Reference Materials for Transcriptomics and Metabolomics Technologies

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OECD
BETTER POLICIES FOR BETTER LIVES

Disclaimer

- The views expressed in this presentation are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

Background / Definitions

- **Transcriptomics** is the study of transcriptomes: i.e. all, or a portion of, the RNAs (typically mRNA) expressed from the genes of an organism, tissue or cell.

- **Reliability** is "the extend of reproducibility of results from a test within and among laboratories over time, when performed using the same standardized protocol" (OECD GD 34). Has been identified as a key criteria for the acceptance and use of NAMs data in regulatory settings (USEPA 2018).

- The **need** and **means** for demonstrating **reliability** of transcriptomics data prior to use in regulatory settings has been recognized in the scientific literature:

  – **MAQC Consortium, 2006:** *"Concerns have raised regarding the **reliability** and consistency and hence potential application of microarray ['omics] technology in clinical and regulatory settings...it follows that before this technology can be applied in clinical practice and **regulatory decision making**, standards, quality measures and consensus on data analysis methods need to be developed...['omics] studies need **unified metrics and standards**, which can be used to identify suboptimal results and monitor performance..."*

  – **Sauer et al., 2017:** *"The establishment of **calibrated RNA samples** and **reference datasets** were identified as crucial for an objective assessment of the performance of different microarray ['omics] platforms.*

What are Reference Materials for 'Omics?

- **Standardized reference materials** are either commercially manufactured or generated in bulk in a research laboratory and contain a mixture of biological molecules in varying amounts that can be measured using an 'omics platform.

  – **Transcriptomics → RNA Species**

  – Metabolomics → Small molecules

  – Proteomics → Proteins / peptides

- *"Expression values generated on different ['omics] platforms cannot be directly compared because unique labeling method and probe sequences will result in variable signals for probes that hybridize to [measure] that same target. Alternatively the relative expression **between a pair of sample types should be maintained across platforms.**"* [MAQC, 2006]

- *The combination of **biologically different RNA sources** and **known titration differences** provides a method for assessing the relative accuracy of an 'omics platform based on differential detection* (MAQC, 2006, adapted).

Key Characteristics of Reference Materials for Transcriptomics

Key Characteristics:

- Paired samples from diverse sources or with diverse gene expression profiles.

- Substantial overlap in the species of biomolecules contained within each sample.

- Large dynamic range in expression levels / constituent concentrations upon comparison of samples.

- Compatibility with 'omics platform

- Qualitatively similar to test samples (i.e. sample matrix)

- Widespread availability.

- Suitability for use across 'omics technologies

| MAQC ID	Description
Sample A	Universal Human Reference RNA (UHRR)
Sample B	Human Brain Reference RNA (HBRR)
Sample C	75 % UHRR | 25 % HBRR
Sample D	25 % UHRR | 75 % HBRR
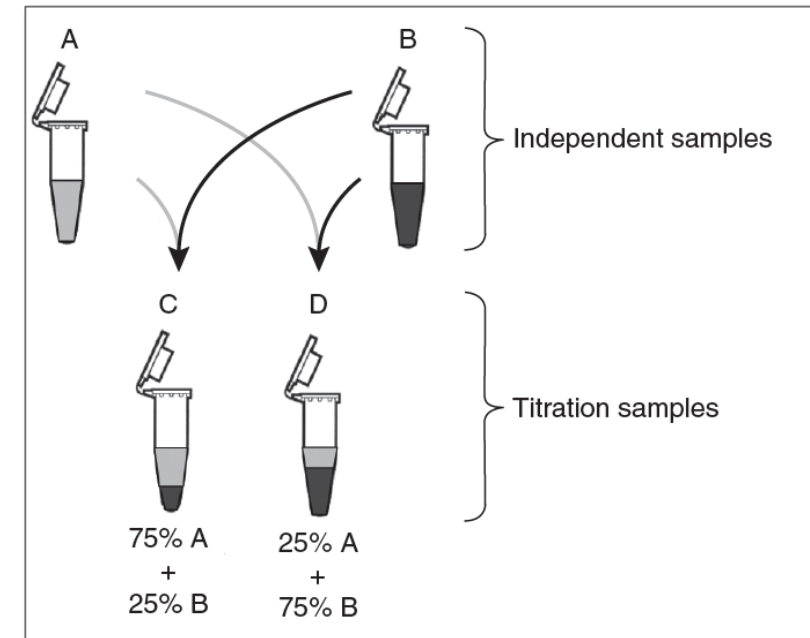
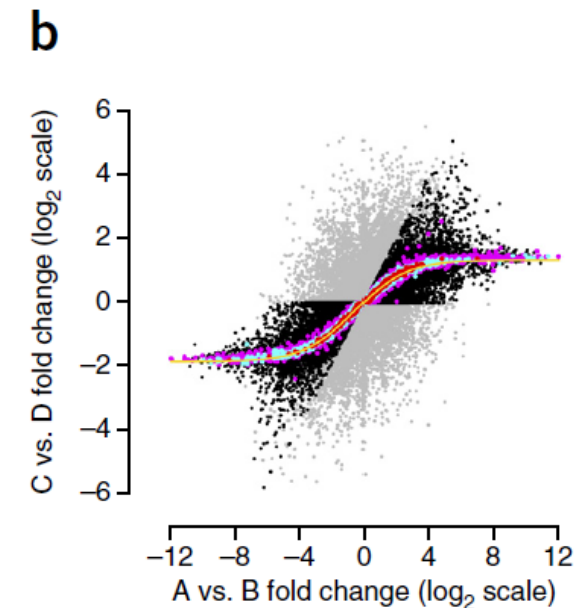*From Shippey et al (2006)*

Properties of MAQC Reference Samples

- Detectable expression of > 12,000 genes in each sample using RNA-Seq.

- Expression of > 10,000 genes in common across the two samples.

- A dynamic range of differential expression spanning greater than 20 $\log_2$ units.
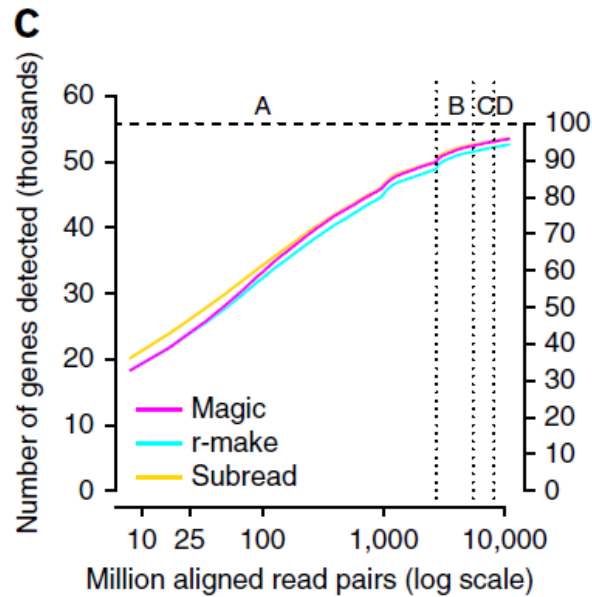
SEQC/MAQC-III Consortium (2014)
Figure 1C.
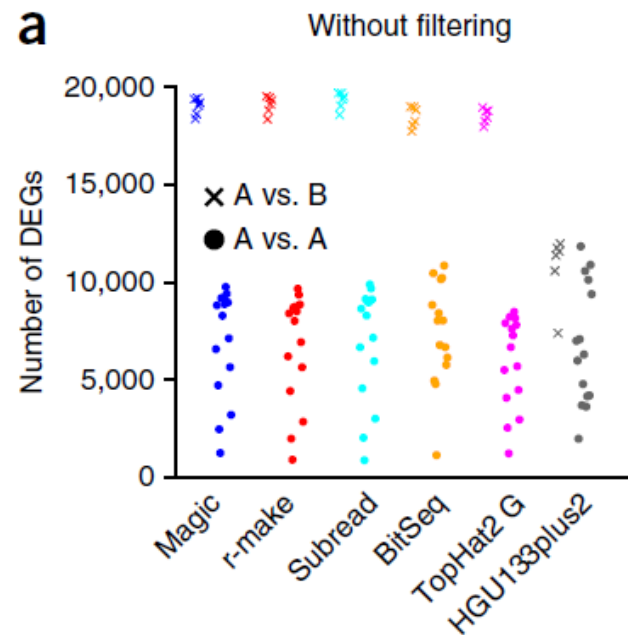
SEQC/MAQC-III Consortium (2014)
Figure 2A.

SEQC/MAQC-III Consortium (2014)
Figure 3B.

Use of Reference Materials In Transcriptomics Studies

- Reference materials are intended to provide objective evaluation(s) of the **technical performance** of an 'omics assay…**NOT the biological response** of an *in vivo* or *in vitro* test system.
    - Use reference treatments for this latter purpose.

- Processed in parallel with test samples → they should be subject to the same manipulations and assay conditions as test samples.

- Implemented in a manner that facilitates monitoring of comparability and consistency of transcriptomics assay results generated within studies, across studies, across laboratories and over time.

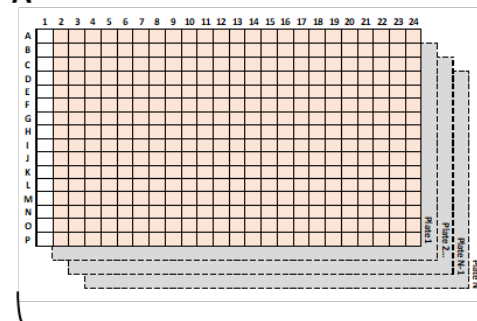# Example of Use of Reference Materials for High Throughput Transcriptomics (HTTr)
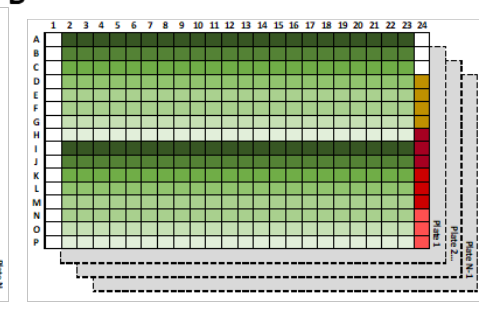
Reference Material Pair 1
- Purified RNAs

Reference Material Pair 2
- Bulk Lysates
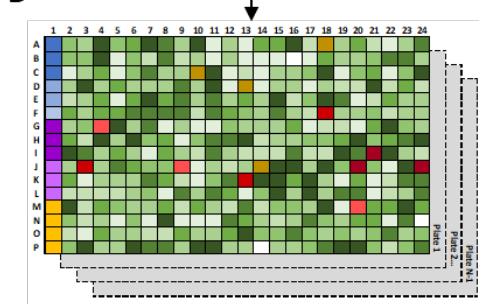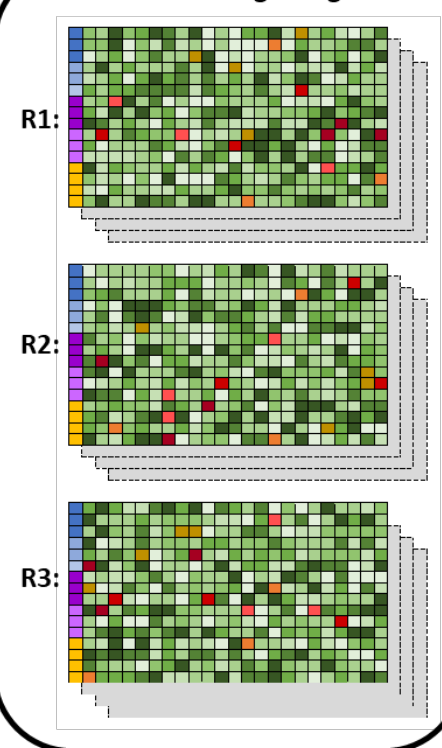
From Harrill et al. (2019)
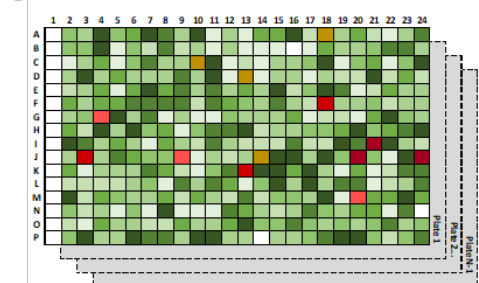
HTTr Screening Study Design

Parameter	Multiplier	Notes
Cell Type(s)	1	U-2 OS
Culture Condition	1	DMEM + 10% HI-FBS
Chemicals	1,218	ToxCast ph1, ph2 Nominated chemicals from e1k / ph3
Time Points:	1	24 hours
Assay Formats:	2	High Throughput Transcriptomics (TempO-Seq) High Throughput Phenotypic Profiling (Cell Painting)
Concentrations:	8	3.5 $\log_{10}$ units; ~half-$\log_{10}$ spacing
Biological Replicates:	3	--
HTTr Assay Plates	87	--
Reference Materials	2 pairs	Takara Bio UHRR (636690) Takara Bio HBRR (636530) DMSO Bulk Lysate Trichostatin A Bulk Lysate

Metrics for Assessing Transcriptomics Assay Reproducibility

- **Sample-Based Metrics**
  - Gene abundance distribution
- **Comparison of Like Sample**
  - Correlation of expression values
  - D-statistic
- **Comparison of Paired Samples**
  - Differential Gene List Overlap**
  - Dynamic range of FC
  - Correlation of L2FC profiles
  - Gene signature / pathway enrichment concordance (*if applicable*)
- **Dilution Series**
  - Repeatability of assay sensitivity

**Lesson Learned from MAQC

> A straightforward approach of FC ranking plus a non-stringent p-cutoff can be successful in identifying reproducible gene lists, whereas ranking and selecting differentially expressed genes solely by the t-test statistic **predestine a poor concordance in results**, in particular for shorter gene lists, due to the relatively unstable nature of the variance (noise) estimate in the t-statistic measure.
>
> Furthermore, the impact of normalization methods on the reproducibility of gene lists becomes minimal when the fold change, instead of the p-value, is used as the ranking criterion for gene selection

- Each of these metrics are either qualitative, quantitative, comparative or some combination of each.
- Interpretation requires defining typical performance ranges.

Sample Based Metrics for Transcriptomics Assay Performance (1)

Correlation of Expression Values

Abbreviation	Description
NMR	Number of mapped reads, defined as a sum of total read counts summed over all detected probes.
FMR	Fraction of uniquely mapped reads
Ncov5	The number of probes with at least 5 uniquely mapped reads
Nsig80	The number of probes capturing the top 80% of signal in a sample
Gini	A measure of inequality in a distribution. Computed based on the distribution of raw counts for all probes including those with 0 aligned reads.

Flagging gates for gene abundance distribution metrics are based on Tukey's Outer Fence principle
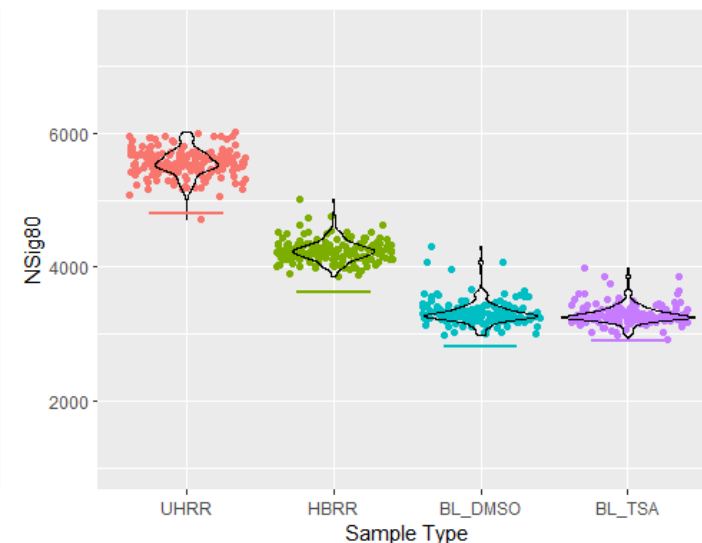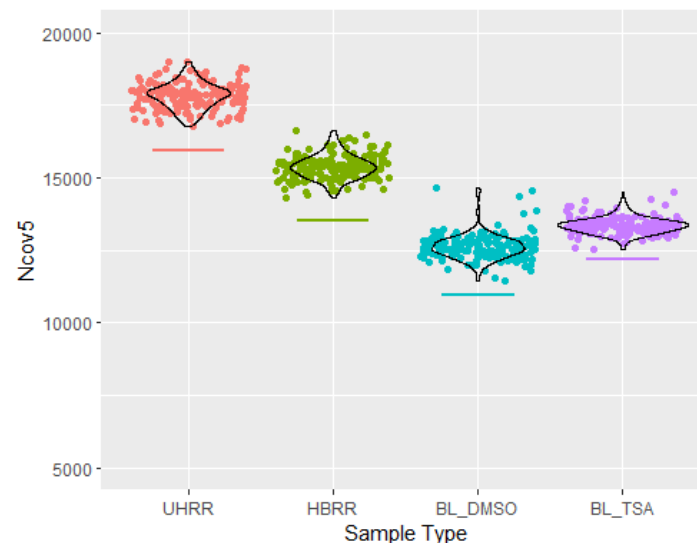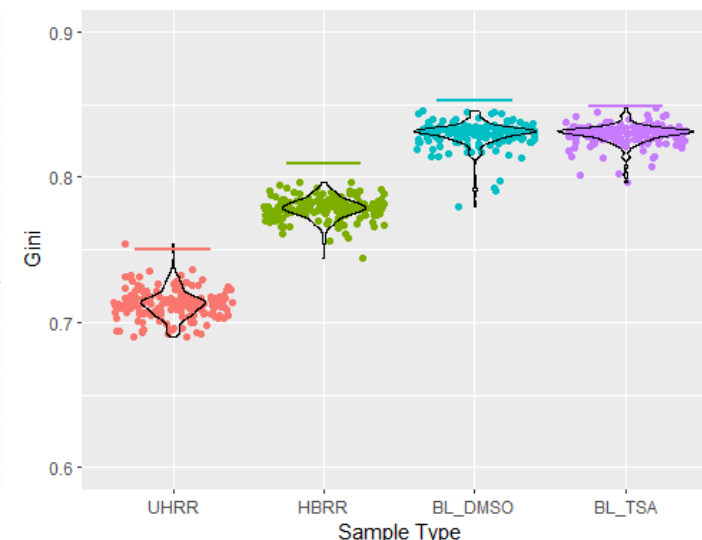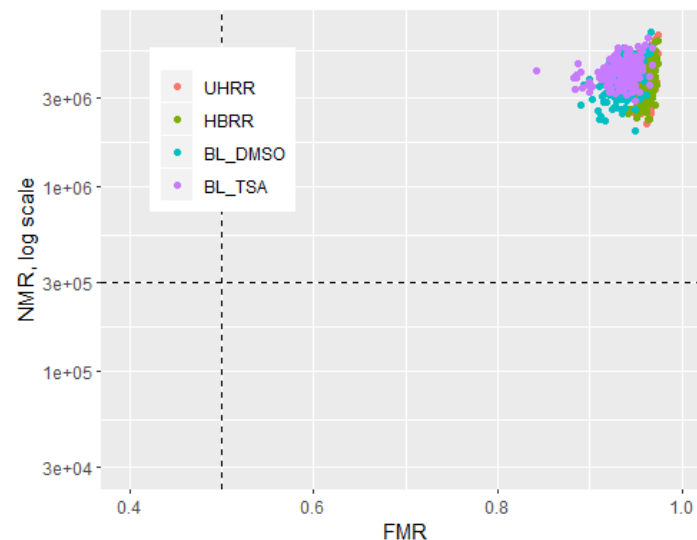
Comparison of Like Samples for Transcriptomics Assay Performance

Correlation of Expression Values

174 samples of each type across 87 assay plates
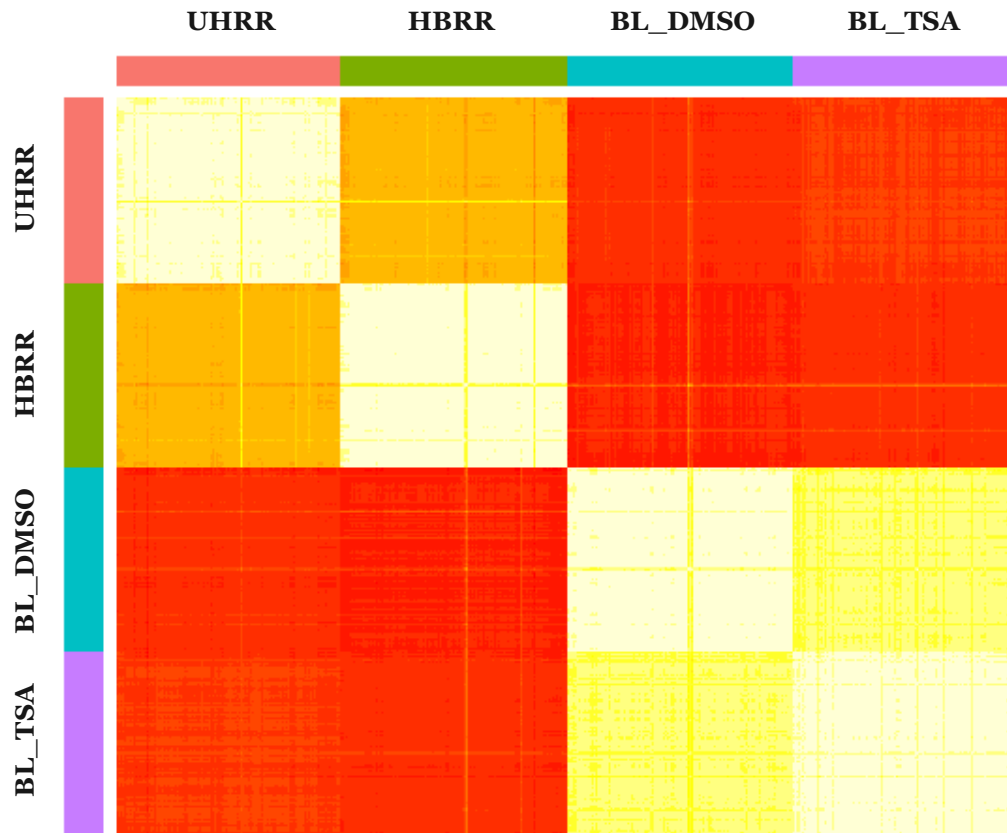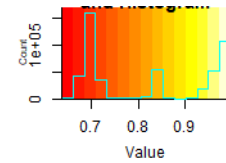
D-Statistic (House et al. 2017)

Mean correlation of CPM to like samples

Reference samples on plate 578 flagged based on D-statistic
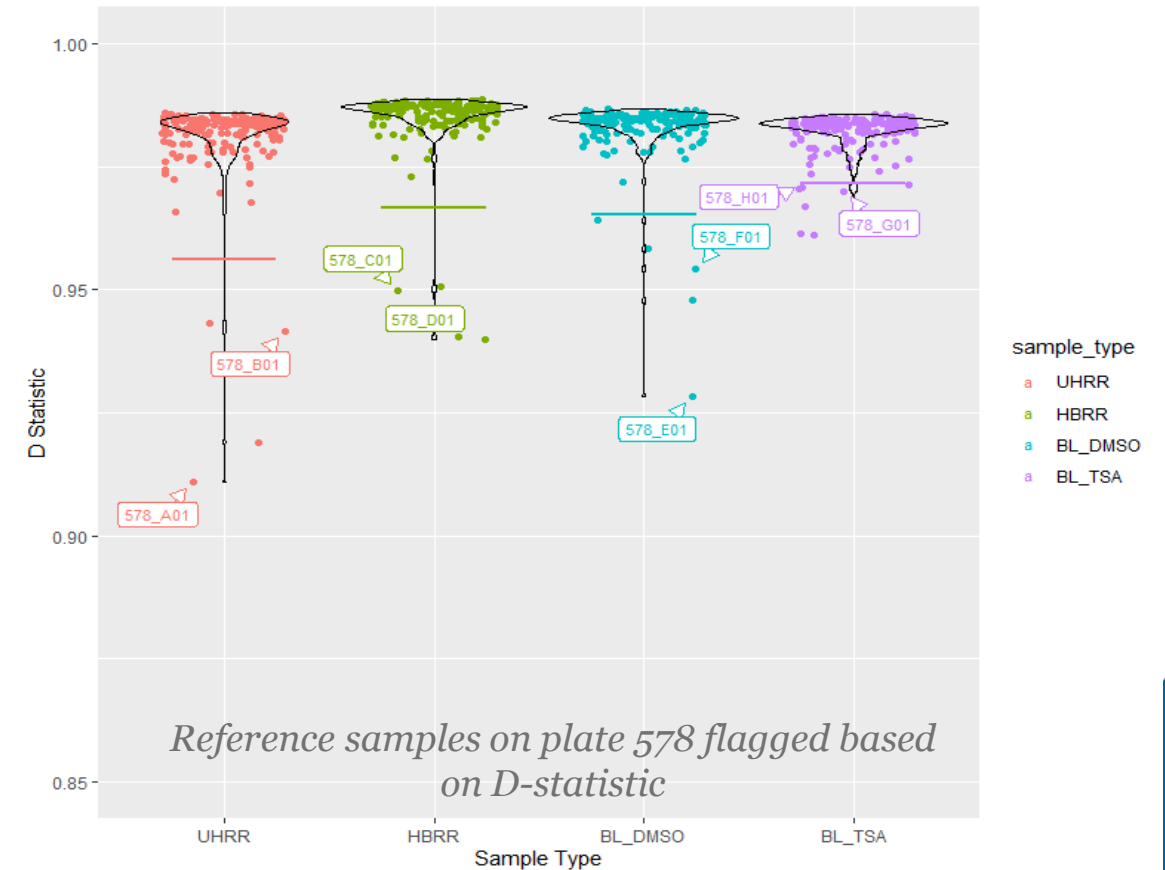
Comparison of Paired Samples for Transcriptomics Assay Performance

Correlation of L2FC Profiles
Pairs of standards across 87 assay plates

D-Statistic
Mean correlation of like FC profiles

RNA Standards **Lysate Standards**
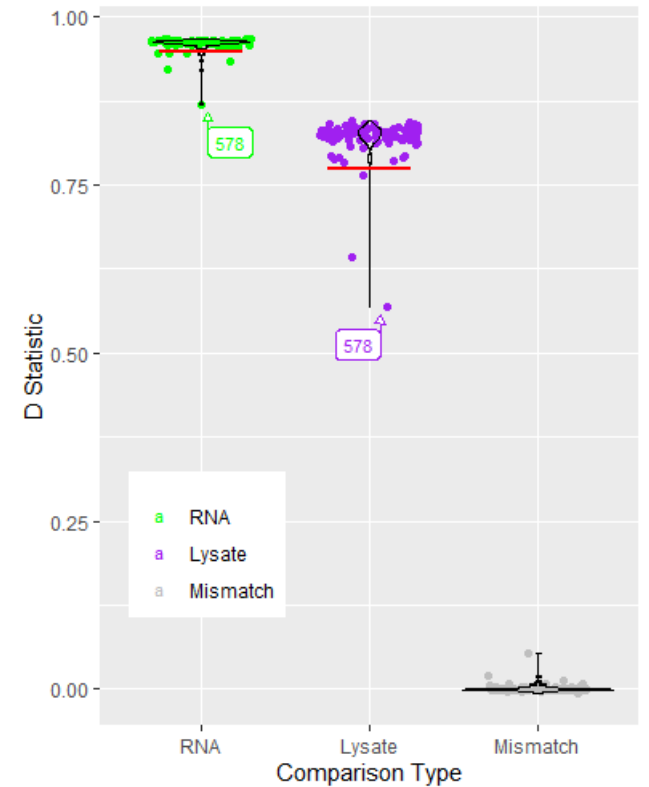
FC profiles of RNA and Lysates were highly dissimilar → very different source materials

Engineering of Transcriptomics Reference Samples (1)

- MAQC and Takara reference materials were prepared as purified RNA samples from tissues of distinct individuals.

  - Not optimal for evaluating performance of cell lysate-compatible transcriptomics assays.

  - Finite resource.

  - Were "selected" not "designed" §

- There is a need to develop **replenishable** human-derived transcriptomics reference materials that are:

  - Compatible with multiple assay modalities

  - Can be produced in sufficient quantities to satisfy the needs of the research community at large

  - Yield reproducible fold-change profiles across production batches

§ personal communication from MAQC / W. Tong

Engineering of Transcriptomics Reference Samples (2)

- Paired reference materials were prepared by combining the genetic material from different human-derived cell lines cultured under different conditions.

- Formulated to mimic the performance characteristics of MAQC samples.

- Prepared as both purified RNAs and cell lysates (*BioSpyder, Inc*).

Sample	Number of Genes >5 counts at a depth of 8M
BURR1	13,962
BURR2	13,779
BURL1	14,919
BURL2	14,565

Samples	Number of Genes >5 counts in Common
BURR1 and BURR2	12,881
BURL1 and BURL2	13,546
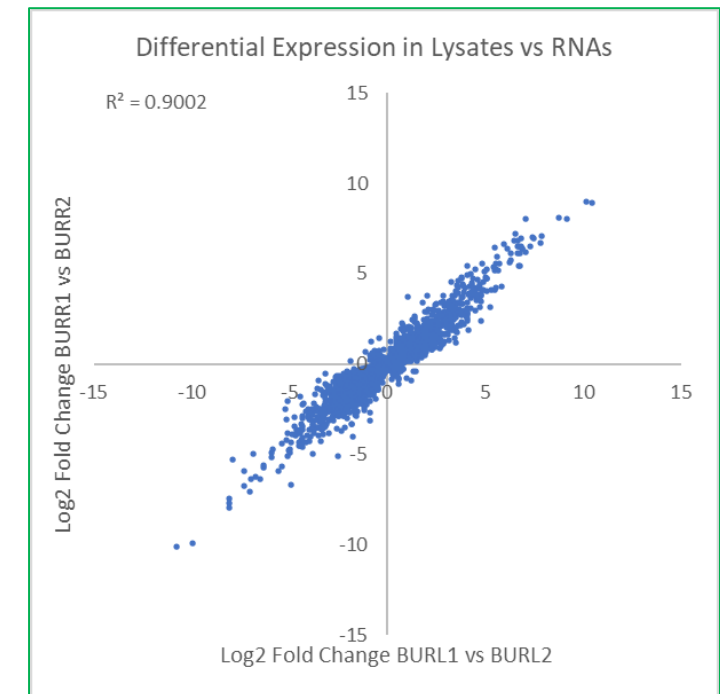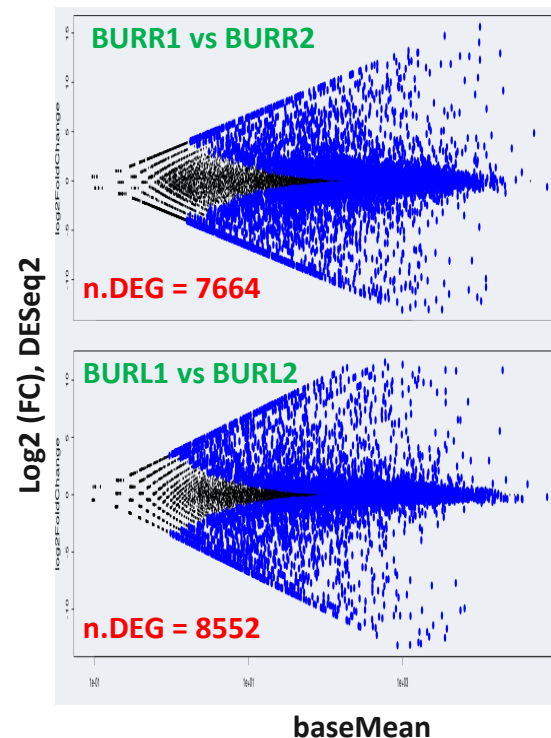
§ Standard formulation, prep and analysis by BioSpyder, Inc.

Perspective & Outlook

- Demonstration of transcriptomics assay reliability using reference materials would support the uptake of 'omics data into regulatory decision making processes.

- Reference materials should be incorporated into transcriptomics experimental design in a manner that would facilitate objective evaluation of assay performance.

- Needs with the research / regulatory community:
  - Replenishable reference materials that are widely available & compatible with multiple 'omics platforms.
  - Best practices for evaluating transcriptomics assay performance in the context of a toxicology study.
  - Reporting mechanisms for use of reference materials → **Transcriptomics Reporting Framework (TRF)**

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- Kyle LeBlanc
- Garrett McComb

Reference Materials for Metabolomics Technologies

- **Two principal uses**
  - Integral part of QA procedures and QC samples in metabolomics
  - Required for the reliable <u>identification and quantification</u> of metabolites

- **Purposes of presentation**
  - High level introduction to topic
  - Reassure EAGMST that progress is being made, internationally
  - Identify near-future needs for *new* references materials, when applying metabolomics in regulatory toxicology

Reference Materials for Metabolite Identification

- Metabolites - the <u>endogenous</u> low molecular weight biochemicals in a biological test system (e.g. cholesterol, T3 and T4 thyroid hormones, etc.)

- Identifying metabolites is a critical step in interpreting metabolomics data
  - to define metabolic Key Events in an AOP
  - to bring confidence to identifying a hazard based on changes in a metabolic biomarker pattern

- **However, identifying thousands of metabolites in a metabolomics study is a major challenge**

- To ensure that we can accurately describe the extent and quality of metabolite identification within a study, the international community defined a series of confidence levels for identification.

International Metabolomics Standards Initiative: Levels of Metabolite Identification

ORIGINAL ARTICLE

Proposed minimum reporting standards for chemical analysis

Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI)

Lloyd W. Sumner · Alexander Amberg · Dave Barrett · Mi...
Richard Beger · Clare A. Daykin · Teresa W.-M. Fan · Oli...
Royston Goodacre · Julian L. Griffin · Thomas Hankemeie...
James Harnly · Richard Higashi · Joachim Kopka · Andre...
John C. Lindon · Philip Marriott · Andrew W. Nicholls · M...
John J. Thaden · Mark R. Viant

Four levels of confidence in metabolite identification

- **Level 1 - <u>Identified</u> metabolite (requires a chemical reference standard for each metabolite)**

- Level 2 - <u>Putatively annotated</u> metabolites (not required)

- Level 3 - <u>Putatively characterized</u> metabolite classes

- Level 4 - <u>Unknown</u> metabolites

Levels of Metabolite Quantification

Three levels of confidence in metabolite quantification

- **Level 1 - <u>Absolute</u> quantification (requires a chemical reference standard for each metabolite)**

- Level 2 - <u>Semi-</u>quantification (requires a metabolite class reference standard)

- Level 3 - <u>Relative</u> quantification (not required)

To Achieve the Highest Confidence in a Metabolomics Study requires Reference Materials

Analytical Standards and Certified Reference Materials

Confidence in Metabolite Identification & Quantification is Deeply Embedded in OECD MRF
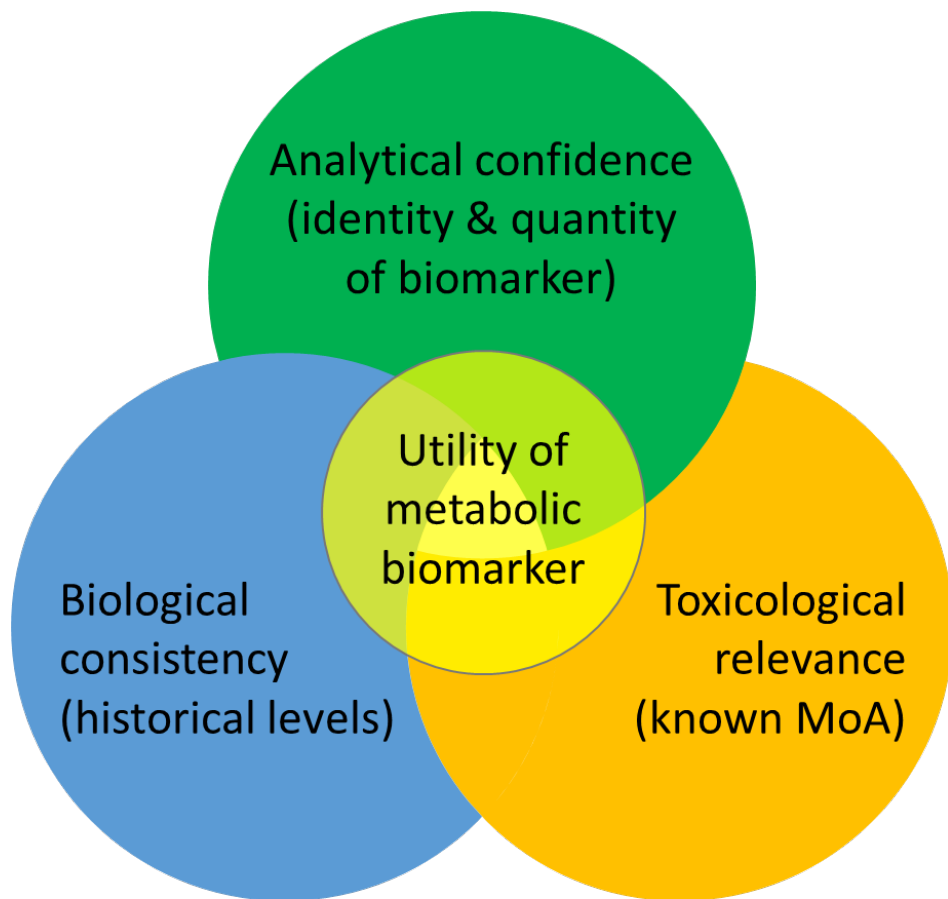
Modules within MRF

1. Summary Report

2. Toxicology Experiment Module

3. MRF Technology-specific Data Acquisition & Processing Reporting Modules

3.1 Mass Spectrometry Metabolomics Module

3.2 NMR Spectroscopy Metabolomics Module

4. Data Analysis Reporting Modules

4.1 Discovery of Differentially Abundant Molecules Module

4.2 Multivariate Statistical Analysis Module

5. Application Reporting Modules

5.1 Chemical Grouping for Read-Across Module

Theoretically a major challenge, but...

- Human Metabolome Database (HMDB 4.0) lists 114,100 metabolites (known, expected or predicted)

- Estimated that <20% can be purchased as reference materials

  ⇨ Most metabolites *cannot* be identified *nor* absolutely quantified (to level 1 confidence).

UhOh

- But do we need to identify *all* metabolites in a toxicology study?

- BASF measure ca. 205 metabolites (rodent metabolomics for grouping/read-across)

- What are the most critical metabolites, which we need to identify confidently using reference materials, in a toxicology study?

ECHA – Michabo Health Science: define a *standardised metabolic biomarker panel for toxicology*

- Conceptually this study is the metabolomics equivalent to the transcriptomics S1500+ study and gene set…

RESEARCH ARTICLE

A hybrid gene selection approach to create the S1500+ targeted gene sets for use in high-throughput transcriptomics

Deepak Mav[1], Ruchir R. Shah[1], Brian E. Howard[1], Scott S. Auerbach[2], Pierre R. Bushel[3], Jennifer B. Collins[4], David L. Gerhold[5], Richard S. Judson[6], Agnes L. Karmaus[6], Elizabeth A. Maull[2], Donna L. Mendrick[7], B. Alex Merrick[2], Nisha S. Sipes[2], Daniel Svoboda[1], Richard S. Paules[2]*

- We are currently reviewing multiple sources of information to extract *all known metabolites* associated with toxicity, adverse outcomes and disease.

ECHA – Michabo Health Science: define a *standardised metabolic biomarker panel for toxicology* (2)

- Current estimate: ca. 700 metabolites in the metabolic biomarker panel (version 1)

- Of these, ca. 40% are commercially available (from two metabolite standards libraries, with many other suppliers to be checked later in 2020)

Discuss with EAGMST:

- Following completion of proposed metabolic panel, request chemical suppliers to synthesize standards for all metabolites in the metabolic biomarker panel?

- Strategies to encourage stakeholder-uptake of this standardised metabolic biomarker panel?

Reference Materials are Integral to QA Procedures and QC Samples in Metabolomics

Metabolomics
Quality Assurance & Quality Control
Consortium (mQACC)

MERIT
MEtabolomics standaRds Initiative in Toxicology
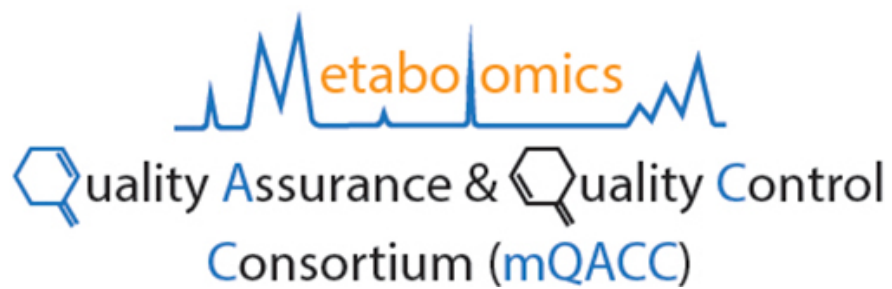
2018-2019

- Established in February 2018
- Tripartite - academic, industry and government
- Goal to address key QA/QC issues in metabolomics
- Reference & Test Material Working Group
- https://epi.grants.cancer.gov/Consortia/mQACC/

nature COMMUNICATIONS

PERSPECTIVE

https://doi.org/10.1038/s41467-019-10900-y   OPEN

Use cases, best practice and reporting standards for metabolomics in regulatory toxicology

Five Types of Quality Control Samples in Metabolomics

1. System Suitability QC
2. Intra-study QC
3. Intra-laboratory QC
4. Inter-laboratory QC
5. Process blank

- Different purposes
- Different compositions
- Different stages of development and hence community uptake

nature
COMMUNICATIONS

PERSPECTIVE

https://doi.org/10.1038/s41467-019-10900-y   OPEN

Use cases, best practice and reporting standards for metabolomics in regulatory toxicology

QA/QC is Deeply Embedded in OECD MRF

Modules within MRF

1. <mark>Summary Report</mark>

2. Toxicology Experiment Module

3. MRF Technology-specific Data Acquisition & Processing
Reporting Modules

   <mark>3.1 Mass Spectrometry Metabolomics Module</mark>

   <mark>3.2 NMR Spectroscopy Metabolomics Module</mark>

4. Data Analysis Reporting Modules

   4.1 Discovery of Differentially Abundant Molecules Module

   4.2 Multivariate Statistical Analysis Module

5. Application Reporting Modules

   <mark>5.1 Chemical Grouping for Read-Across Module</mark>

Inter-laboratory QC (as example)

Principal use: provides a measure of inter-laboratory analytical reproducibility

Cefic LRI-C8: Assessing the Repeatability of Metabolomics within a Regulatory Context through a Multi-Laboratory Ring-Trial (2020-22)

- Grouping/read-across ring-trial is based on *rodent plasma*
- We will use NIST Standard Reference Material 1950 - *Metabolites in Frozen Human Plasma* – as our inter-laboratory QC

Discuss with EAGMST:

- Should we have toxicologically relevant reference materials? Rodent plasma? HepaRG and other cell extracts?
- Request NIST to produce these?