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Determining Transcriptional Points of Departure Using a Whole Transcriptome Screening Assay

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Disclaimer

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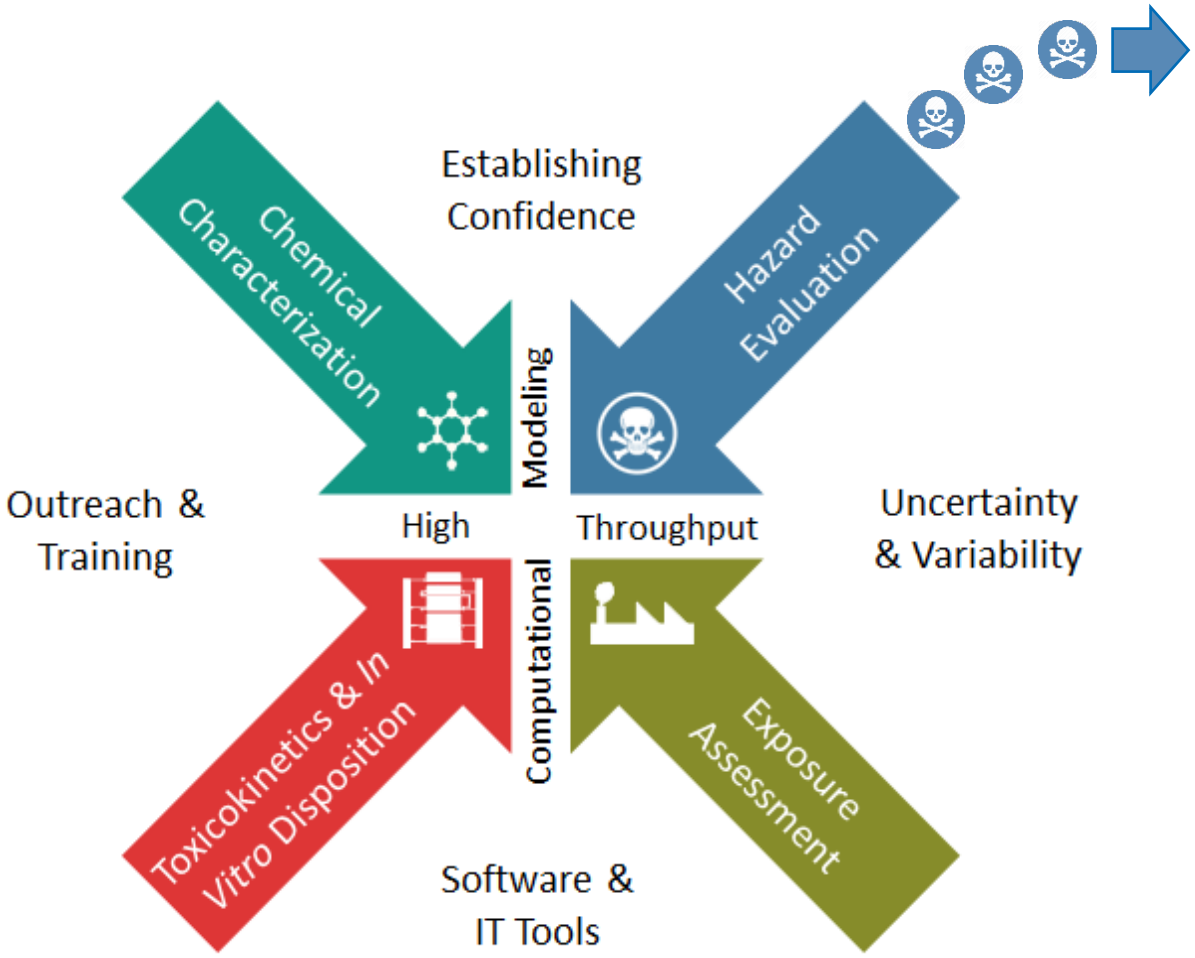
Computational Toxicology Research Areas

CCTE research programs focus on developing the **tools, approaches and data** needed to accelerate the pace of chemical risk assessment and foster incorporation of non-traditional toxicity testing data into regulatory decision-making processes.



The NexGen Blueprint of CompTox as USEPA
Tox. Sci. 2019; 169(2):317-322

- **ToxCast:** Use of targeted high-throughput screening (HTS) assays to expose living cells or isolated proteins to chemicals and assess bioactivity and potential toxic effects.

	# of assays	# of chemicals	Types of chemicals
Phase 1 ***(2007 – 2009)***	500	300	Mostly pesticides
Phase 2 ***(2009 – 2013)***	700	2,000	Industrial, consumer product, food use, "green" chemicals

- Mostly targeted assays (*chemical X → target Y*)
- Incomplete coverage of biological space.

- **New Approach for Hazard Evaluation:** Employ broad-based (i.e. non-targeted) **profiling assays** that cast the broadest net possible for capturing the potential molecular and phenotypic responses of human cells in response to chemical exposures.

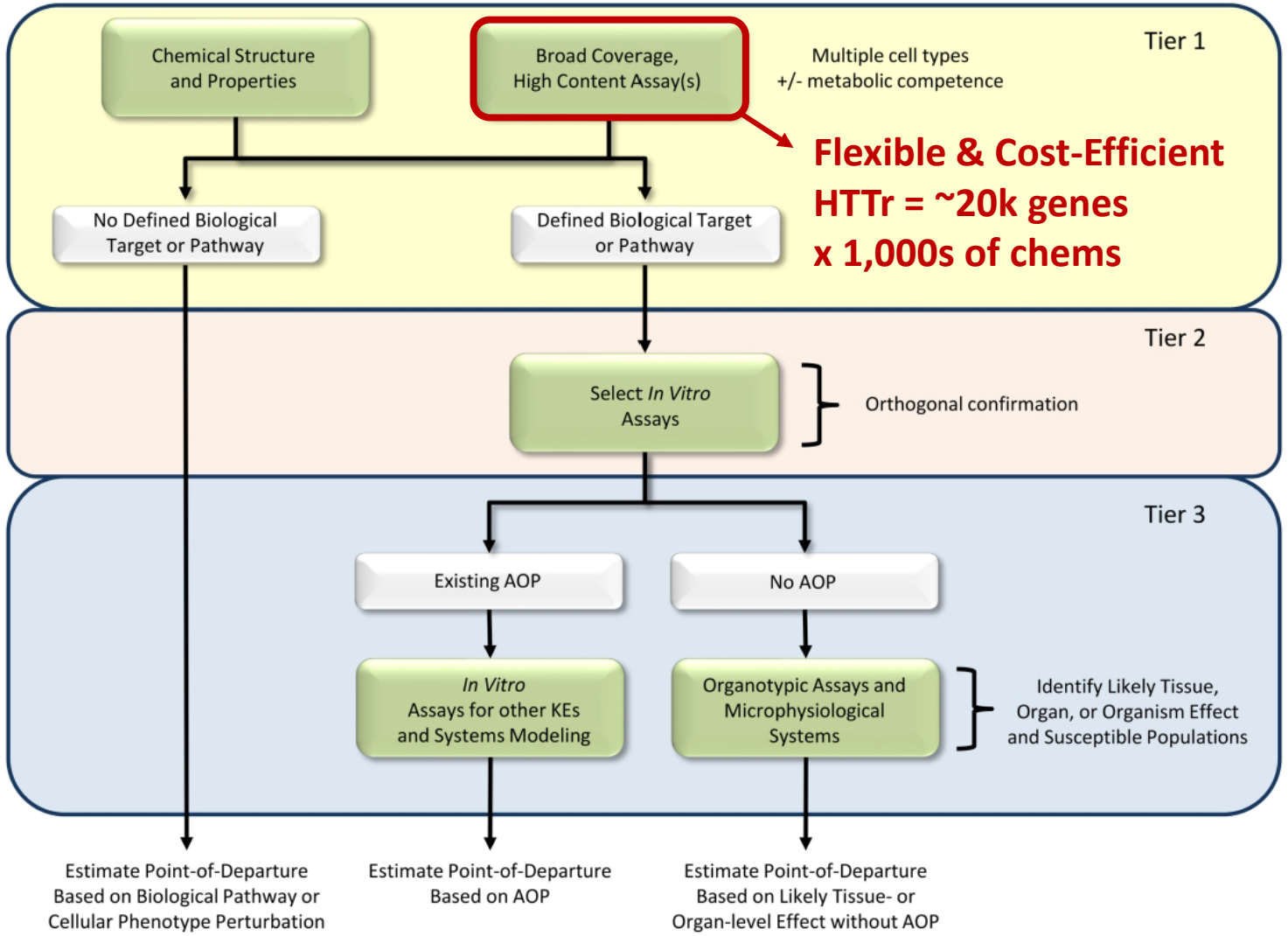
Tiered Chemical Safety Testing Strategy

Tier 1 Primary Goals:

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

HTTr Key Challenges:

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



Thomas, et al. Toxicol Sci 2019

Automated *in vitro* Chemical Screening

Cryopreserved Cell Stocks

Cell Expansion

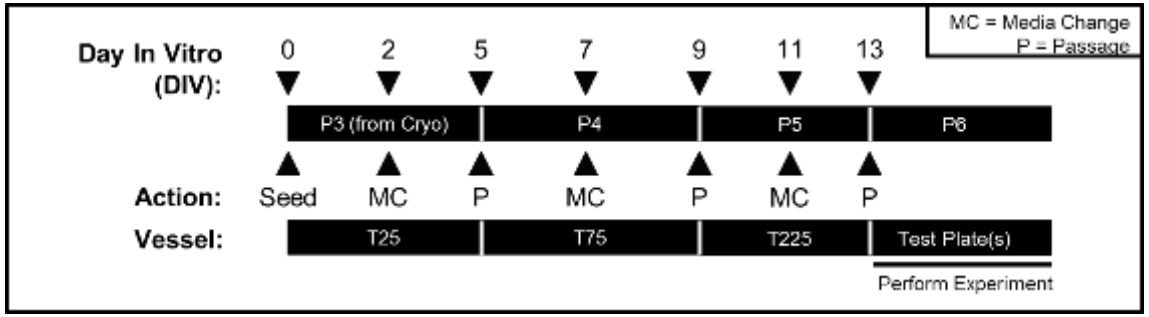
Cell Plating

BioTek
MultiFlo ™ FX

Dispensing Test Chemicals

LabCyte Echo® 550
Liquid Handler

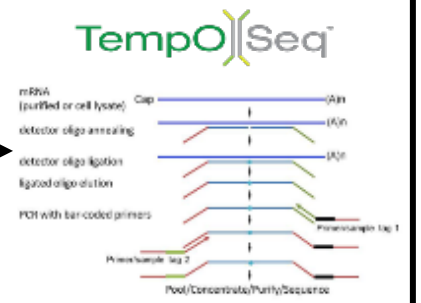
Standardized Expansion Protocol



Track 1: Targeted RNA-Seq

Generating Cell Lysates

TempO-Seq WT



Track 2: Apoptosis / Cell Viability

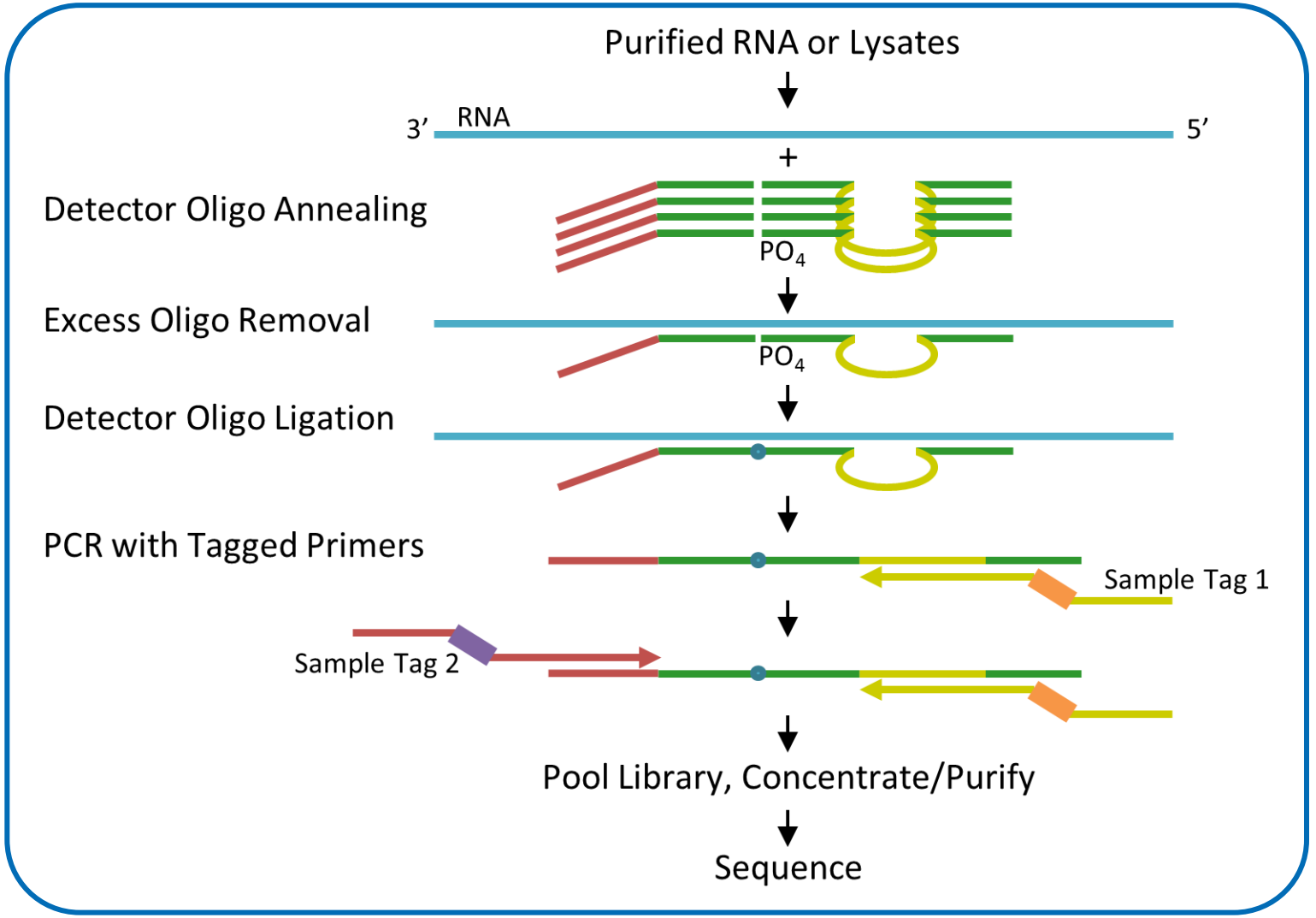
Reagent Dispensing

High Content Imaging

Perkin Elmer
Opera Phenix™
High Content Screening System

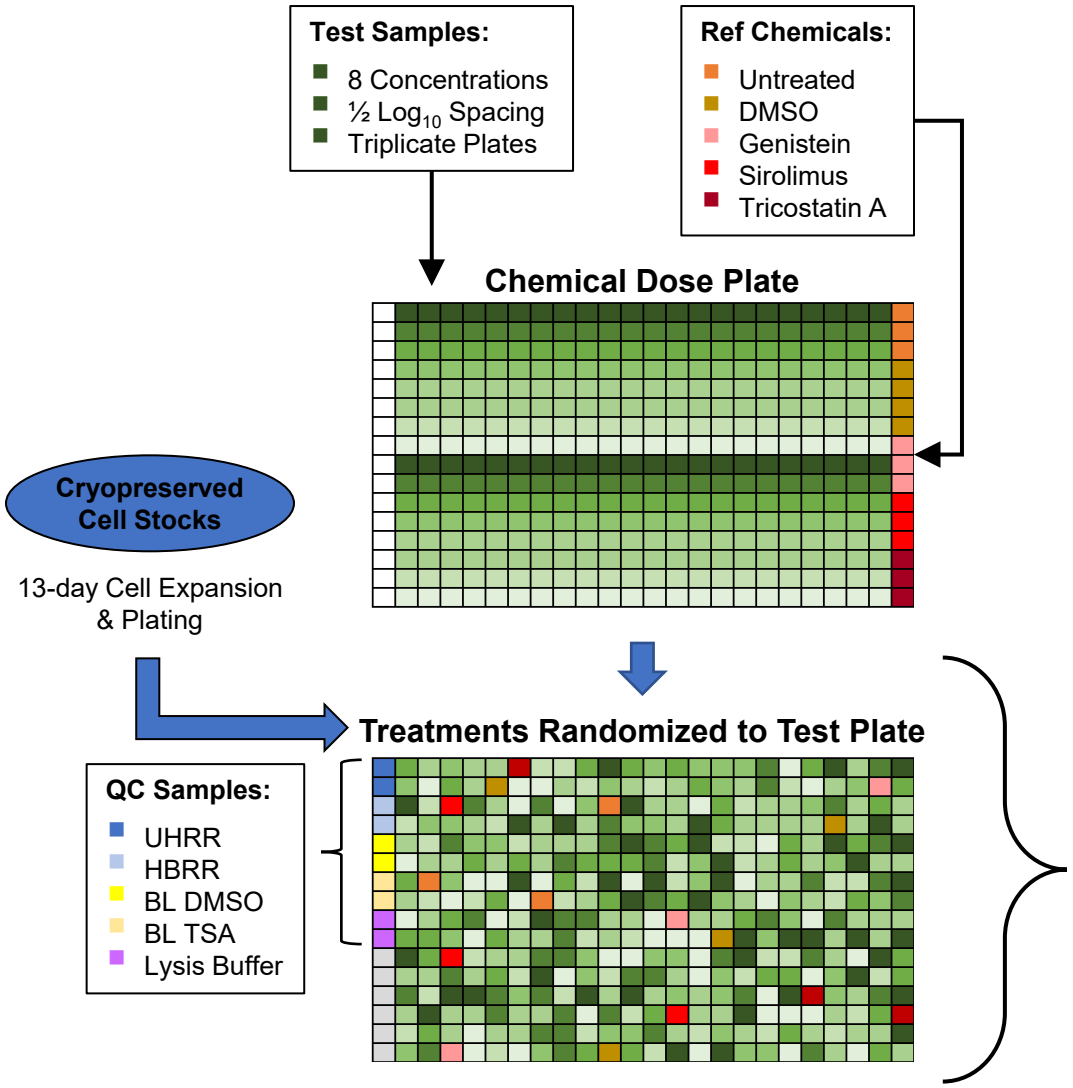
High-Throughput Transcriptomics (HTTr) Assay

- Targeted RNA-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

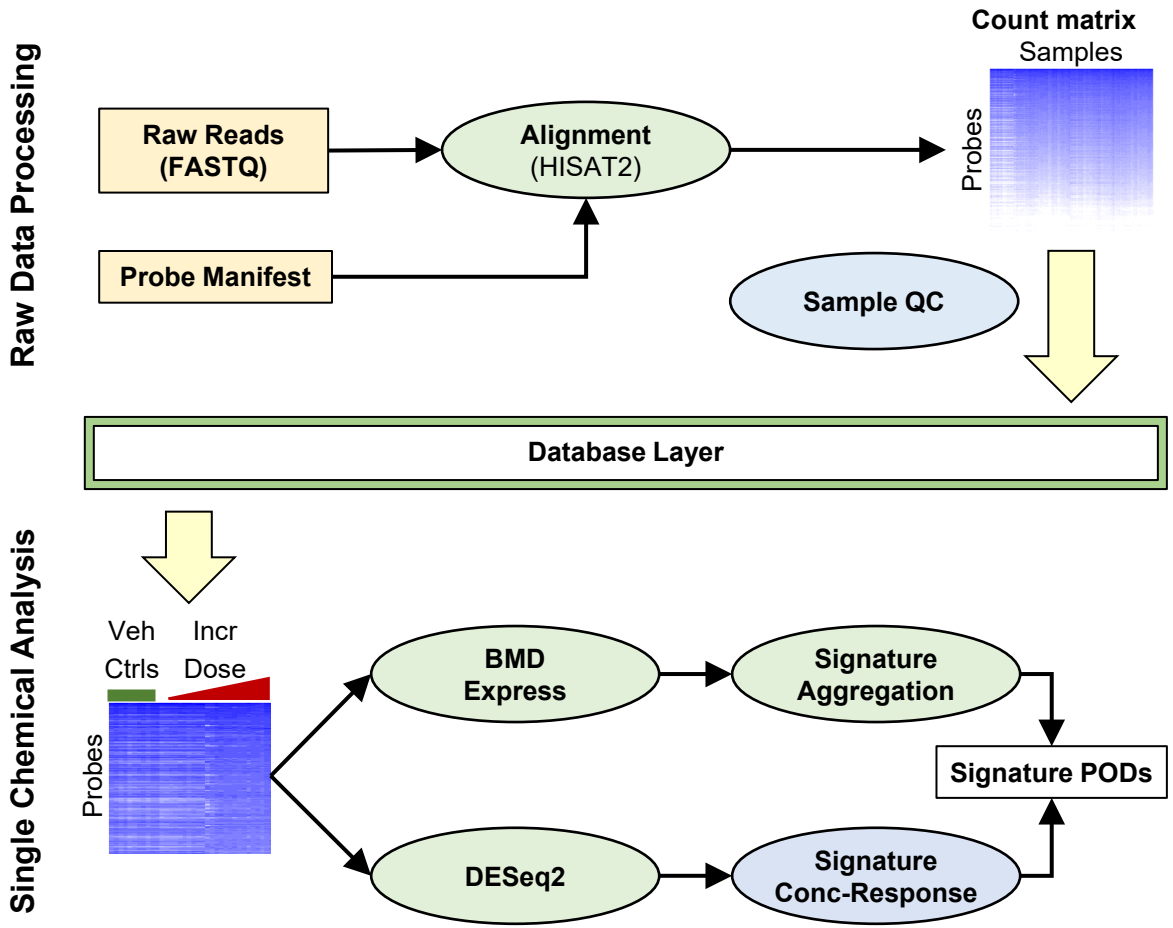
HTTr Study Design



- 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

HTTr Bioinformatics Pipeline



- 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 MCF-7 Screen: Experimental Design

Parameter	Multiplier	Notes
Cell Type(s)	1	MCF-7
Culture Condition	1	DMEM + 10% HI-FBS [a]
Chemicals	2,112[b]	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; ~half-$\log_{10}$ spacing
Biological Replicates:	3	--

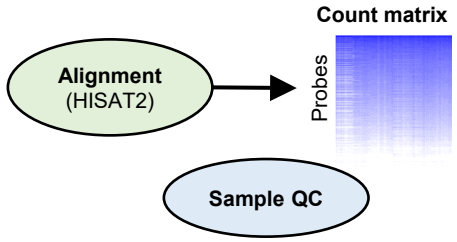
Reference **Samples** and Reference **Chemicals**:

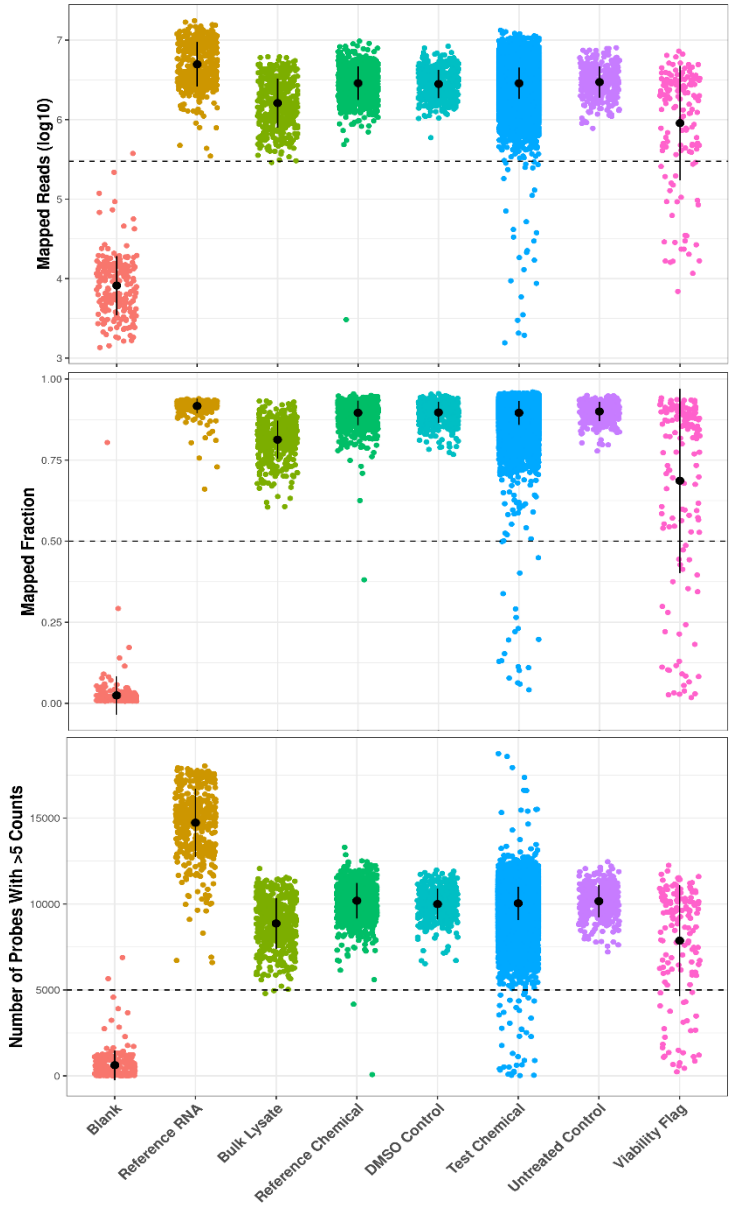
- **Reference RNA** – UHRR and HBRR
- **Bulk Lysate Preparations** – DMSO vehicle control and Trichostatin A
- **Reference Chemicals** – Genistein, Sirolimus, and Trichostatin A

[a] MCF7 cells cultured in DMEM + 10% HI-FBS was selected

[b] Due to reagent error, one experimental block was removed leaving **1577 unique chemicals** across 37 triplicate test plates

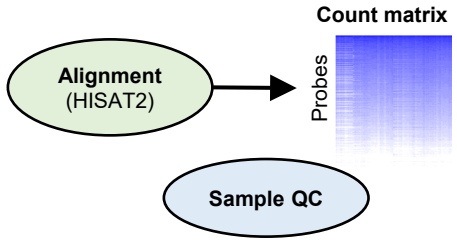
MCF-7 Screen Sample Quality



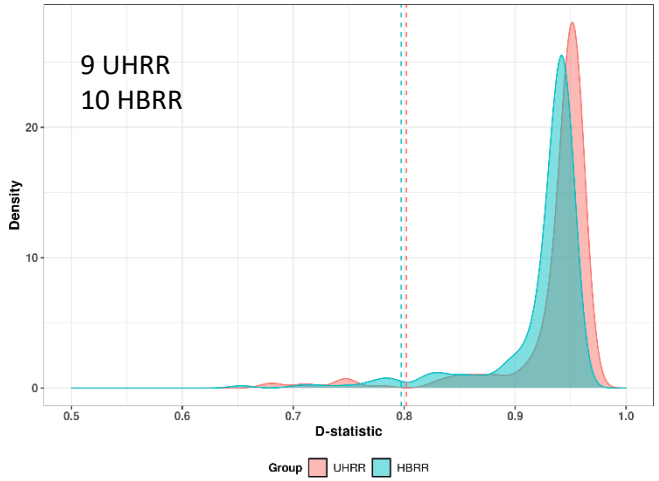


- Five alignment-based quality control metrics are estimated from raw count data
- Sample below thresholds are removed from analysis
- The parallel cell viability/apoptosis assay is used to remove samples due to cytotoxicity
- A total of **~98%** of all samples passed initial QC

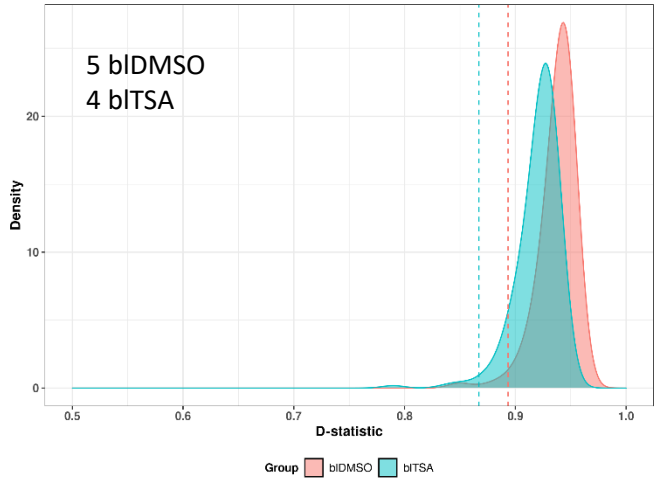
MCF-7 Reference Count Reproducibility



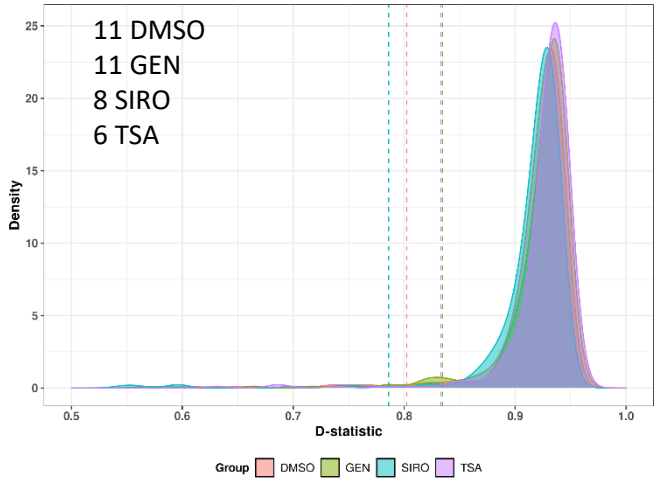
Reference RNA



Bulk Lysate



Reference Chemicals



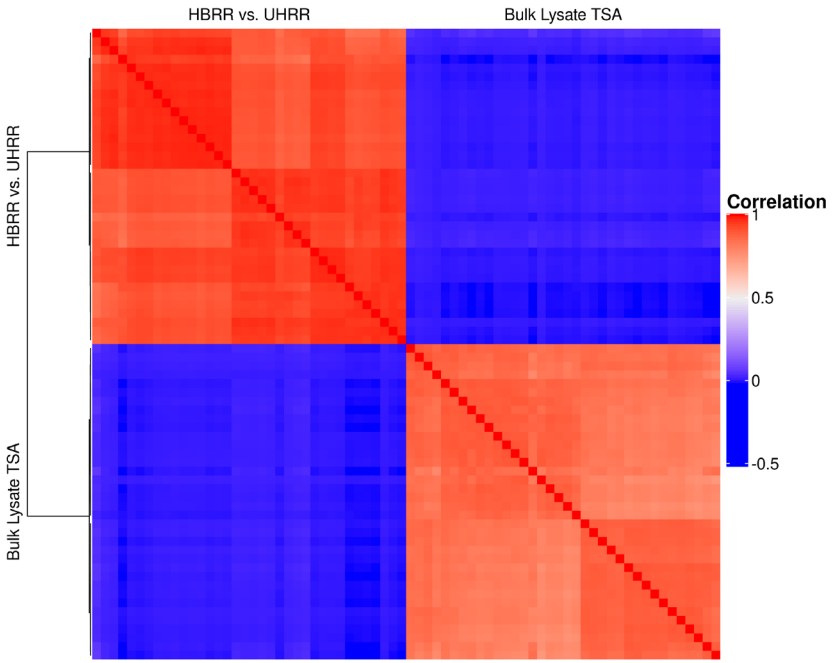
D-Statistic for Outlier Identification (*House et al., Front Genet 2017*)

- Counts were converted to $\log_2$ counts-per-million (CPM)
- D-statistic calculated as the average correlation of a reference sample or chemical against all other replicate wells of the same sample type

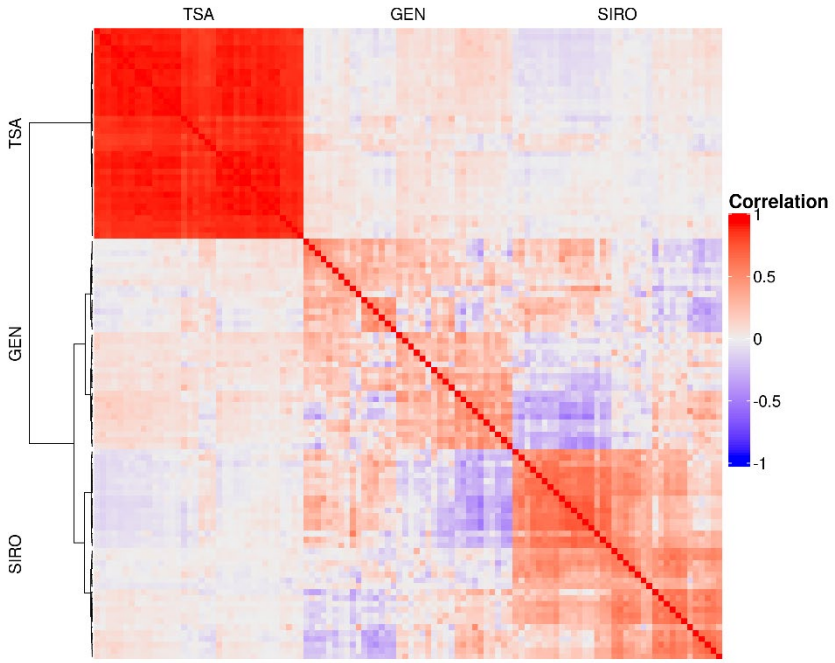
- Computed distribution of the D-statistic for each reference sample and chemical type
- Outliers defined as 3 SDs below median D-statistic
- Count-level quality metrics alongside the added D-statistic approach demonstrated **96.8%** of all reference samples and chemicals passed quality control

HTTr Fold Change Estimation

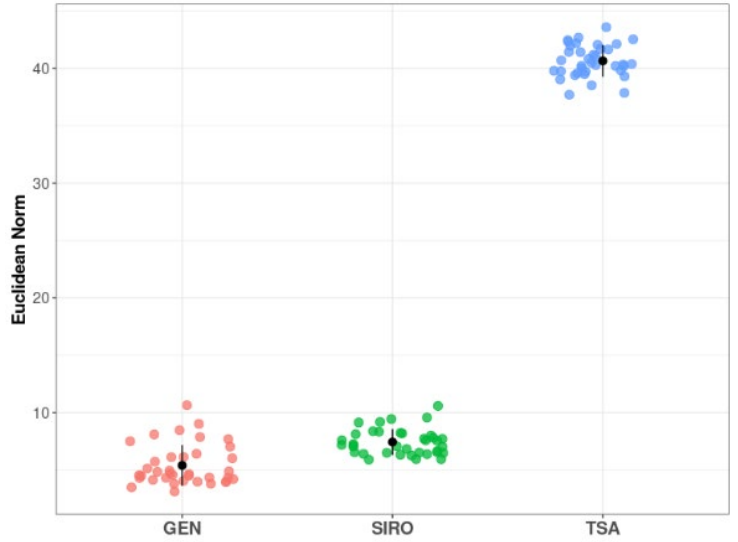
Reference Samples



Reference Chemicals



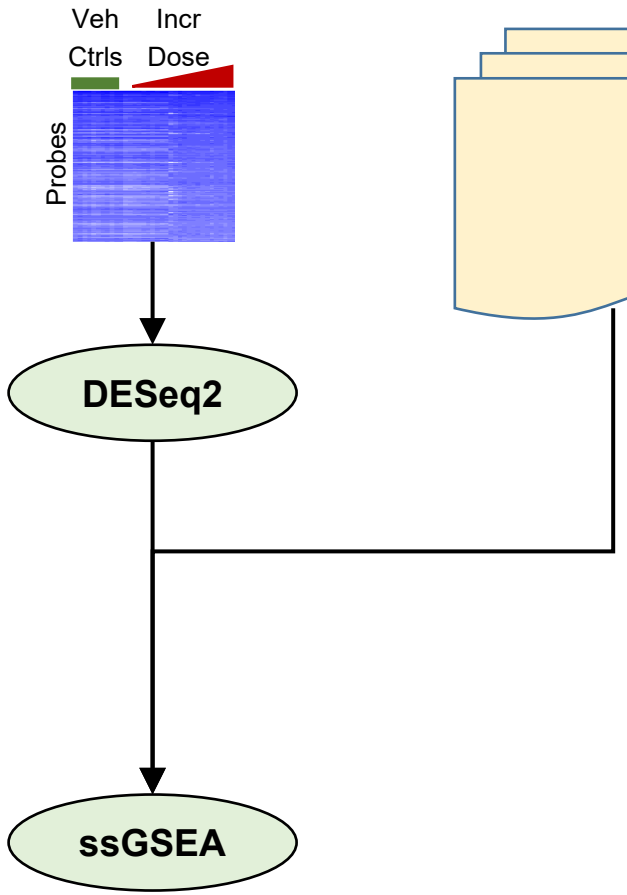
Transcriptional Signal Strength



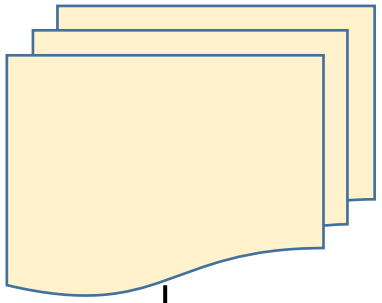
- Estimated moderated fold changes using DESeq2 with default parameters and including plate
- Determined correlation of DESeq2 moderated $\log_2$ FC values for QC samples (left) and reference chemicals (right)
- Compared correlation in $\log_2$ FC to transcriptional signal strength of reference chemicals

HTTr Signature Scoring

Count data per chemical



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

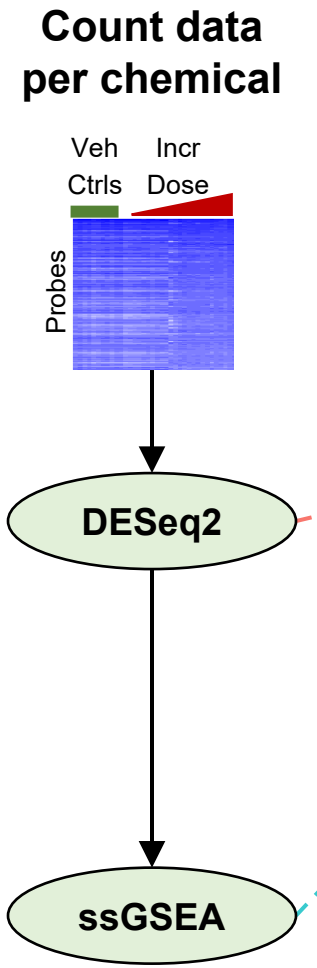


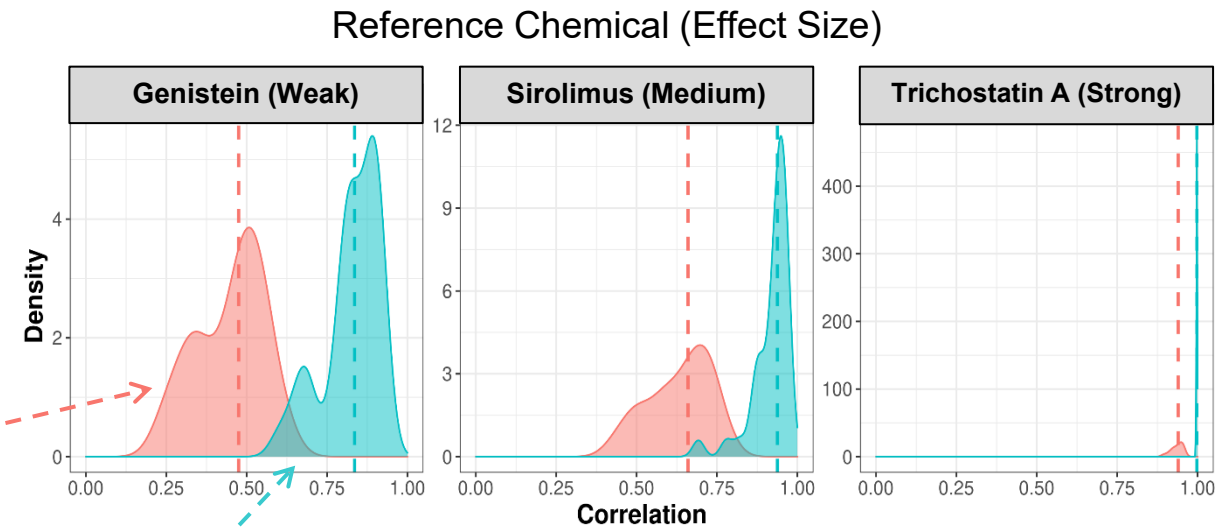
- **Bioplanet** *(Huang, et al. Front Pharmacol 2019)*
- **CMap** *(Subramanian, et al. Cell 2017)*
- **DisGeNET** *(Pinero, et al. Database 2015)*
- **MSigDB** *(Liberzon, et al. Cell Syst 2015)*

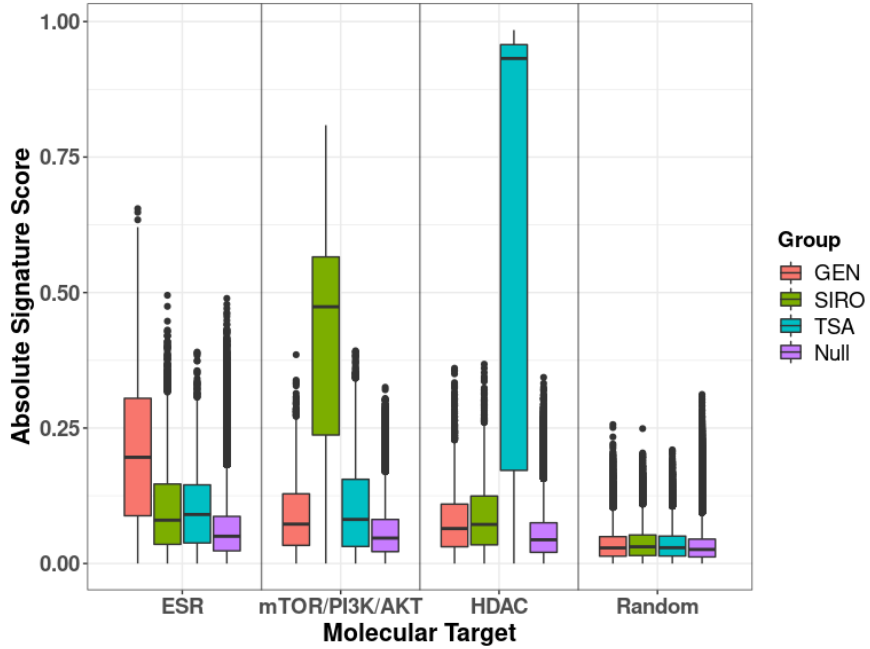
Single-Sample Gene Set Enrichment Analysis (ssGSEA) ***(Barbie et al., Nature 2009)***

- 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:
  mean(gene set log2FC) – mean(background log2FC)

MCF-7 Screen Reference Chemical Signature Scores



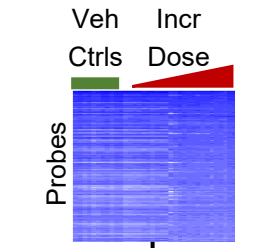




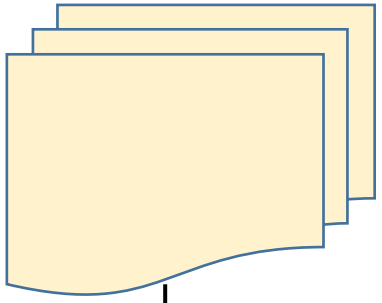
- Differential expression analysis of 3 reference chemicals replicated 37 times (MCF-7 large screen)
- Computed distribution of correlations between each replicate analysis
- Categorized signature scores based on relevant molecular target for each reference chemical

HTTr Signature Scoring

Count data per chemical



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



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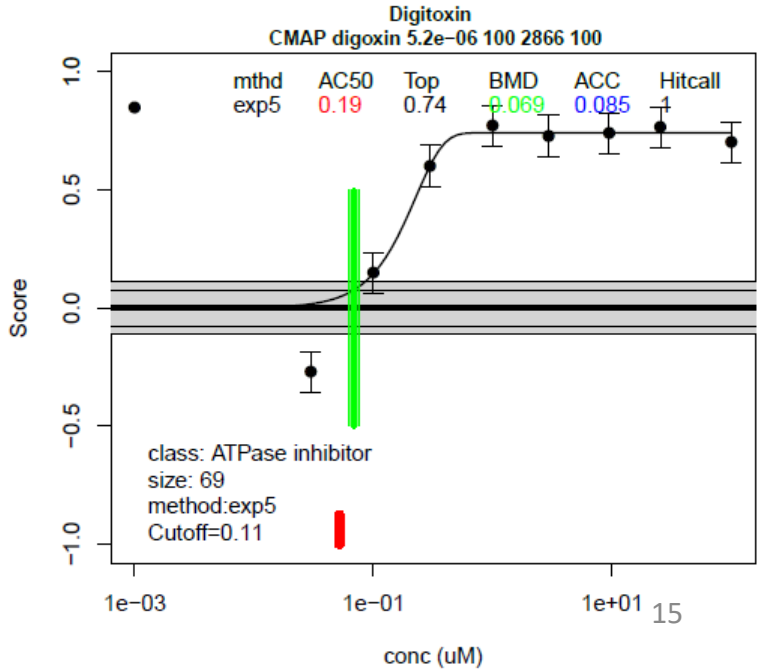
- **DisGeNET** *(Pinero et al., Database 2015)*
- **MSigDB** *(Liberzon et al., Cell Syst 2015)*

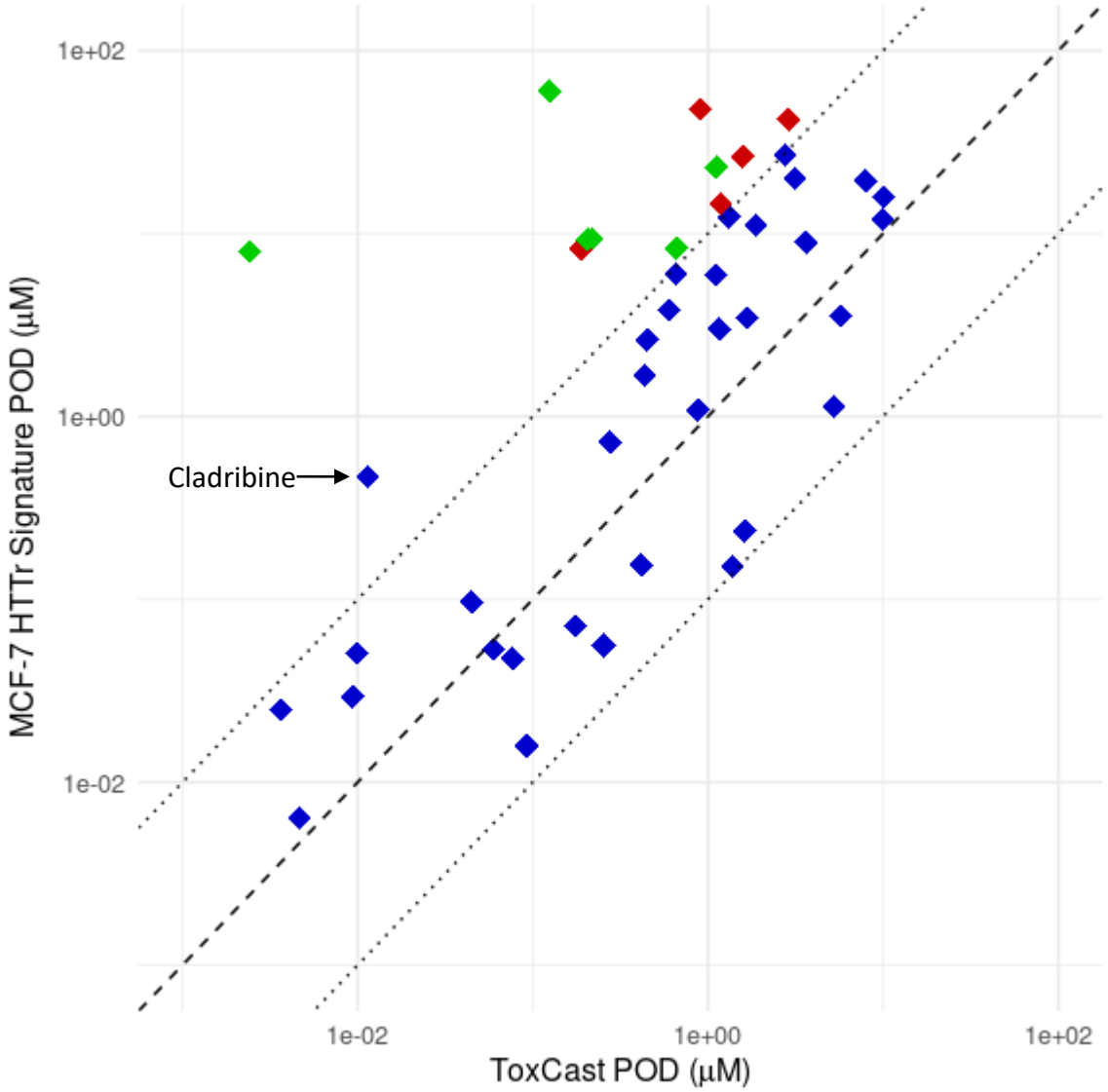
DESeq2

ssGSEA

Concentration-Response Curve Fitting (*tcplFit2*)



MCF-7 Pilot Point of Departure Analysis



- Pilot study of 44 well-characterized chemicals ***(Harrill et al., Toxicol Sci, In Press)***
- Compared HTTr-derived PODs from MCF-7 cells to previous ToxCast HTS assay results (***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
  - 6 chemicals with targets that have low/absent expression in MCF-7 cells
  - 5 chemicals show off-target hit as most potent assay in ToxCast
  - Cladribine is a non-specific DNA synthesis inhibitor

Summary and Future Directions

- CCTE has developed reliable and cost-efficient workflow for generating HTTr data from thousands of chemicals across multiple cell lines
- Correlation and reproducibility of reference samples and chemicals in a large MCF-7 screen demonstrate the experimental design and TempO-Seq HTTr platform to be robust
- Functional analysis of reference chemicals shows the benefit of signature-level analysis compared to probe-level and fold-change estimates, with signature scores reflecting the biological targets of the reference chemicals
- Preliminary/pilot analysis demonstrates that overall results are concordant with previous assays (ToxCast/HTS) and known chemical targets

- Future research efforts focus on:
  - Data generation in complementary cell models (e.g. HepaRG and U2OS screens)
  - Validation by orthogonal assays
  - Methods to summarize signature-level/overall PODs from high-dimensional data
  - Predictive models of MIEs/pathways relevant to toxicity
  - Coupling HTTr-derived PODs with HTTK/IVIVE work to predict *in vivo* safety levels

Acknowledgments

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- Richard Judson
- Logan Everett
- Imran Shah
- Beena Vallanat
- Woody Setzer
- Thomas Sheffield
- Joseph Bundy
- Bryant Chambers
- Rusty Thomas

Questions?

Orion and surrounding nebulae. B Everett Jordan Dam, April 3rd, 2021.

HTTr QC Metrics: Overview

Abbreviation	Description	Threshold	Additional Information
FrVC	Fraction of viable cells (**assay varies by cell type/study**)	Reject < 50%	Highly cytotoxic conditions no longer represent molecular initiating event
NMR	# of uniquely mapped reads	Reject < 300,000	Threshold =10% of target depth
FMR	Fraction of uniquely mapped reads	Reject < 50%	Majority of reads must align to a single probe sequence
$Ncov_5$	The number of probes with at least 5 uniquely mapped reads	Reject < 5,000	Based on Tukey's Outer Fence (3*IQR) of all viable samples cultured on each plate (test samples, vehicle controls, and reference chemical treatments)
$Nsig_{80}$	# of probes capturing the top 80% of signal in a sample	Reject < 1,000	
GiC	Gini coefficient computed on count vector for each sample	Reject > 0.95	

Adapted from MCF7 Pilot Manuscript (Harrill, et al., 2021) – larger screens also include QC flags for errors on LabCyte Echo indicating problems with chemical dispensing; these data streams not well standardized or fully captured