Using ERCC Spike-In Control Transcripts Provides Confidence in Agilent Microarray and RNA-Seq Gene Expression Data

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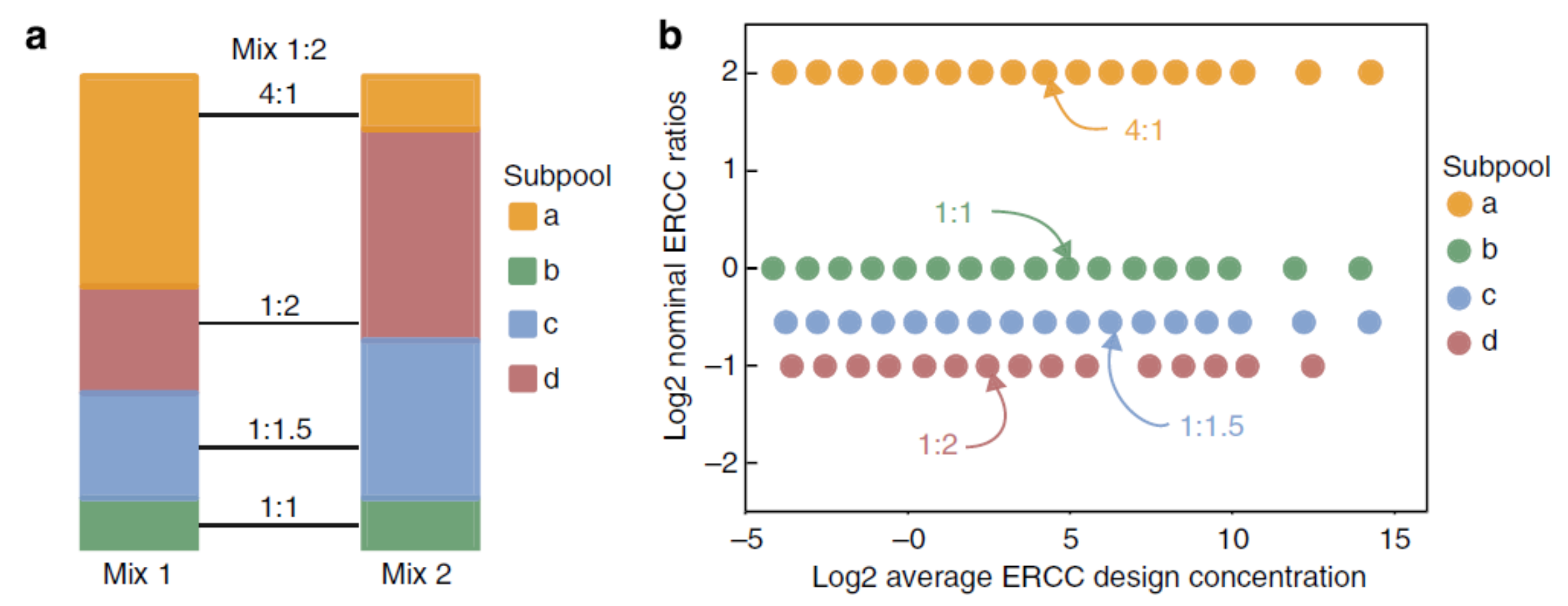
Introduction

Gene expression profiling is being widely applied in cancer research to identify biomarkers for clinical endpoint prediction such as diagnosis, prognosis, or prediction of therapeutic response. The advent of RNA deep sequencing (RNA-Seq) technologies has revolutionized eukaryotic transcriptome analysis by providing the ability to decipher global gene expression patterns beyond the limitation of microarrays, including the ability to discover novel genes and splice variants. However, data generated by the Sequencing Quality Control (SEQC) consortium has shown that RNA-Seq and microarray-based gene expression models are comparable in clinical endpoint prediction performance, even when the additional information unique to RNA-Seq is included in the prediction models.

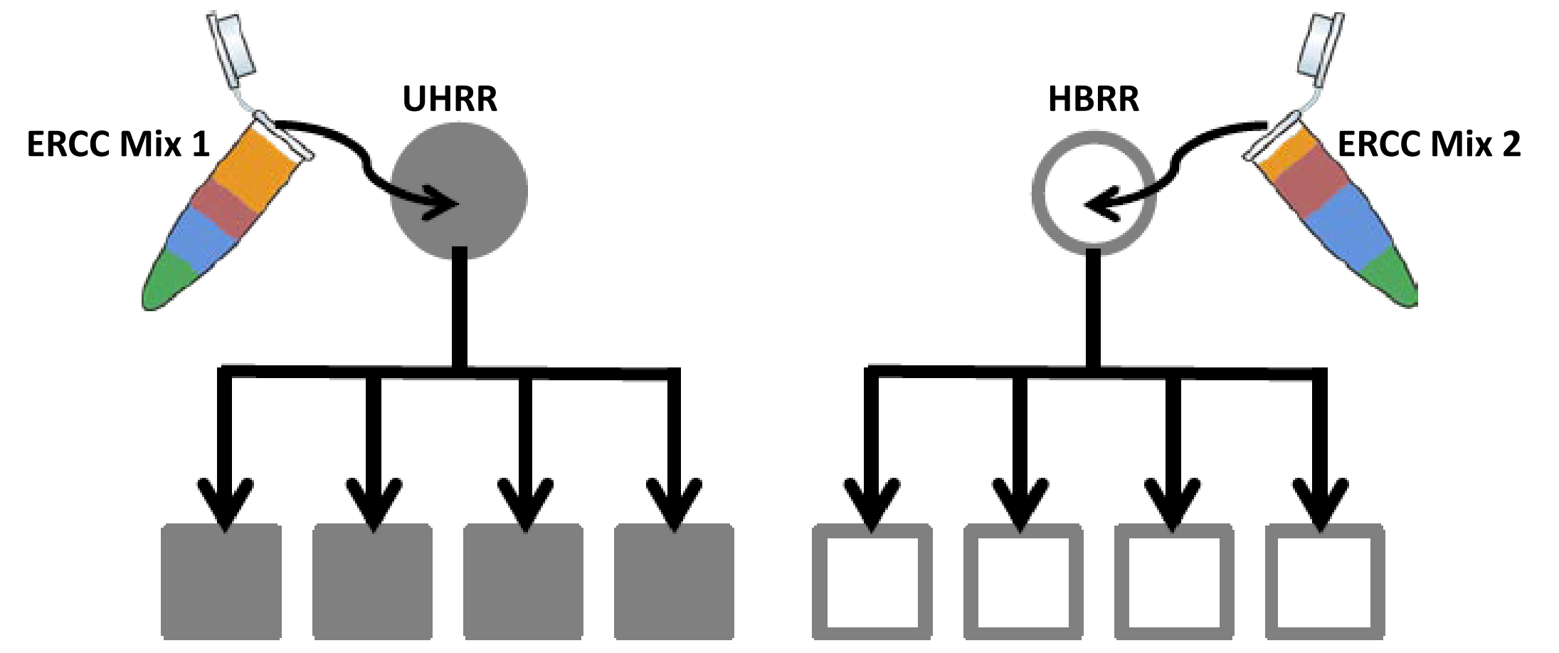
In order to understand and characterize the reproducibility and validity of gene expression experiments, exogenous RNA spike-in controls and associated ratio performance metrics have been introduced by the External RNA Controls Consortium (ERCC). The ERCC developed a library of 96 exogenous control transcripts from synthetic or bacterial sequences that are spiked into RNA at defined abundances and ratios, providing data that measures the technical performance of gene expression profiling methods. Scientists from the National Institute of Standards and Technology (NIST) have created a Bioconductor software tool called "ERCC*dashboard*" that produces ERCC performance metrics from expression values such as microarray signals or RNA-Seq read counts. Using the ERCC spike-in control transcripts and the "ERCC*dashboard*" analysis tool, we compare the performance of Agilent gene expression microarray and Agilent RNA-Seq platforms run with the same total RNA samples, and highlight performance differences between the two different Agilent gene expression measurement technologies.

ERCC RNA Ratio/Abundance in Mixtures



Two mixtures (Mix 1 and Mix 2) were prepared from 92 ERCC RNA control transcripts that were placed in different subpools (a to d) in four defined abundance ratios of 4:1, 1:2, 1:1.5 and 1:1 between the mixtures (Figure a). Within each subpool there are 23 controls that span a large dynamic range of 2^{20} or 6 orders of magnitude (Figure b). These ERCC control transcript mixes are technology-independent and can be spiked into the RNA at the beginning of any gene expression analysis experiment. (Munro, S., *et al.* **Assessing technical performance in differential gene expression experiments with external spike-in RNA control ratio mixtures**. (2014) *Nature Communications*, 5:5125)

Experimental Design

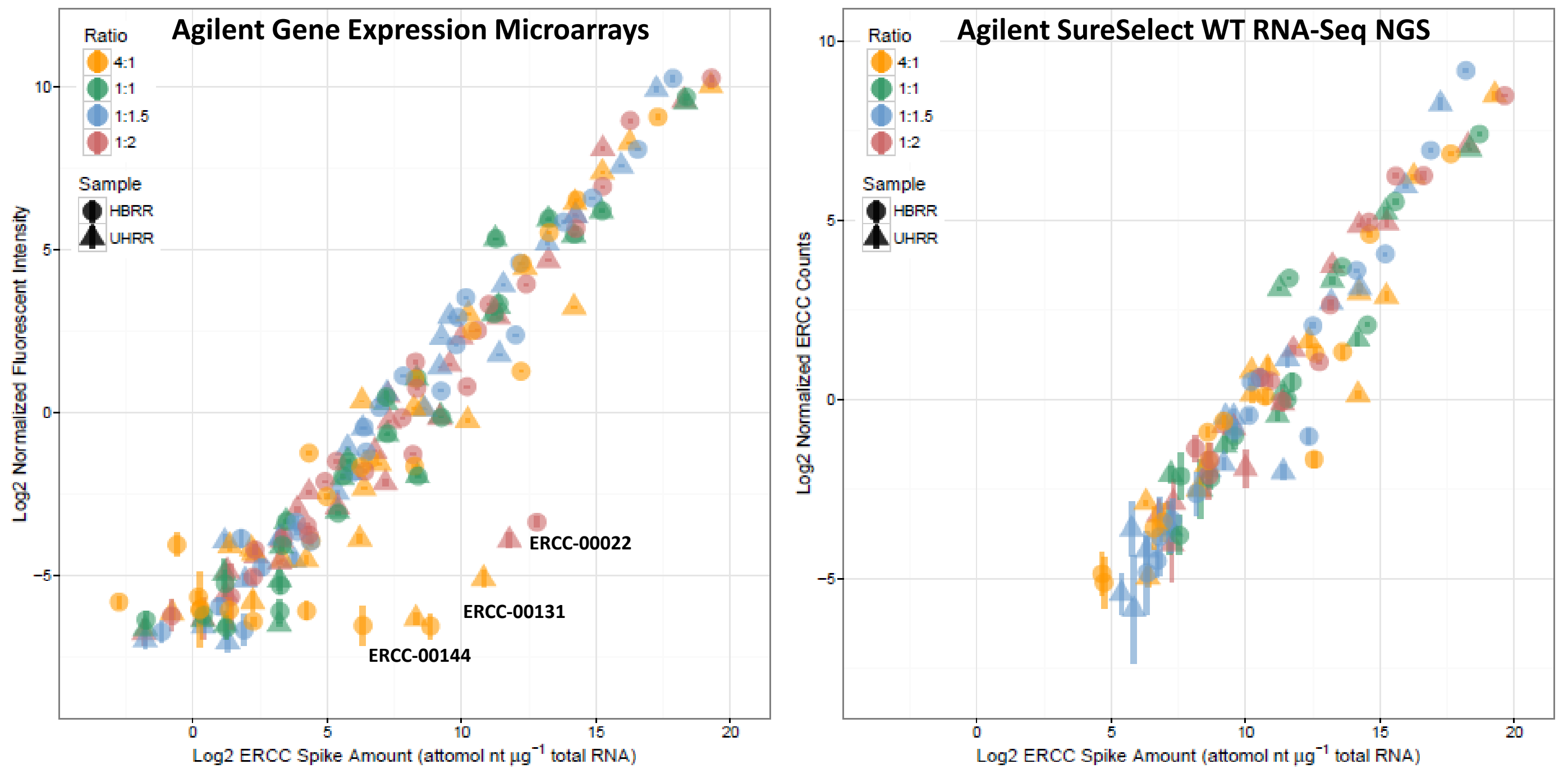


Two ERCC spike-in RNA transcript mixtures (Mix 1 and Mix 2) were obtained from Ambion (p/n 4456739) and 2 µl of a 1:10 dilution of each respective spike-in mix was added to 10 µg of either the Universal Human Reference RNA (UHRR, Agilent p/n 740000) or to the Human Brain Reference RNA (HBRR, Ambion p/n AM6050) as noted in the figure above.

Four (4x) technical replicate 100 ng aliquots of total RNA from each sample were prepared for Agilent One-Color Gene Expression microarray analysis using the Agilent Low Input Quick Amp kit (p/n 5190-2305) and run on the SurePrint G3 Human Gene Expression v3 8x60K Microarrays (p/n G4851C).

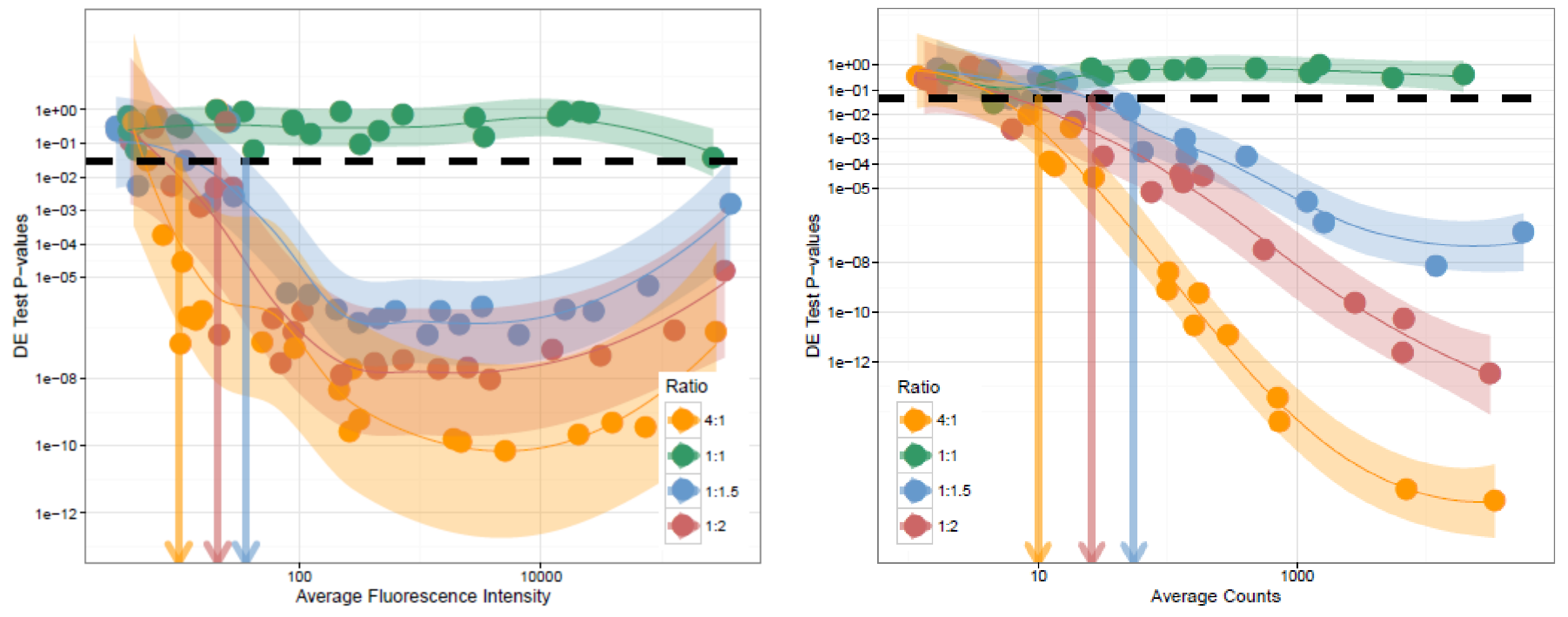
Three (3x) technical replicate 2 µg aliquots of total RNA from each sample (one less technical replicate as compared to the figure above) were prepared for RNA-Seq analysis using the Agilent SureSelect Strand-Specific RNA Library Preparation Kit kit (p/n G9691A/G9692A). The barcoded libraries for both the UHRR and HBRR technical replicates were pooled and prepared for paired-end multiplexed sequencing on the Illumina platform. The mean number of mapped reads per indexed library was 151,155,130 for UHRR samples and 115,602,753 for HBRR samples.

ERCC Signal-Abundance Plot Comparisons



ERCC*dashboard* derived signal-abundance plots compare the dynamic range capabilities of the two different gene expression experimental platforms using the same RNA samples. In these figures the points are colored by ratio subpool, error bars represent the standard deviation of the replicate ratio measurements, and shape represents the sample type (UHRR or HBRR). In the RNA-Seq results, ERCC controls that did not have at least one count in three libraries for either sample were not included in the signal-abundance plot. There are several ERCC control transcripts that consistently give outlier signals (ERCC-00022, ERCC-00131, ERCC-00144) on the microarray experiments. Note that the linear dynamic range on these plots spans 2^{20} (microarrays) or 2^{16} (RNA-Seq).

Estimating Limit of Detection Rates (LODR)



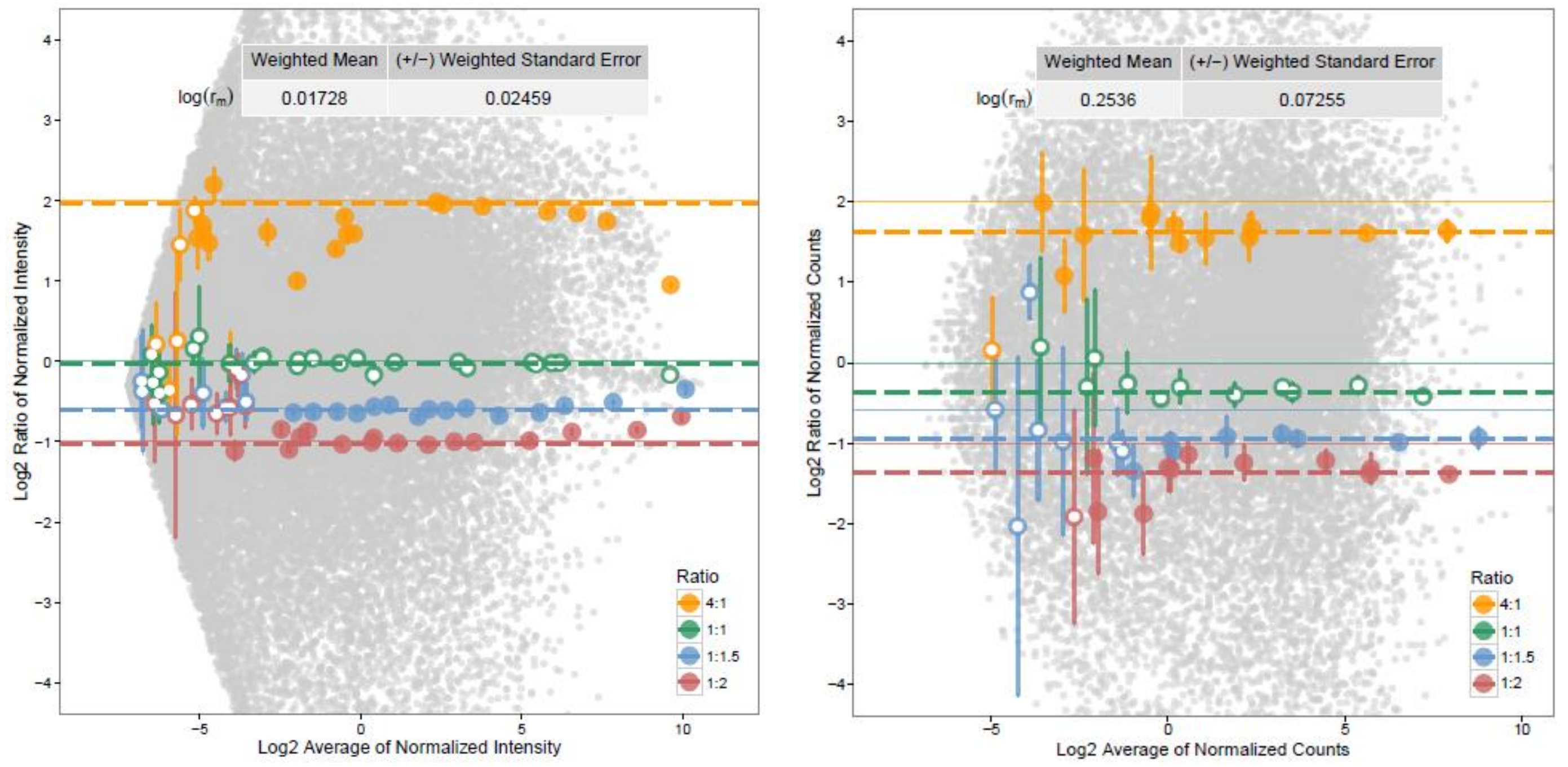
Ratio	LODR Estimate	90% CI Lower Bound	90% CI Upper Bound
4:1	10	7.2	13
1:1.5	36	20	43
1:2	21	12	24

Ratio	LODR Estimate	90% CI Lower Bound	90% CI Upper Bound
4:1	10	6.3	11
1:1.5	55	30	62
1:2	26	12	32

Agilent Gene Expression Microarrays **Agilent SureSelect WT RNA-Seq NGS**

This figure demonstrates the ability of each platform to detect small changes in ERCC spike-in concentration between the UHRR and HBRR samples. Shown are the average fluorescence intensity (microarrays) or the average counts (RNA-Seq) versus the differential expression test P-values calculated by the *ERCCdashboard* program. The heavy black dashed line indicates the false discovery rate (FDR) cutoff of FDR = 0.01 chosen for this analysis. Colored arrows indicate the LODR estimate (average intensity or average counts) for each fold change that crosses the line indicating the upper boundary of the 90% confidence interval. LODR results and confidence intervals derived from the plots are shown in the annotation tables below the plots.

MA Plots Demonstrate Measurement Variability



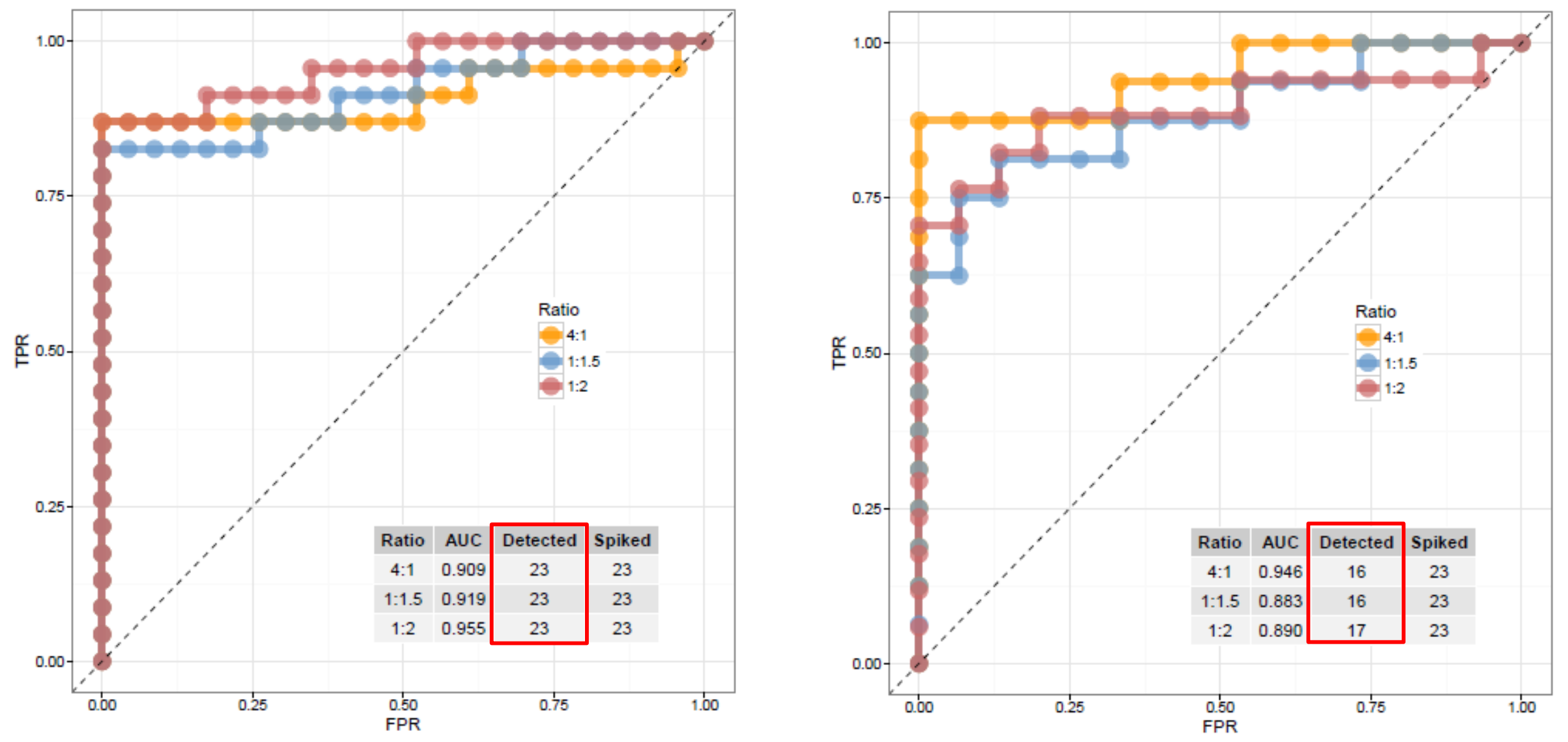
	Weighted Mean	(+/−) Weighted Standard Error
$\log(r_m)$	0.01728	0.02459

	Weighted Mean	(+/−) Weighted Standard Error
$\log(r_m)$	0.2536	0.07255

Agilent Gene Expression Microarrays **Agilent SureSelect WT RNA-Seq NGS**

Magnitude of fold-change (log ratio) versus average signal/count (MA) plots contain ERCC data points colored by expected ratio with error bars that represent the standard deviation of the replicate ratio measurements. Filled circles indicate ERCC ratios above the limit of detection (LODR) for the 4:1, 1:2, and the 1:1.5 ratios. Endogenous transcript ratio measurements are shown as gray points. The estimate of mRNA fraction differences between the UHRR and HBRR RNA samples (r_m) with weighted standard errors is provided in an inset table and is used to adjust the nominal ERCC ratios. The nominal ratios are annotated with the light solid color lines for each ratio subpool and the adjusted ratios are annotated with the heavy dashed lines.

ROC Curves and AUC Statistics



Ratio	AUC	Detected	Spiked
4:1	0.909	23	23
1:1.5	0.919	23	23
1:2	0.955	23	23

Ratio	AUC	Detected	Spiked
4:1	0.946	16	23
1:1.5	0.883	16	23
1:2	0.890	17	23

Agilent Gene Expression Microarrays **Agilent SureSelect WT RNA-Seq NGS**

Using the *ERCCdashboard* program, true-positive and true-negative ERCC control ratios can be used in receiver operator characteristic (ROC) curve analysis of the rank-ordered differential expression test *P*-values. ROC curves and the corresponding area under the curve (AUC) statistics change based on the discrimination of true-positive values and true-negative values, where ideal performance is represented by AUC = 1.0 for all 23 controls spiked at a given ratio. Note that the microarray experiments were able to detect all 23 ERCC spike-in controls at each fold change tested with AUCs >0.90 whereas the RNA-Seq experiments at a read depth of 100M reads detected 16 (or 17) of the 23 spike-in controls with AUCs of <0.90 for the smaller fold changes (1:2 fold and 1:1.5 fold) tested.

Conclusions

- The technology-independent spike-in control transcripts developed by the ERCC can serve as a truth set to benchmark the accuracy of endogenous transcript ratio measurements.
- The ERCC*dashboard* tool enables rapid, reproducible, and automated analysis of the ERCC spike-in control transcripts in any differential expression experiment.
- The ERCC controls demonstrate that both the Agilent One-Color Gene Expression Microarray platform and the Agilent RNA WT NGS platform can generate linear gene expression data spanning 5 orders of magnitude with the ability to detect sensitive 1.5-fold changes at levels as low as:
  - 36 counts with the microarray workflow
  - 55 mapped reads with the RNA-Seq workflow

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