High-Throughput Transcriptomics (HTTr) for Toxicological Testing

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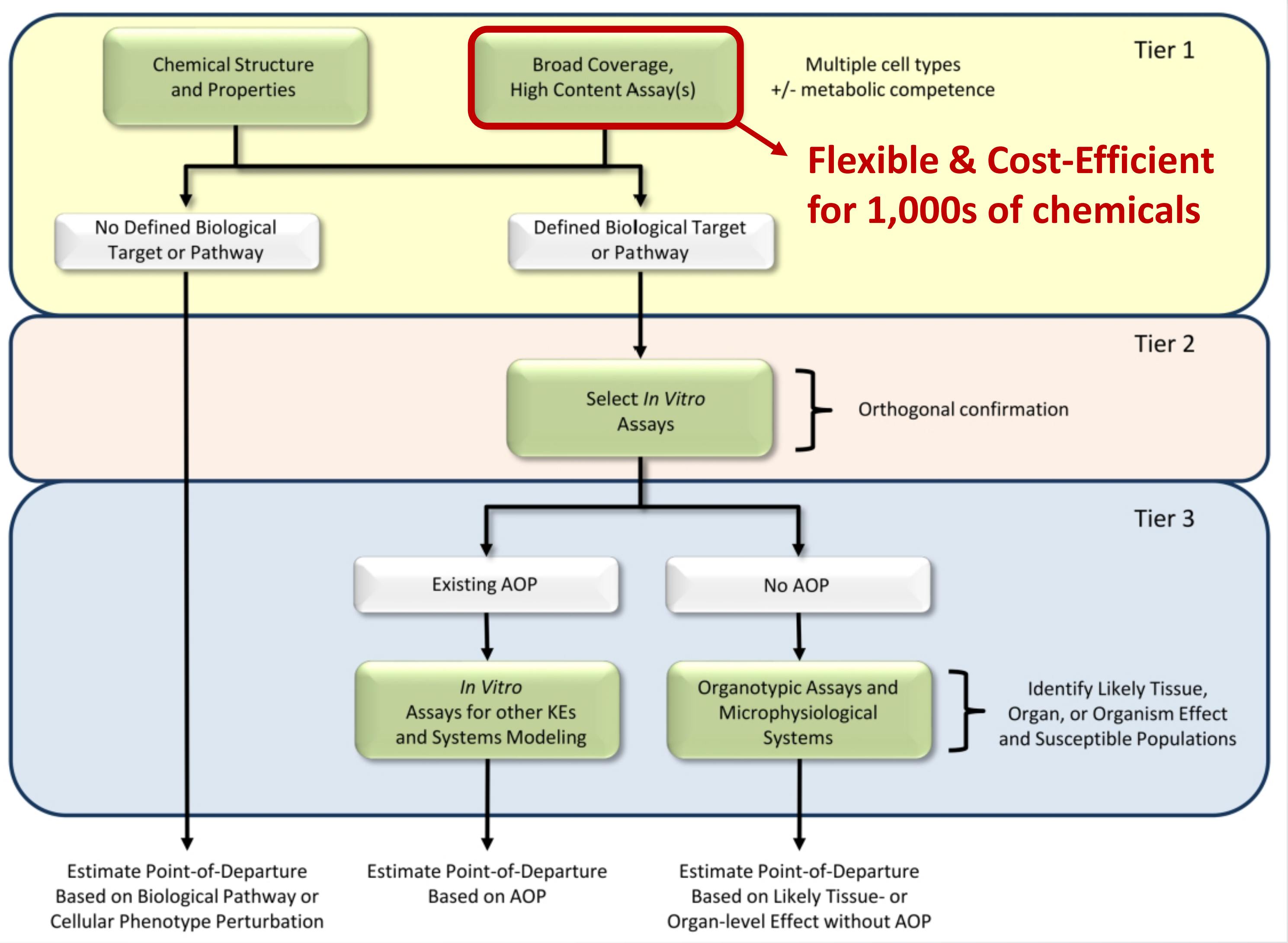
Tiered Chemical Safety Testing Strategy

Tier 1 Primary Goals:

- Prioritize chemicals by bioactivity & potency
- Predict biological targets for chemicals

Key Challenges:

- Curve-fitting on count-based data
- Summarization at pathway/chemical level



Tier 1

Chemical Structure and Properties

Broad Coverage, High Content Assay(s)

Multiple cell types +/- metabolic competence

Flexible & Cost-Efficient for 1,000s of chemicals

No Defined Biological Target or Pathway

Defined Biological Target or Pathway

Tier 2

Select *In Vitro* Assays

Orthogonal confirmation

Tier 3

Existing AOP

No AOP

In Vitro Assays for other KEs and Systems Modeling

Organotypic Assays and Microphysiological Systems

Identify Likely Tissue, Organ, or Organism Effect and Susceptible Populations

Estimate Point-of-Departure Based on Biological Pathway or Cellular Phenotype Perturbation

Estimate Point-of-Departure Based on AOP

Estimate Point-of-Departure Based on Likely Tissue- or Organ-level Effect without AOP

Thomas, et al. Toxicol Sci 2019

Automated *in vitro* Chemical Screening

Cryopreserved Cell Stocks

Cell Expansion

Cell Plating

BioTek MultiFlo ™ FX

Dispensing Test Chemicals

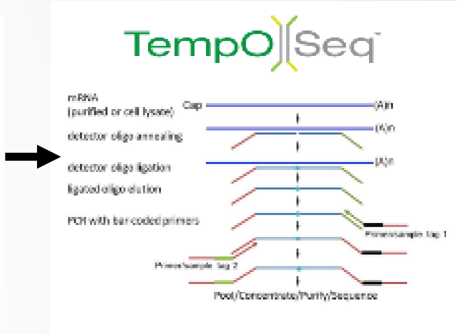
LabCyte Echo® 550 Liquid Handler

6 or 24 Hour Exposures

Track 1: Targeted RNA-Seq

Generating Cell Lysates

TempO-Seq WT



Track 2: Apoptosis / Cell Viability

Reagent Dispensing

High Content Imaging

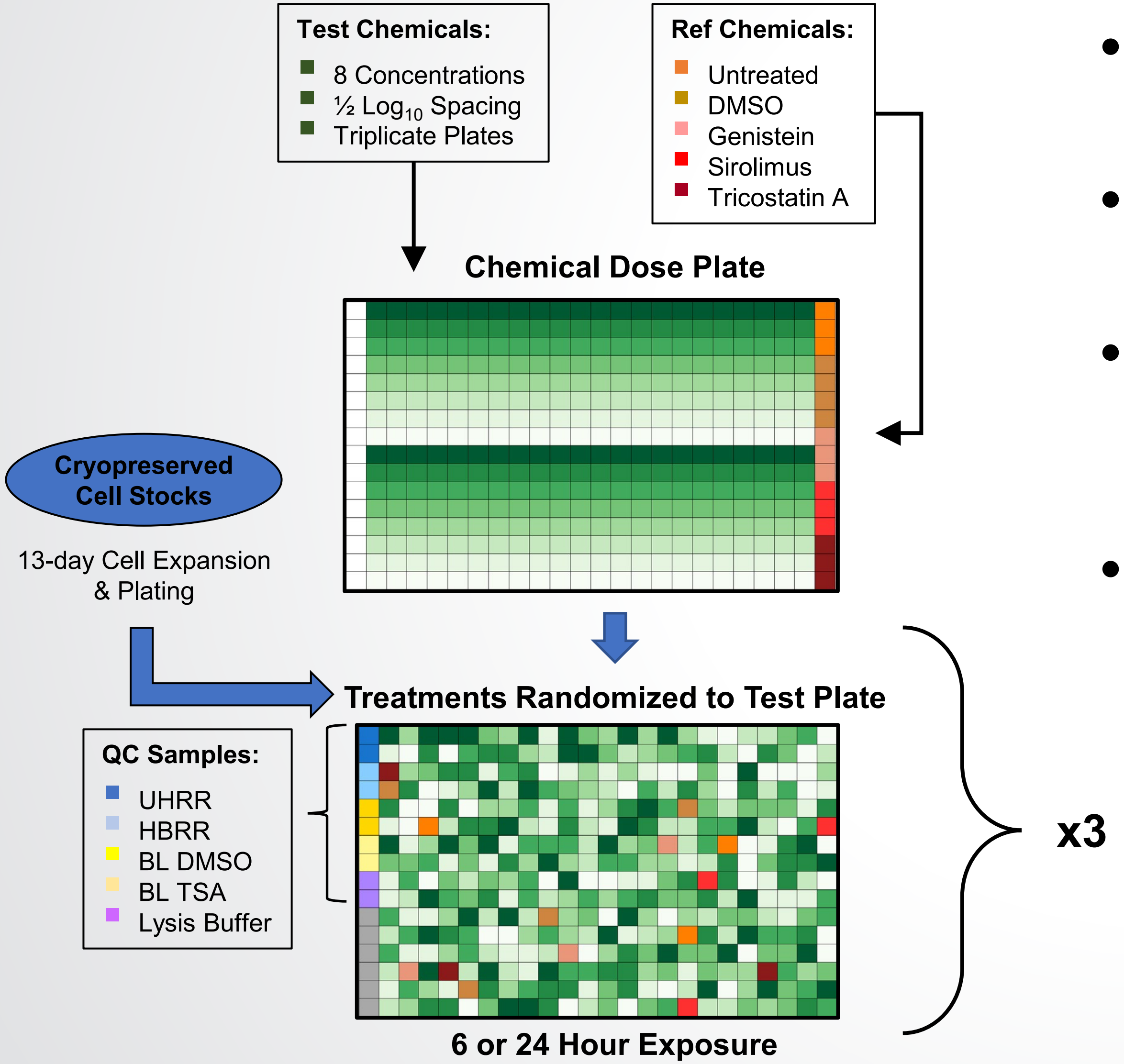
Perkin Elmer Opera Phenix™ High Content Screening System

Standardized Expansion Protocol

MC = Media Change
P = Passage

Day In Vitro (DIV):	0	2	5	7	9	11	13
	P3 (from Cryo)		P4		P5		P6
Action:	Seed	MC	P	MC	P	MC	P
Vessel:	T25		T75		T225		Test Plate(s)
							Perform Experiment

HTTr Study Design



Test Chemicals:
- 8 Concentrations
- ½ Log_{10} Spacing
- Triplicate Plates

Ref Chemicals:
- 🟧 Untreated
- 🟨 DMSO
- 🟥 Genistein
- 🟥 Sirolimus
- 🟥 Tricostatin A

Chemical Dose Plate

Cryopreserved Cell Stocks

13-day Cell Expansion & Plating

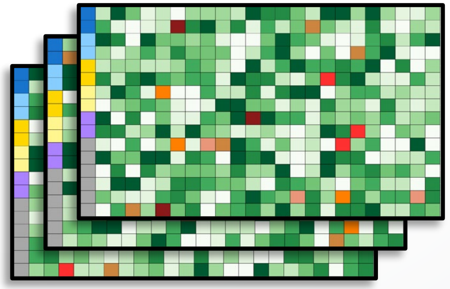
Treatments Randomized to Test Plate

QC Samples:
- 🟦 UHRR
- 🟦 HBRR
- 🟨 BL DMSO
- 🟨 BL TSA
- 🟪 Lysis Buffer

6 or 24 Hour Exposure

x3

- High-throughput *in vitro* screens performed on 384 well plates
- Standardized dilution series for every test sample
- Multiple QC and reference chemicals included on every plate to track assay performance
- Triplicate Test Plates:

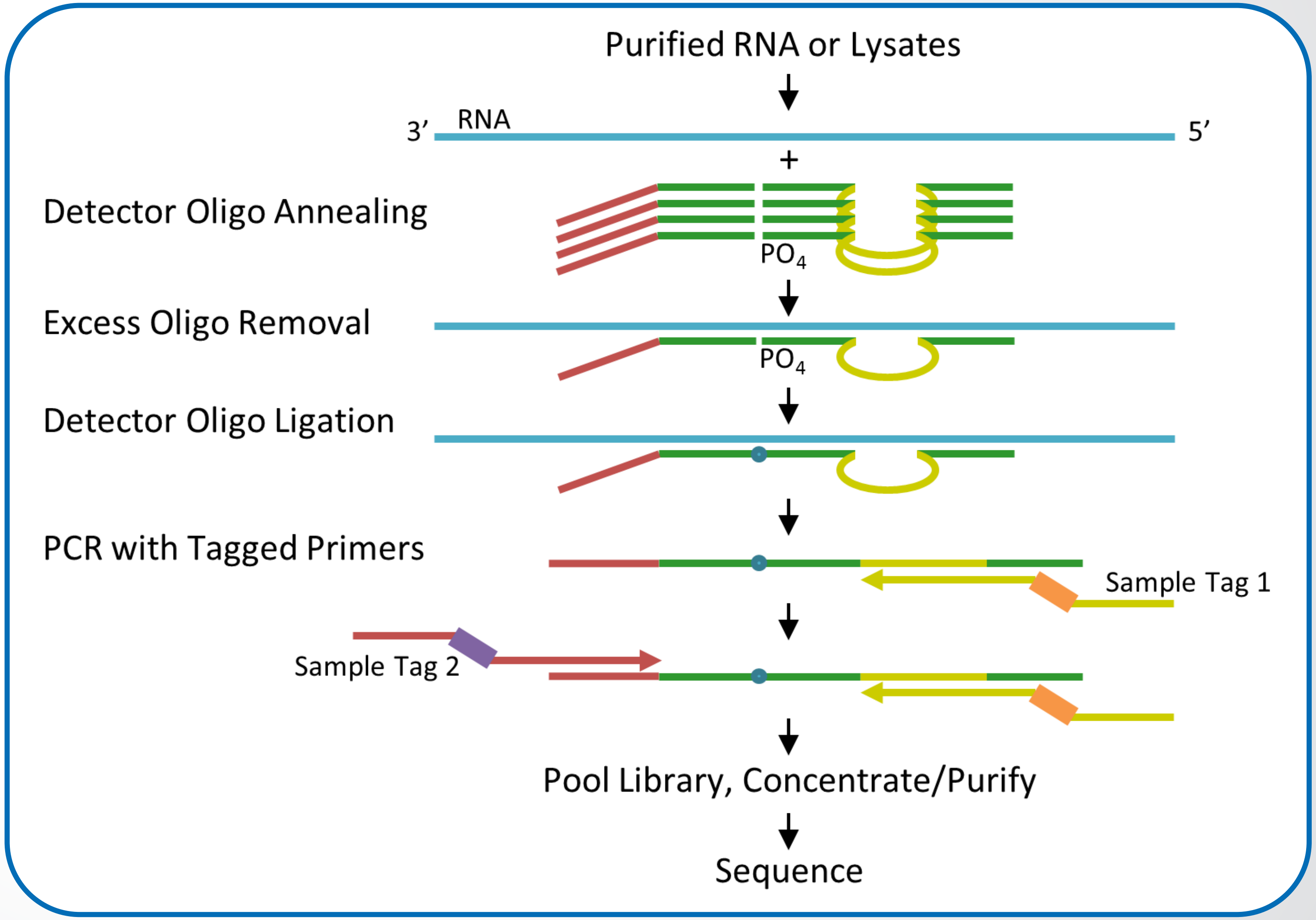


- ➢ Randomized independently
- ➢ Separate cell culture batches

Harrill, et al. Toxicol Sci 2021

TempO-seq Assay

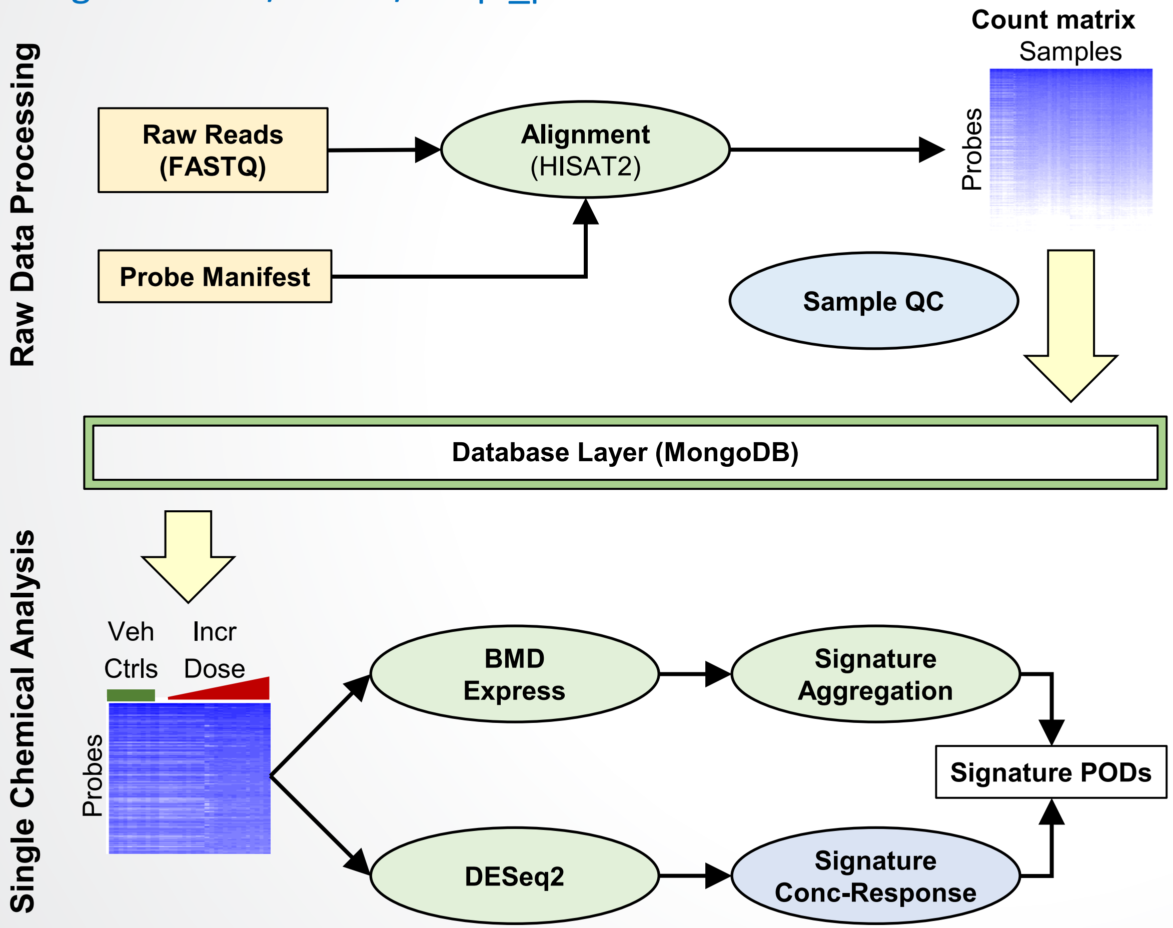
- Targeted RNA-seq (TempO-seq) enables high-throughput profiling of **cell lysates** or purified RNA
- Probe set for whole human transcriptome targets ~21,000 human genes
- Captures majority of signal with much lower sequencing depth (~3M reads with attenuation)
- Barcoding and pooling allows multiplexing of hundreds of samples



Yeakley, et al. PLoS ONE 2017

HTTr Bioinformatics Pipeline

github.com/USEPA/httrpl_pilot

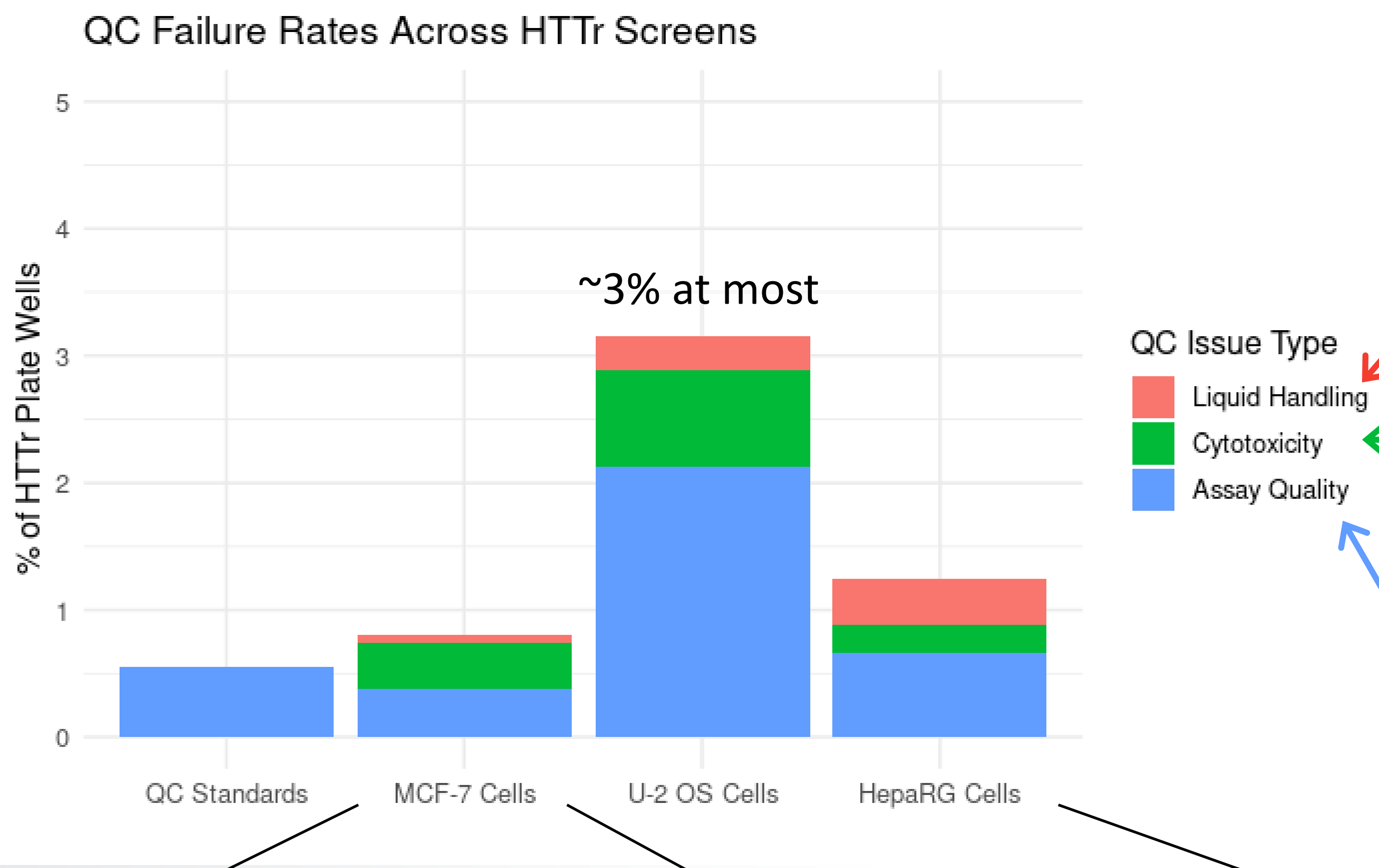


github.com/USEPA/CompTox-httrpathway

- Rapid processing for large screens
- Many data steps performed independently for each test chemical:
  - Removal of low signal probes
  - Normalization
  - DESeq2 analysis
- Exploring multiple analysis strategies for curve-fitting and signature & chemical-level summarization

Harrill, et al. Toxicol Sci 2021

HTTr Quality Control



Acoustic dispenser logs identify problems with chemical handling

Apoptosis/cell viability assays identify >50% cytotoxic concentrations

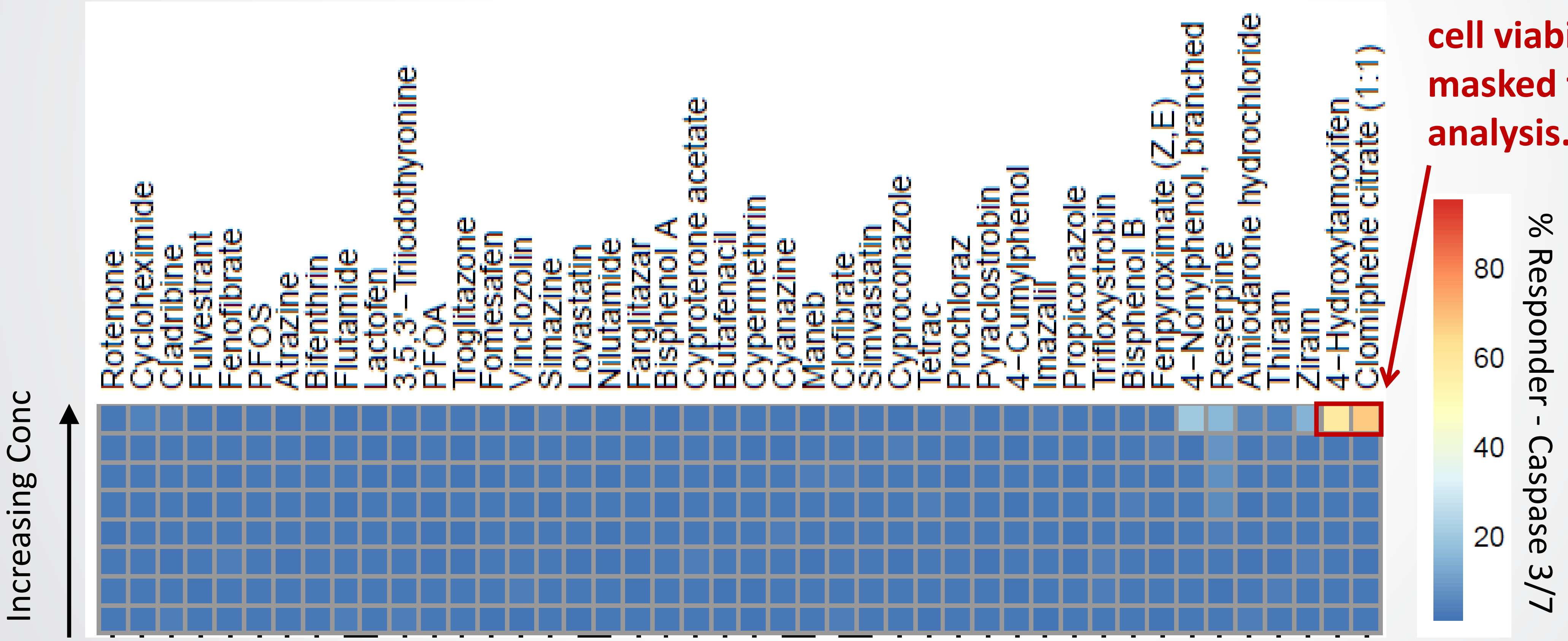
Bioinformatic QC checks remove:

- Low read depth samples
- High rate of alignment failure
- Samples with low gene coverage
- Samples with irregular count distributions

- 44 Chemical Pilot Study
- Screened 1,577 ToxCast chemicals

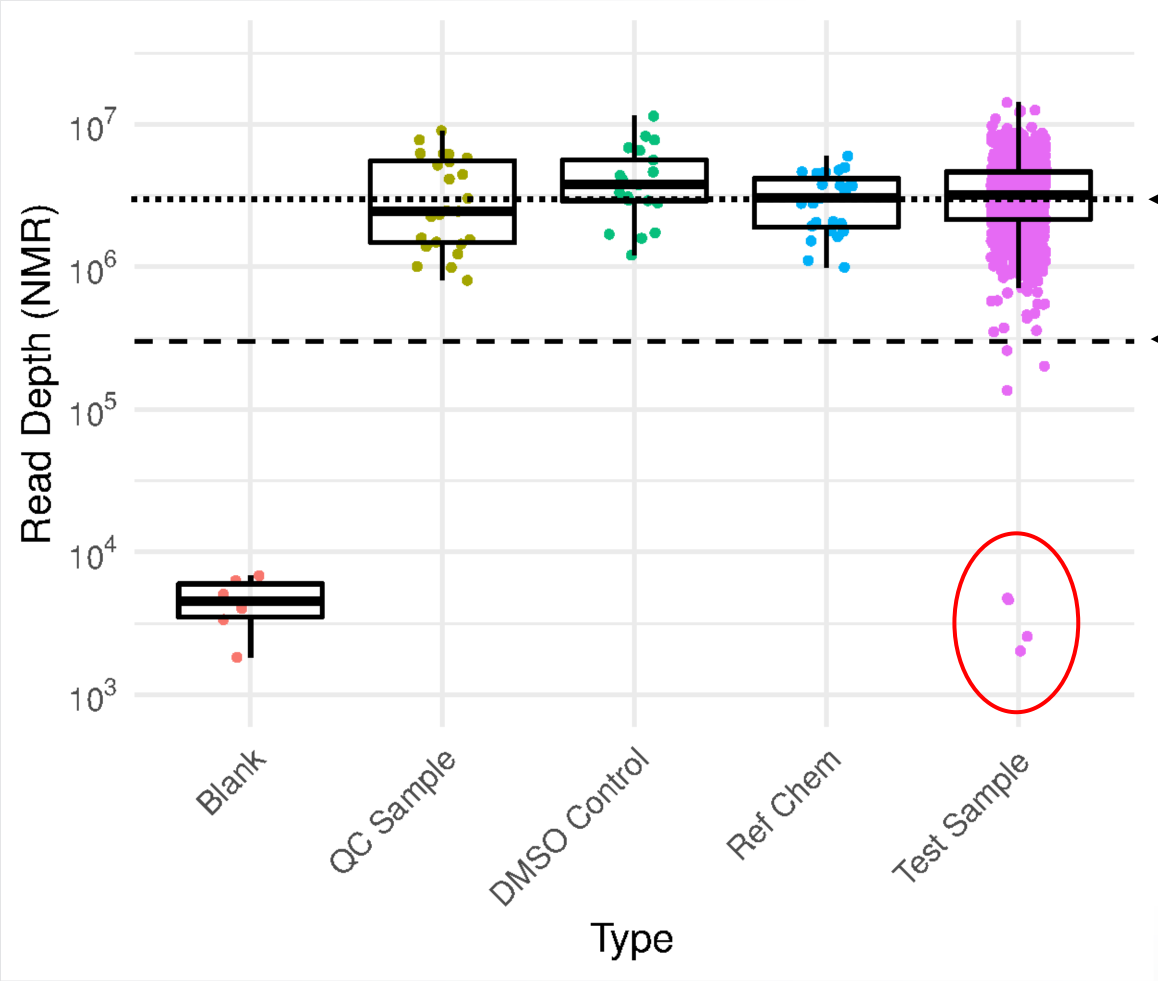
- Screened 1,201 ToxCast chemicals
- Screened 137 PFAS

Cell Viability



Conditions causing cell viability loss >50% masked from further analysis.

QC Metrics: Read Depth



Depends on probe set, cell type, attenuation

Target Depth = 3M Reads

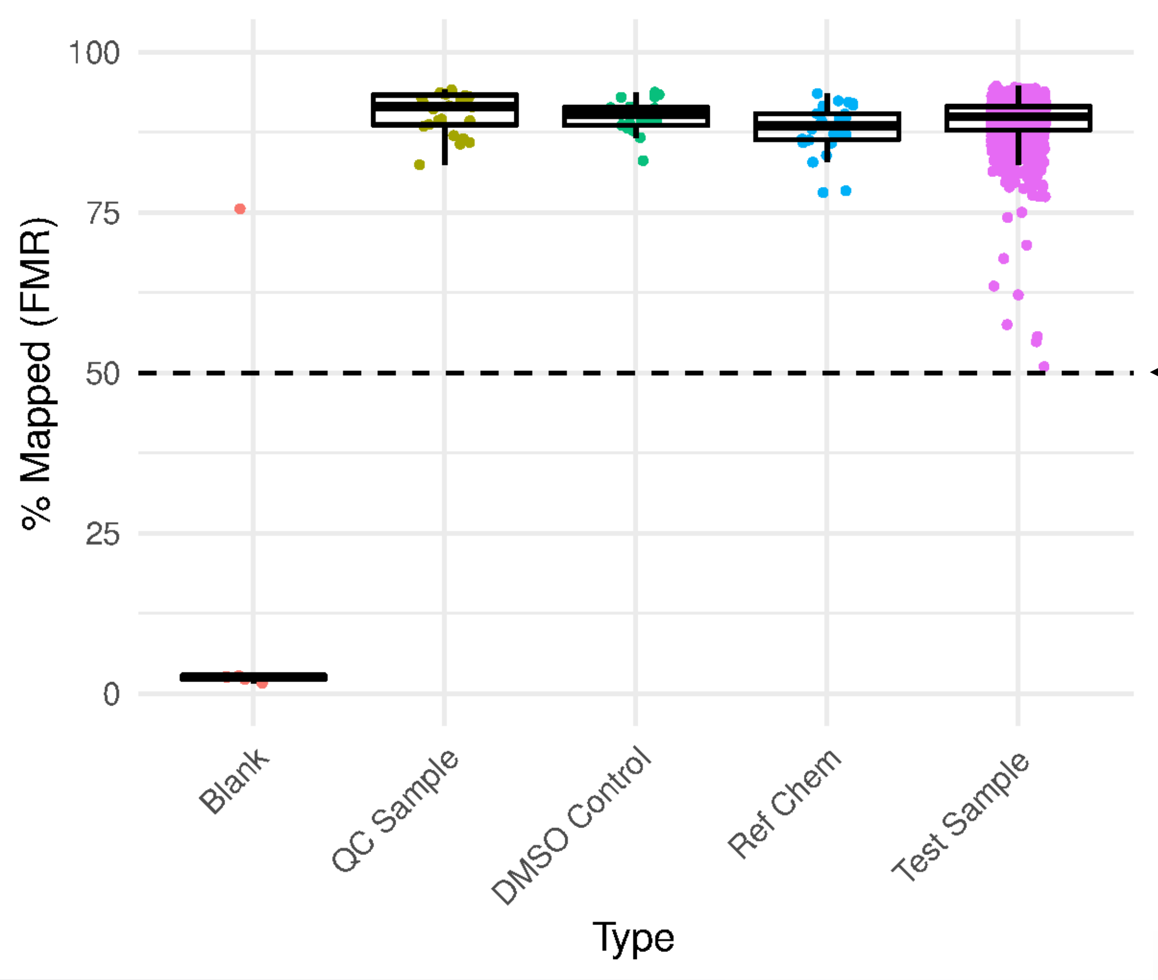
Threshold = 10% of Target Depth

More variability for cell lysates than purified RNA

Reasons for low read depth:

- **Cytotoxicity**
- **Sample degradation**
- **Low input**
- **Assay failure**

QC Metrics: Mapping Rate



- **Each read mapped to known probe sequences**
- **Only uniquely mapped reads used for analysis**

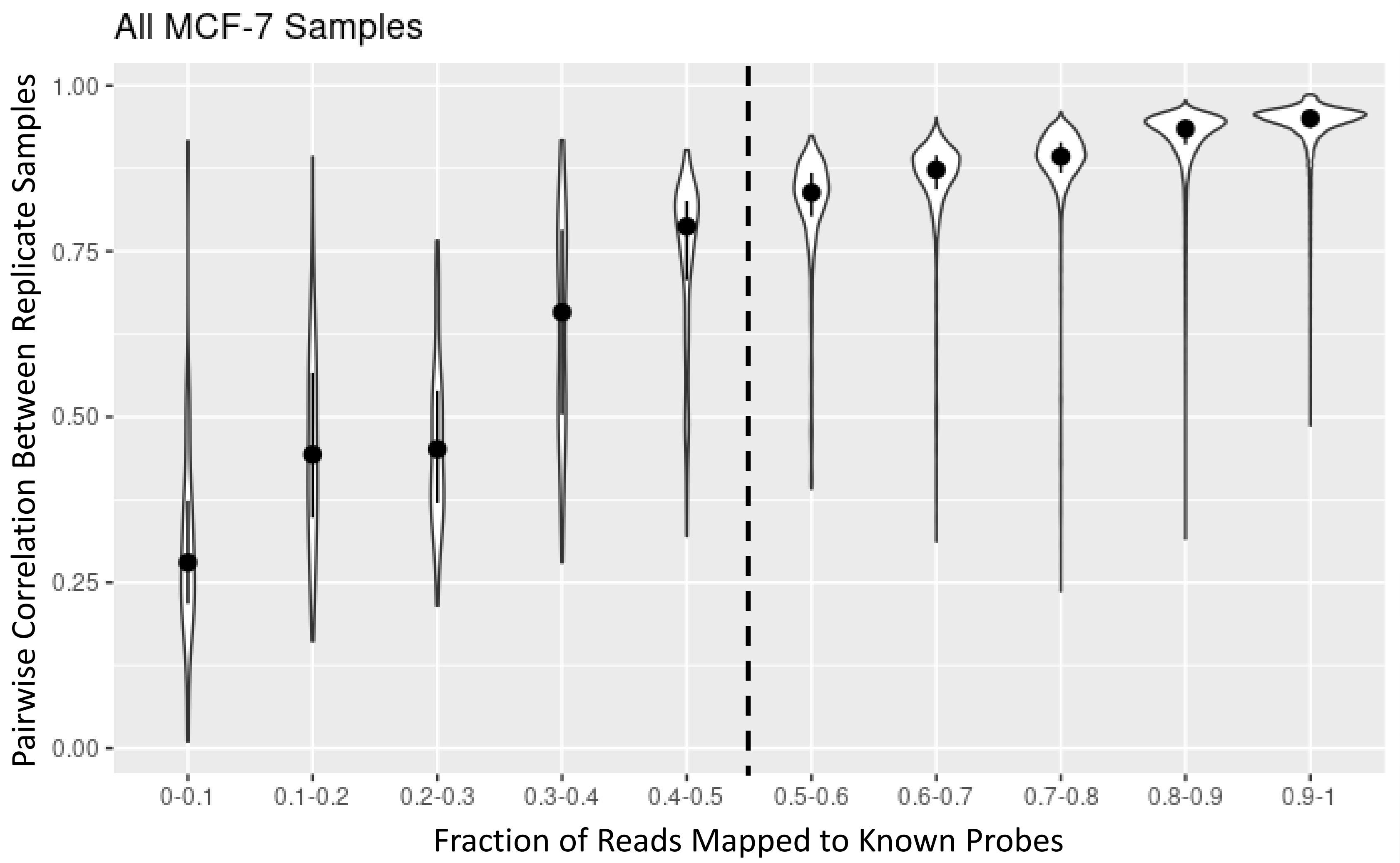
Threshold = 50% Mapping Rate

May depend on media/lysate condition, cell type

Reasons for low mapping rate:

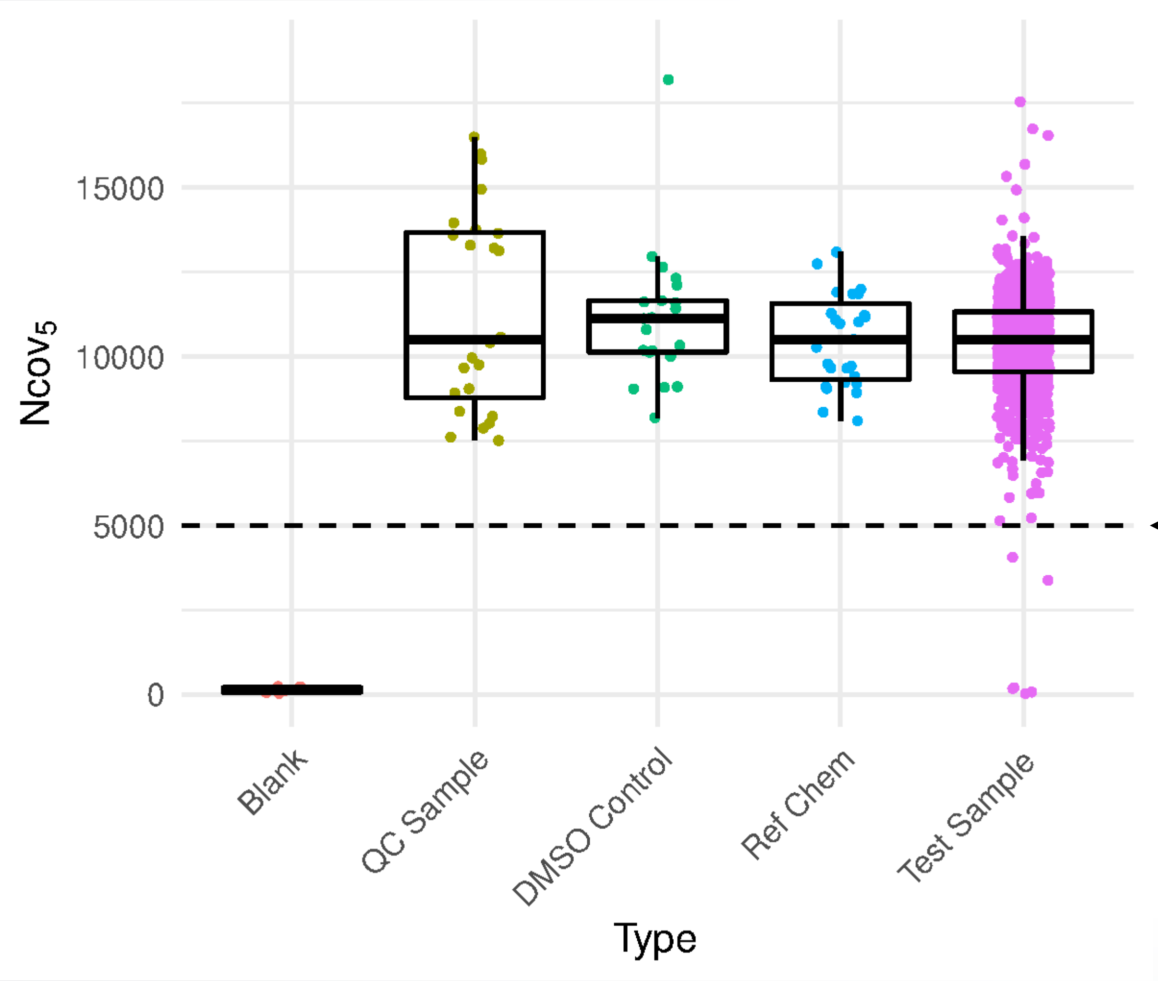
- **Cytotoxicity**
- **Sample degradation**
- **Low input**
- **Assay failure**

QC Metrics: Mapping Rate



- Replicate correlation drops off when <50% of reads mapped uniquely to probe sequences
- Lower mapping rate leads to lower depth
- May also indicate sample quality issues (e.g. RNA degradation or incomplete cell lysis)

QC Metrics: Transcriptome Coverage



$Ncov_5$ = # of probes with at least 5 reads

Threshold = 5,000 Probes (MCF-7)

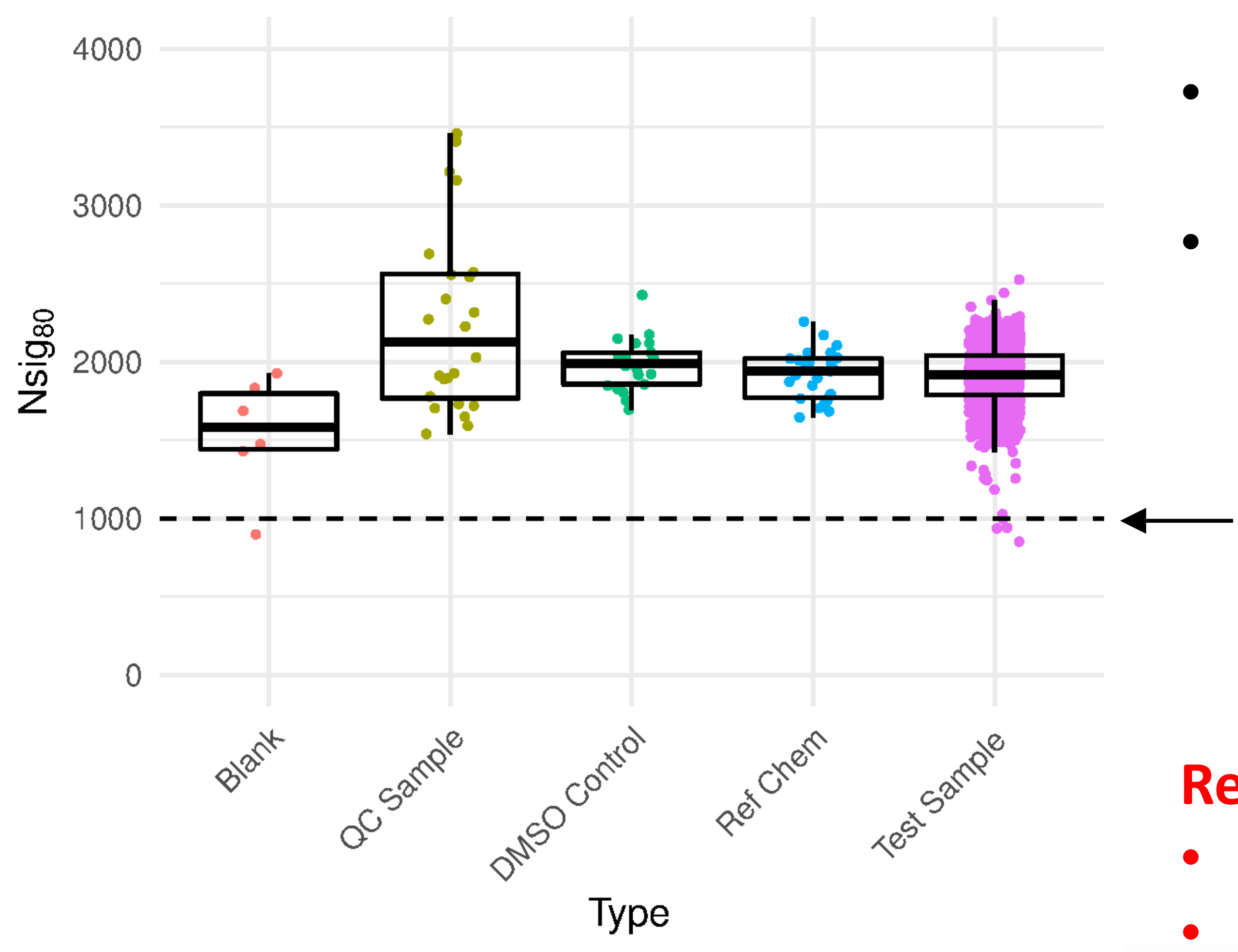
Based on "outer fence" principle (Tukey, 1976)

Re-evaluated on new cell types, probe sets, and attenuation strategies

Reasons for low coverage samples:

- **Low read depth**
- **Sample degradation**
- **Low input**
- **Assay failure**

QC Metrics: Signal Distribution



- **Nsig_{80} = # of probes capturing top 80% of signal**
- **Low values = reads highly concentrated among small number of probes**

Threshold = 1,000 Probes (MCF-7)

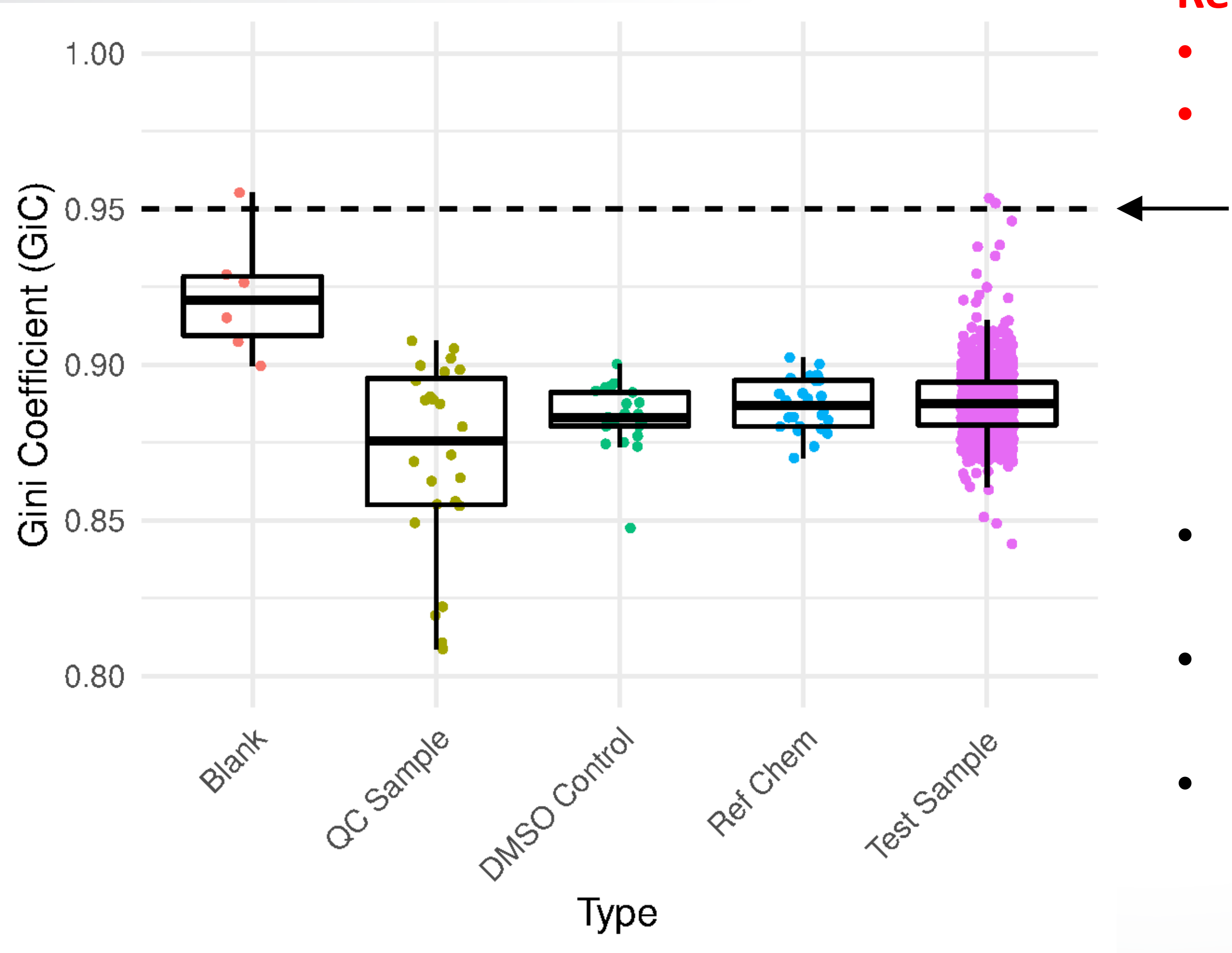
Based on "outer fence" principle (Tukey, 1976)

Should be re-evaluated on new cell types, probe sets, and attenuation strategies

Reasons for low values:

- **Sample degradation**
- **Low input**
- **Assay failure**

QC Metrics: Signal Distribution



Reasons for high values:

- **Sample degradation**
- **Low input**

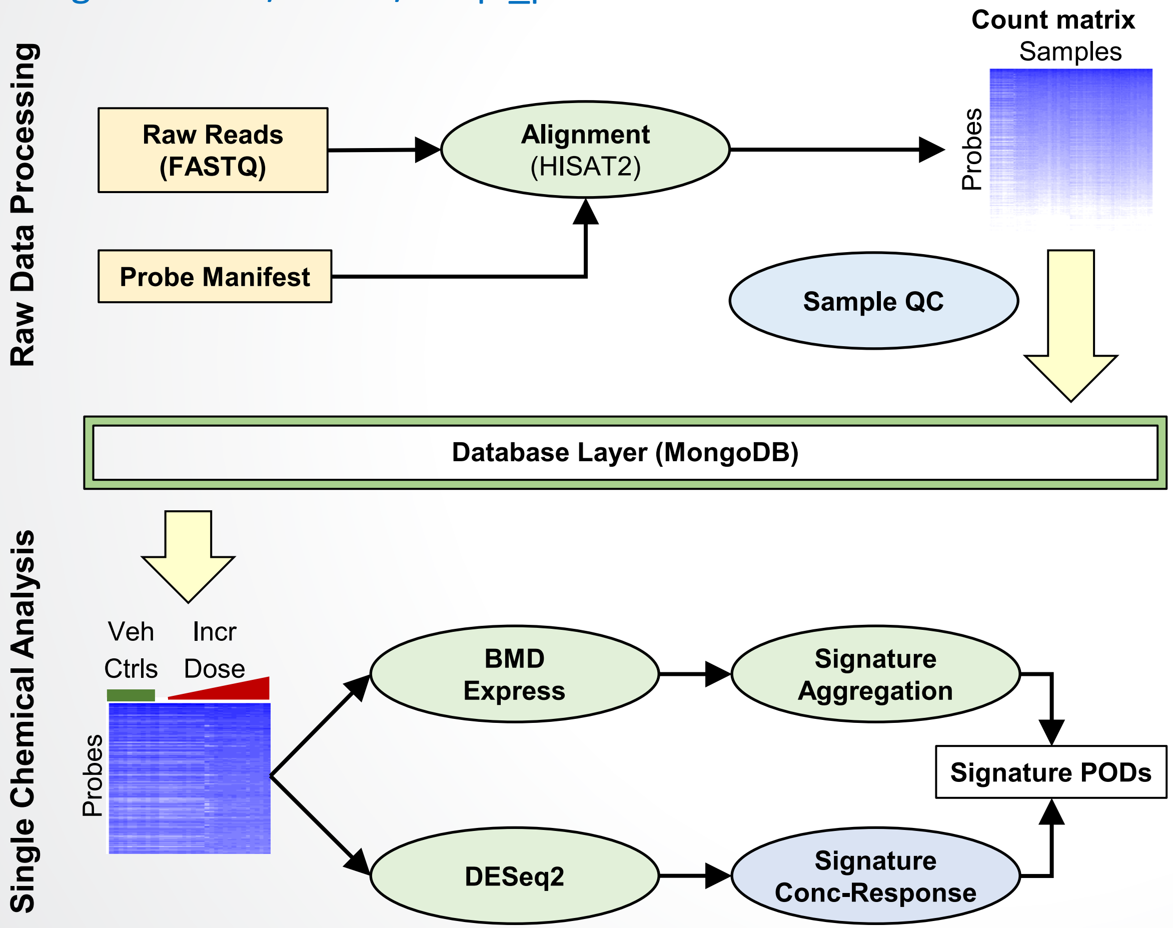
Threshold = 0.95

Based on "outer fence" principle (Tukey, 1976)
Should be re-evaluated on new cell types, probe sets, and attenuation strategies

- **Gini coefficient = measure of inequality or skewness in a distribution**
- **High values = most reads coming from few probes (Max 1: All reads from 1 probe)**
- **Lower values = closer to uniform distribution of reads across all probes (Min 0, not expected for expression data)**
- **Expect samples from same cell type to be similar**

HTTr Bioinformatics Pipeline

github.com/USEPA/httrpl_pilot



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- Rapid processing for large screens
- Many data steps performed independently for each test chemical:
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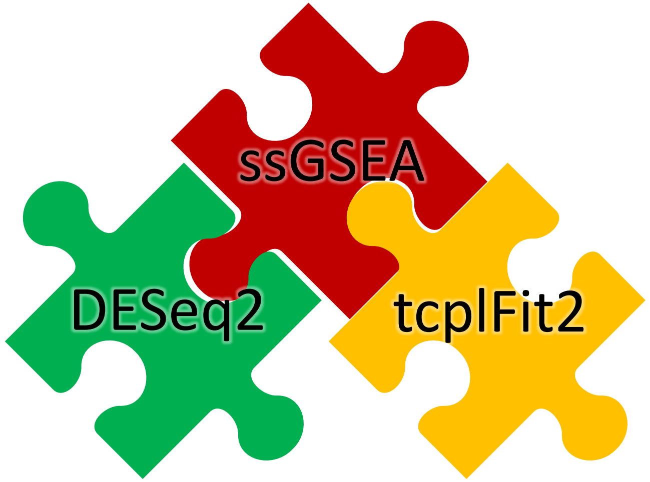
Harrill, et al. Toxicol Sci 2021

3 Flavors of Bioinformatic Approaches

Gene-First Approaches

- BMDExpress (NTP)
- tcplFit2 (CCTE)
- BIFROST (Unilever)

Signature Conc-Response



github.com/USEPA/CompTox-httrpathway

(Richard Judson)

Latent Variable Methods

- Many possible tools, e.g. PLIER, WGCNA
- Identify latent variables in data that capture primary response patterns
- Annotate biological relevance of LVs by gene components
- Perform curve-fitting on LVs
  - Fewer features to fit
  - Compatible with BMDExpress & tcplFit2

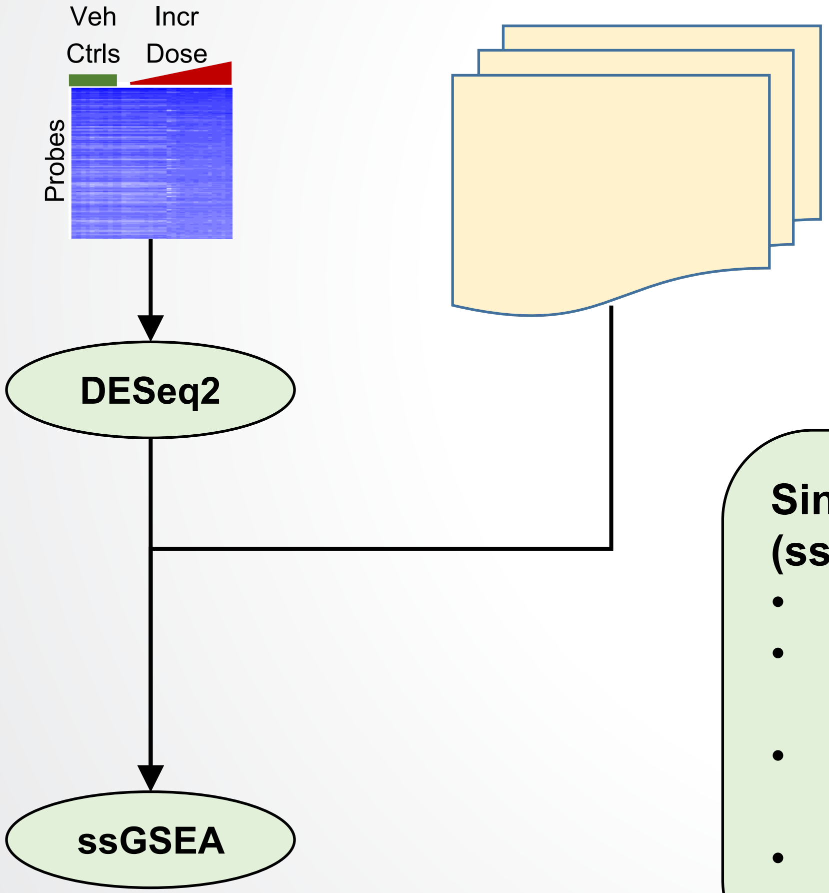
Improved integration through HTTr pipeline & database development

Signature Scoring

Count data per chemical

Catalog of signatures with toxicological relevance, annotated for known molecular targets

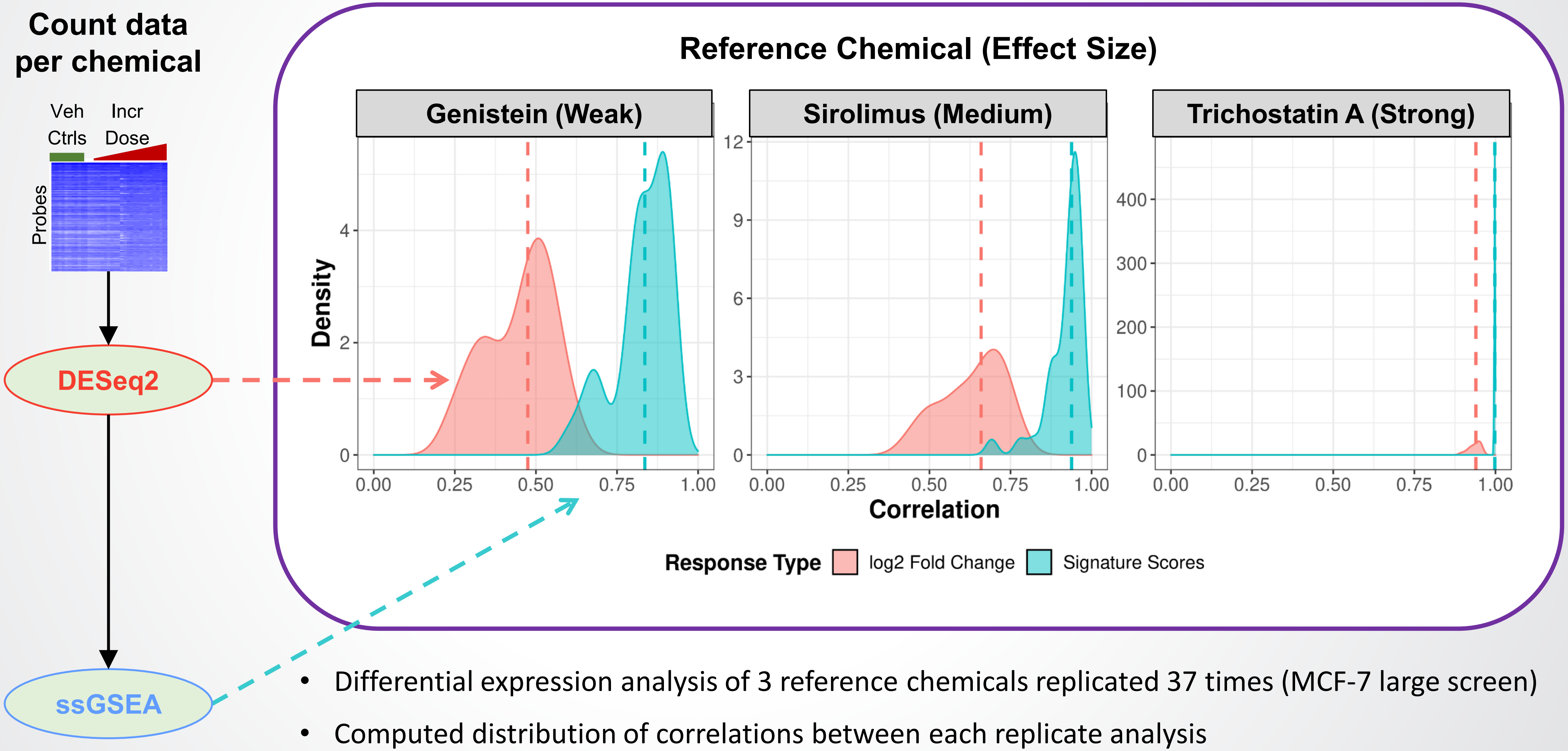
- **Bioplanet** *(Huang, et al. Front Pharmacol 2019)*
- **CMap** *(Subramanian, et al. Cell 2017)*
- **DisGeNET** *(Pinero, et al. Database 2015)*
- **MSigDB** *(Liberzon, et al. Cell Syst 2015)*



Single-Sample Gene Set Enrichment Analysis (ssGSEA) ***(Barbie, et al. Nature 2009)***

- Score coordinated responses at each concentration
- Use moderated log2 FC values from DESeq2 as input (no thresholds)
- Null distributions constructed by resampling log2 FC values from whole screen
- Alternate scoring function: $\text{mean(gene set log2FC)} - \text{mean(background log2FC)}$

Signature Scoring

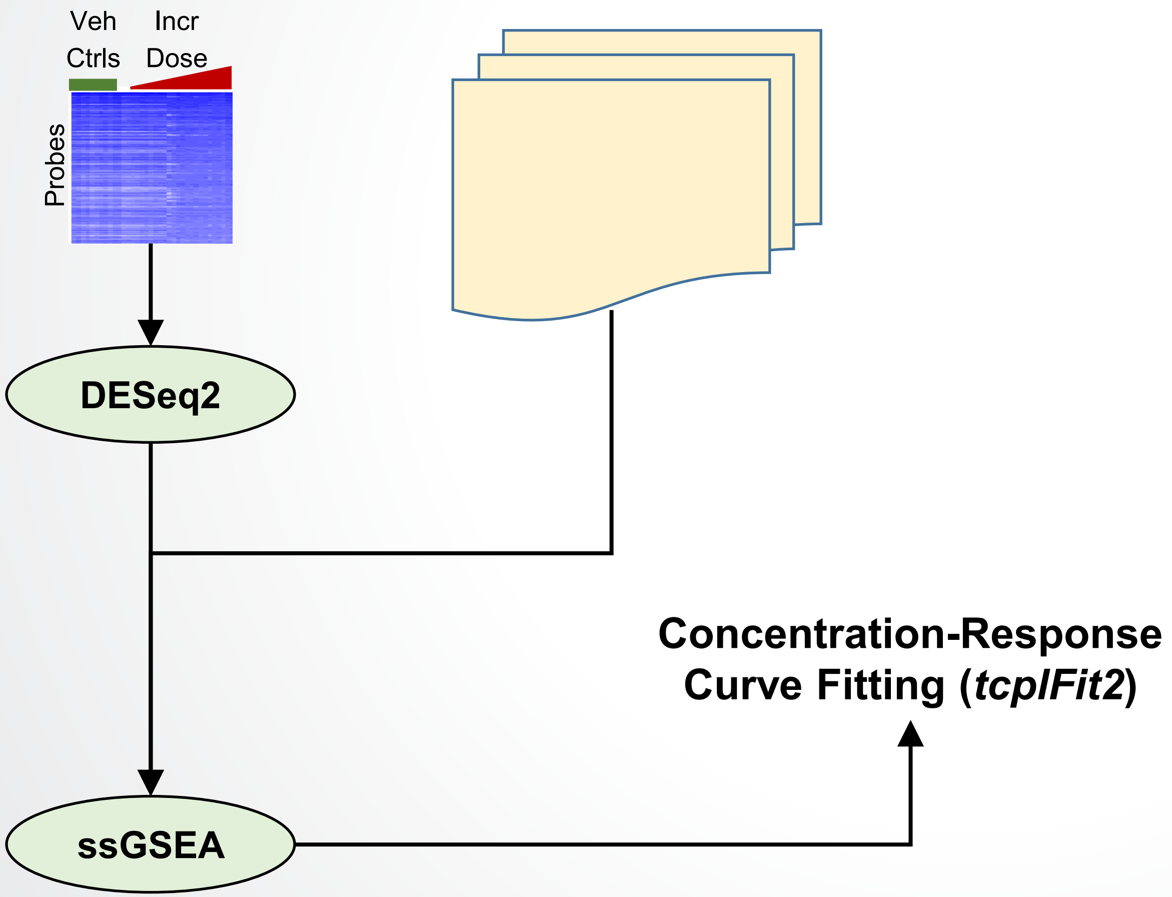


- Differential expression analysis of 3 reference chemicals replicated 37 times (MCF-7 large screen)
- Computed distribution of correlations between each replicate analysis
- **Signature scores have higher reproducibility than fold-changes, especially for weaker effect sizes**

Signature Scoring

Count data per chemical

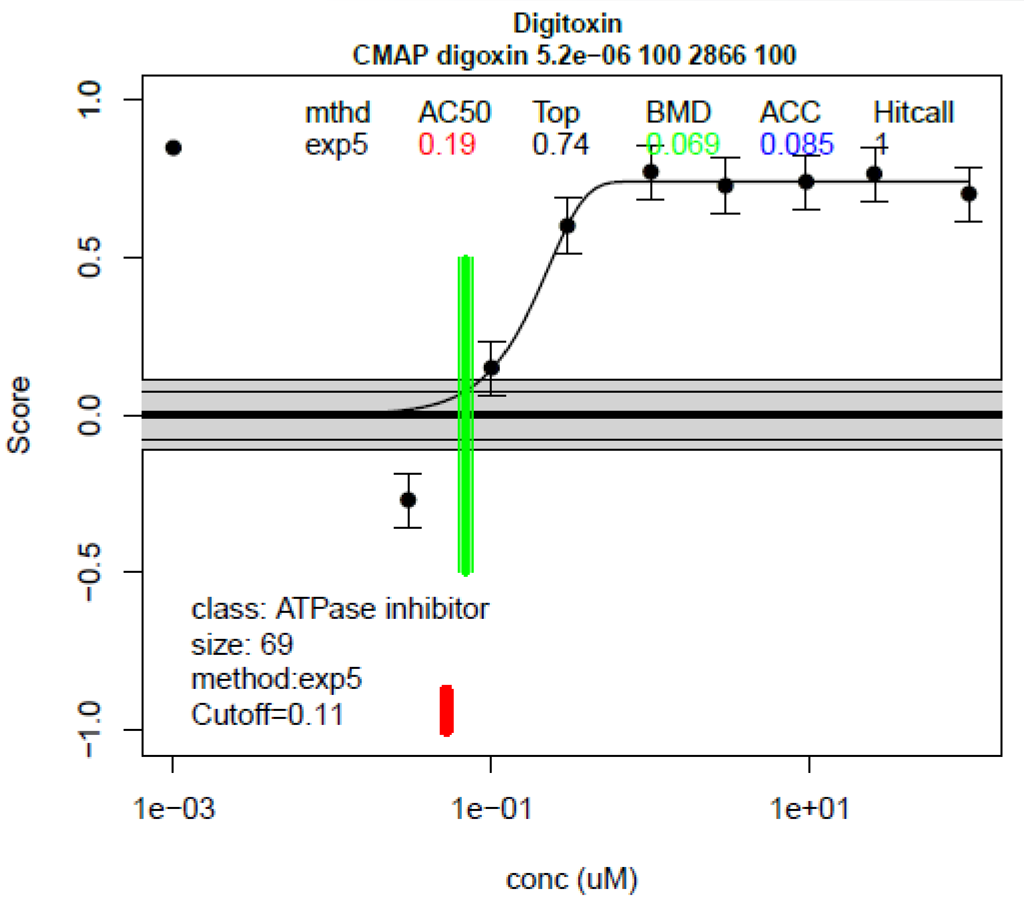
Catalog of signatures with toxicological relevance, annotated for known molecular targets



DESeq2

ssGSEA

Concentration-Response Curve Fitting (*tcplFit2*)



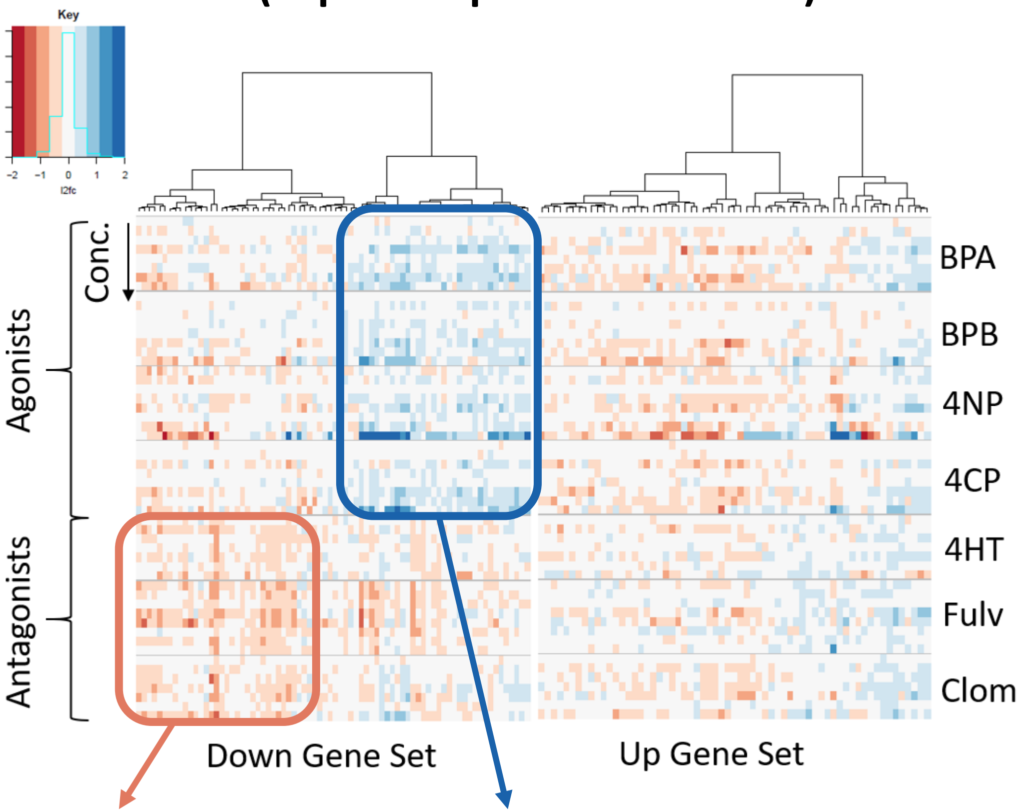
class: ATPase inhibitor
size: 69
method:exp5
Cutoff=0.11

Richard Judson

Directionality of Signature Scores

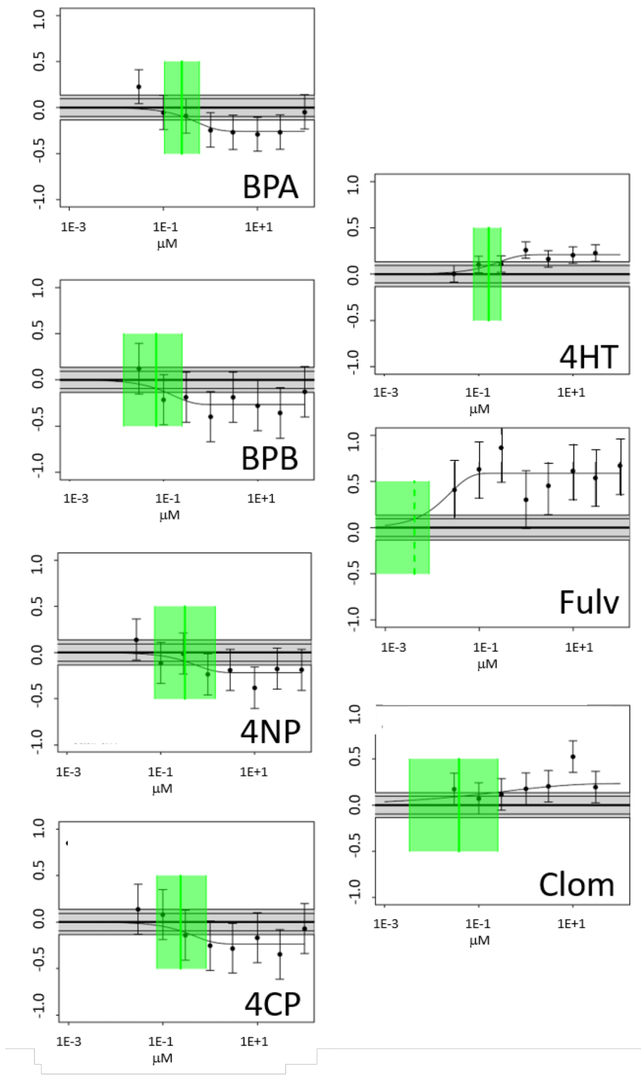
Fulvestrant Signature from CMap (Top 100 Up & Down Genes)

Gene level data are noisy!



The expression of some fulvestrant signature “down” genes goes <u>down</u> following ER antagonist treatment

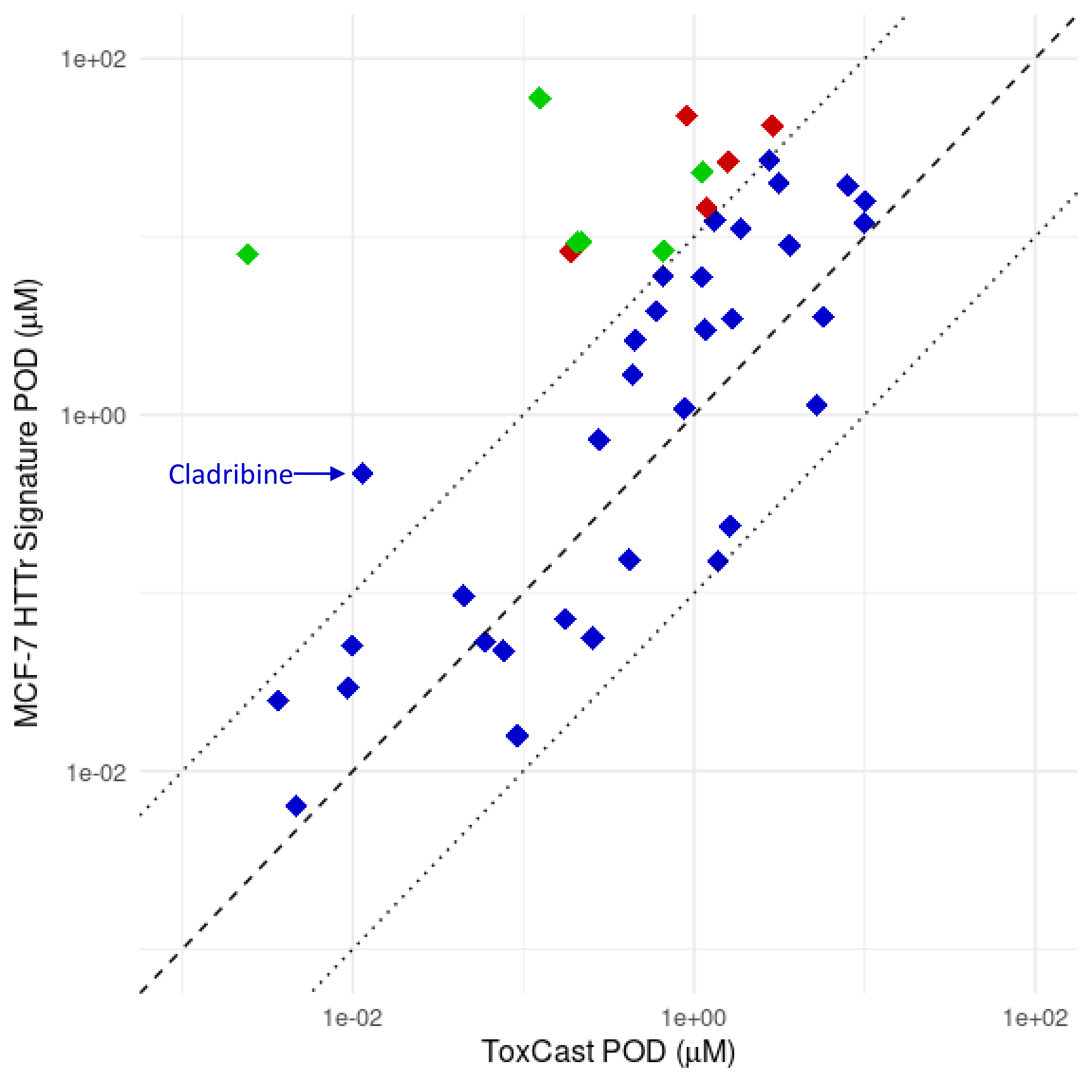
The expression of some fulvestrant signature “down” genes goes <u>up</u> following ER agonist treatment



Signature level results display correct directionality!

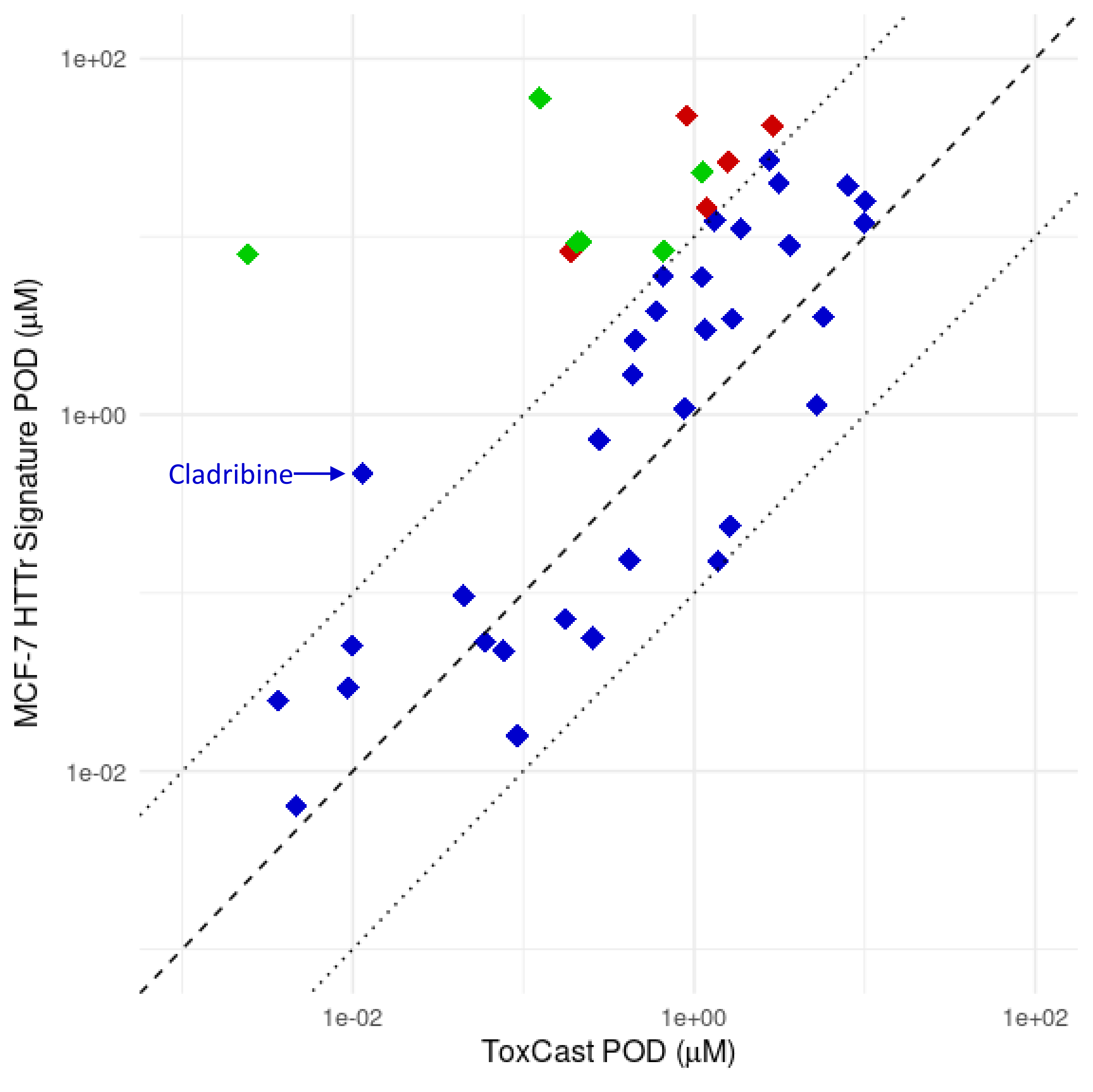
Harrill et al. (2021) DOI: 10.1093/toxsci/kfab009

HTTr MCF-7 Pilot Analysis



- Pilot study of 44 well-characterized chemicals in MCF-7 cells, 6h exposure ***(Harrill, et al. Toxicol Sci, 2021)***
- Compared HTTr-derived PODs to previous ToxCast HTS assay results *(multiple cell types, assays, and exposure lengths)* (***Paul-Friedman, et al. Toxicol Sci 2020)***
- Signature-based POD are highly concordant with ToxCast results for the majority of test chemicals in pilot study

HTTr MCF-7 Pilot Analysis



- 6 chemicals with targets that have low/absent expression in MCF-7 cells
  - 3,5,3’-triiodothyronine (Thyroid Receptor)
  - Cyproconazole (pan-CYP inhibitor)
  - Butafenacil (pan-CYP inhibitor)
  - Prochloraz (pan-CYP inhibitor)
  - Imazalil (pan-CYP inhibitor)
  - Propiconazole (pan-CYP inhibitor)
- 5 chemicals where most potent assays in ToxCast do not match known target(s)
  - Lovastatin
  - Clofibrate
  - Maneb
  - Lactofen
  - Vinclozolin
- Cladribine (2-chloro-2’-deoxyadenosine) is a DNA synthesis inhibitor
- All other PODs within 1 order of magnitude

Conclusions

- EPA/ORD has developed reliable and cost-efficient workflow for generating HTTr data from thousands of chemicals across multiple cell lines

- Preliminary/pilot analysis demonstrates that overall results are concordant with previous assays (ToxCast/HTS) and known chemical targets
  Harrill, et al. Toxicol Sci 2021

- Ongoing research efforts focused on:
  - Methods to summarize signature-level/overall PODs from high-dimensional data
  - Inference of underlying mechanism (e.g. Connectivity Mapping)
  - Comparative evaluation of methods on simulated/synthetic conc-response data

Acknowledgements

Questions?

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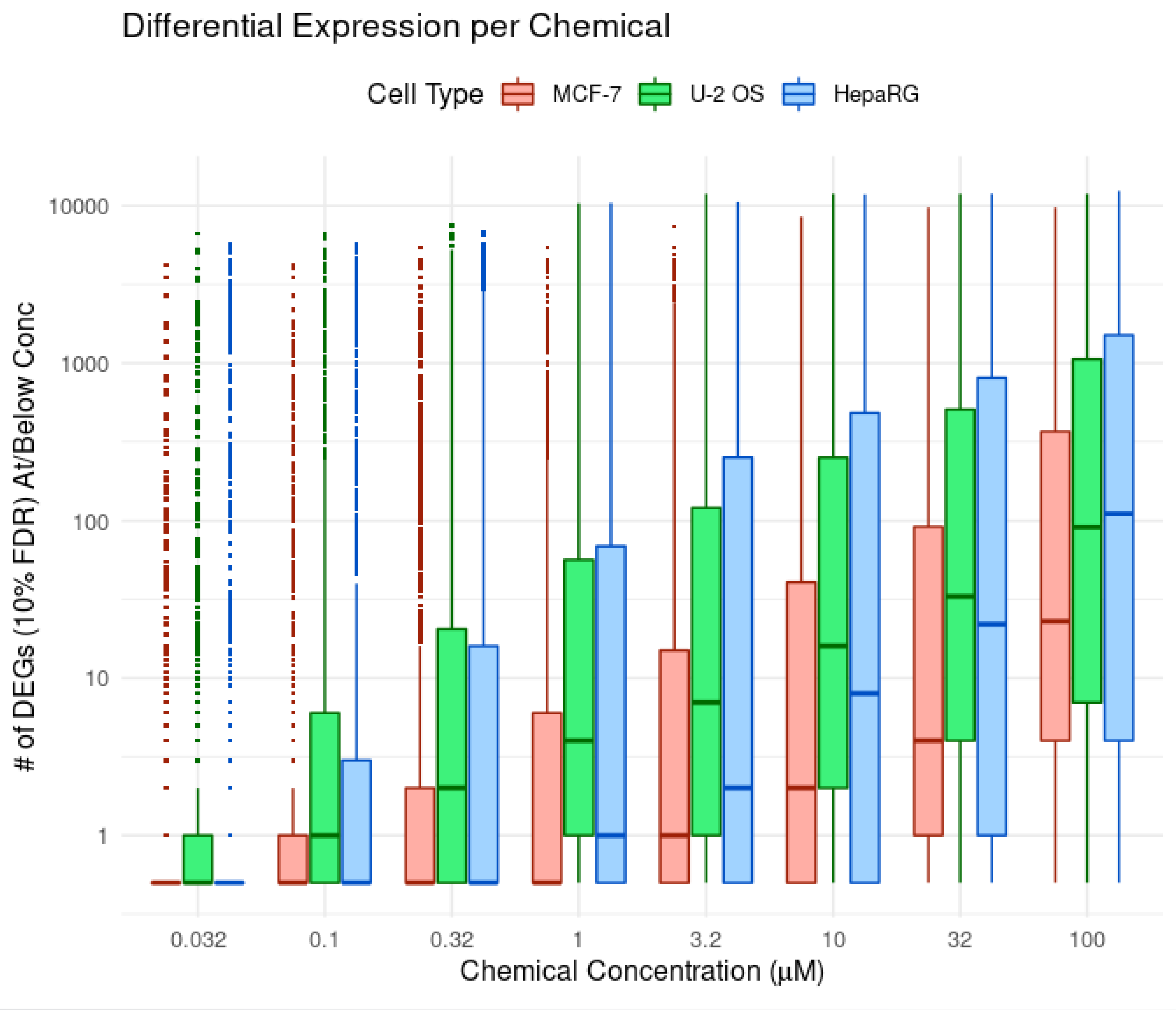
CCTE Leadership

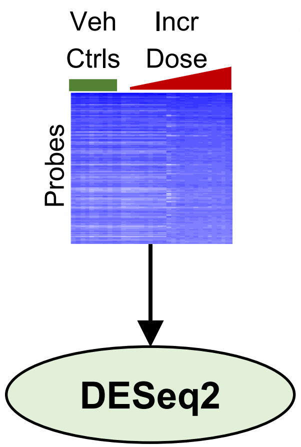
Rusty Thomas
Sid Hunter
Andrew Watkins
John Cowden
Kimberly Slentz-Kesler

Center for Computational Toxicology and Exposure
Biomolecular and Computational Toxicology Division

Global View of Bioactivity



Count data for single chemical (vehicle controls + 8 concs x 3 reps)

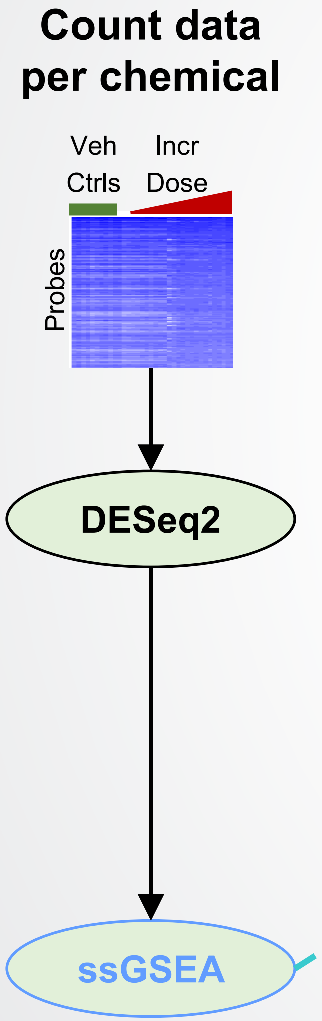


- Statistical model tailored to *-seq data
- Remove plate-level effects
- Smooths noise across depth & expression levels

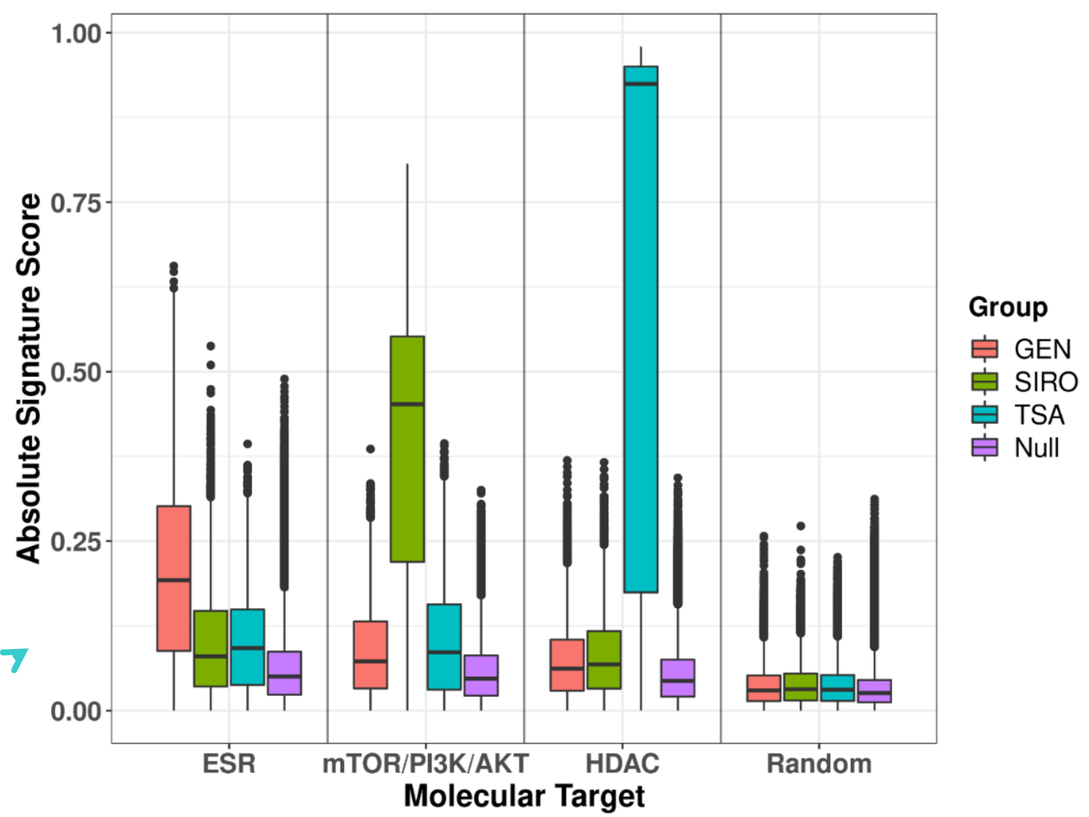
(Love, et al. Genome Biol 2014)

- Each boxplot shows distribution of Differentially Expressed Gene (DEG) count per chemical
- **Primarily interested in transcriptional changes that:**
  - **Are coordinated across known pathways/gene sets**
  - **Fit standard curve-models across all concentrations**

Signature Scoring

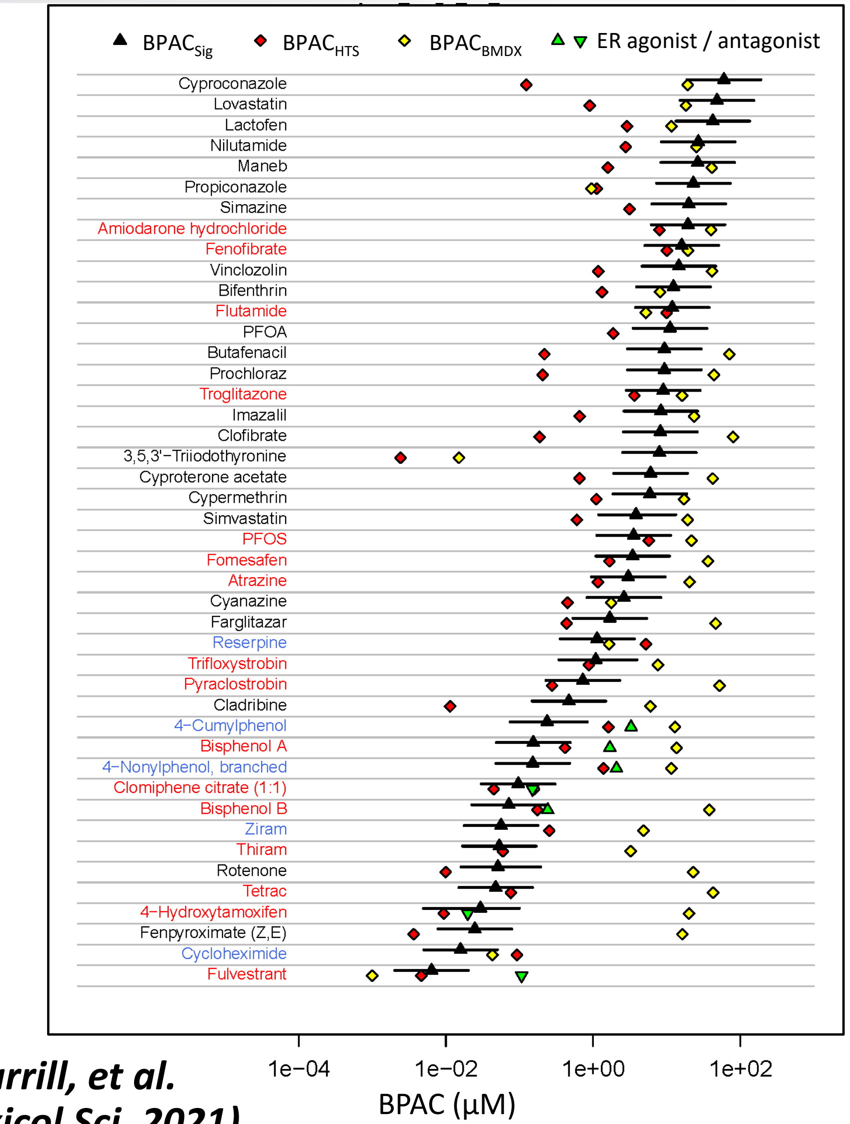


Reference Chemical Signature Specificity



- **Higher scores for signatures corresponding to known targets of each reference chemical**

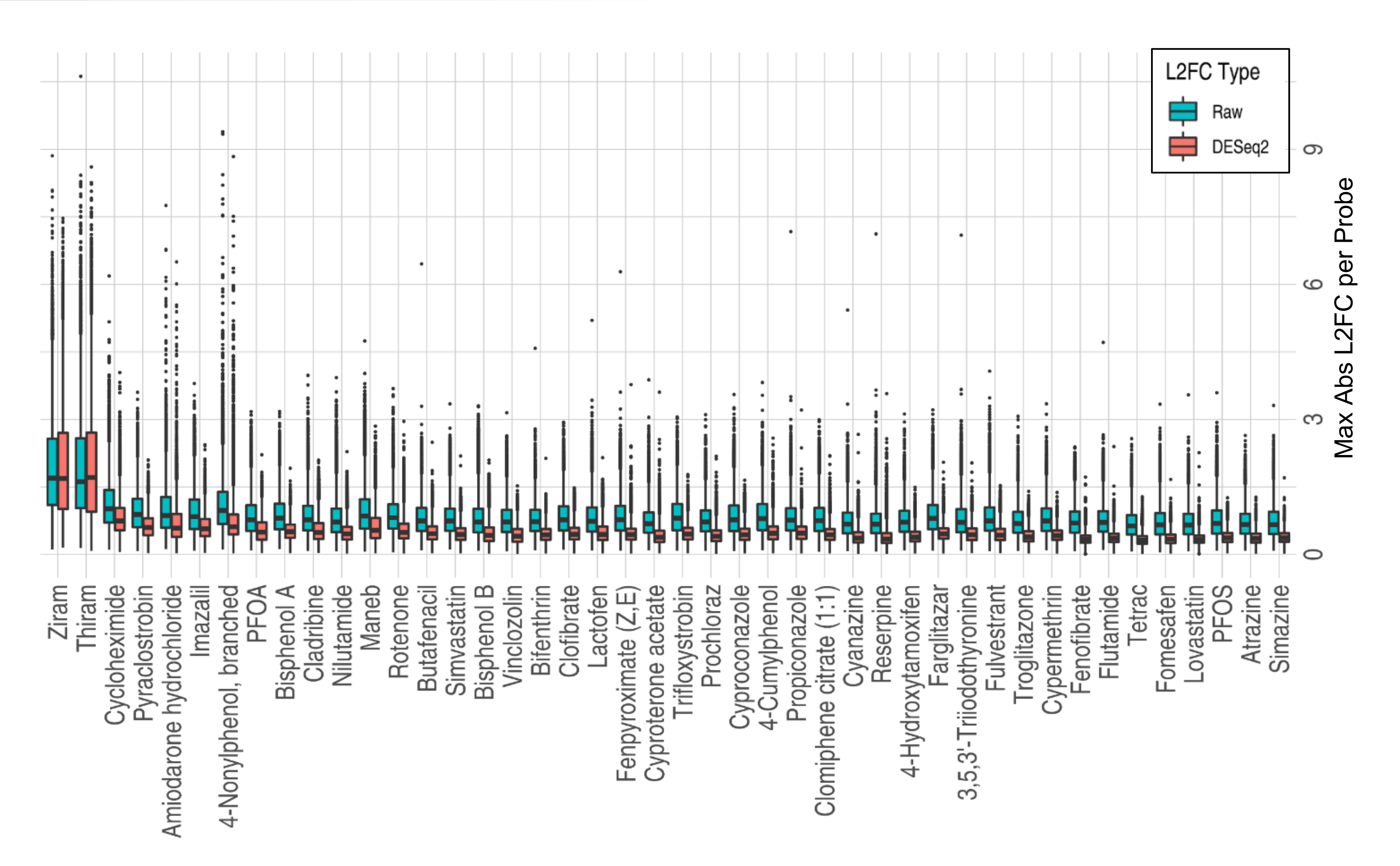
HTTr MCF-7 Pilot Analysis



(Harrill, et al. Toxicol Sci, 2021)

- Also calculated BPAC/PODs using NTP approach with BMDExpress2 ***(NTP Research Report 5, 2018; Phillips, et al. 2019)***
- BPAC$_{BMDX}$ (◇) tended to be higher and less concordant with ToxCast PODs
  - Poor signal:noise at gene-level is likely cause
- ***We continue to use BMDExpress for other transcriptomics applications and continue to explore this issue***

HTTr MCF-7 Pilot Analysis

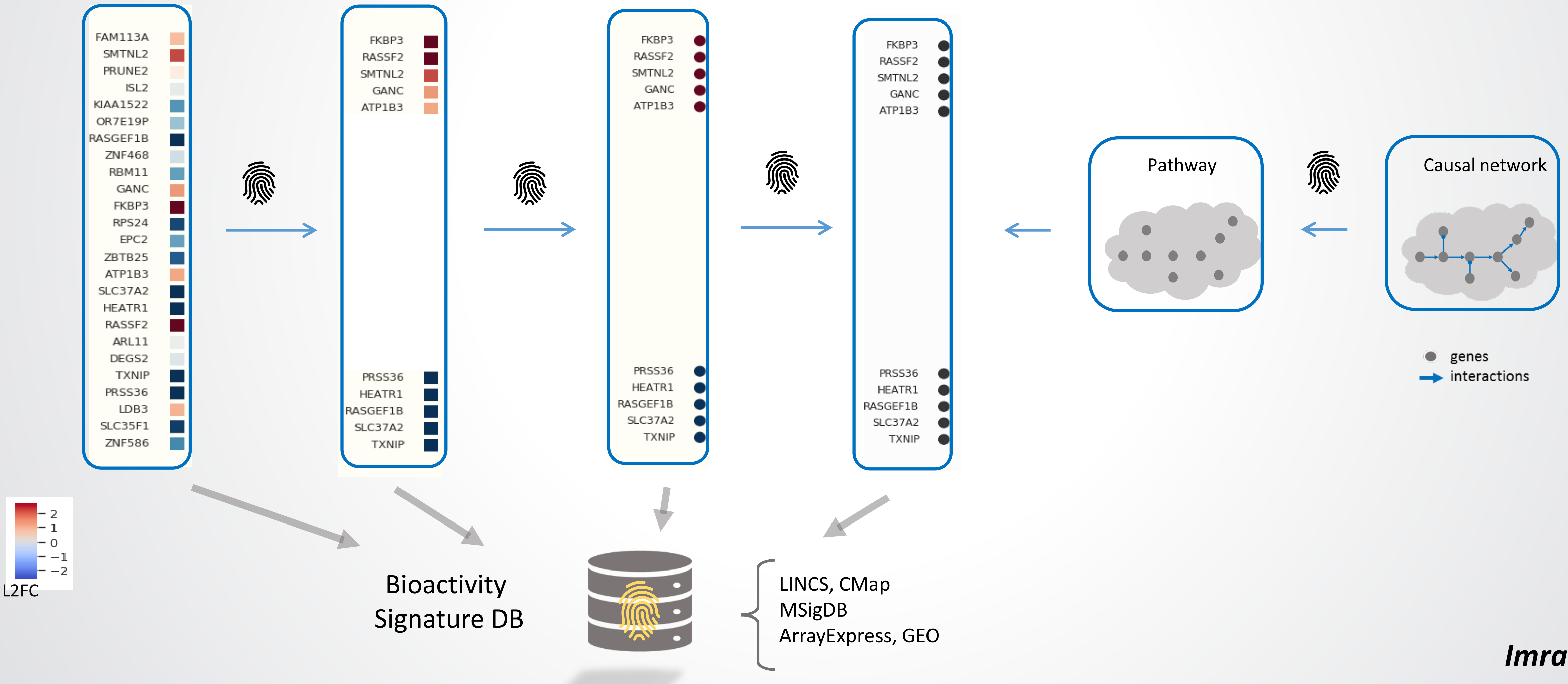


(Harrill, et al. Toxicol Sci, 2021)

- Majority of differential expression is weak (2-4x) for most chemical treatments
  - DESeq2 dampens these further in most cases
- Consistent with previous studies using MCF-7 cells
- Lower effect size results in lower signal:noise
- Signature-level scores (e.g. GSEA) may perform better than probe-level when this is the case

“Fingerprinting” bioactivity via gene signatures

Reference Profiles | Top N Up-/Dn-genes | Directional Signature | Signature | Pathway | Network

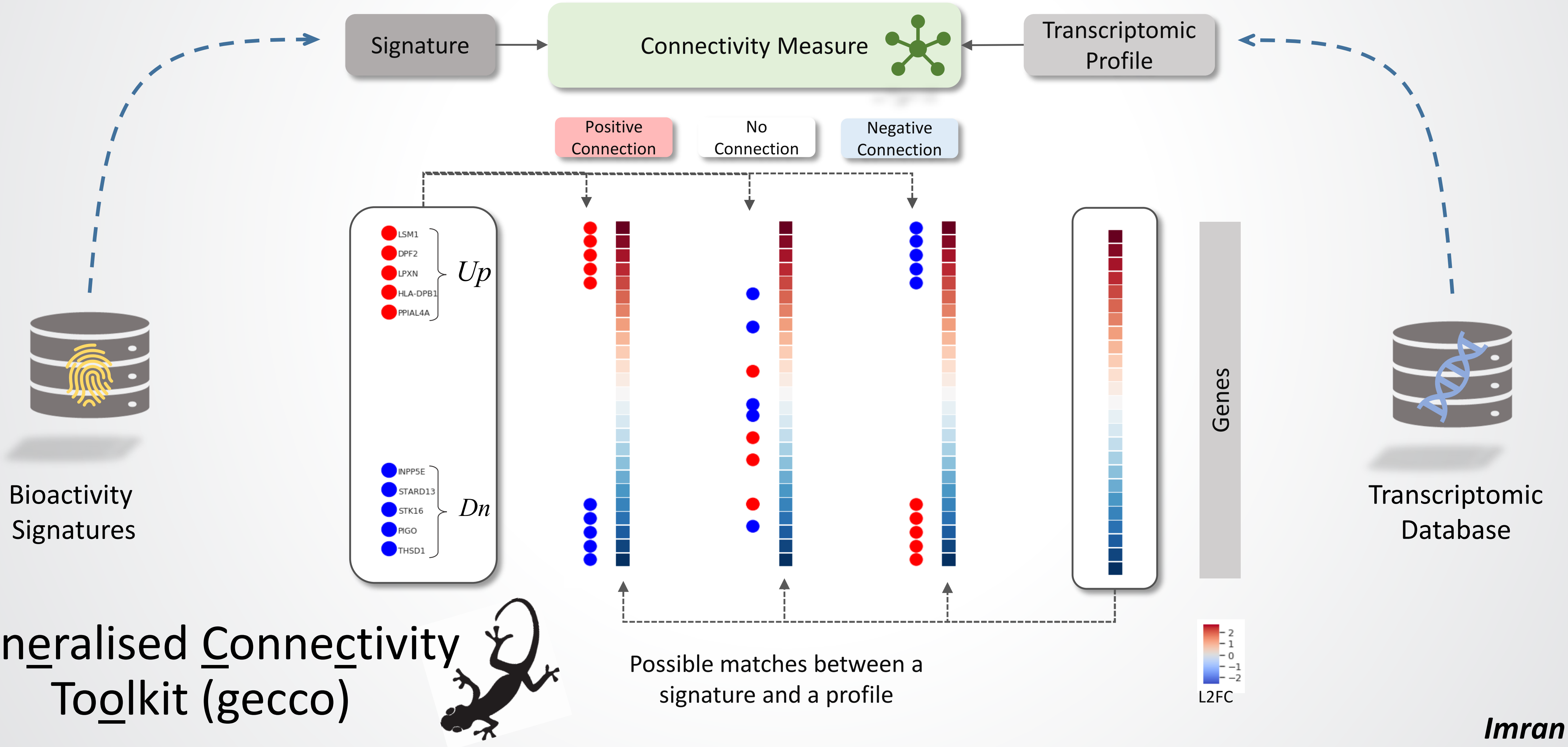


Bioactivity Signature DB

LINCS, CMap
MSigDB
ArrayExpress, GEO

Imran Shah

Connectivity-mapping with gene signatures



Possible matches between a signature and a profile

Generalised Connectivity Toolkit (gecco)

Imran Shah