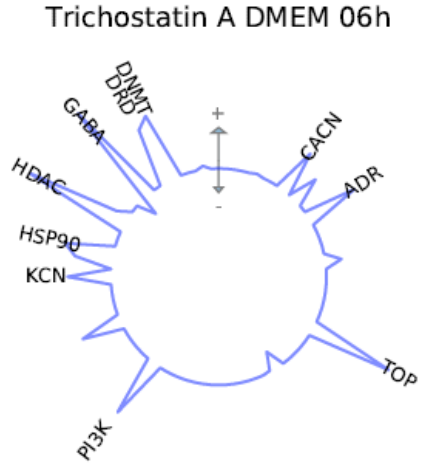
- The ability to detect ERa antagonists (particularly SERMs) was decreased by use of charcoal stripped serum.

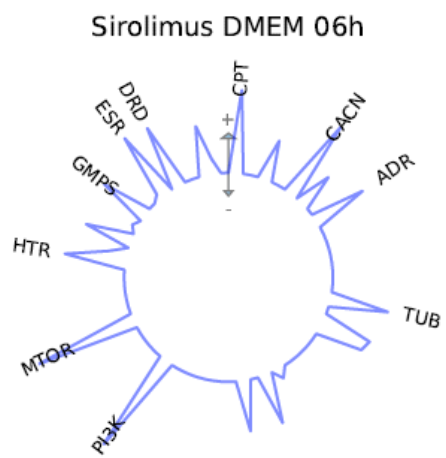
Connectivity Mapping

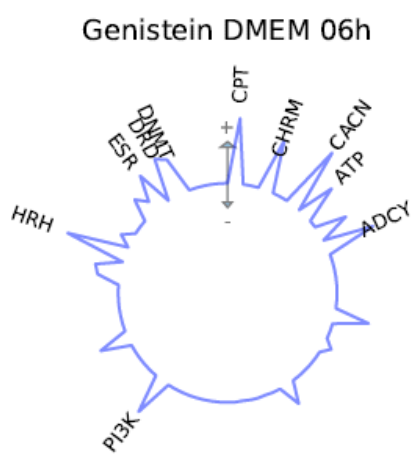












- Differential gene expression observed with reference chemicals.
- Putative targets identified using Connectivity Mapping
- Large degree of promiscuity of predicted targets observed.
- Currently evaluating additional methods for MIE prediction

Benchmark Dose Modeling

Parameter	Criteria [a]
Pre-filter:	ANOVA (p_{raw} < 0.05 & |FC| ≥ 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 ≥ 5% Gene Set Coverage Fisher's Exact Two Tailed ≤ 0.05
Gene Set Collections [c]:	MSigDB_C2 MSigDB_H Reactome

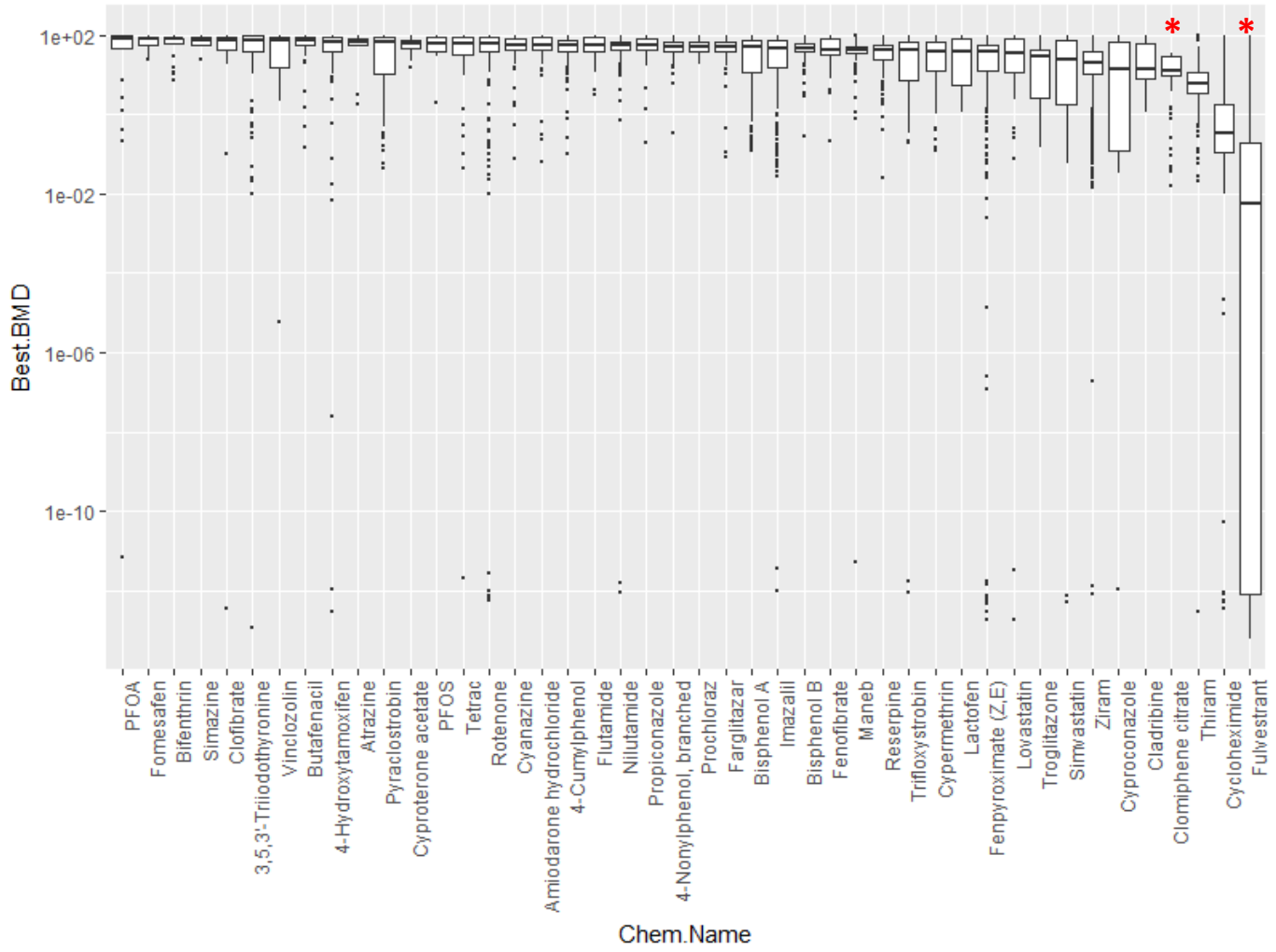
[a] Exploratory analysis – modeling criteria not finalized

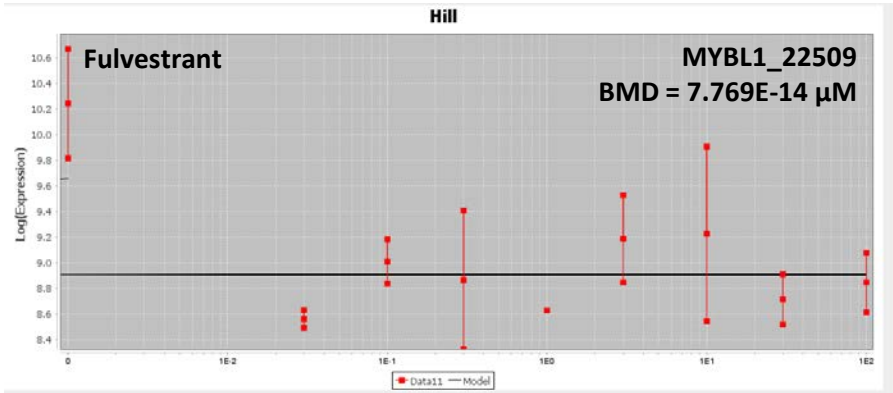
[b] Flagged Hill Models were retained to illustrate a specific point regarding concentration range selection

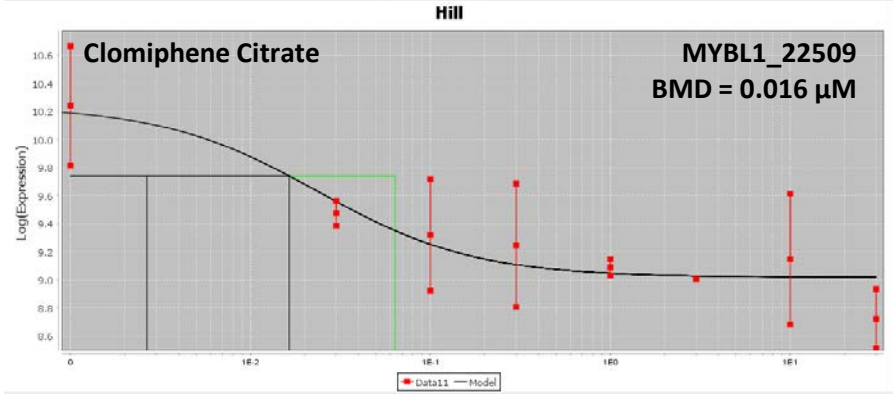
[c] Gene Set Collections:

- **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.

Benchmark Dose Modeling Results

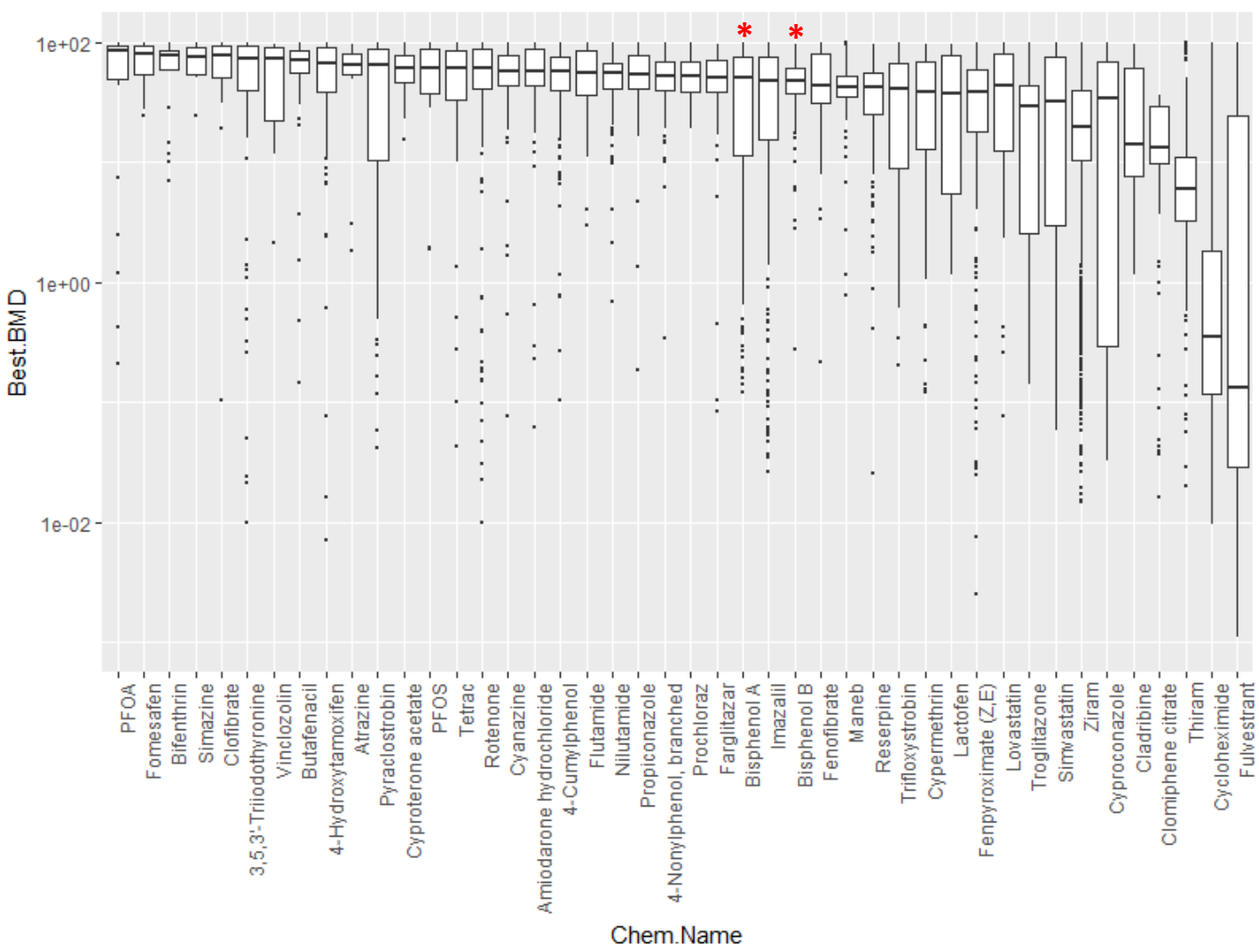


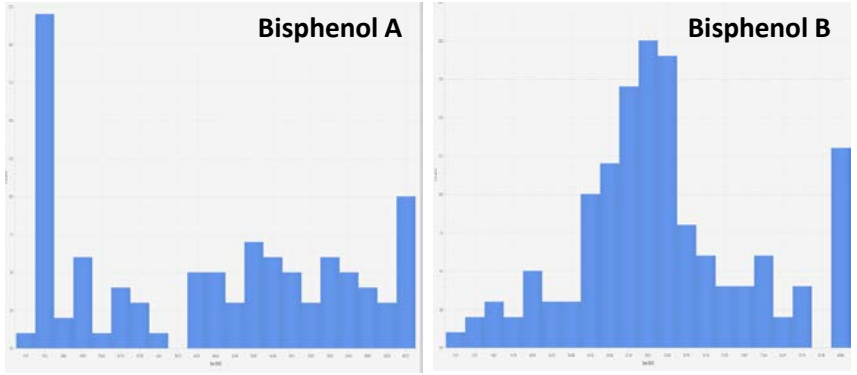


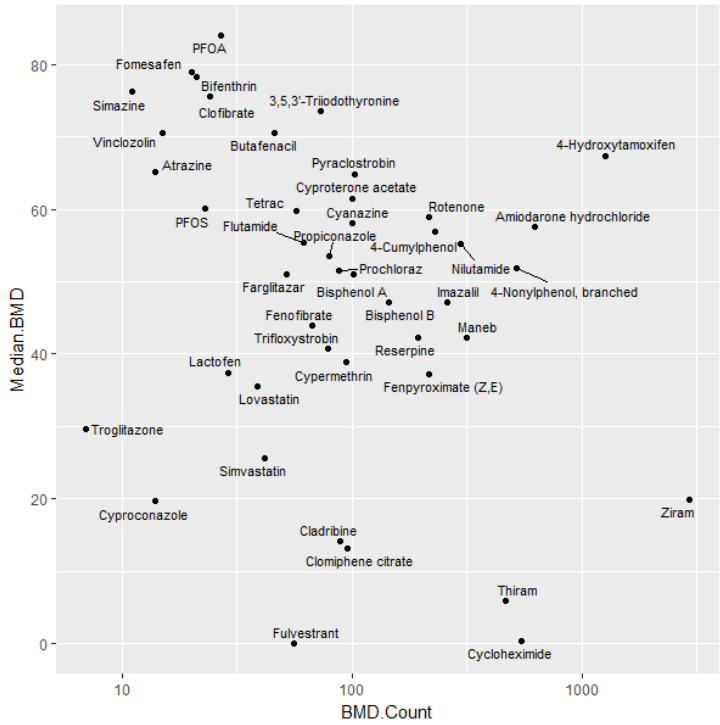


- A high occurrence of flagged Hill fits with unreasonably low BMDs may indicate the concentration range was not low enough.
- Flagged BMDs were observed with low frequency in this dataset.
- The identify of genes with flagged hill models was inconsistent across chemicals. Not driven by DMSO controls.

Benchmark Dose Modeling Results







- Wide range of chemical potencies at the probe level.
- The distribution of probe level BMDs vary from chemical to chemical.
- No apparent relationship between potency and number of probes affected (?).

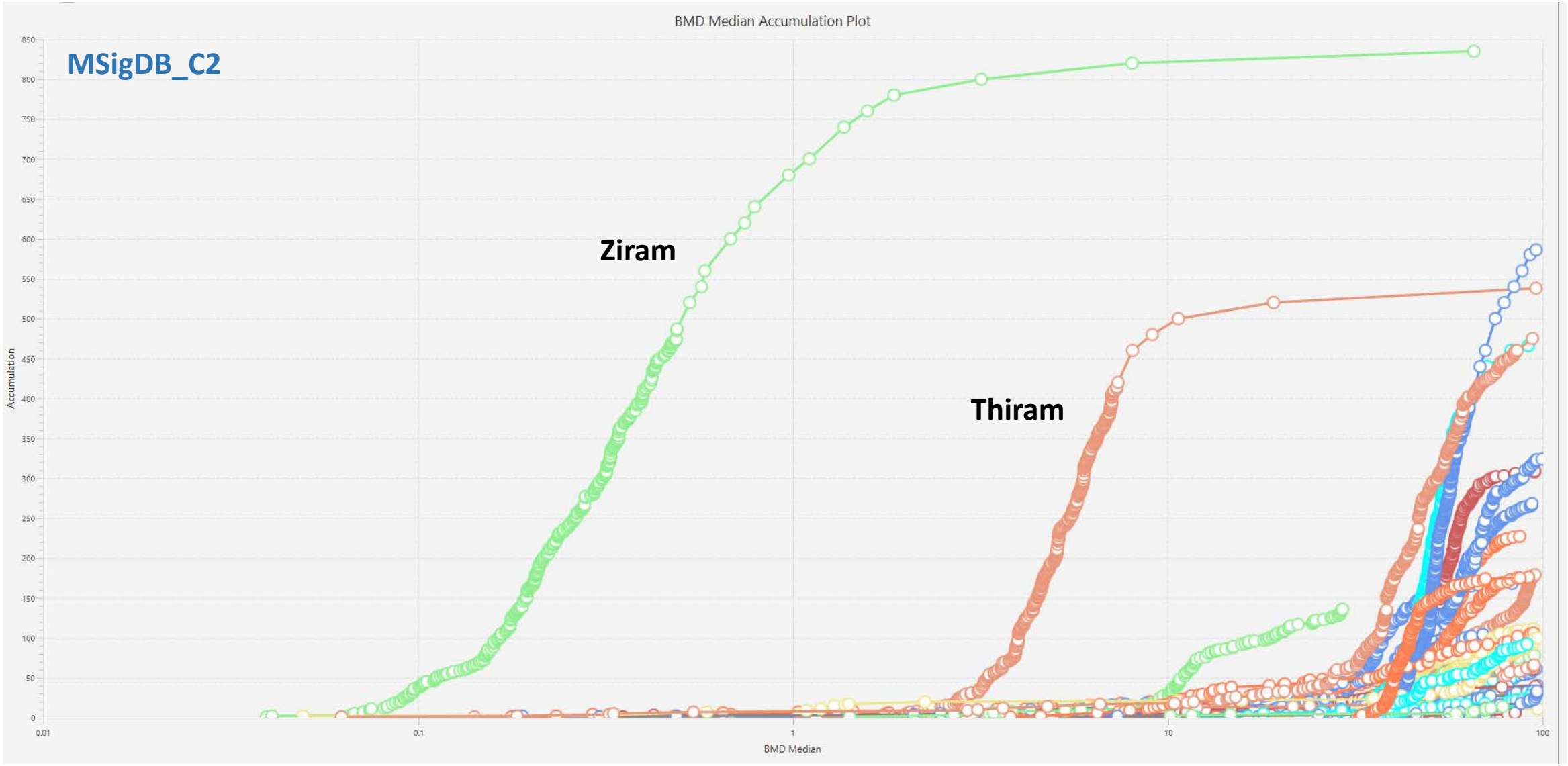
Pathway Enrichment

Numbers of Pathways Enriched

Chemical Name	MSigDB_C2	MSigDB_H	Reactome	Chemical Name	MSigDB_C2	MSigDB_H	Reactome
Ziram	1834	44	366	Bisphenol A	5	0	0
4-Hydroxytamoxifen	739	28	148	Flutamide	5	0	0
Cycloheximide	252	6	80	Lovastatin	4	0	0
Amiodarone hydrochloride	228	21	16	Pyraclostrobin	4	0	1
Reserpine	131	1	6	Imazalil	4	0	1
4-Nonylphenol, branched	83	6	9	Propiconazole	3	0	0
Fenpyroximate (Z,E)	63	0	1	Tetrac	2	0	1
Clomiphene Citrate	50	2	0	3,5,3'-Triiodothyronine	2	0	0
Prochloraz	37	0	0	Simvastatin	1	0	0
Cyproterone acetate	33	1	0	Cyproconazole	0	0	0
Cladribine	33	0	70	Cypermethrin	0	0	1
Rotenone	24	0	2	Clofibrate	0	0	0
4-Cumylphenol	18	0	0	PFOS	0	0	0
Bisphenol B	14	0	1	Simazine	0	0	0
Thiram	13	4	4	Vinclozolin	0	0	0
Maneb	13	0	4	Fomesafen	0	0	0
Farglitazar	11	1	0	Lactofen	0	0	0
Fenofibrate	8	0	0	Troglitazone	0	0	0
Fulvestrant	8	1	0	PFOA	0	0	0
Nilutamide	7	0	2	Atrazine	0	0	0
Cyanazine	6	0	0	Bifenthrin	0	0	0
Trifloxystrobin	5	0	0	Butafenacil	0	0	0

- Heterogeneity in the amount and type of pathways enriched.
- Changing filtering stringency and BMD modeling strategy affects these results.

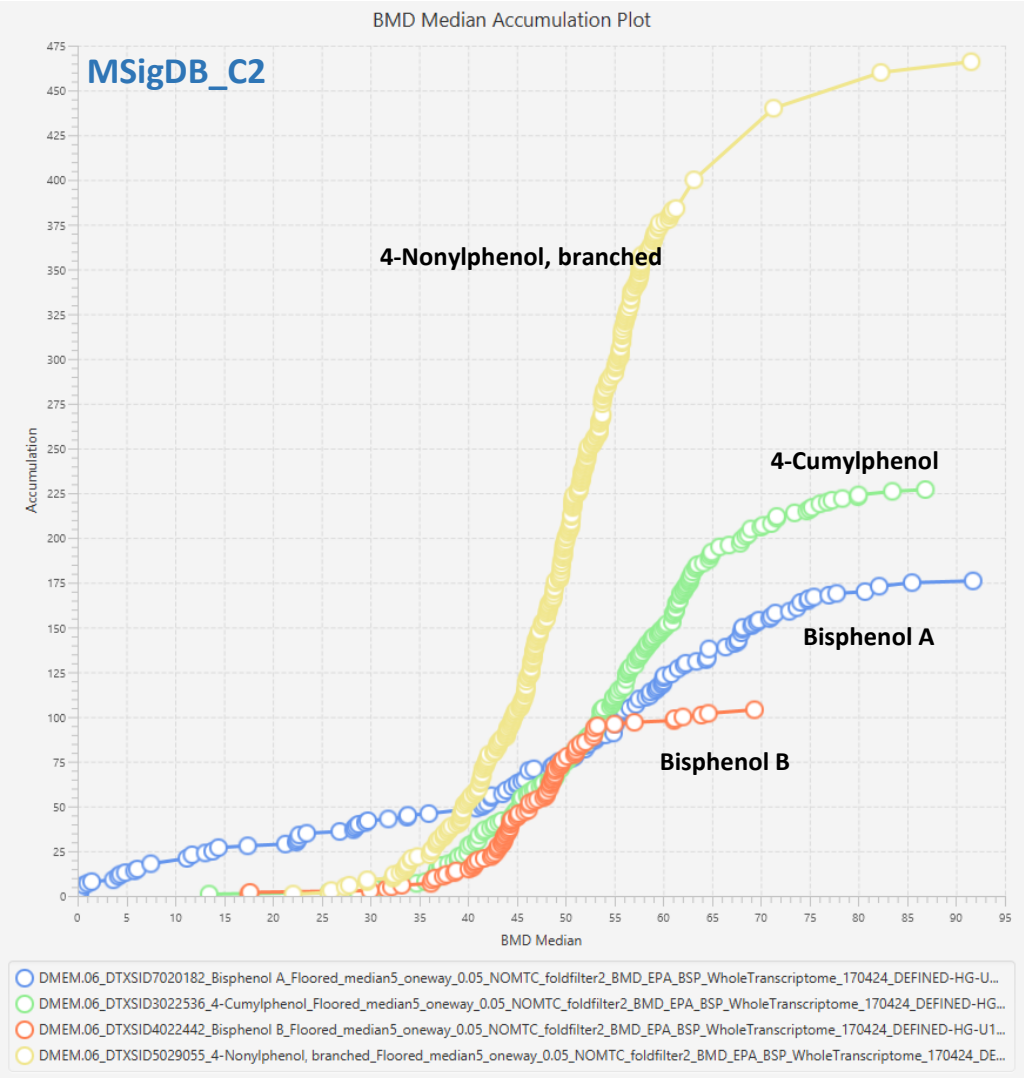
Pathway Potencies



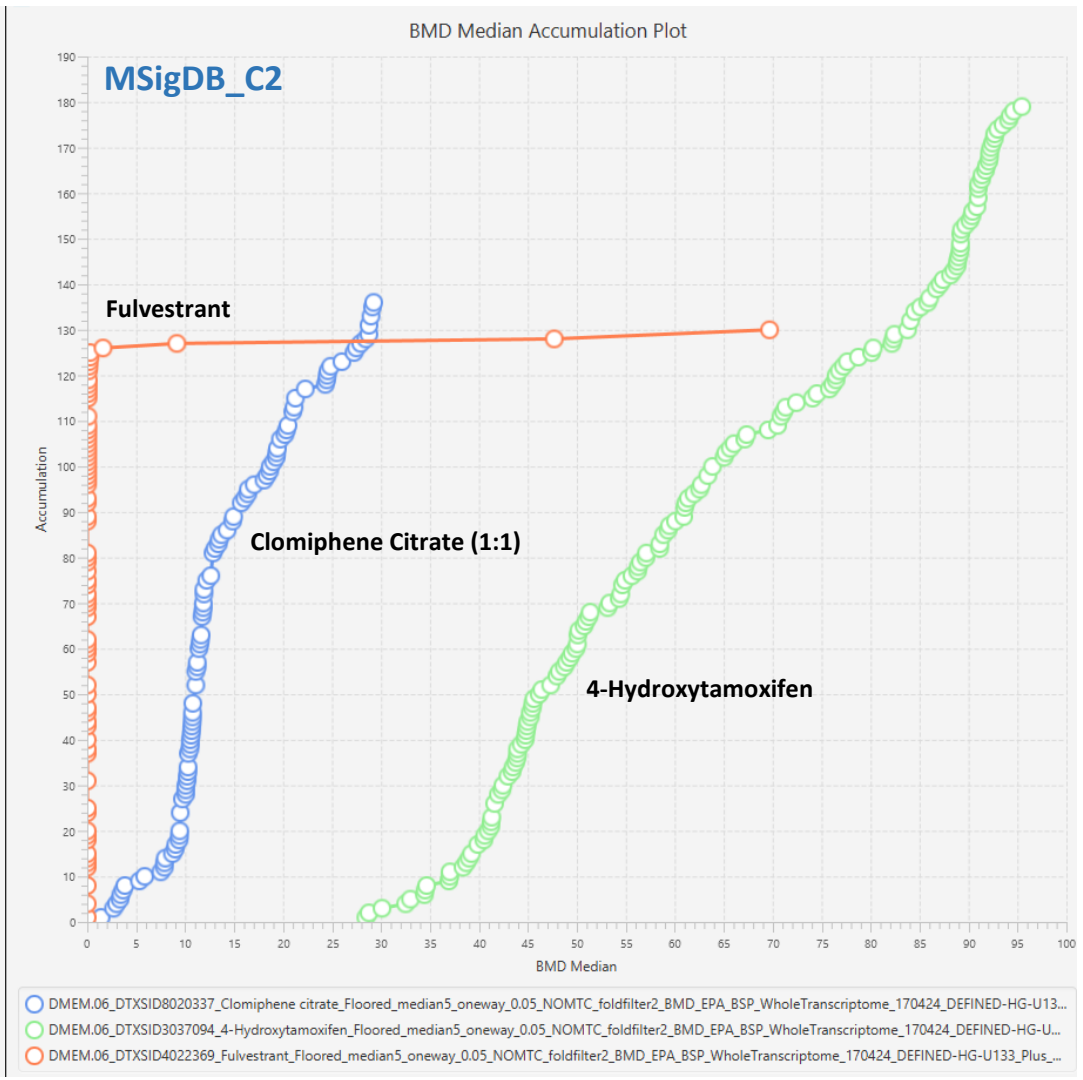
- Broad range of pathway level potency estimates and number of pathways affected across chemicals.

Pathway Potencies

ER Agonist

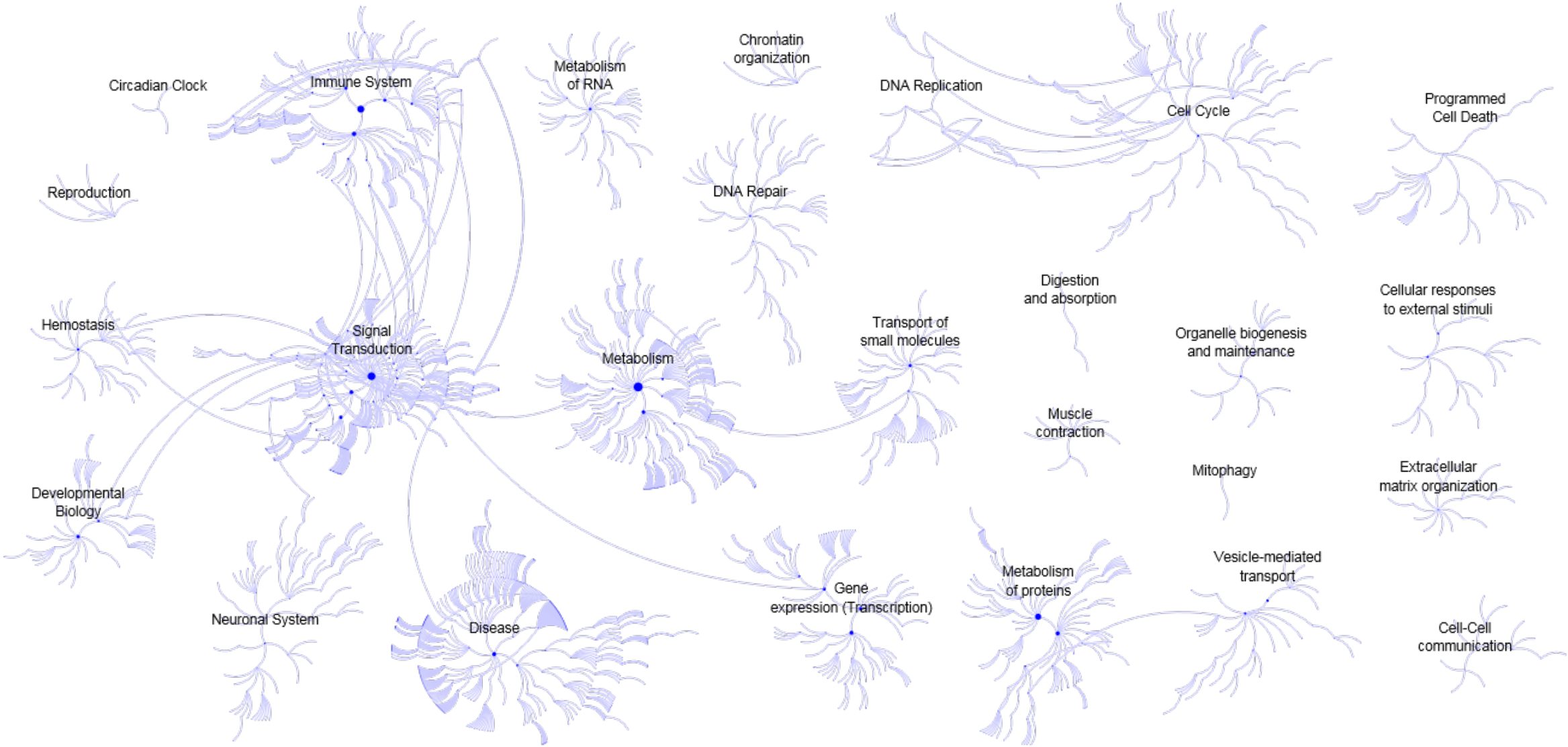


ER Antagonist



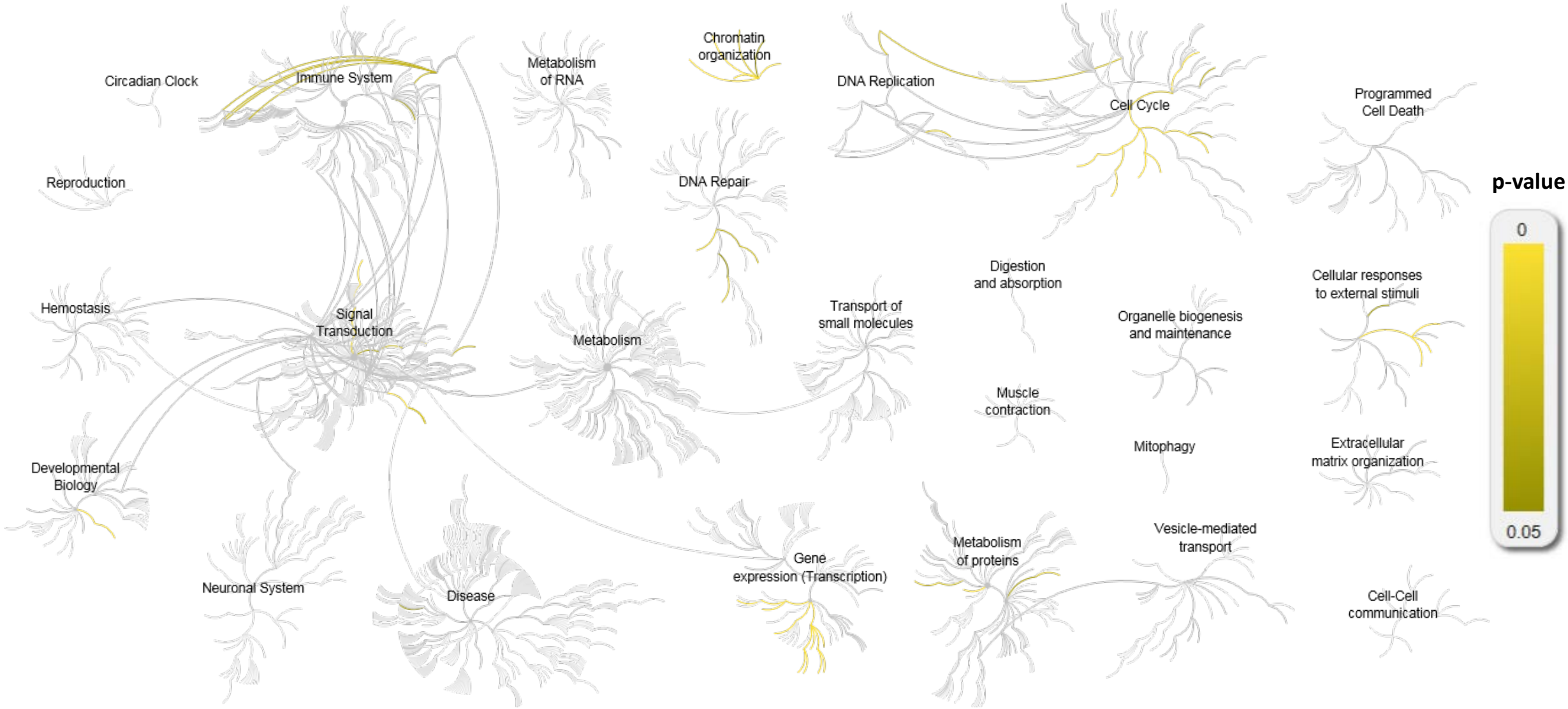
- Heterogeneity in pathway levels potency estimates and number of pathways affected within chemical class.

Network Mapping



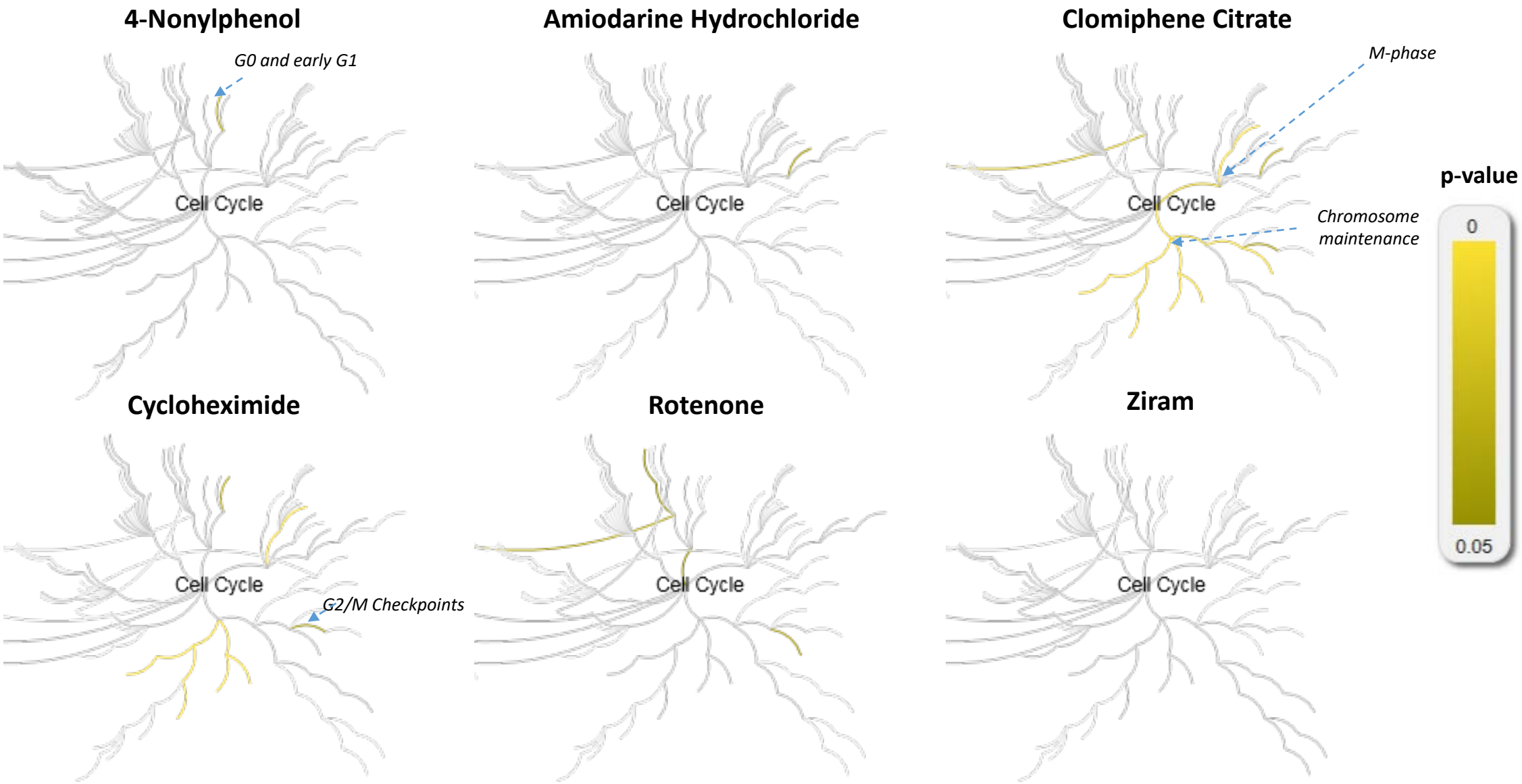
- Reactome (v60) Pathway Hierarchy

Network Mapping [Clomiphene Citrate]

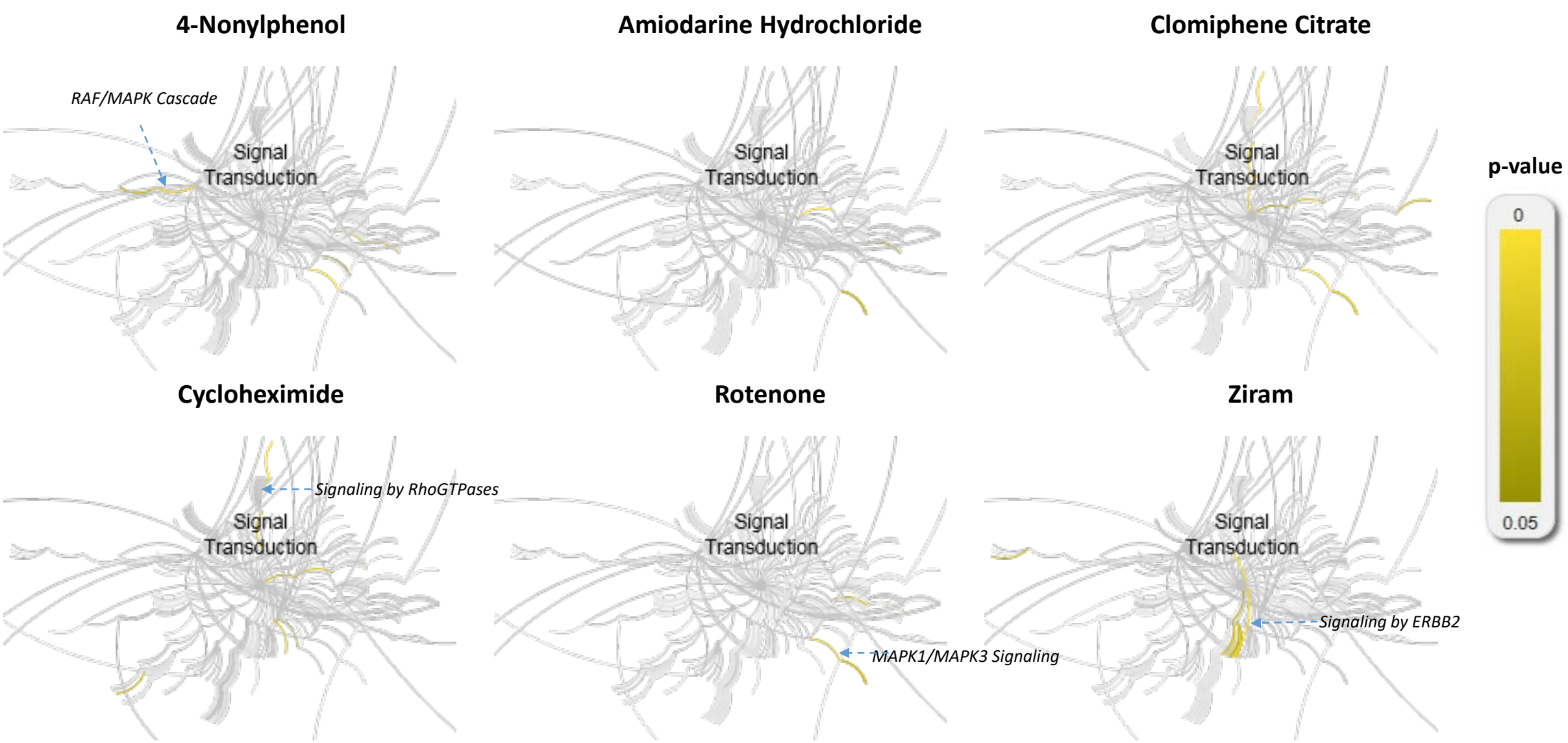


- Reactome (v60) Pathway Hierarchy → Overlaid with enrichment scores based on probes with acceptable BMD model fit
- Highlights different areas of biology affected by a chemical

Diversity in Response of Cell Cycle Networks



Diversity in Response of Signal Transduction Networks



Current Activities & Future Directions

- **Fall 2017:**
  - Refining data analysis pipeline and BMD modeling approach.
  - Exploring methods for MIE prediction & characterization of biological responses.
  - Prepping initial publication.
  - Conducting concentration-response screening of 2,200 chemicals in MCF7 cell model (8 conc., 6 HR exposure).

- **Beyond 2017:**
  - Tox21 reference chemical partner project
  - Screening in additional cell lines.
  - Coupling with image-based phenotypic screening assay.

Acknowledgements

- **NCCT**
  - Clinton Willis
  - Danielle Suarez
  - Chad Deisenroth
  - Imran Shah
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  - Matt Martin
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  - Rusty Thomas
- **BioSpyder**
  - Joel McComb
  - Jo Yeakley
  - Jason Downing
  - Milos Babic
  - Kyle LeBlanc
  - Harper Van Steenhouse

Questions ?

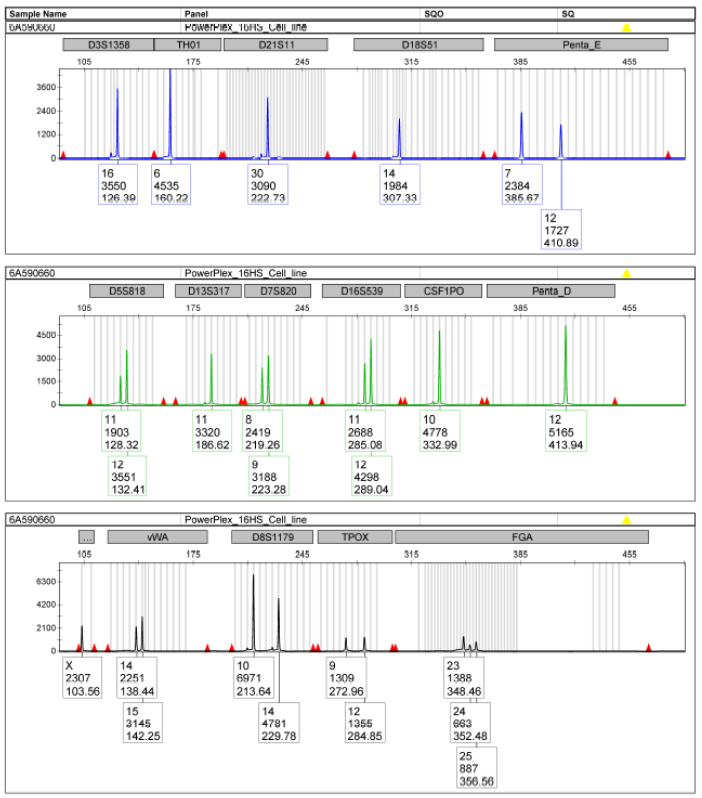
Bonus Slides

MCF7 Cell Line Cryopreserved Stocks & Authentication

Cell Sourcing: ATCC® HTB-22™

Cryo Stock Expansion Strategy:
Procured 5 vials of cells
Expanded in parallel to internal Passage 3.
Pooled cells prior to cryopreservation (~120 vials @ $2x10^6$ cells / vial)

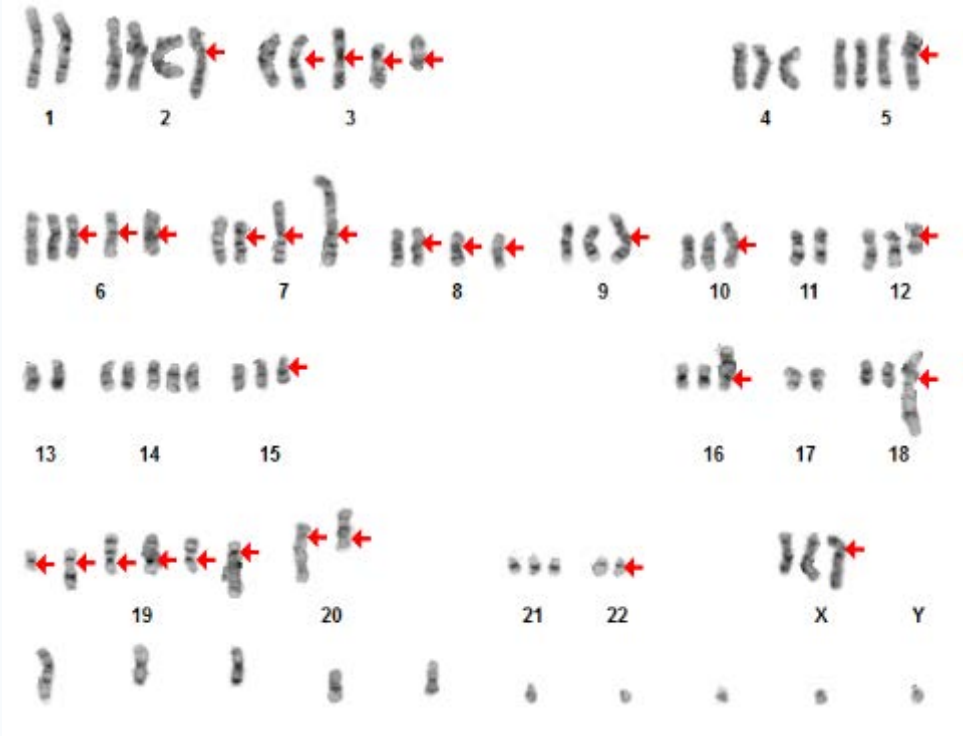
Authentication 1: STR Profiling



Loci	MCF7 Ref.	MCF7 NCCT
D5S818	11,12	11,12
D13S317	11	11
D7S820	8.9	8,9
D16S539	11,12	11,12
vWA	14,15	14,15
TH01	6	6
AMEL	X	X
TPOX	9.12	9,12
CSF1PO	10	10

100% Concordance with Reference Profile

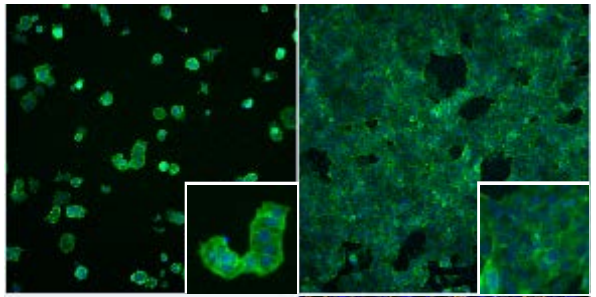
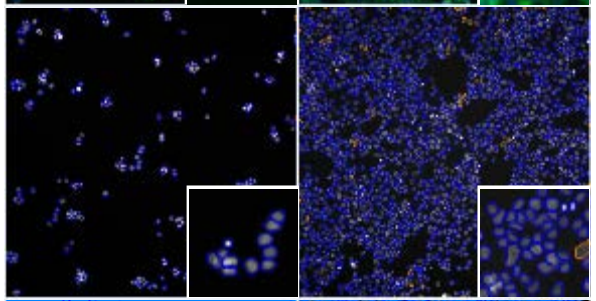
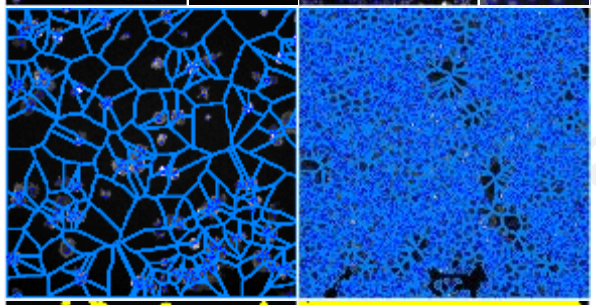
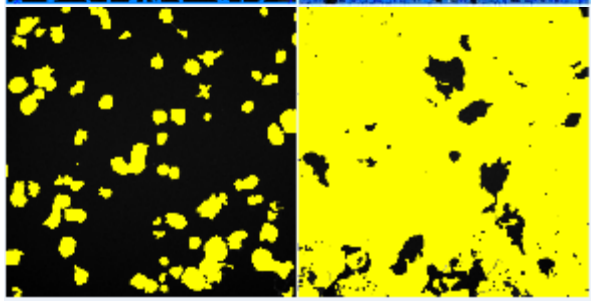
Authentication 2: Karyotyping



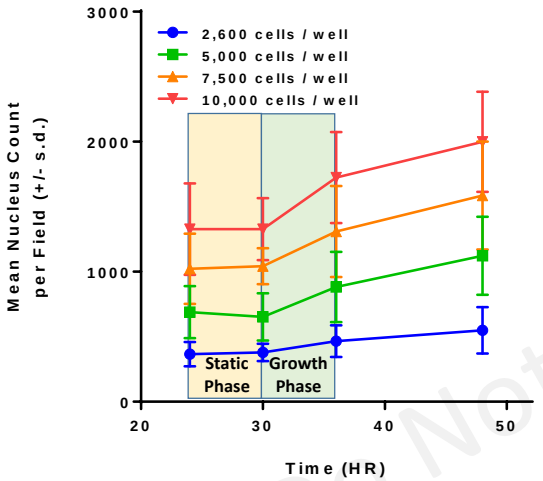
NCCT MCF7 Karyotype similar (but not identical) to reference profile.

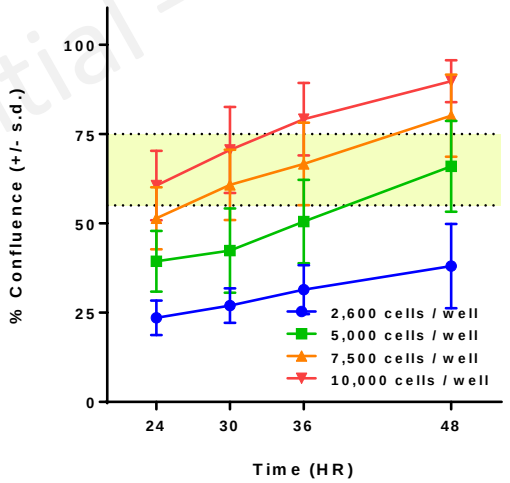
Quantification of Growth in NCCT MCF7 Cells & Selection of Seeding Densities

24 HR

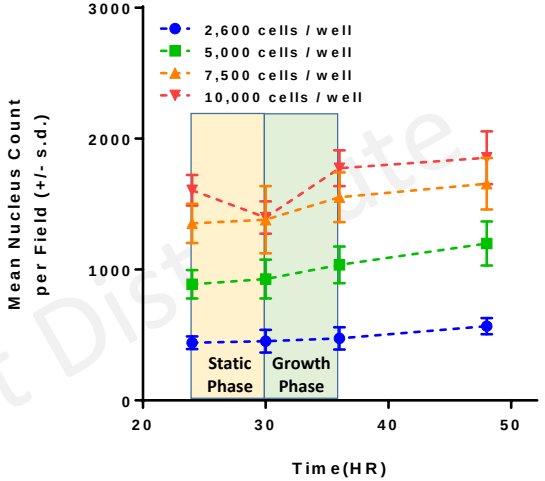
	2,600 cells / well	10,000 cells / well
Composite		
Nucleus Trace		
ZOI Masking		
Pixel Detection		

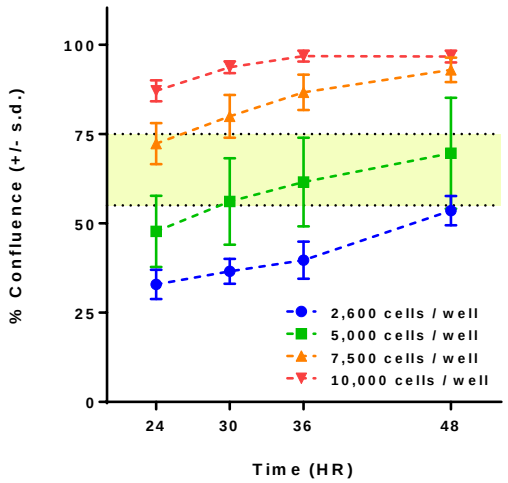
DMEM + 10% HI-FBS [a,c]





PRF-DMEM + 10% CS-HI-FBS [b,c]





- Increases in cell proliferation do not begin until 30 hours post-plating.
- Proliferation is greater in DMEM+10% HI-FBS versus PRF-DMEM + 10% CS-HI-FBS
- Cells seeded at concentrations yielding 55-75% confluence at time of sampling.
- **DMEM + 10% HI-FBS:**
  - 30 HR → 10,000 cells
  - 36 HR → 7,500 cells
  - 48 HR → 5,000 cells
- **PRF-DMEM + 10% CS-HI-FBS:**
  - All Times → 5,000 cells.

[a] Dulbecoo's Modified Eagle's Media (MediaTech 10-013) + Heat-Inactivated FBS (Sigma-Aldrich F4135)
[B] Phenol Red Free Dulbecco's Modified Eagle's Media (MediaTech 17-205) + Charcoal-Stripped Heat-Inactivated FBS (Sigma-Aldrich 6765)
[C] n = 72 replicate wells across two independent cultures.

Study Design: TempO-Seq Attenuation in MCF7 Cells

Study Component 1:
6 HR Exposure, Static Phase

Cell Type:	MCF7
Media Type:	DMEM + 10% HI-FBS PRF-DMEM + 10% CS-HI-FBS
Treatments:	DMSO (0.5%) Trichostatin A (1 µM)
Replicates:	3
Dose Time:	24 hr post-plating
Sample Time:	30 hr post-plating
Total # of Samples:	12

Media Type, Exposure Window

Study Component 2:
6 HR Exposure, Growth Phase

Cell Type:	MCF7
Media Type:	DMEM + 10% HI-FBS PRF-DMEM + 10% CS-HI-FBS
Treatments:	DMSO (0.5%) Trichostatin A (1 µM)
Replicates:	3
Dose Time:	30 hr post-plating
Sample Time:	36 hr post-plating
Total # of Samples:	12

Media Type, Exposure Window

Study Component 3:
Time Course, Untreated Cells

Cell Type:	MCF7
Media Type:	DMEM + 10% HI-FBS PRF-DMEM + 10% CS-HI-FBS
Treatments:	None
Replicates:	3
Dose Time:	n/a
Sample Time:	30, 36, 48 hr post-plating
Total # of Samples:	18

Media Type, Time Course of Cell Growth

Lysis Option 1:
40 µL Media: 40 µL 2X Lysis Buffer

Lysis Option 2:
Drain to 10 µL Media: 10 µL 2X Lysis Buffer

Lysis Option 3:
Complete Drain → 10 µL 1X Lysis Buffer

- Each study component was performed using each lysis option.
- Samples from **Lysis Option 2** (n = 42) were used for identification of candidate Detector Oligos (DOs) for attenuation.

Results

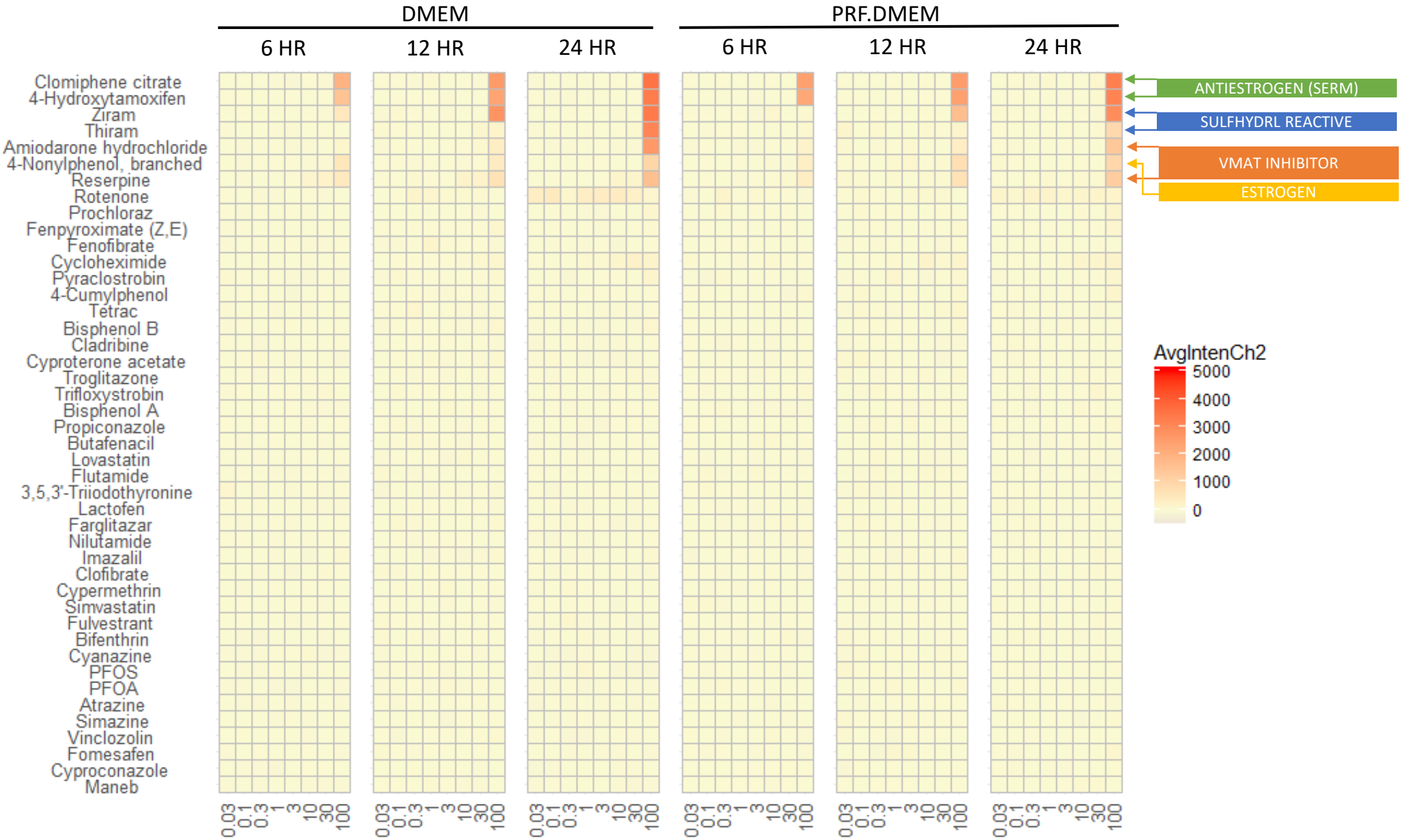
- **Apoptosis & Cytotoxicity Assays**
- **Transcriptomics Data Analysis Pipeline**
- **HTTr Assay Performance Metrics**
- **Concentration Response Modeling**

Top 12 Candidates for Attenuation

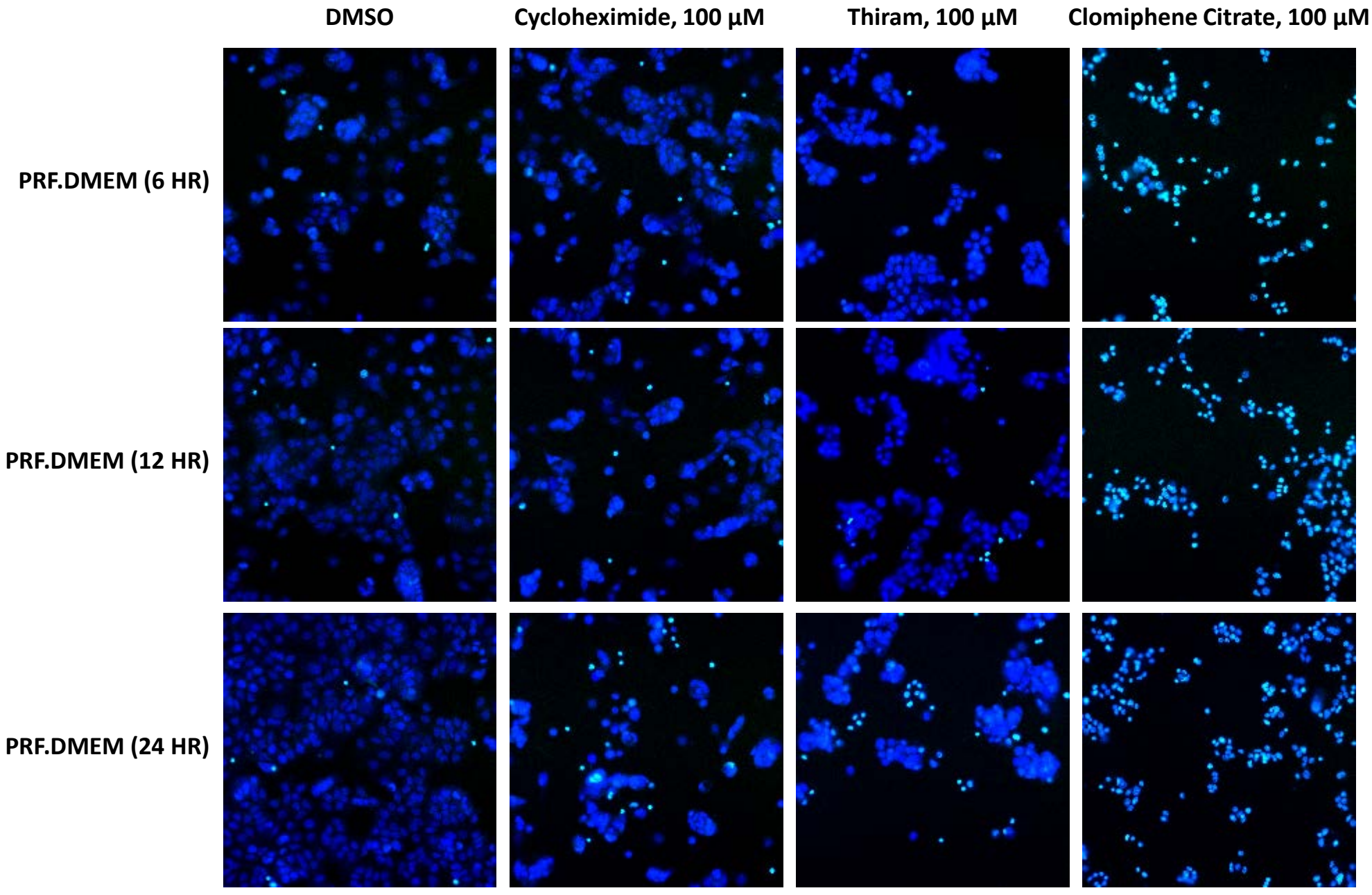
Gene Symbol	Official.Full.Name	Category	Description
TMSB4X	thymosin beta 4, X-linked	Cytoskeleton	This gene encodes an actin sequestering protein which plays a role in regulation of actin polymerization.
KRT8	keratin 8	Cytoskeleton	This gene is a member of the type II keratin family clustered on the long arm of chromosome 12. Type I and type II keratins heteropolymerize to form intermediate-sized filaments in the cytoplasm of epithelial cells.
ACTG1	actin gamma 1	Cytoskeleton	Actin, gamma 1, encoded by this gene, is a cytoplasmic actin found in non-muscle cells.
KRT18	keratin 18	Cytoskeleton	KRT18 encodes the type I intermediate filament chain keratin 18. Keratin 18, together with its filament partner keratin 8, are perhaps the most commonly found members of the intermediate filament gene family.
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Energy Metabolism	This gene encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family. The product of this gene catalyzes an important energy-yielding step in carbohydrate metabolism
HIST2H4B	histone cluster 2 H4 family member b	Histone	This gene is intronless and encodes a replication-dependent histone that is a member of the histone H4 family
ATP5E	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	Mitochondrial	This gene encodes a subunit of mitochondrial ATP synthase.
RPS3	ribosomal protein S3	Ribosomal	This gene encodes a ribosomal protein that is a component of the 40S subunit, where it forms part of the domain where translation is initiated.
RPL37	ribosomal protein L37	Ribosomal	This gene encodes a ribosomal protein that is a component of the 60S subunit.
RPL4	ribosomal protein L4	Ribosomal	This gene encodes a ribosomal protein that is a component of the 60S subunit.
PABPC1	poly(A) binding protein cytoplasmic 1	Ribosomal Transport	This gene encodes a poly(A) binding protein. The protein shuttles between the nucleus and cytoplasm and binds to the 3' poly(A) tail of eukaryotic messenger RNAs via RNA-recognition motifs.
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	Ribosomal Transport	This gene encodes an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome.

- The most highly expressed genes in the attenuation set are “housekeeping” genes.

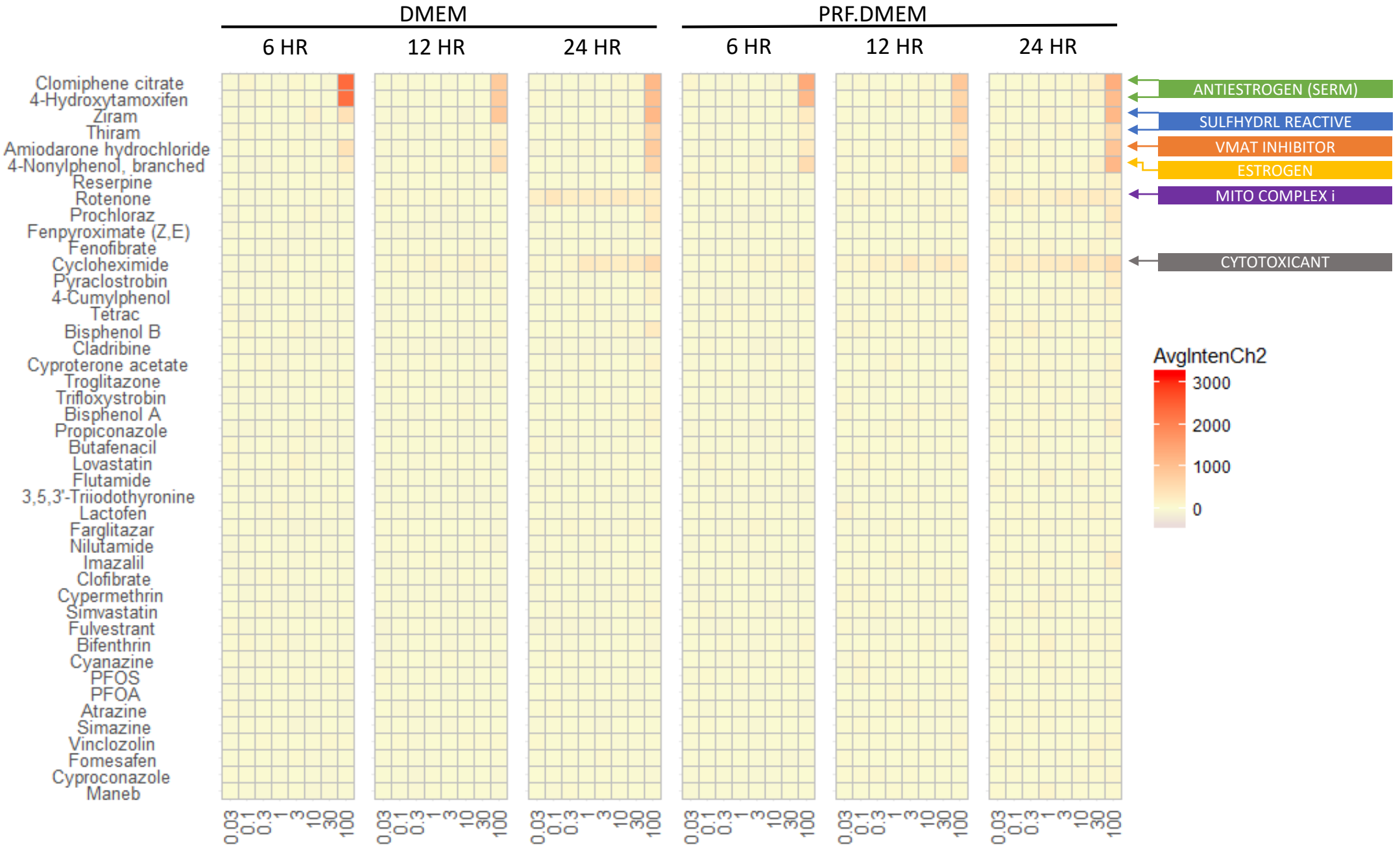
Apoptosis Assay Results



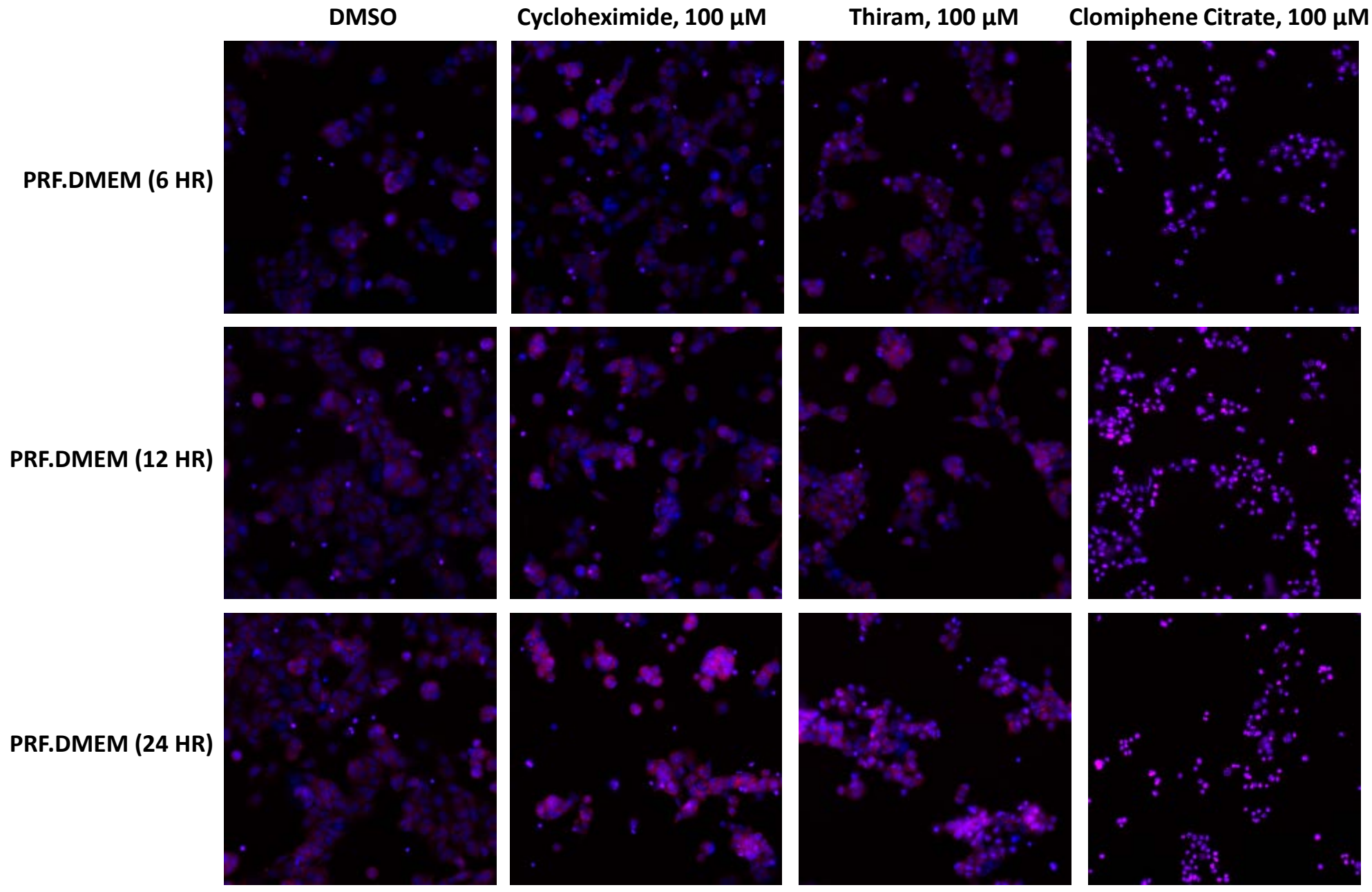
Apoptosis Assay, Ground Truth



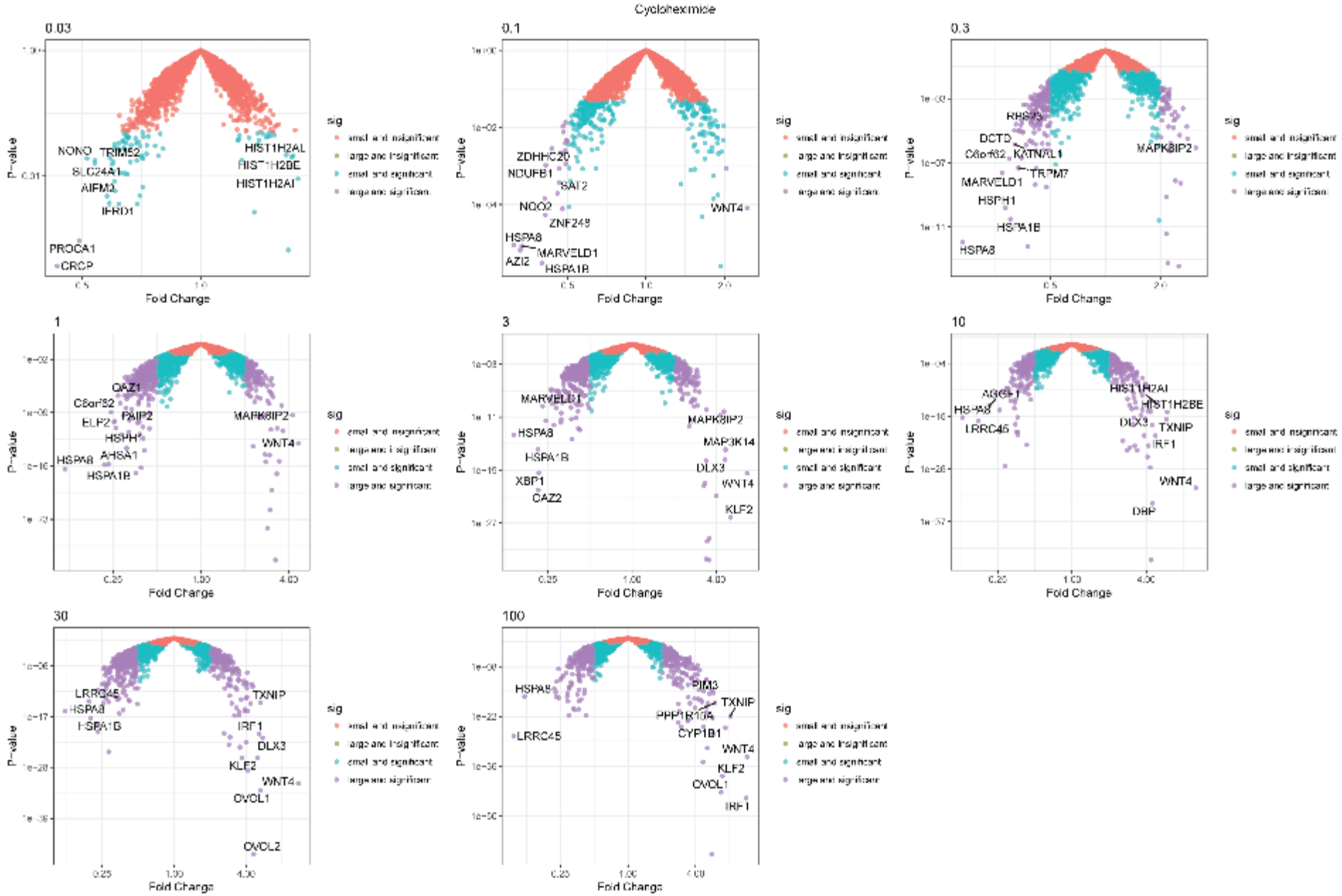
Cell Viability Assay Results



Cell Viability Assay, Ground Truth

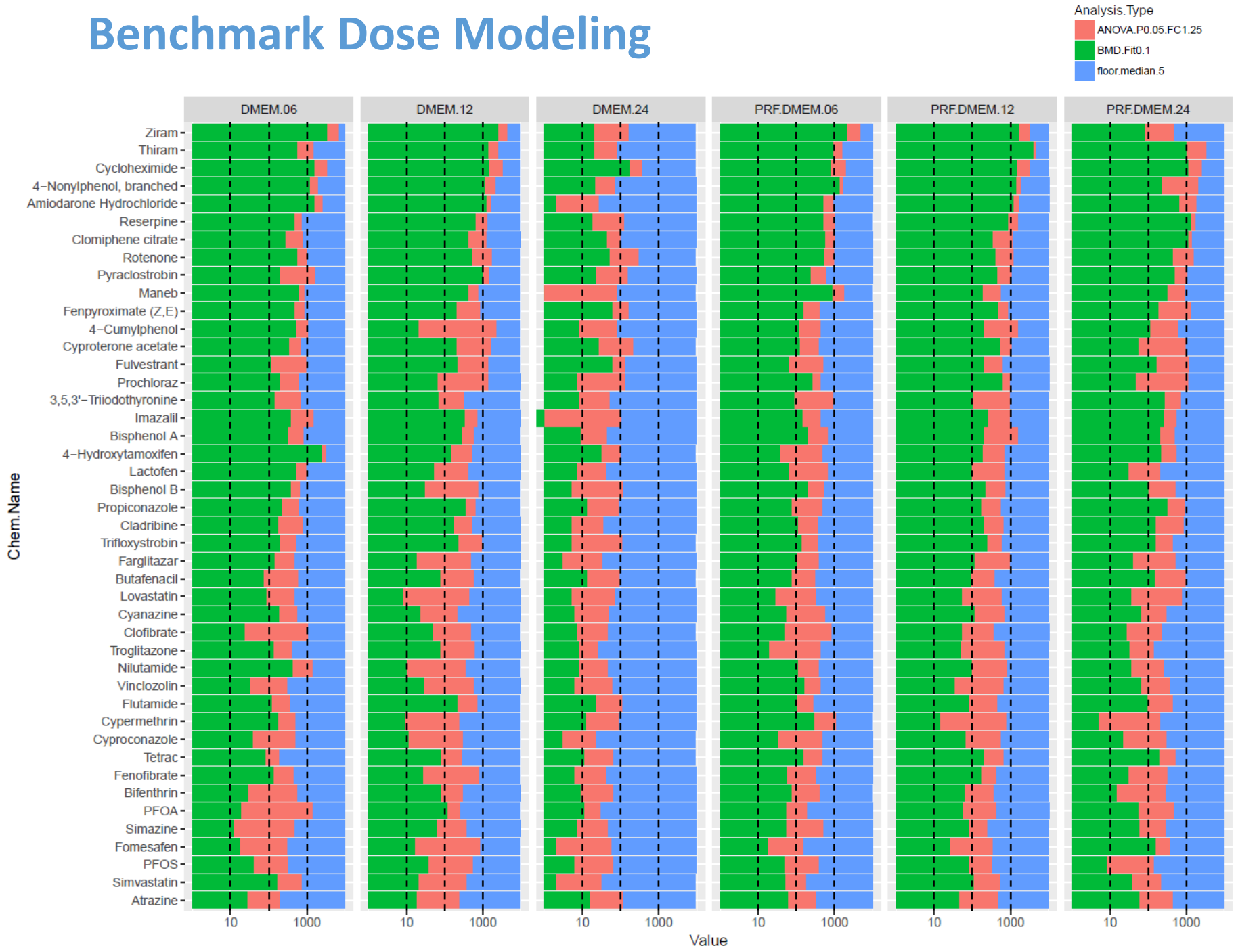


Concentration Dependent Increases in Transcriptional Response

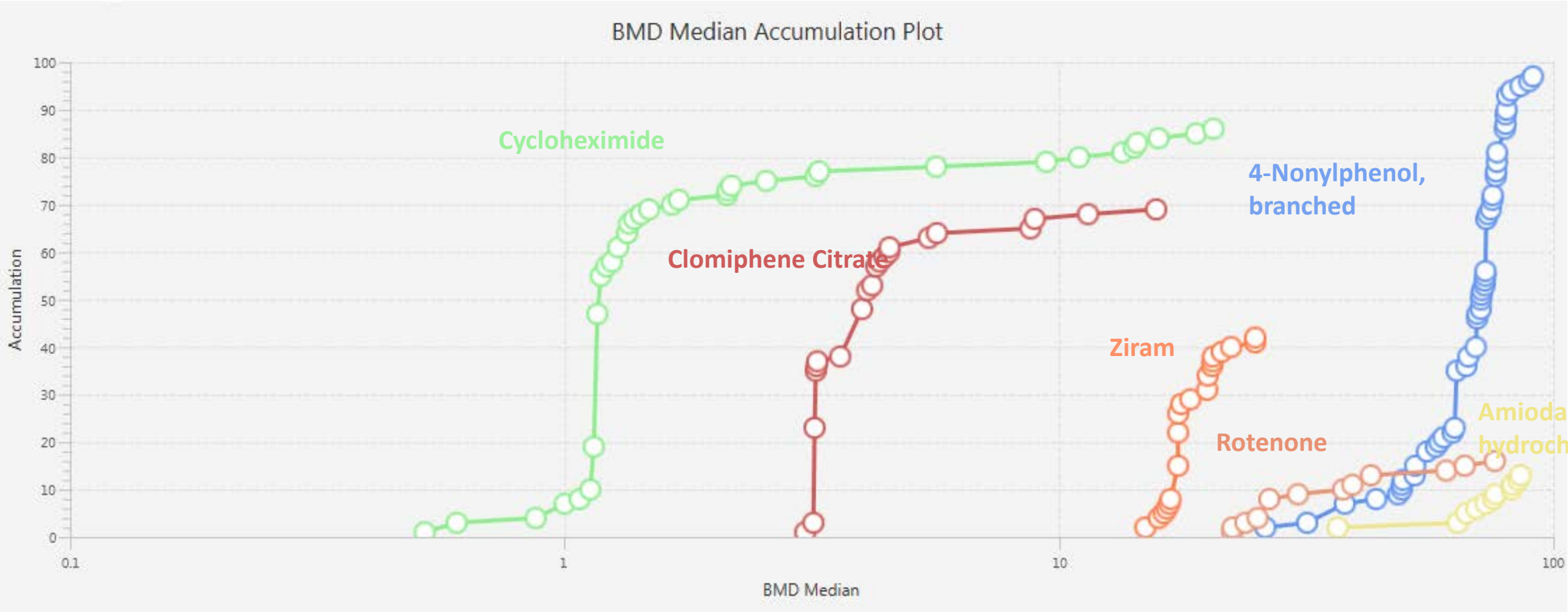


Benchmark Dose Modeling

Parameter	Criteria
Pre-filter	ANOVA ($p_{raw} < 0.05$ & $\lvert FC \rvert \geq 1.25$)
Models	Hill, power, linear, poly 2, exponential 2
BMR Factor:	1.349 (10 %)
Best Model Selection:	Lowest AIC
Hill Model Flagging:	'k' < 1/3 Lowest Positive Dose Select next best model with p > 0.05



Benchmark Dose Modeling



- Enrichment using Reactome Pathway Database
- Observed broad range of thresholds for chemical bioactivity.