



# Dose-Response Modeling for Determining Transcriptomic Reference Values

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## Disclaimer

*The views expressed in this presentation are those of the presenter and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Company or product names do not constitute endorsement by US EPA.*



# About The Speaker

## Education

- Bachelors in Computer Science from Binghamton University
- Ph.D. in Genomics & Computational Biology from UPenn
- Postdoc fellowships at UPenn and NC State



## Work Experience

- Worked as Bioinformatics Scientist at Sciome, LLC, supporting research at U.S. National Toxicology Program and in industry
- Joined U.S. EPA Center for Comp Tox & Exposure in 2019
  - EPA Transcriptomics Assessment Product (ETAP)
  - High-Throughput Transcriptomics (HTTr) screening project





# Learning Objectives

- Introduce motivation for 'omics-based toxicity testing
- Overview of transcriptomic technologies
- Understand specific challenges and approaches for analyzing high-dimensional toxicogenomics data
- Demonstrate the use of BMDExpress v2 and potential application for EPA regulatory assessment product

Course Material Available Online:

[doi.org/10.23645/epacomptox.23786334.v1](https://doi.org/10.23645/epacomptox.23786334.v1)



# Motivation for Developing 'Omics-based Toxicity Testing



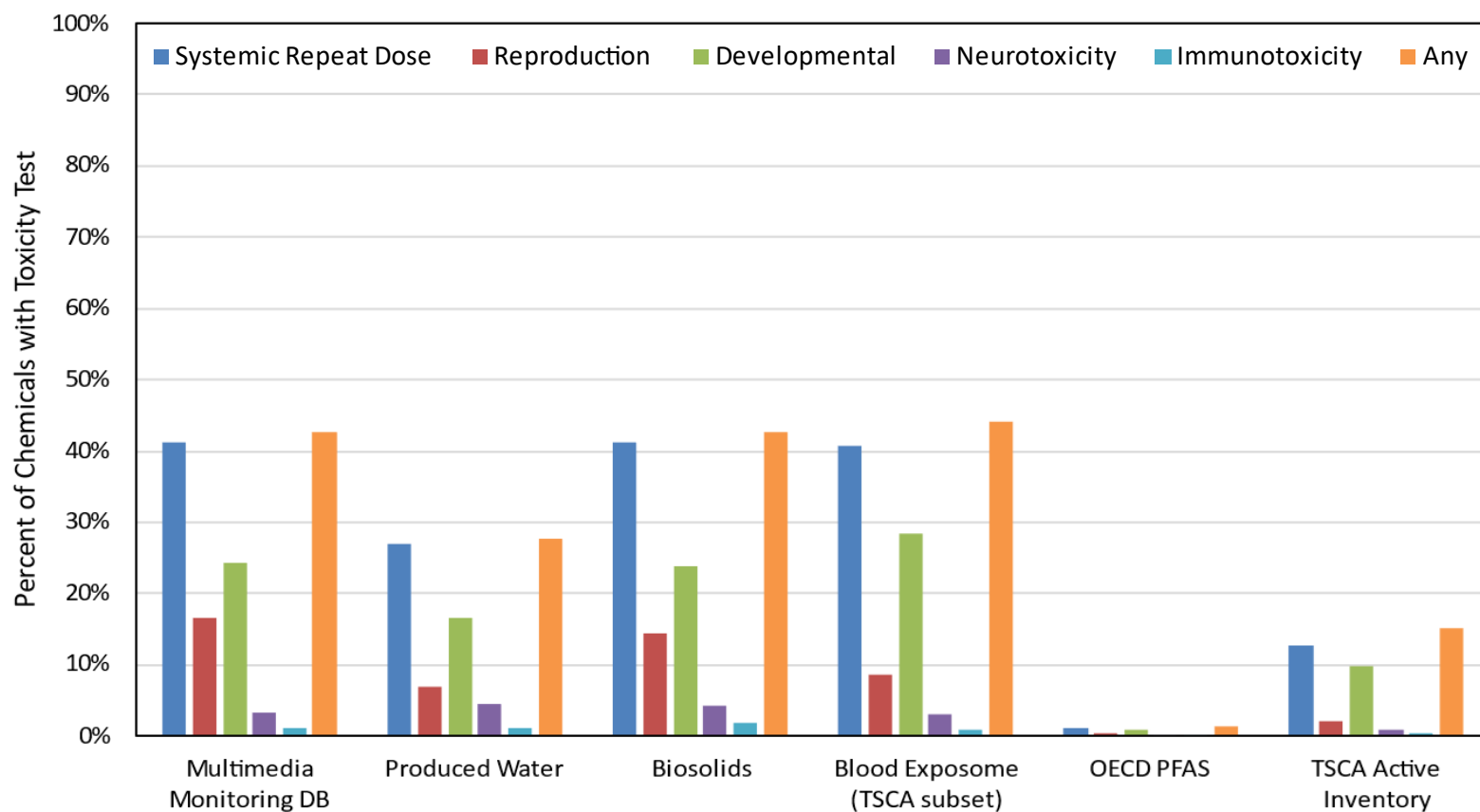
# Motivation for Using 'Omics in Toxicity Testing

## Rationale: Too Many Chemicals, Too Little Data!

- 1,000s of chemicals lack safety data for human health impacts
- Traditional toxicity testing is costly and slow
  - *2-year rodent cancer bioassay costs over \$1 million per substance*
- Fast, flexible, cost-effective methods needed to fill gaps in safety data
- Molecular effects of chemical exposure can be detected much earlier than apical effects → shorter & more cost-effective toxicity studies



# % of Chemicals with Available Toxicity Data



- Majority of chemicals across a range of exposure and regulatory contexts lack traditional toxicity data

Chemicals in Environment

(3,270)

Chemicals in Waste Streams

(1,936)

Chemicals in Human Body

(4,896)

Contaminants of Emerging or Immediate Concern

(4,729)

Chemicals in Commerce

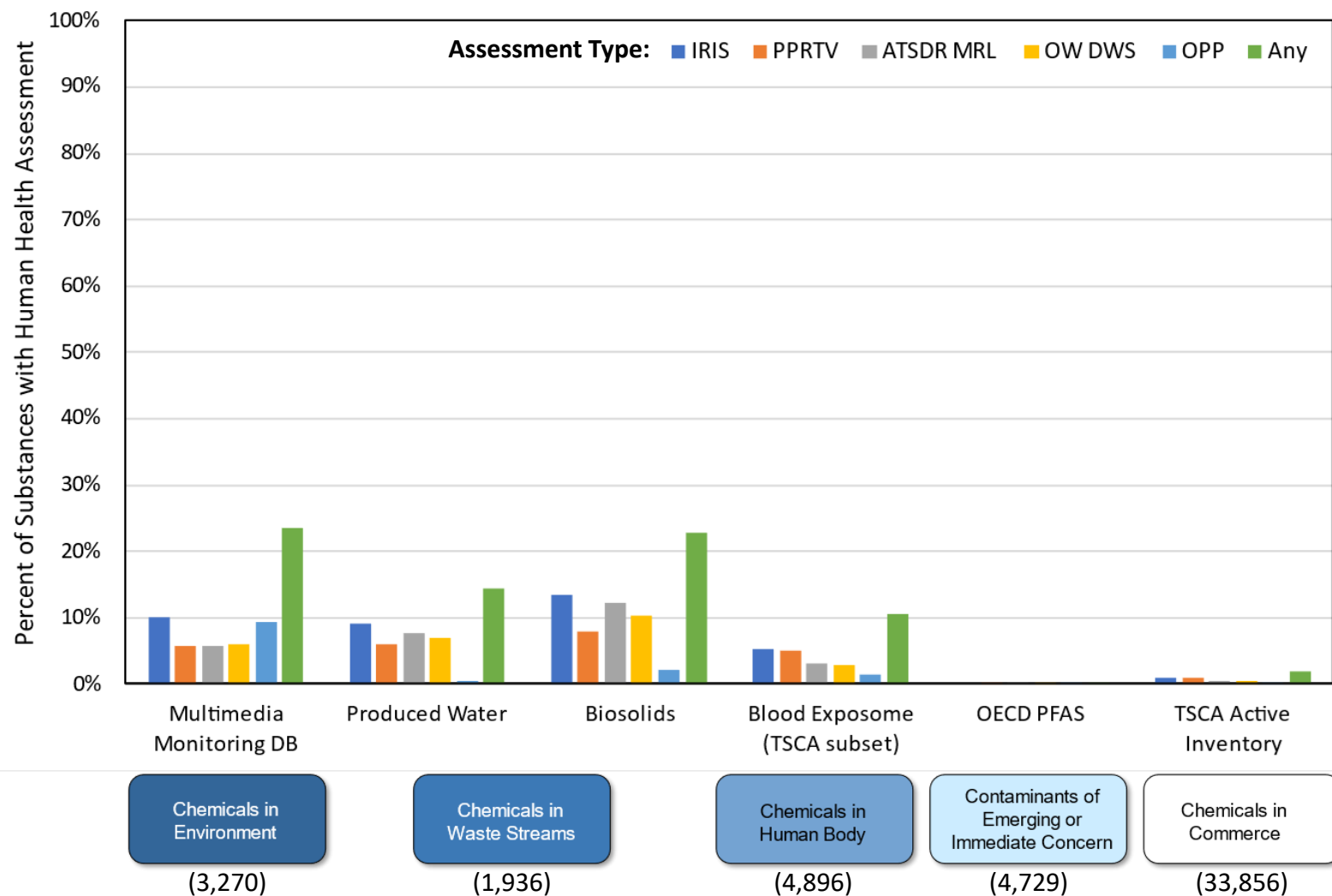
(33,856)

Figure 2-1 from: *Scientific Studies Supporting Development of Transcriptomic Points of Departure for EPA Transcriptomic Assessment Products* (Draft status)

[epa.gov/bosc/etap-july-11-12-2023-meeting](https://epa.gov/bosc/etap-july-11-12-2023-meeting)



# % of Chemicals with Available Toxicity Data

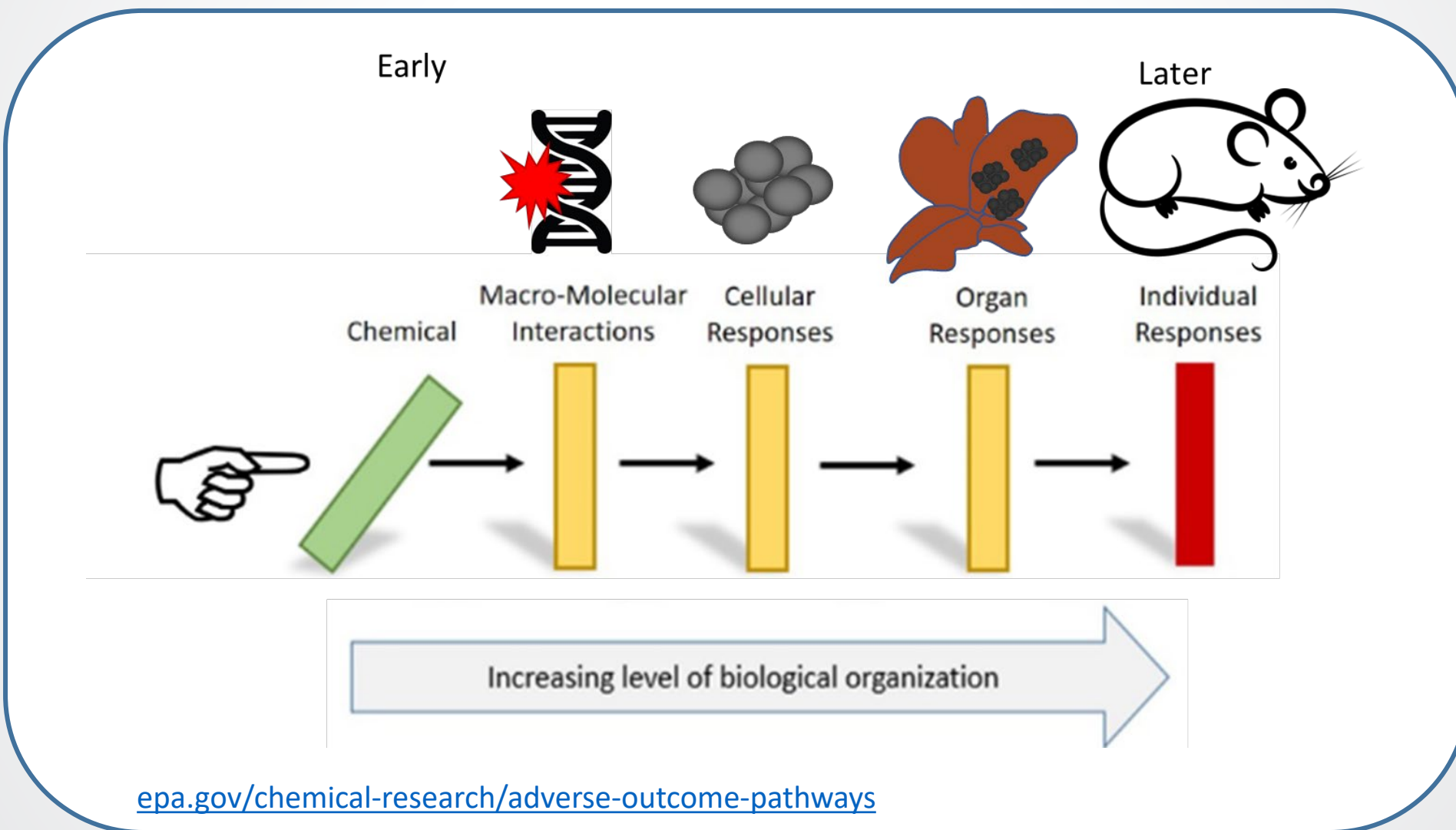


- Majority of chemicals across a range of exposure and regulatory contexts lack traditional toxicity data
- **Even fewer chemicals have human health assessments**
- Takes  $\geq 4$  years to develop human health assessment for industrial and commercial chemicals  
(Krewski et al. 2020)

Figure 2-2 from: *Scientific Studies Supporting Development of Transcriptomic Points of Departure for EPA Transcriptomic Assessment Products* (Draft status)  
[epa.gov/bosc/etap-july-11-12-2023-meeting](https://epa.gov/bosc/etap-july-11-12-2023-meeting)



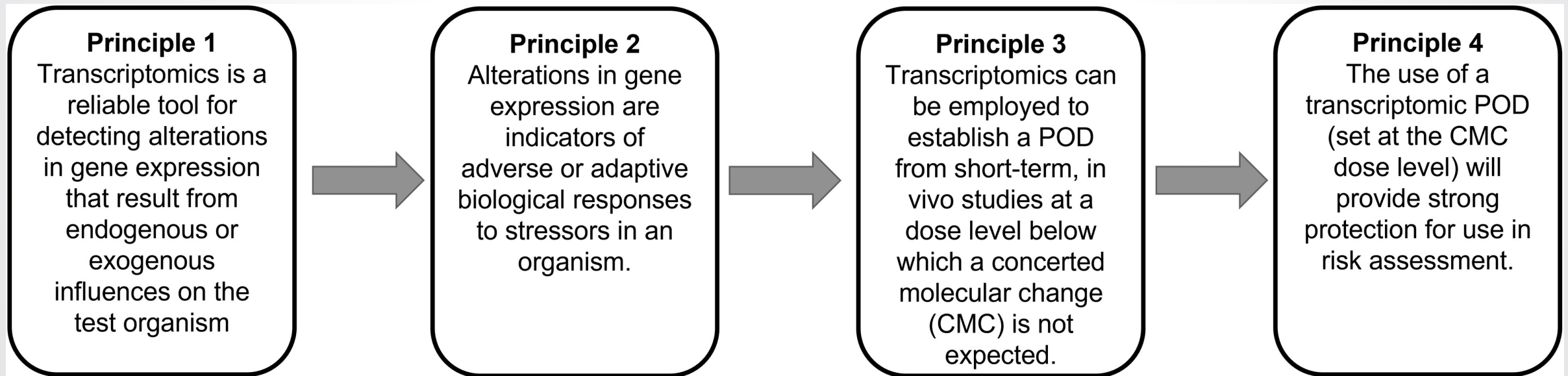
# Molecular Points of Departure





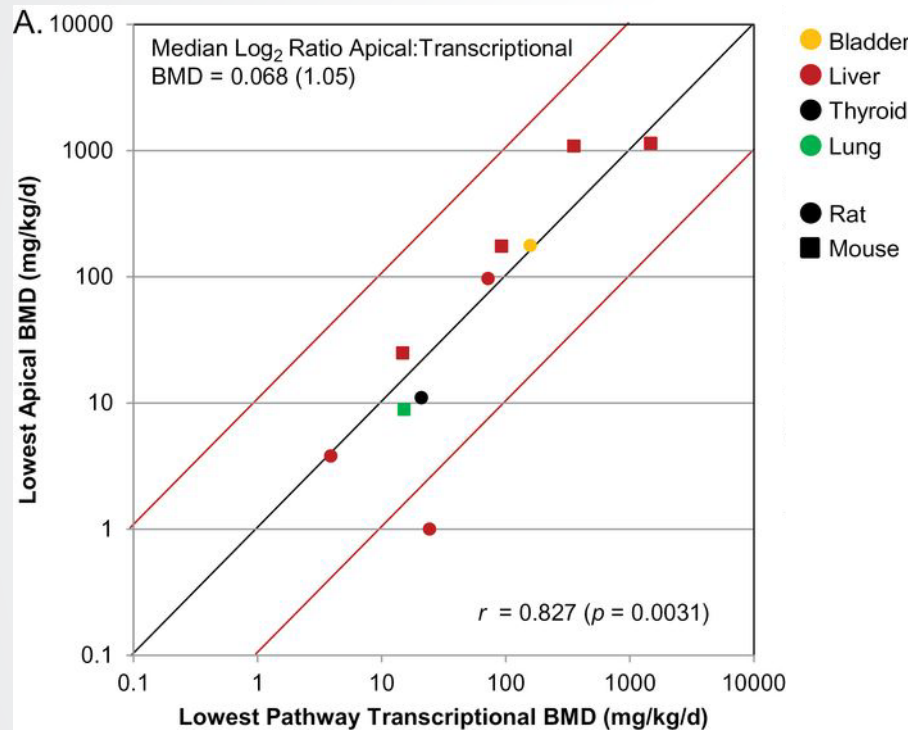
# Molecular Points of Departure

Principles underlying the development of pathway agnostic transcriptomic-derived points of departure (PODs)  
*Johnson, et al. Toxicol Sci 2022, DOI:[10.1093/toxsci/kfac097](https://doi.org/10.1093/toxsci/kfac097)*

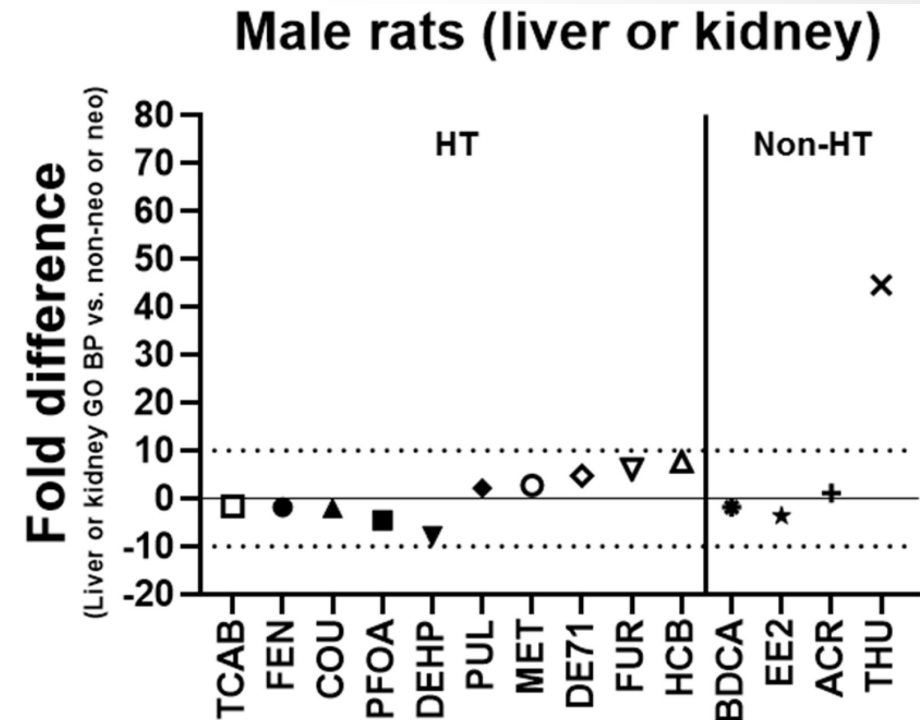




# Concordance with Chronic Apical POD



Thomas et al. 2013 DOI:[10.1093/toxsci/kft094](https://doi.org/10.1093/toxsci/kft094)

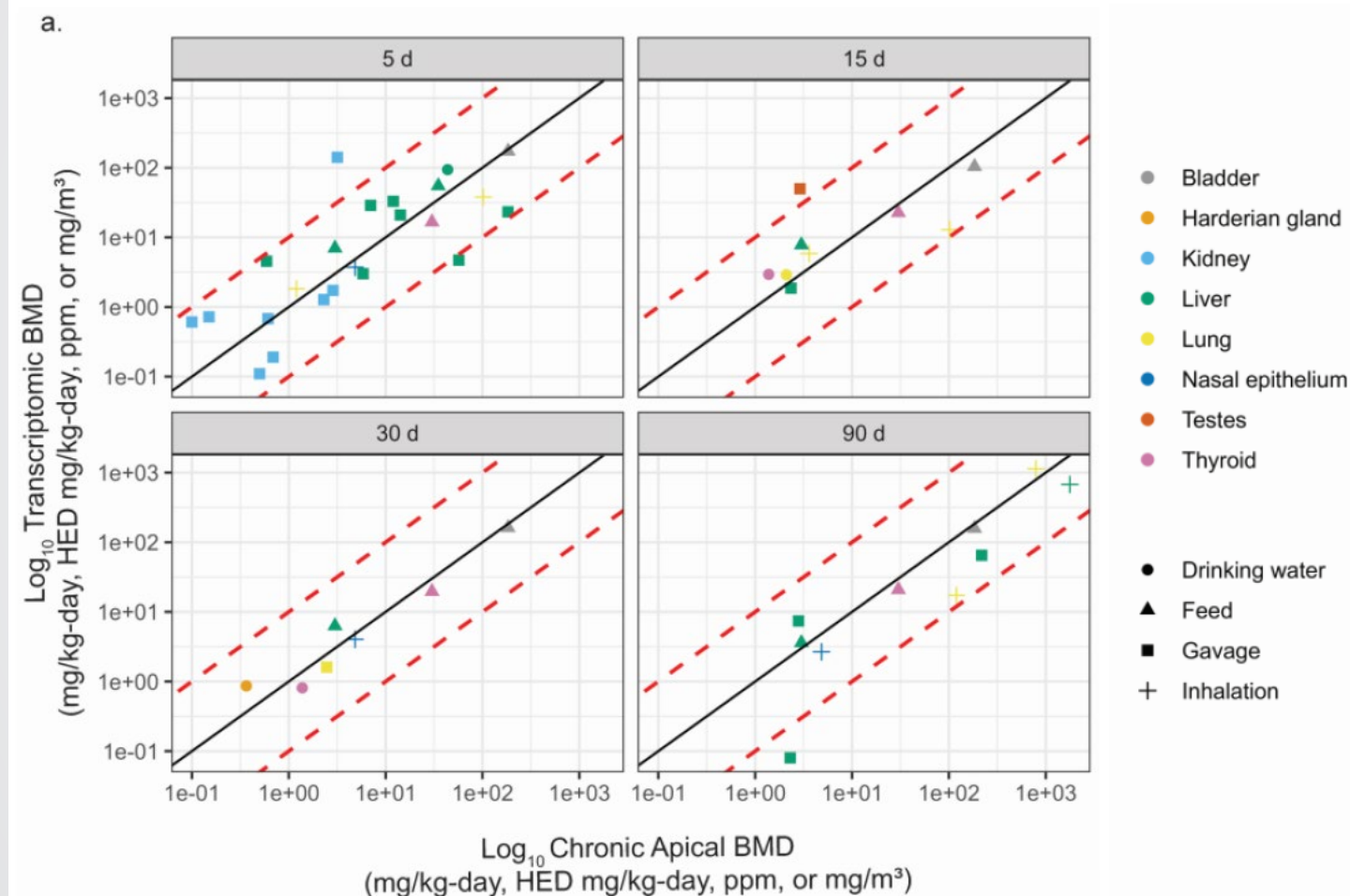


Gwinn et al. 2020 DOI:[10.1093/toxsci/kfaa081](https://doi.org/10.1093/toxsci/kfaa081)

Previous studies have shown that short-term transcriptomic PODs are concordant with chronic apical PODs (within 10x for most cases) across a range of chemicals, tissues, cancer & non-cancer effects.



# Concordance with Chronic Apical PODs



- Literature survey summarizing concordance across studies:
  - Multiple transcriptomic platforms
  - Multiple target tissues
  - Multiple routes of exposure
- Concordance similar to interstudy variability of LOAELs for traditional toxicity studies  
(See: *Pham et al. 2020*  
DOI: [10.1016/j.comtox.2020.100126](https://doi.org/10.1016/j.comtox.2020.100126))
  - Median abs ratio =  $1.9 \pm 0.7$  (MAD)
  - RMSD = 0.56 (log<sub>10</sub> mg/kg-day)
  - Pearson correlation = 0.83



# Overview of Transcriptomics

Profiling Genome-Wide Gene Expression

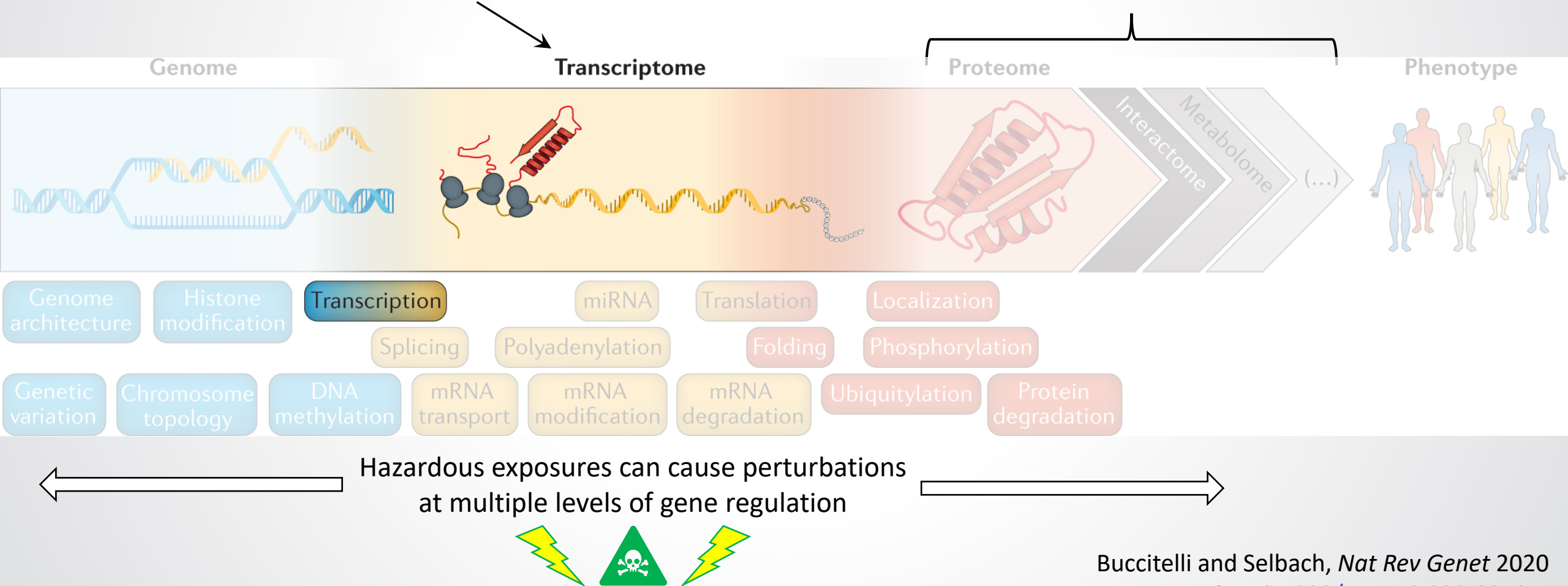




# Why Transcriptomics?

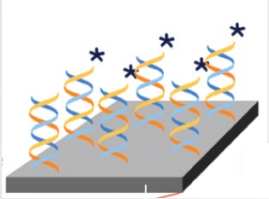
Highly dynamic in response to environmental stimuli,  
Well-established, cost-effective, high-throughput methods

Broad profiling methods are newer  
or less cost-effective



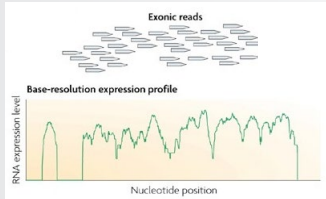
Buccitelli and Selbach, *Nat Rev Genet* 2020

DOI: [10.1038/s41576-020-0258-4](https://doi.org/10.1038/s41576-020-0258-4)



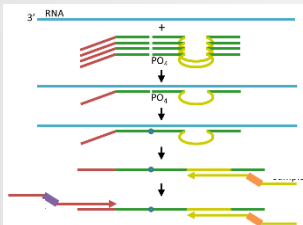
## ➤ Microarrays

- Uses targeted probes and fluorescent dyes to measure transcript levels
- Many commercially available options and established protocols
- Limited dynamic range compared to other technologies



## ➤ RNA-seq

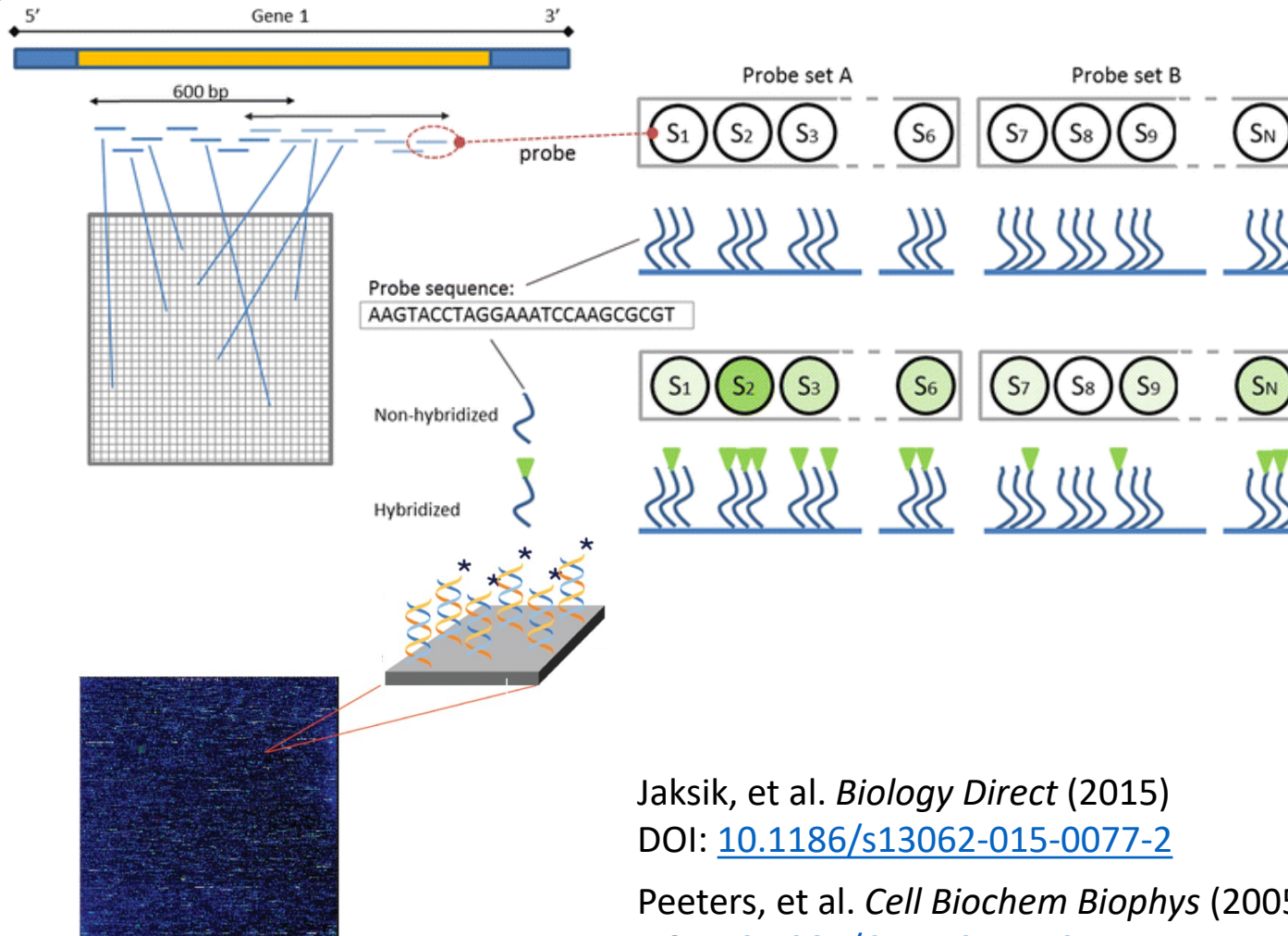
- Uses next-gen sequencing of cDNA to measure transcript levels
- Wide array of protocols for capturing different classes of RNA
- Can capture richer information than other technologies (e.g. splicing)



## ➤ Targeted RNA-seq, e.g. TempO-seq

- Uses targeted probes plus next-gen sequencing
- More amenable to high-throughput transcriptomics than RNA-seq
- Scalable: can measure ~1,000 genes or whole transcriptome

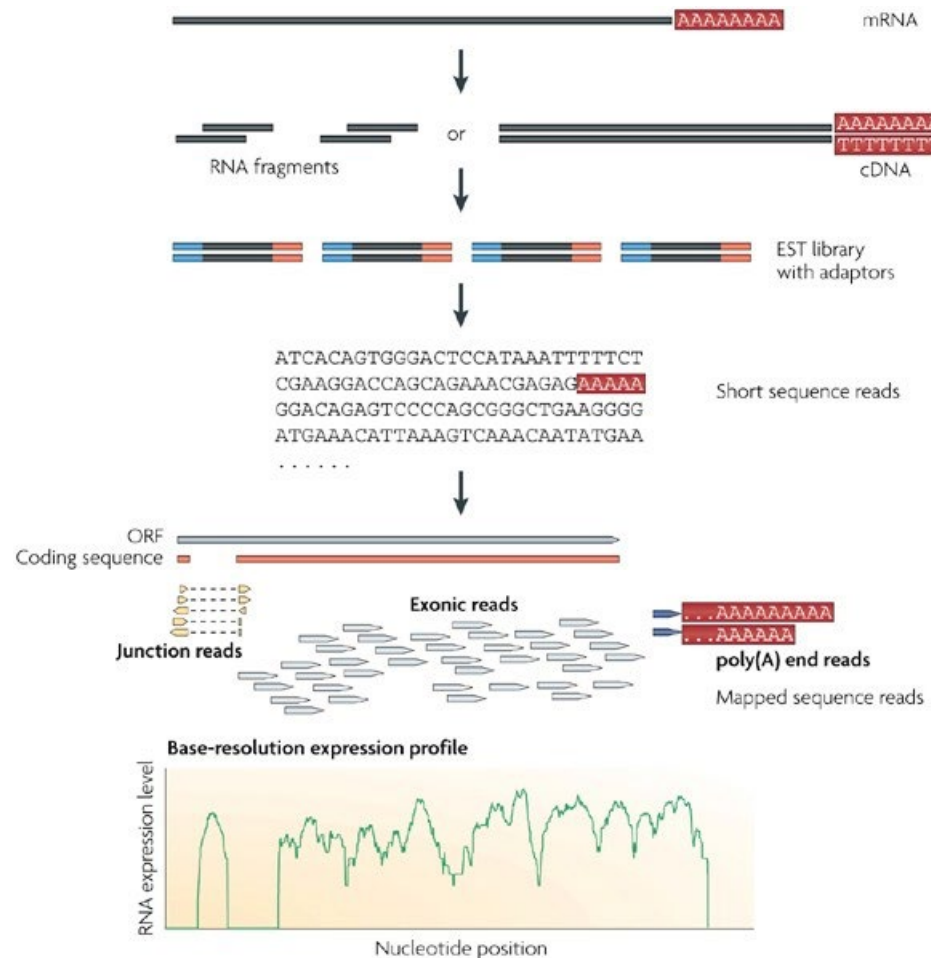
# Microarrays



- Fragments of expressed transcripts hybridized to pre-defined probe sequences
- Fluorescent labeling of hybridized probes
- **Label intensity** measures relative expression of probe target
- **Limited dynamic range** compared to other technologies
- Many commercially available options, well-established protocols



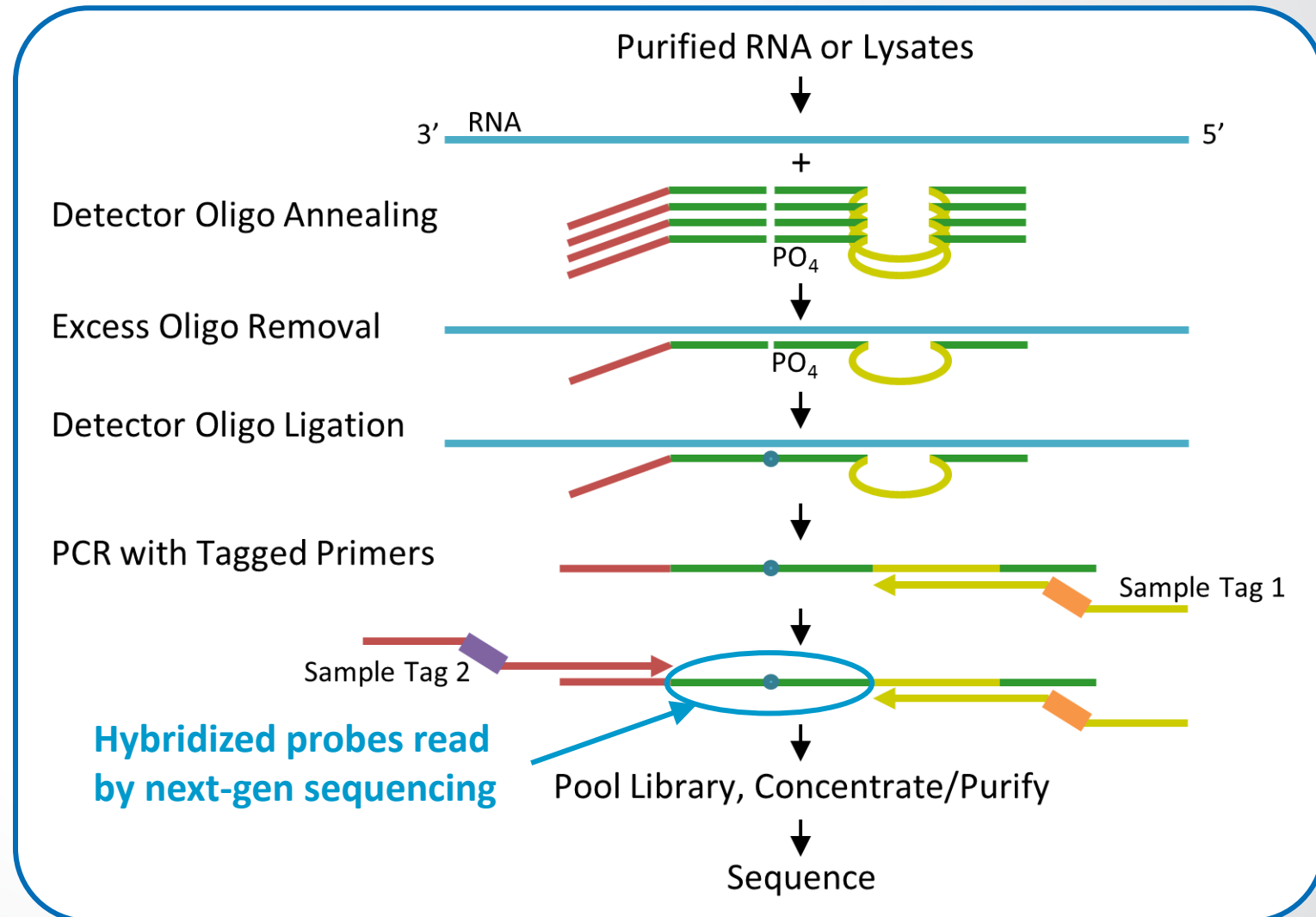
# RNA-sequencing



- Quantifies mRNA abundance by counting tens of millions of short reads
- Most common platform is Illumina next-gen sequencing
- Greater dynamic range than microarrays
- Can capture more information than other technologies (e.g. splicing, lncRNA, etc.)
- Requires more complex statistical models for count-based measurements

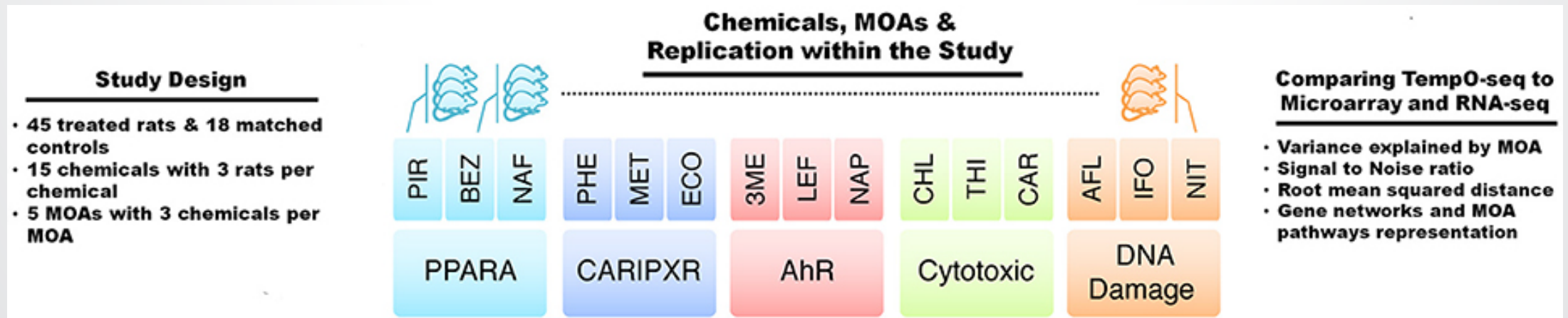
# Targeted RNA-seq Assay (TempO-seq)

- Next-Gen sequencing of targeted probes hybridized to expressed transcripts
- Scalable: whole transcriptome or reduced representation probe sets (S1500+)
  - Mav, et al. PLoS ONE 2018, DOI: [10.1371/journal.pone.0191105](https://doi.org/10.1371/journal.pone.0191105)
- Captures gene expression at lower cost than RNA-seq or microarrays
- Using same assay technology for both *in vivo* & *in vitro* screening
  - J Harrill, et al. Tox Sci 2021, DOI: [10.1093/toxsci/kfab009](https://doi.org/10.1093/toxsci/kfab009)



# Reproducibility of Transcriptomic Data

- Consortium approach to assessing reproducibility of transcriptomic platforms ([themaqc.org/papers](http://themaqc.org/papers))
  - MicroArray Quality Control (MAQC) started in 2006: [DOI:10.1038/nbt1239](https://doi.org/10.1038/nbt1239)
  - SEQC established to assess NGS technologies, including RNA-seq: [SEQC 2014 DOI:10.1038/nbt.2957](https://doi.org/10.1038/nbt.2957) [Mercer, et al. 2021](#)
- Comparison of all 3 platforms for toxicogenomics:
  - *Bushel, et al. 2018* ([DOI:10.3389/fgene.2018.00485](https://doi.org/10.3389/fgene.2018.00485))





# Transcriptomics Knowledgebases

**Signature Databases – any collection that links sets of genes to specific biological categories or patterns (e.g. functions, pathways, responses):**

- Gene Ontology ([geneontology.org](http://geneontology.org)) - *Nucleic Acids Res* (2021) DOI:[10.1093/nar/gkaa1113](https://doi.org/10.1093/nar/gkaa1113)
- MSigDB ([gsea-msigdb.org](http://gsea-msigdb.org)) - *Bioinformatics* (2011) DOI:[10.1093/bioinformatics/btr260](https://doi.org/10.1093/bioinformatics/btr260)
- Reactome ([reactome.org](http://reactome.org)) - *Nucleic Acids Res* (2022) DOI:[10.1093/nar/gkab1028](https://doi.org/10.1093/nar/gkab1028)

**Databases of Toxicogenomic/Transcriptomic Profiles:**

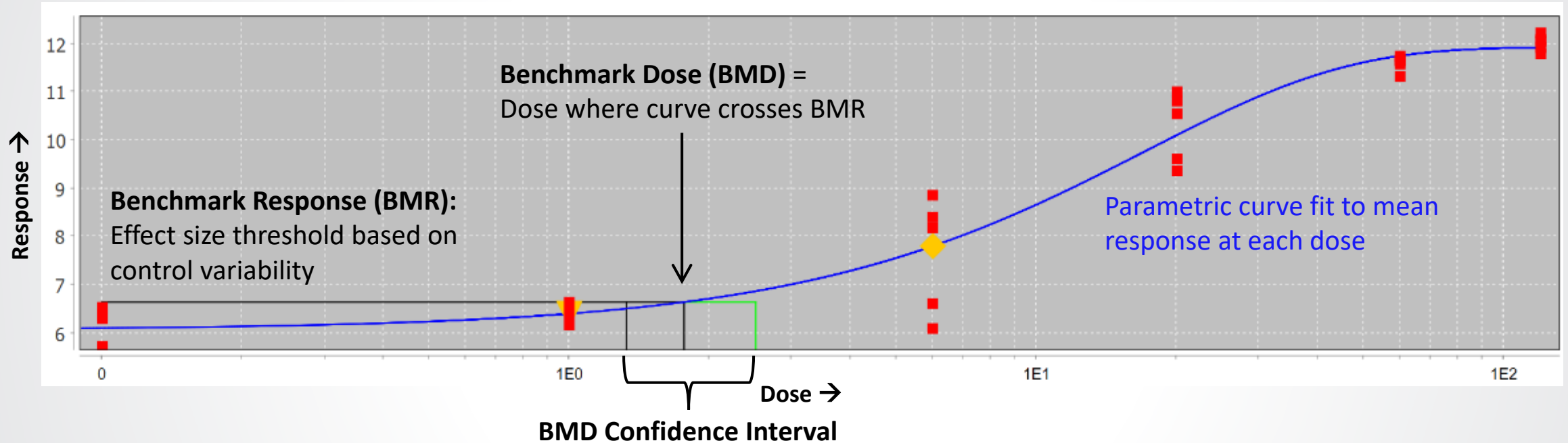
- TG-GATES ([biosciencedb.jp](http://biosciencedb.jp)) - *Nucleic Acids Res* (2015) DOI:[10.1093/nar/gku955](https://doi.org/10.1093/nar/gku955)
- Connectivity Map ([clue.io](http://clue.io)) – *Cell* (2018) DOI:[10.1016/j.cell.2017.10.049](https://doi.org/10.1016/j.cell.2017.10.049)
- General Transcriptomic DBs: [Gene Expression Omnibus](#) (NCBI) and [ArrayExpress](#) (EMBL)



# Dose-Response Modeling of Transcriptomic Data



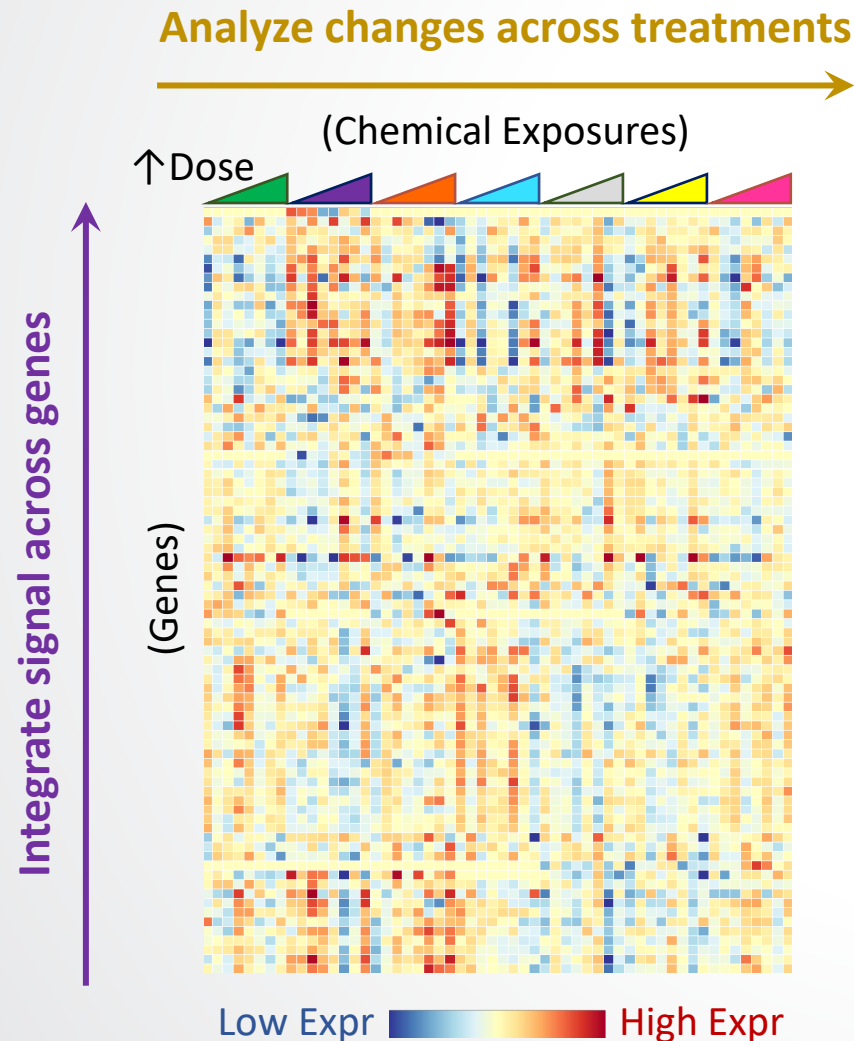
# Dose-Response Models



- Widely used approach for apical endpoint data
- EPA Benchmark Dose Software (BMDS): [www.epa.gov/bmds](http://www.epa.gov/bmds)



# Transcriptomic Dose-Response Models



- Different genes may respond at different doses of a given exposure!
- Need to analyze both:
  - Dose-responsive trends
  - Coordinated changes in gene expression
- Gene-level data noisier in transcriptomics than targeted measurements (e.g. RT-qPCR)
- Dose-response modeling thousands of features (e.g. mRNA levels) leads to computational & statistical challenges

# Many Analysis Choices!



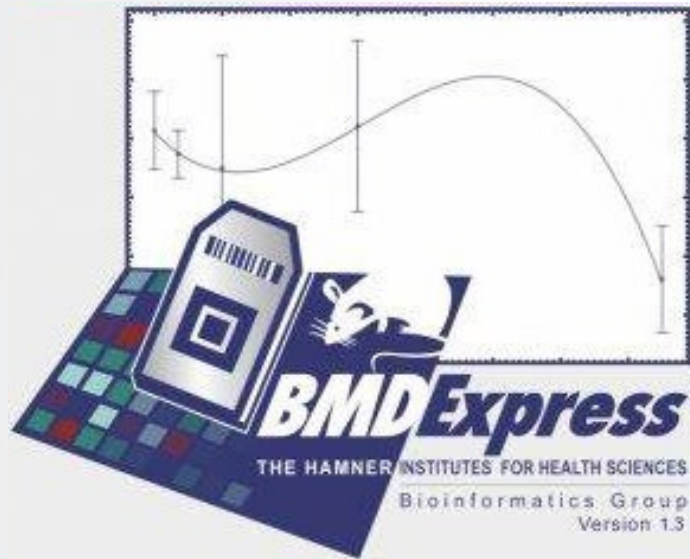
**No single “best” method for analyzing high-throughput transcriptomic data**

- Are you interested in mechanism, or just want a threshold for general bioactivity?
- Is it more important to be **predictive** or **protective** of hazard level *in vivo*?
- What other data is available for the same/analogous chemicals?
- Different technologies require different statistical models, quality control, etc.
- Experimental design (*# of replicates, doses, etc.*) impacts analysis choices!





2007



2015

Hamner to NTP/DTT

2018



## BMC Genomics



Software

**BMDExpress: a software tool for the benchmark dose analyses of genomic data**

Longlong Yang<sup>1</sup>, Bruce C Allen<sup>2</sup> and Russell S Thomas<sup>\*1</sup>

Address: <sup>1</sup>The Hamner Institutes for Health Sciences, 6 Davis Drive, Research Triangle Park, NC 27709-2137, USA and <sup>2</sup>Bruce Allen Consulting, 101 Corbin Hill Circle, Chapel Hill, NC 27514, USA

Email: Longlong Yang - lyang@thehamner.org; Bruce C Allen - bruce\_allen@verizon.net; Russell S Thomas<sup>\*</sup> - rthomas@thehamner.org

<sup>\*</sup> Corresponding author

Open Access

Gene expression

## BMDExpress 2: enhanced transcriptomic dose-response analysis workflow

Jason R. Phillips<sup>1</sup>, Daniel L. Svoboda<sup>1</sup>, Arpit Tandon<sup>1</sup>, Shyam Patel<sup>1</sup>, Alex Sedykh<sup>1</sup>, Deepak Mav<sup>1</sup>, Byron Kuo<sup>2</sup>, Carole L. Yauk<sup>2</sup>, Longlong Yang<sup>3</sup>, Russell S. Thomas<sup>4</sup>, Jeff S. Gift<sup>5</sup>, J. Allen Davis<sup>6</sup>, Louis Olszyk<sup>7</sup>, B. Alex Merrick<sup>8</sup>, Richard S. Paules<sup>8</sup>, Fred Parham<sup>8</sup>, Trey Saddler<sup>8</sup>, Ruchir R. Shah<sup>1</sup> and Scott S. Auerbach<sup>8,\*</sup>

*Bioinformatics*, 35(10), 2019, 1780–1782

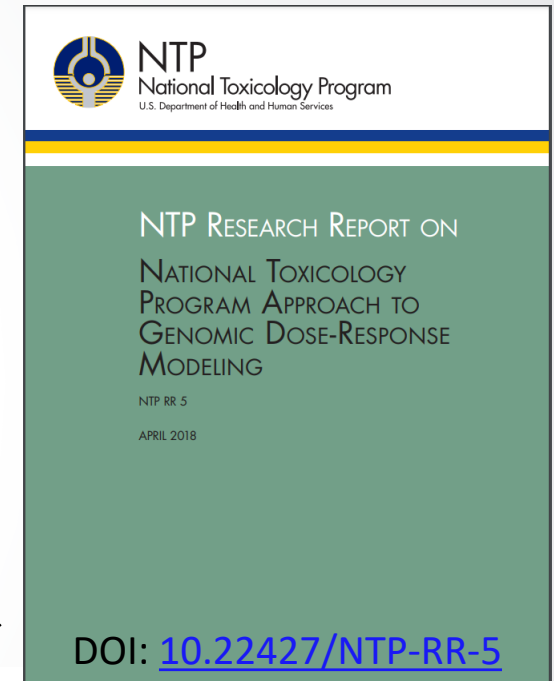
doi: 10.1093/bioinformatics/bty878

Advance Access Publication Date: 17 October 2018

Applications Note



- National Toxicology Program (NTP) approach to dose-response modeling of transcriptomic data
- Focused on determining overall transcriptomic POD (tPOD) for a chemical exposure (mainly chronic *in vivo*)
- First test case was for MCHM after Elk River spill in January 2014
- Expert panel review in 2017 led to guideline report →
- Data analysis based around BMDExpress v2 → [github.com/auerbachs/BMDExpress-2/](https://github.com/auerbachs/BMDExpress-2/)

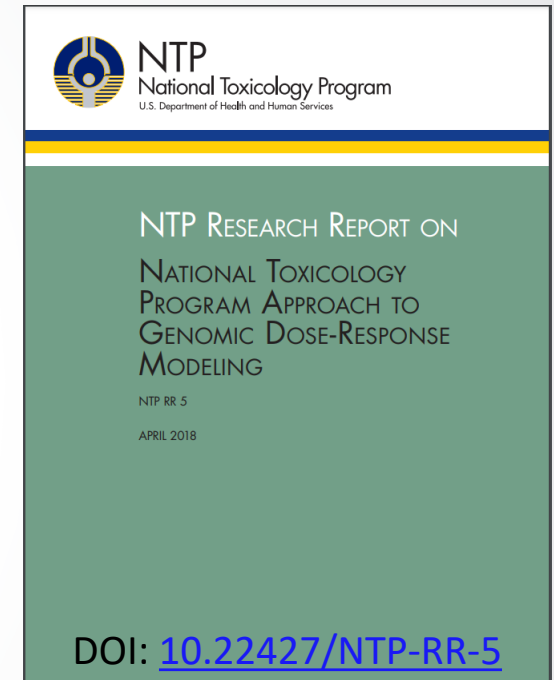




# NTP Genomic Dose-Response Method

## *in vivo* Study Design Parameters:

- 5 day repeat exposures in rats
- At least 5 dose levels x 3 animals per dose group
  - # of dose levels more critical than # of replicates for modeling BMD (see [Slob 2005](#) & [Ewald 2022](#))
- Multiple organs: liver and expected target organs
  - Oral exposures generally expected to impact liver
- Top of dose range = 5-day maximum tolerated dose



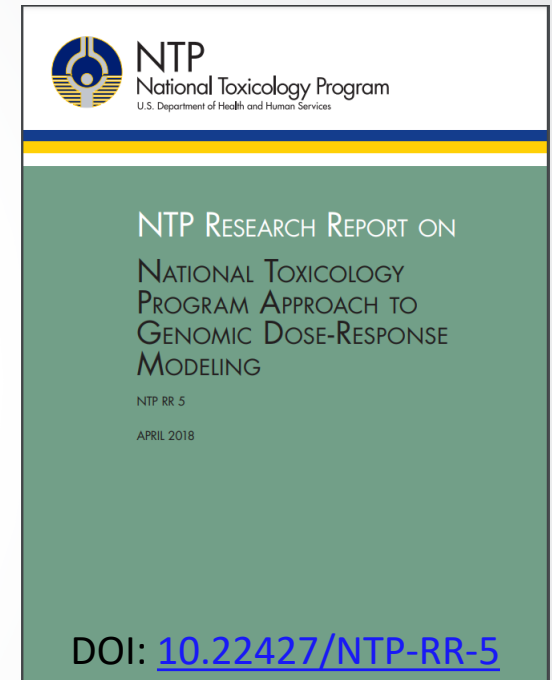


# NTP Genomic Dose-Response Method

Four main steps to data analysis (BMDExpress v2)

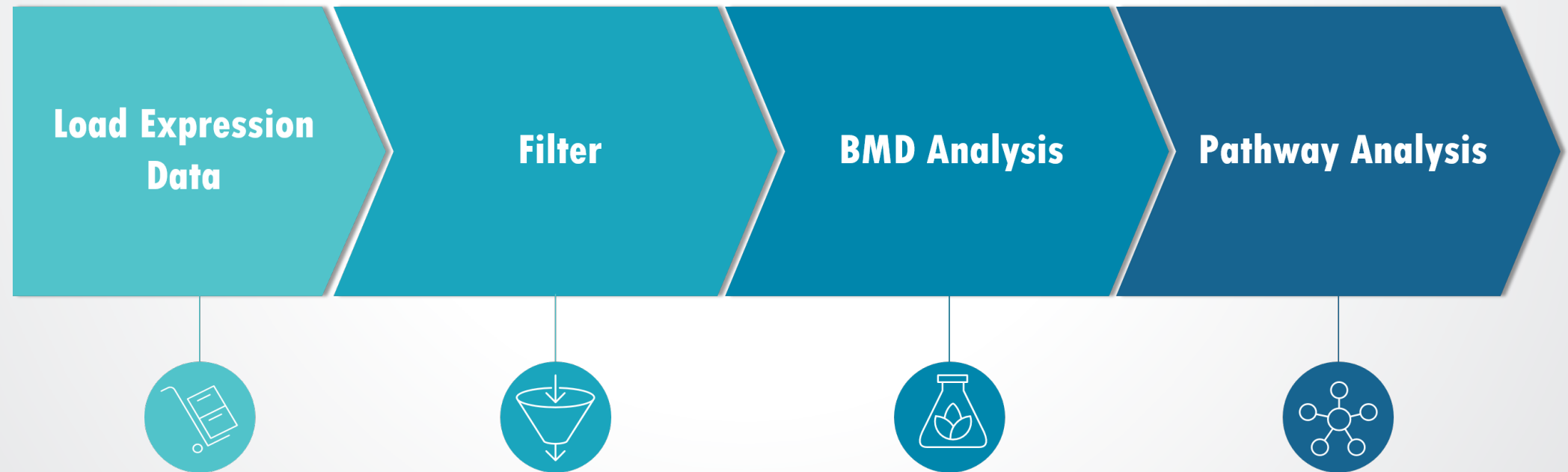
1. Evaluate dataset for adequate signal (ANOVA test)
2. Pre-modeling filter for dose-responsive probes
3. Dose-response modeling of individual probes  
*(Continuous parametric models from BMDS)*
4. Summarization of BMD(L) for known gene sets

*Not all parameters standardized – some noted as platform-dependent*

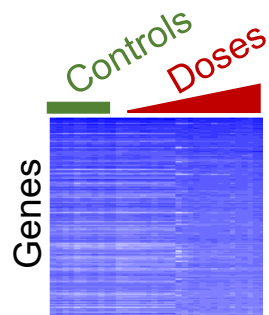




# BMDExpress Software



# BMDExpress Software



- Input all replicates of controls and doses for a **single chemical exposure and tissue**
- Compatible with many transcriptomic technologies
- Data should be normalized, with low expression genes and samples failing QC removed prior to input

Load Expression  
Data

Filter

BMD Analysis

Pathway Analysis





# BMDExpress Software

First determine if **any** genes show significant effect at **any** dose

- ANOVA test on each feature with multiple testing correction
- If **at least 1** feature passes at stringent p-value cutoff, then proceed with analysis of **whole data set**



# BMDExpress Software

Next identify **which** genes are **most likely** to be dose-responsive:

- Remove genes with weak fold-change at **all** doses vs controls
- Williams Trend Test for significant monotonic changes in response
- Pre-modeling filter reduces unreliable curve fits & speeds up analysis



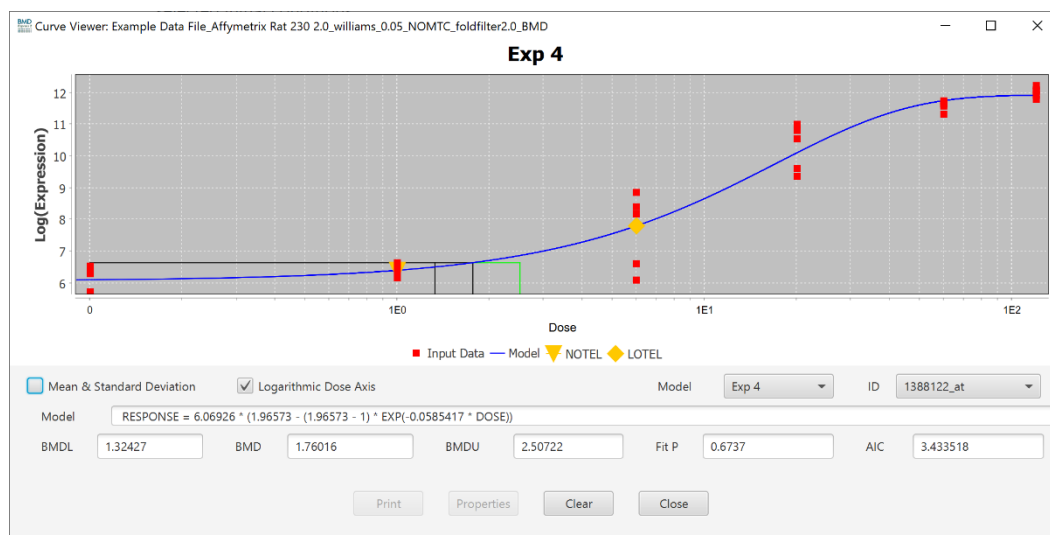




# BMDExpress Software

Run independently for each probe/gene:

- Fit multiple parametric models to data
- Select best-fit model



Many tunable parameters for model fitting and active gene identification

- Perform dose-response analysis on individual probes/genes
- Filter to **active** genes based on quality of curve-fit

## BMD Analysis



*Run time scales with number of features and models*

## Pathway Analysis





# BMDExpress Software

Many Gene Set Collections:

- **Gene Ontology**
- Reactome
- *MSigDB, custom...*



Summarize dose-response models for **biologically related** sets of genes

- Identify gene sets with multiple dose-responsive genes
- Gene set-level POD = median of active gene-level PODs
- Overall tPOD = most sensitive gene set POD

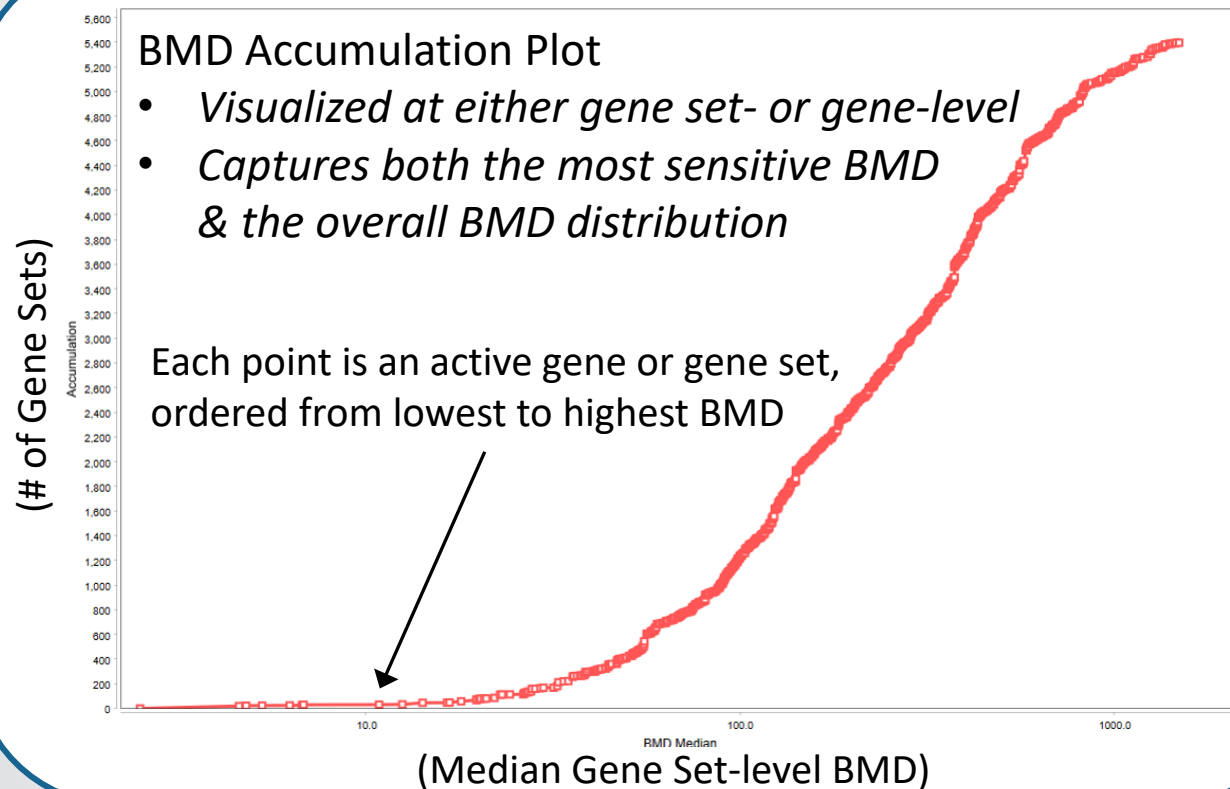




# BMDExpress Software

Summarize dose-response models for **biologically related** sets of gene

- Identify gene sets with multiple dose-responsive genes
- Gene set-level POD = median of active gene-level PODs



BMD Analysis

Pathway Analysis

(Gene Set Summarization)





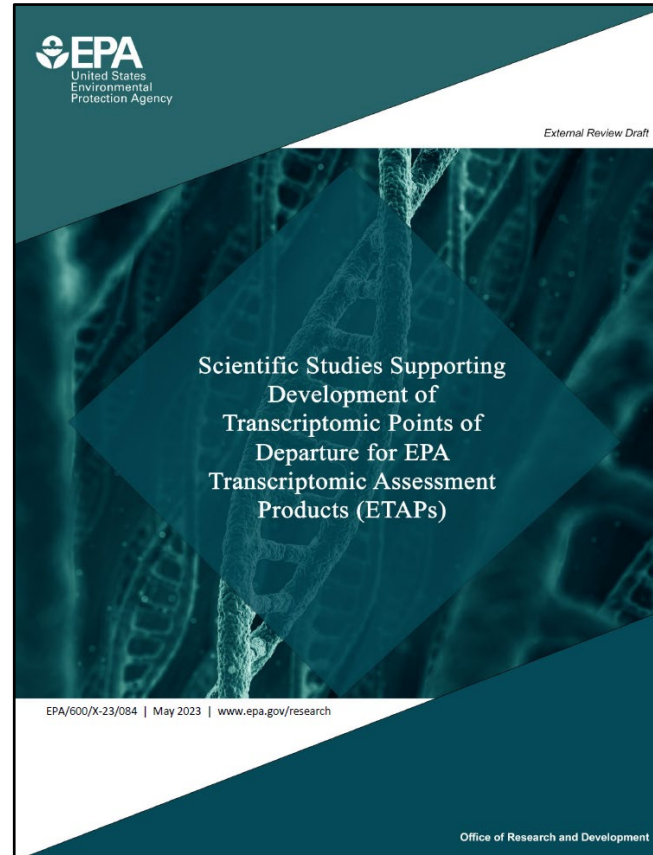
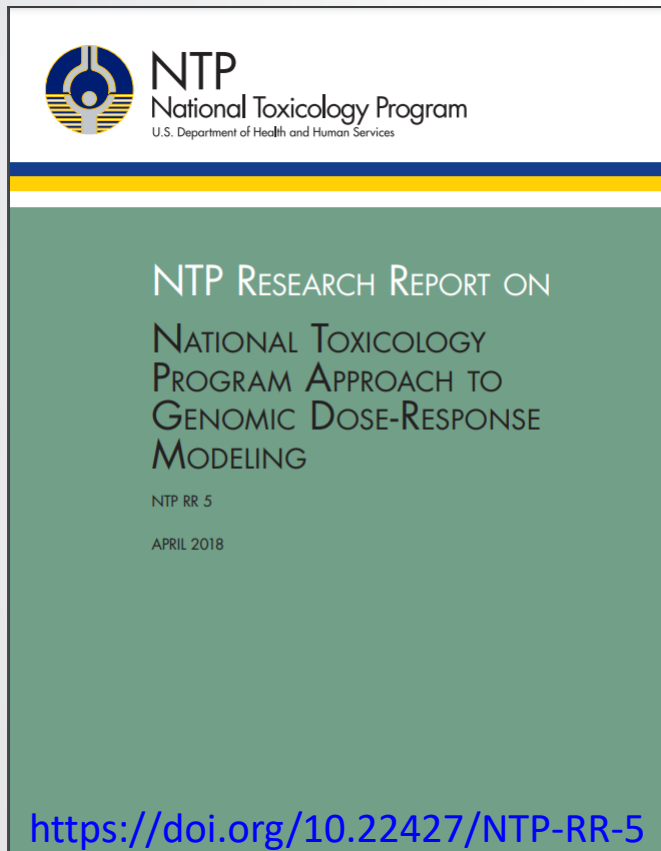
# Development of the EPA Transcriptomic Assessment Product (ETAP)

Proposal for standardized method to derive overall tPOD and convert to TRV



# ETAP Overview

Building on NTP expert panel review feedback from 2017, tailoring to regulatory assessment use-case at EPA



Draft proposals under panel review

See: [epa.gov/bosc/etap-july-11-12-2023-meeting](https://www.epa.gov/bosc/etap-july-11-12-2023-meeting)



# ETAP Overview

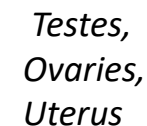
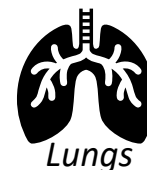
- EPA Office of Research & Development has proposed a new assessment product based on 5-day transcriptomic studies in rats
  - Proposal currently under review by Board of Scientific Counselors  
[www.epa.gov/bosc/epa-transcriptomic-assessment-products-etap-panel](http://www.epa.gov/bosc/epa-transcriptomic-assessment-products-etap-panel)
- Intended for data-poor chemicals – systematic review performed first to confirm there is no data to support other assessments
- Primary goal is to derive a **Transcriptomic Reference Value (TRV)**
  - Estimate of daily oral dose that is unlikely to have risk of adverse effects following chronic exposure (analogous to PPRTV and other reference dose values)
  - Mechanism-agnostic, does **not** predict specific hazards
  - Convert tPOD from 5-day rodent study → Human Equivalent Dose (HED)
  - Apply Uncertainty Factors to HED → TRV





# ETAP Study Design

- Study performed on male and female Sprague Dawley rats
- Daily dose of test chemical for 5 days, harvest tissues on day 6
- Minimum of 8 dose groups plus vehicle control group
  - Anchored to 5-day maximum tolerated dose
  - Span at least 4 orders of magnitude
  - Ideally test lowest positive dose below the BMD
- Minimum of 4 animals per sex per dose group
- Transcriptomic profiling of 12 tissues using TempO-seq S1500+





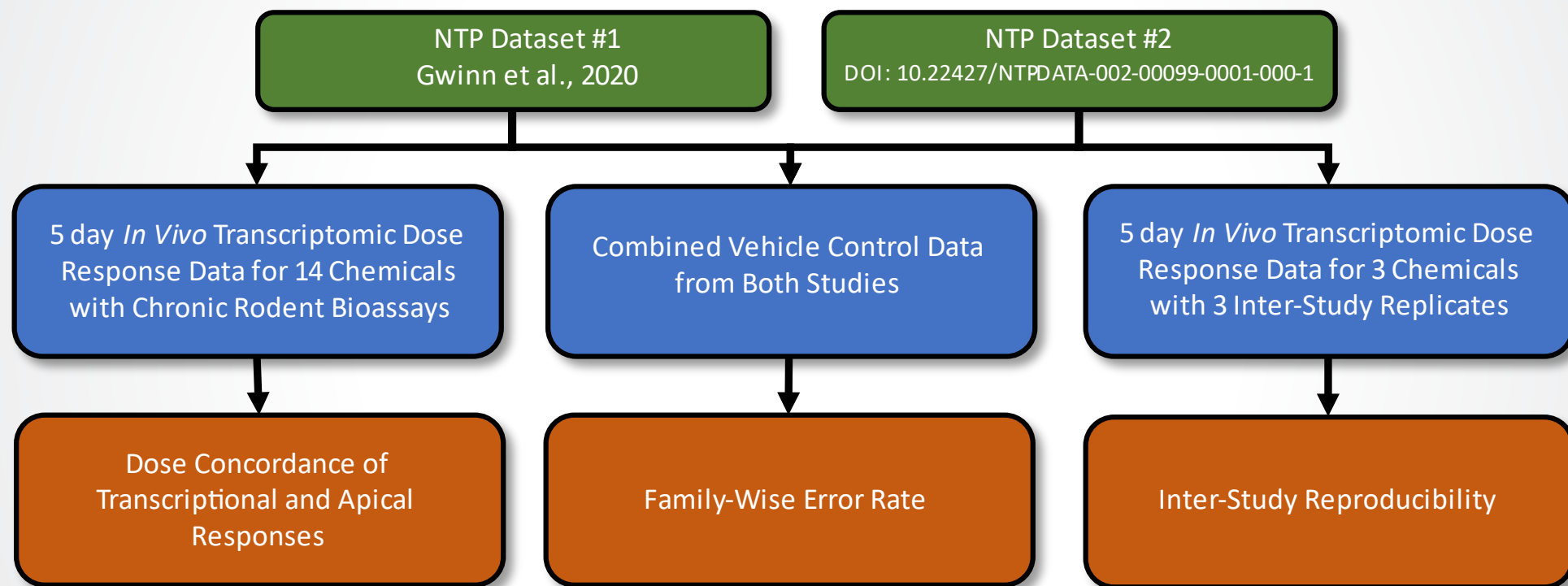
# ETAP Data QC & Normalization

- Reproducible pipeline with rigorous QC criteria previously developed for TempO-seq based on *in vitro* screening work:  
*Harrill et al. Tox Sci 2021* DOI: [10.1093/toxsci/kfab009](https://doi.org/10.1093/toxsci/kfab009)
- Important to remove samples with potential technical issues prior to dose-response modeling:
  - Multiple quality metrics computed, remove low quality samples
  - Detailed outlier review using principal component analysis (PCA) plots and structured criteria for flagging problematic samples
- Remove low expression genes for each tissue and sex
- Normalize data to counts per million (CPM) and convert to  $\log_2(\text{CPM}+1)$





# ETAP Scientific Support Analyses



Analyses focused on validating and refining the dose-response modeling methodology for this specific use-case



# ETAP Scientific Support Analyses

## Re-analysis of data from Gwinn, et al. 2020

- 5-day repeat dose exposure in rats following recommendations from NTP report
- 14 chemicals with chronic apical benchmark dose (BMD) established from 2-year study
- 8+ dose groups per chemical + matched vehicle controls, 4 replicates per group
- Transcriptome profiled from liver and kidney in each animal using TempO-seq S1500+



Chemicals Tested	
Acrylamide <sup>NC</sup>	Hexachlorobenzene <sup>NC</sup>
Bromodichloroacetic acid <sup>NC</sup>	Methyl eugenol <sup>C</sup>
Coumarin <sup>NC</sup>	Perfluorooctanoic acid <sup>NC</sup>
Pentabromodiphenyl ether mixture (DE71) <sup>NC</sup>	Tris(2-chloroisopropyl) phosphate <sup>*,C</sup>
Di(2-ethylhexyl) phthalate <sup>*,C</sup>	Pulegone <sup>NC</sup>
Ethinyl estradiol <sup>C</sup>	3,3',4,4',-Tetrachloroazobenzene <sup>C</sup>
Furan <sup>NC</sup>	α,β-Thujone <sup>NC</sup>

<sup>C</sup> indicates cancer endpoint was most sensitive BMD

<sup>NC</sup> indicates non-cancer endpoint was most sensitive BMD

\* indicates updated 2-year study results after 2020 publication



# Transcriptomic vs Chronic Apical BMDs

- Transcriptomic BMD(L) = minimum gene set BMD from either tissue (liver, kidney) and corresponding BMDL
- Apical BMD(L) = minimum BMD from either cancer or non-cancer endpoint in chronic (2-year) apical study and corresponding BMDL
- Concordance evaluated by Root-Mean-Square Difference (RMSD):

$$RMSD = \sqrt{\frac{\sum_{i=1}^N (Y_i - X_i)^2}{N}}$$

- $X_i$  = log10 transcriptomic BMD(L)
- $Y_i$  = log10 chronic apical BMD(L)
- $N$  = 14 chemicals

*Also assessed Pearson Correlation of log10 BMD(L)s*



# BMDExpress Parameter Space

**Tested 48 different combinations of analysis parameters**, focused on those most likely to be dependent on platform & study design:

➤ Pre-modeling probe filtering

- William's Trend Test p-value  $\leq 0.05$  or  $0.1$
- Minimum absolute fold-change  $\geq 1.5$  or  $2$

➤ Dose response modeling

- BMR =  $1.349 * \text{S.D.}$  (10% increase in risk when direction is unknown *a priori*)
- Maximum uncertainty:  $\text{BMD}/\text{BMDL} \leq 20$  or  $\text{BMDU}/\text{BMDL} \leq 40$

➤ Gene set (GO Biological Process) summarization

- Minimum genes per set:  $3$  or  $5$
- Minimum percent coverage:  $0\%$ ,  $3\%$ , or  $5\%$



# Transcriptomic vs Chronic Apical BMDs

- 13 of 48 parameter combinations produced transcriptomic BMD values for all 14 chemicals
  - *Focused on these combinations to ensure sufficient sensitivity*
- Computed RMSD and correlation for all 13 combinations of BMDExpress parameters
- RMSD values ranged from **0.567** to **0.958** (log<sub>10</sub> mg/kg-d)
- Pearson correlations ranged from **0.804** to **0.917**



# Parameters Maximizing Concordance

Rank	Pre-Modeling Probe Filtering, BMD Modeling, and Gene Set Summarization Parameter Combination	Pearson Correlation Coefficient (PCC)	RMSD ( $\log_{10}$ mg/kg-d)
1	<b>Williams <math>p &lt; 0.05</math>; FC <math>&gt; 1.5</math>; BMD/BMDL <math>&lt; 20</math>; min 3 genes; min 0%</b>	<b>0.910</b>	<b>0.567</b>
2	Williams $p < 0.1$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 0%	0.907	0.571
3	Williams $p < 0.1$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 3%	0.905	0.578
4	Williams $p < 0.1$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 5%	0.906	0.581
5	Williams $p < 0.05$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 3%	0.905	0.593





# Parameters Maximizing Concordance

Rank	Pre-Modeling Probe Filtering, BMD Modeling, and Gene Set Summarization Parameter Combination	Pearson Correlation Coefficient (PCC)	RMSD ( $\log_{10}$ mg/kg-d)
1	<b>Williams <math>p &lt; 0.05</math>; FC <math>&gt; 1.5</math>; BMD/BMDL <math>&lt; 20</math>; min 3 genes; min 0%</b>	<b>0.910</b>	<b>0.567</b>
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5	Williams $p < 0.05$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 3%	0.905	0.593

## Consistent parameters:

- Pre-filter for probes with maximum fold change (FC)  $> 1.5$
- Maximum uncertainty in best-fit model: BMD/BMDL  $< 20$
- Valid gene set BMD must have minimum of 3 valid gene BMDs



# Parameters Maximizing Concordance

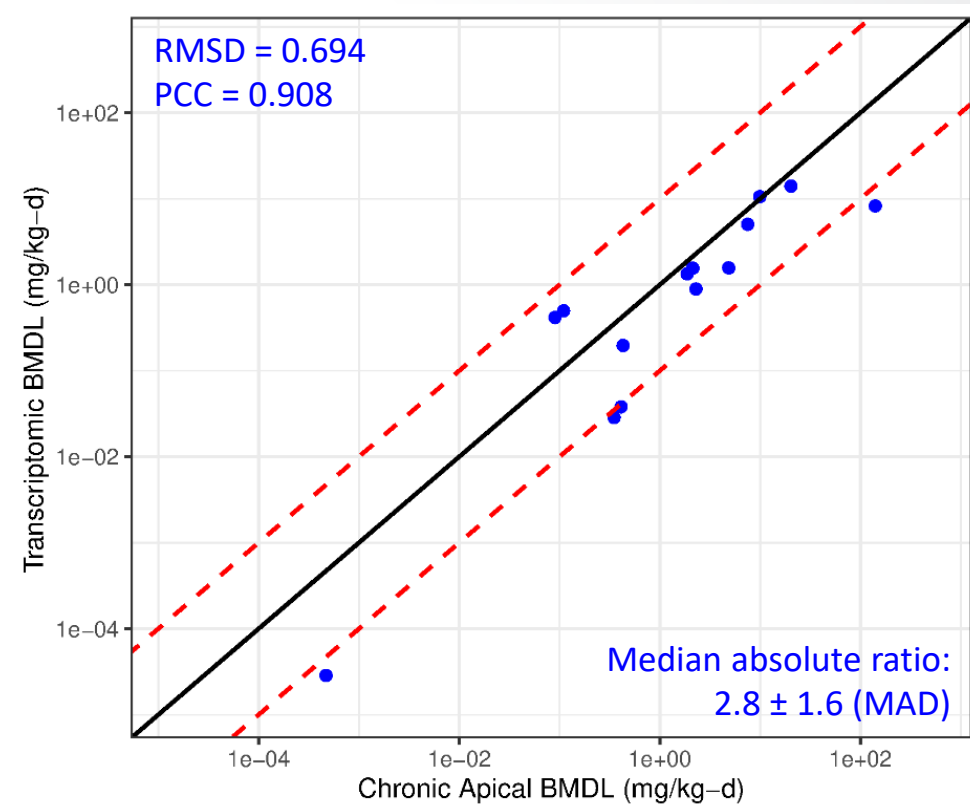
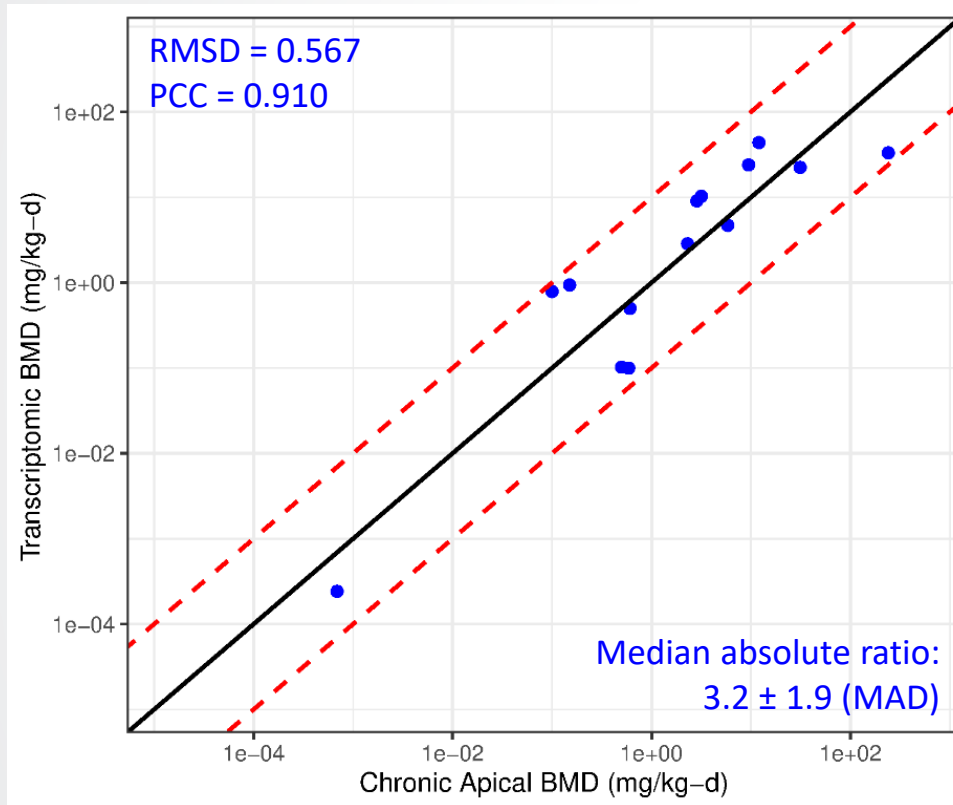
Rank	Pre-Modeling Probe Filtering, BMD Modeling, and Gene Set Summarization Parameter Combination	Pearson Correlation Coefficient (PCC)	RMSD ( $\log_{10}$ mg/kg-d)
1	<b>Williams p &lt; 0.05; FC &gt; 1.5; BMD/BMDL &lt; 20; min 3 genes; min 0%</b>	<b>0.910</b>	<b>0.567</b>
2	Williams p < 0.1; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 0%	0.907	0.571
3	Williams p < 0.1; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 3%	0.905	0.578
4	Williams p < 0.1; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 5%	0.906	0.581
5	Williams p < 0.05; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 3%	0.905	0.593

Variable parameters:

- William's Trend Test p-value cutoff for probe pre-filtering
- Minimum percent coverage of valid gene set (0, 3, or 5%)



# Transcriptomic vs Chronic Apical BMDs



- Concordance of transcriptomic BMD(L) vs chronic apical BMD(L) values for all 14 chemicals
- Using top ranked combination of parameters proposed for ETAP standard methods

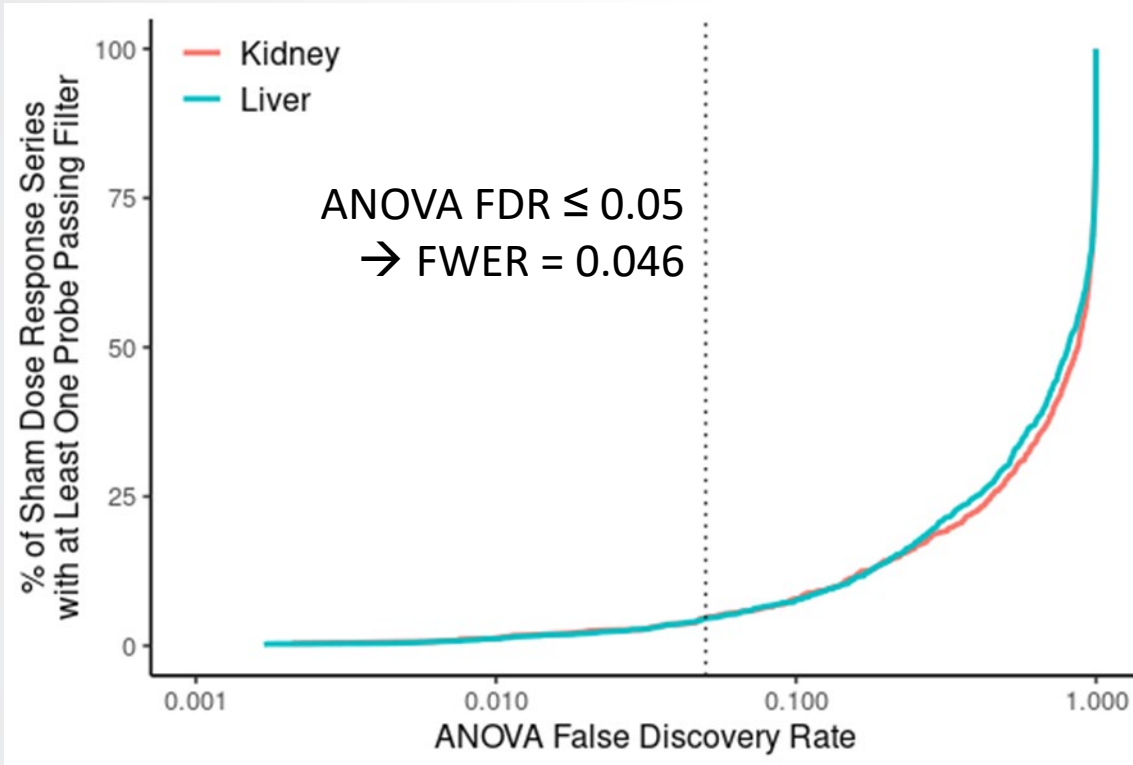


# Family-Wise Error Rate Estimation

- If there is ***no*** real dose-dependent effect, how often would a data set:
  - Pass the first data set prefiltering criteria?
  - Produce an overall tPOD?
- Randomly sampled vehicle control replicates across studies to generate “sham” dose-response data where no dose-responsive genes expected
  - Performed 1,000 times for same tissue and vehicle type
  - Generated series with same design as ETAP studies (8 doses x 4 replicates each)
- Family-wise error rate (FWER) = % of sham data sets passing our filters



# Family-Wise Error Rate Estimation



## Overall FWER:

Sham dose response series with at least one probe passing ANOVA 5% FDR filter were run through complete workflow to determine % of sham series producing at least one valid gene set BMD/L  
(for the top 5 parameter combinations)

- Dataset-level FWER = % of sham dose-response series with 1+ probe passing ANOVA test
- Probe-level False Discovery Rate (FDR) based on Benjamini-Hochberg corrected p-values
- Dotted line marks FDR  $\leq 0.05$ , corresponding FWER = 0.046



# Overall Family-wise Error Rate (FWER)

Rank	Pre-Modeling Probe Filtering, BMD Modeling, and Gene Set Summarization Parameter Combination	Overall FWER
1	<b>Williams <math>p &lt; 0.05</math>; FC <math>&gt; 1.5</math>; BMD/BMDL <math>&lt; 20</math>; min 3 genes; min 0%</b>	<b>0.006</b>
2	Williams $p < 0.1$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 0%	0.009
3	Williams $p < 0.1$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 3%	0.002
4	Williams $p < 0.1$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 5%	0.002
5	Williams $p < 0.05$ ; FC $> 1.5$ ; BMD/BMDL $< 20$ ; min 3 genes; min 3%	0.001

- Overall FWER: If dataset contains no real dose-dependent effect, how often would we assign a final BMD from complete workflow?
- **Top 5 parameter combinations all have overall FWER  $< 1\%$**





# Inter-Study Reproducibility

- Furan, PFOA, BDCA studies replicated from Gwinn, et al. (2 additional replicate studies per chemical)
- Computed overall BMD(L) for each replicate study based on most sensitive gene set in either tissue
- Evaluated standard deviation (SD) based on all unique pairs of replicate studies for same chemical

$$SD = \sqrt{\frac{\sum_{i=1}^N (Y_i - X_i)^2}{2N}}$$

Chemicals Replicated	
Acrylamide	Hexachlorobenzene
<b>Bromodichloroacetic acid</b>	Methyl eugenol
Coumarin	<b>Perfluorooctanoic acid</b>
Pentabromodiphenyl ether mixture (DE71)	Tris(2-chloroisopropyl) phosphate*
Di(2-ethylhexyl) phthalate*	Pulegone
Ethinyl estradiol	3,3',4,4',-Tetrachloroazobenzene
<b>Furan</b>	α,β-Thujone

Data DOI: [10.22427/NTPDATA-002-00099-0001-000-1](https://doi.org/10.22427/NTPDATA-002-00099-0001-000-1)

*All replicate studies performed with same doses in same contract lab over several years*



# Inter-Study Reproducibility

Rank	Pre-Modeling Probe Filtering, BMD Modeling, and Gene Set Summarization Parameter Combination	Log <sub>10</sub> BMD SD (log <sub>10</sub> mg/kg-day)	Log <sub>10</sub> BMDL SD (log <sub>10</sub> mg/kg-day)
1	<b>Williams p &lt; 0.05; FC &gt; 1.5; BMD/BMDL &lt; 20; min 3 genes; min 0%</b>	<b>0.242</b>	<b>0.295</b>
2	Williams p < 0.1; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 0%	0.247	0.292
3	Williams p < 0.1; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 3%	0.245	0.290
4	Williams p < 0.1; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 5%	0.241	0.289
5	Williams p < 0.05; FC > 1.5; BMD/BMDL < 20; min 3 genes; min 3%	0.242	0.289

- Evaluated Standard Deviation (SD) for the top 5 configurations from concordance analysis
- Differences in SD were negligible between these configurations
- Inter-study reproducibility is comparable to that of traditional repeat-dose studies



# BMDExpress v2 Demo

(The following slides just document the settings used for the demo)

Course material here: [doi.org/10.23645/epacomptox.23786334.v1](https://doi.org/10.23645/epacomptox.23786334.v1)

Expression file: NTP\_Gwinn\_DEHP\_Male\_Liver\_S1500.txt

- Generated by processing raw data from Gwinn, et al. DOI: [10.1093/toxsci/kfaa081](https://doi.org/10.1093/toxsci/kfaa081)
- Load with Platform: TempO-seq Rat S1500+, Base: Log2

Complete analysis file: NTP\_Gwinn\_DEHP\_Liver\_Demo.bm2



# BMDExpress v2 Demo

BMD Express v2 One Way ANOVA

One Way Anova

Expression Data: NTP\_Gwinn\_DEHP\_Male\_Liver\_S1500

P-Value Cutoff: 0.05

Multiple Testing Correction: ☒ Benjamini & Hochberg (FDR)

Filter Out Control Genes: ☒ (probes starting with AFFX...)

Fold Change

☐ Use Fold Change Filter

NOTEL/LOTEL Determination

P-Value: 0.05 ☐ Dunnett's Test

Fold Change Value: 2.0 ☒ T-Test

Execution Parameters

Number of Threads: 8

Start Save Settings Cancel



# BMDExpress v2 Demo

Williams Trend Test

Expression Data: NTP\_Gwinn\_DEHP\_Male\_Liver\_S1500

P-Value Cutoff: 0.05

Number of Permutations: 1000

Multiple Testing Correction: ☐ Benjamini & Hochberg (FDR)

Filter Out Control Genes: ☒ (probes starting with AFFX...)

Fold Change

☒ Use Fold Change Filter

Fold Change Val... 1.5

NOTEL/LOTEL Determination

P-Value: 0.05 ☐ Dunnett's Test

Fold Change Value: 1.5 ☒ T-Test

Execution Parameters

Number of Threads: 8

Start Save Settings Cancel



# BMDExpress v2 Demo

BMD Analysis

Data Options

Expression Data: NTP\_Gwinn\_DEHP\_Male\_Liver\_S1500

Prefilter: NTP\_Gwinn\_DEHP\_Male\_Liver\_S1500\_williams\_...

Continuous Models

<input checked="" type="checkbox"/> Exp 2	<input checked="" type="checkbox"/> Exp 3	<input checked="" type="checkbox"/> Exp 4	<input checked="" type="checkbox"/> Exp 5
<input checked="" type="checkbox"/> Linear	<input checked="" type="checkbox"/> Poly 2	<input type="checkbox"/> Poly 3	<input type="checkbox"/> Poly 4
<input checked="" type="checkbox"/> Hill	<input checked="" type="checkbox"/> Power		

Parameters

Maximum Iterations: 250

Confidence Level: 0.95

☒ Constant Variance

BMR Type: Standard Devia...

BMR Factor: 1.349 (10%)

Restrict Power:  $\geq 1$

Model Selection

BMDL and BMDU: Compute and utiliz...

Best Poly Model Test: Lowest AIC

☒ Flag Hill Model with 'k' Param...

Best Model Selection with Flagged Hill Model

Modify BMD of flagged Hill as Best Models with Fraction of Minimum BMD: 0.5

P-Value Cutoff: 0.1

1/3 of Lowest Positive Dose

Select Next Best Model with P-Value > 0.05

Multiple Threads

Number of Threads: 8

Model Execution Timeout (secs): 600 (default)

Start Save Settings Cancel





# BMDExpress v2 Demo

BMDExpress Gene Ontology Category Analysis

Category Analysis **IVIVE**

Benchmark Dose Data:

GO Categories **biological pr...**

☐ Is the Data In Vitro

☒ Remove Promiscuous Probes

☒ Remove BMD > Highest Dose from Category Descript...

☒ Remove BMD with p-Value < Cutoff:

☒ Remove Genes with BMD/BMDL >

☐ Remove Genes with BMDU/BMD >

☐ Remove Genes with BMDU/BMDL >

☐ Remove Genes With BMD Values > N ...

☒ Remove Genes With Max Fold Change <

☒ Remove Genes With Prefilter p-Value >

☐ Remove Genes With Prefilter Adjusted ...

☐ Eliminate Gene Set Redundancy

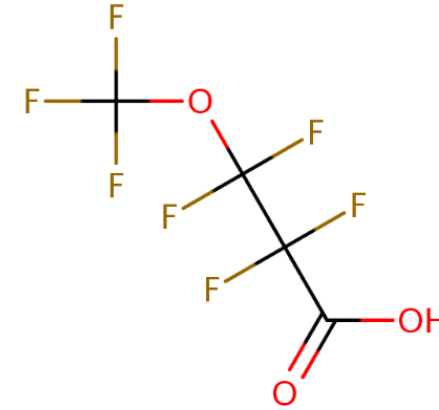
Probe Set to Gene Conversion

☒ Identify Conflicting Probe Sets

Correlation Cutoff for Conflicting Probe Sets:

Tested Chemical:

Perfluoro-3-Methoxypropanoic Acid (MOPA)



- Tested at 9 doses ranging from 0.01 to 300.0 mg/kg-day
  - 104 animals profiled total (52 male, 52 female)
  - TempO-seq S1500+ profiling of 12 tissues
- 17 / 1,092 samples (<2%) removed during QC process



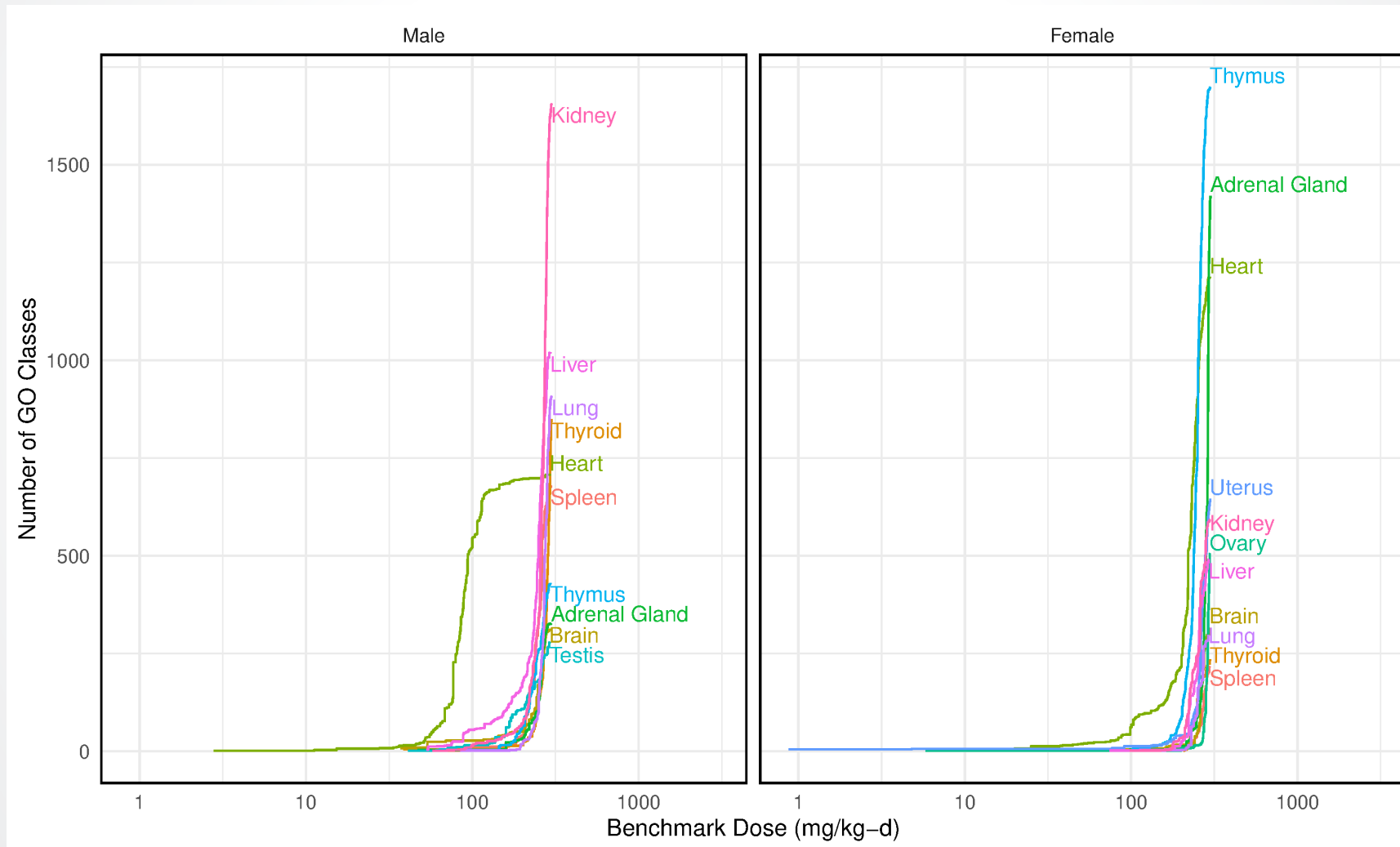
# Dose Response Modeling

1. Evaluate dataset for adequate signal
  - Evaluated separately for each tissue, sex
  - All tissues profiled passed ANOVA test for at least 1 probe
2. Pre-modeling filtering for dose-responsive probes →
3. Dose-response modeling of individual probes
4. Summarization of BMD(L) for known gene sets

Tissue	Male	Female
Adrenal Gland	170	327
Brain	103	110
Heart	252	296
Kidney	540	153
Liver	347	158
Lung	340	86
Ovary	NA	168
Spleen	163	89
Testis	120	NA
Thymus	121	419
Thyroid	277	166
Uterus	NA	183



# BMD Accumulation Plot



Accumulation plots of GO biological process classes by median benchmark dose value for each tissue



# POD Identification and Considerations

## **Select GO:BP class with lowest BMD across all tissues, both sexes**

- Only consider GO:BP classes with 3 or more dose-responsive genes
- If there are multiple most sensitive GO:BP classes by BMD, select GO:BP class with lowest median BMDL

## **“No Value” ETAP declared if:**

- No tissue produces any valid GO:BP classes
- Most sensitive GO:BP class BMD > 3-fold below lowest positive dose

*(Report would include dose range tested in both cases)*



# Lowest BMD by Tissue (Males)

Tissue	GO Accession	Gene Ontology Biological Process Class	# of Genes with BMD	BMD (mg/kg-day)	BMDL (mg/kg-day)
Adrenal Gland	GO:0051248	negative regulation of protein metabolic process	3	$4.68 \times 10^1$	$1.08 \times 10^1$
Brain	GO:0033365	protein localization to organelle	3	$3.70 \times 10^1$	$8.35 \times 10^0$
Heart	<b>GO:0048608</b>	<b>reproductive structure development</b>	<b>3</b>	<b><math>2.80 \times 10^0</math></b>	<b><math>8.93 \times 10^{-1}</math></b>
Kidney	GO:1901568	fatty acid derivative metabolic process	3	$5.76 \times 10^1$	$3.77 \times 10^1$
Liver	GO:0006656	phosphatidylcholine biosynthetic process	3	$5.30 \times 10^1$	$3.53 \times 10^1$
Lung	GO:0070374	positive regulation of ERK1 and ERK2 cascade	4	$8.45 \times 10^1$	$1.13 \times 10^1$
Spleen	GO:0007519	skeletal muscle tissue development	4	$6.30 \times 10^1$	$3.87 \times 10^1$
Testis	GO:0090304	nucleic acid metabolic process	4	$4.11 \times 10^1$	$5.63 \times 10^0$
Thymus	GO:0010629	negative regulation of gene expression	3	$6.64 \times 10^1$	$4.09 \times 10^1$
Thyroid	GO:0071320	cellular response to cAMP	3	$3.85 \times 10^1$	$1.01 \times 10^1$

Lowest GO biological process class median benchmark dose values across tissues in male rats.

**Heart was the most sensitive tissue in males with BMD = 2.8 mg/kg-day and BMDL = 0.893 mg/kg-day.**





# Lowest BMD by Tissue (Females)

Tissue	GO Accession	Gene Ontology Biological Process Class	# of Genes with BMD	BMD (mg/kg-day)	BMDL (mg/kg-day)
Adrenal Gland	GO:1901655	cellular response to ketone	5	$9.78 \times 10^1$	$5.15 \times 10^1$
Brain	GO:0097305	response to alcohol	3	$8.09 \times 10^1$	$4.49 \times 10^1$
Heart	GO:1901216	positive regulation of neuron death	3	$1.86 \times 10^1$	$3.15 \times 10^0$
Kidney	GO:0042594	response to starvation	3	$9.79 \times 10^1$	$7.96 \times 10^1$
Liver	GO:0034641	cellular nitrogen compound metabolic process	5	$7.44 \times 10^1$	$4.50 \times 10^1$
Lung	GO:0032355	response to estradiol	3	$1.20 \times 10^2$	$5.39 \times 10^1$
Ovary	GO:0060612	adipose tissue development	3	$5.80 \times 10^0$	$6.38 \times 10^{-1}$
Spleen	GO:0045597	positive regulation of cell differentiation	3	$1.53 \times 10^2$	$1.10 \times 10^2$
Thymus	GO:0060070	canonical Wnt signaling pathway	3	$9.00 \times 10^1$	$4.97 \times 10^1$
Thyroid	GO:0045597	positive regulation of cell differentiation	3	$2.06 \times 10^2$	$1.41 \times 10^2$
Uterus	<b>GO:0051271</b>	<b>negative regulation of cellular component movement</b>	<b>3</b>	<b><math>8.72 \times 10^{-1}</math></b>	<b><math>1.21 \times 10^{-1}</math></b>

Lowest GO biological process class median benchmark dose values across tissues in female rats.

**Uterus was the most sensitive tissue in females with BMD = 0.872 mg/kg-day and BMDL = 0.121 mg/kg-day. This was the most sensitive tissue overall, and was therefore used to determine the overall POD.**



## Conversion to TRV

- Transcriptomic POD (tPOD) = 0.121 mg/kg-d
  - Median BMDL from most sensitive gene set, detected in uterus
- Convert to Human Equivalent Dose (HED) based on standard factor

$$BMDL_{HED} = BMDL \times \frac{BW_{Animal}^{1/4}}{BW_{Human}^{1/4}} = 0.121 \times \frac{0.227 \text{ kg}^{1/4}}{80 \text{ kg}^{1/4}} = 0.0279 \text{ mg/kg} - d$$

- Apply uncertainty factors (UF) to derive TRV
  - Current draft method proposes a standard composite UF = 300

$$TRV = \frac{BMDL_{HED}}{\text{Composite UF (300)}} = 0.00009 \text{ mg/kg} - d$$

# Future Research Directions



**No single “best” method for analyzing high-throughput transcriptomic data**

- Alternate methods used in other contexts
- Important to benchmark methods for the intended purpose, platform, and study design!

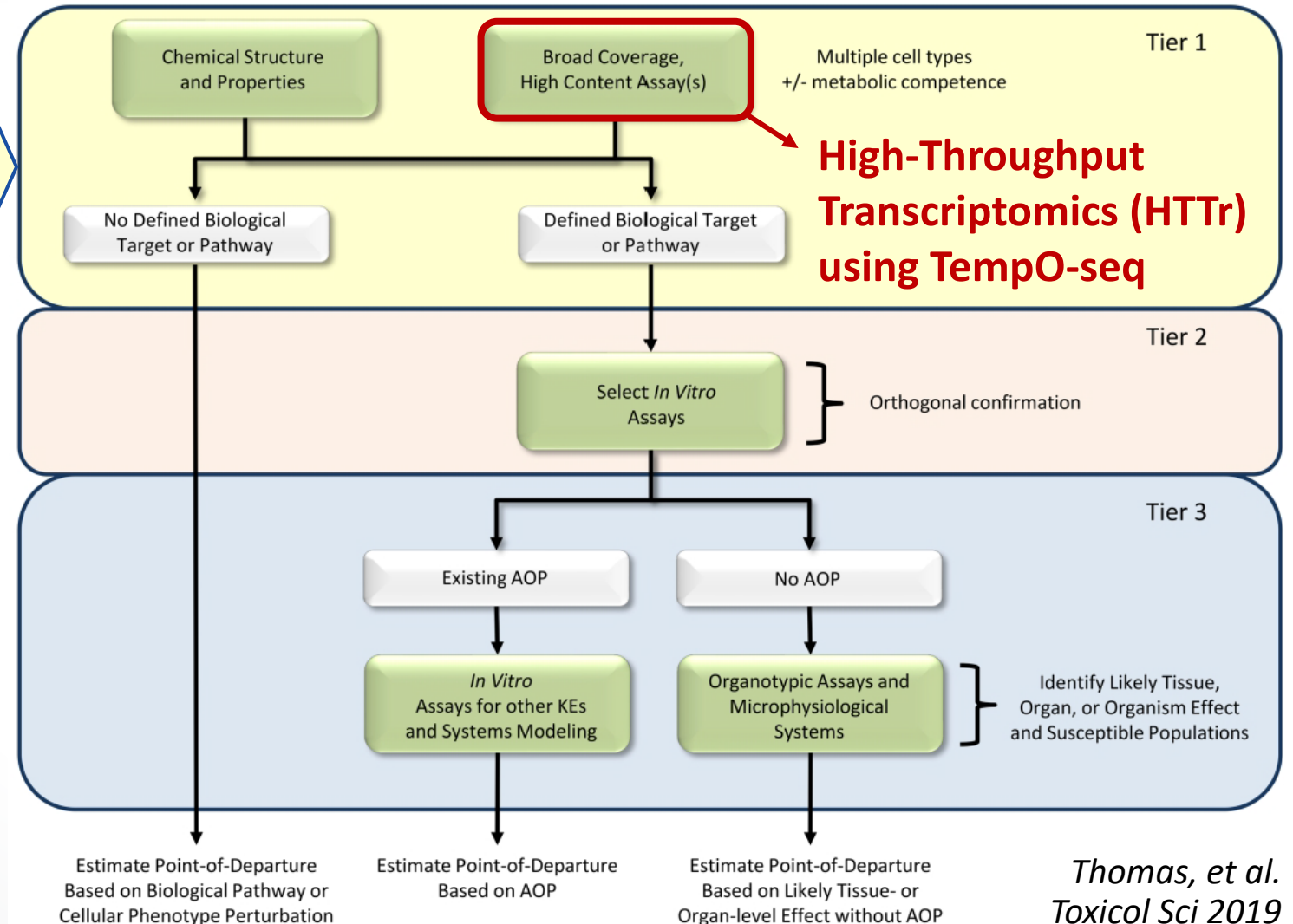




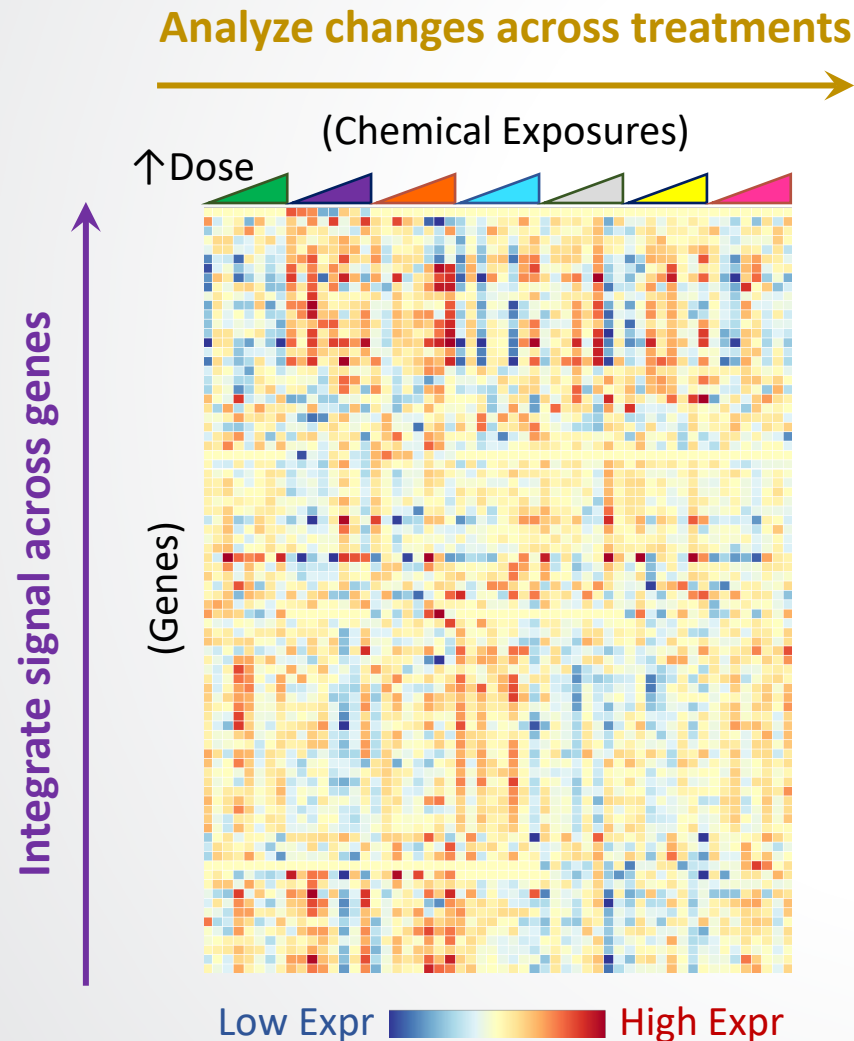
# *in vitro* Tiered Testing Strategy

## Tier 1 Primary Goals:

- Acute (6-24h) exposures *in vitro*
- Prioritize chemicals by bioactivity & potency
- Predict biological targets for chemicals



# Transcriptomic Dose-Response Models



- Different genes may respond at different doses of a given exposure!
- **Need to analyze both:**
  - **Dose-responsive trends**
  - **Coordinated changes in gene expression**
- Gene-level data noisier in transcriptomics than targeted measurements (e.g. RT-qPCR)
- Dose-response modeling thousands of features (e.g. mRNA levels) leads to computational & statistical challenges





# Alternate Dose-Response Modeling Methods

**Many other analysis methods proposed, this is an active area of research!**

Bayesian Methods:

- BIFROST – *Reynolds, et al. 2020* DOI: [10.1016/j.comtox.2020.100138](https://doi.org/10.1016/j.comtox.2020.100138)
- BBMD – *Shao & Shapiro, 2018* URL: [benchmarkdose.com](https://benchmarkdose.com)
- ToxicR – *Wheeler, et al. 2022* GitHub: [NIEHS/ToxicR](https://github.com/NIEHS/ToxicR)

Dose-response modeling of aggregate gene signal:

- Signature scores - *Harrill, et al. 2021* DOI: [10.1093/toxsci/kfab009](https://doi.org/10.1093/toxsci/kfab009)
- Latent variables – *Basili, et al. 2022* DOI: [10.1021/acs.chemrestox.1c00444](https://doi.org/10.1021/acs.chemrestox.1c00444)





# Alternative Methods for Deriving tPOD

ETAP/NTP  
Methods →

Gene-based Methods	
Nth Percentile (e.g. 5 <sup>th</sup> %ile) BMD	<a href="#">Reardon, et al. <i>Tox Sci</i> 2021</a>
Nth Lowest (e.g. 25 <sup>th</sup> ) BMD	
Gene Set/Category-based Methods	
Lowest Active Gene Set BMD	<a href="#">Gwinn, et al. <i>Tox Sci</i> 2020</a>
5 <sup>th</sup> Percentile Gene Set BMD	<a href="#">Harrill, et al. <i>Tox Sci</i> 2021</a>
Global Methods:	
Multi-dimensional POD (e.g. Mahalanobis Distance)	<a href="#">Nyffeler, et al. <i>SLAS Discov</i> 2021</a>

*in vitro*  
Methods

For recent assessment of multiple tPOD metrics in the context of in vitro transcriptomic studies, see:  
*Reardon, et al. 2023* DOI:[10.3389/ftox.2023.1194895](https://doi.org/10.3389/ftox.2023.1194895)



# Summary

- Transcriptomic profiling captures many types of bioactivity in one assay
- No single best way to analyze the data – depends on use-case
  - Important to benchmark methods for each purpose, platform, and study design
- EPA Transcriptomic Assessment Product (ETAP) based on 5-day transcriptomic study in rats with many doses and tissues
- Transcriptomic Reference Value corresponds to BMDL of most sensitive gene set across all tissues
  - Mechanism agnostic, does not predict specific hazards



# Acknowledgements

## Questions?

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## Course Material Available Online:

[doi.org/10.23645/epacomptox.23786334.v1](https://doi.org/10.23645/epacomptox.23786334.v1)

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