

## **Whole Transcript (WT) Sense Target Labeling Assay Performance**

### **SUMMARY:**

Using a mini-Latin square experiment with RNA spikes in the background of HeLa total RNA, sensitivity and specificity were evaluated using WT target generated from total RNA with and without reduction of ribosomal RNA (rRNA). The protocol employing the rRNA reduction step started with 1 $\mu$ g of input total RNA and the protocol without rRNA depletion included a range of input total RNA from 50-300 ng. Gene-level analysis was carried out on both the Human WT Gene 1.0 ST Array (Gene Array) and the Human Exon 1.0 ST Array (Exon Array) and exon-level performance was analyzed on the Exon Array. The gene-level array performance specification was met on the two arrays using both versions of the labeling protocol across nearly all of the total RNA input amounts tested. Furthermore, analysis of the array data showed no significant benefit of the rRNA depletion step for gene-level analysis on either the Gene or Exon Arrays. However, for analysis at the exon-level on the Exon Array, the rRNA depleted samples had a clear advantage in array performance.

### **INTRODUCTION:**

Expression analyses typically focus on the relative level of mRNA from different transcripts or genes. However, total RNA samples consist of a combination of ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA), and other small RNA species with the rRNA fraction constituting the vast majority. With some methodologies such as random cDNA priming, ribosomal RNA can participate in the labeling reaction potentially increasing non-specific background and decreasing the signal to noise ratio of target generated from mRNA.

The RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen – P/N K1550-02) is designed to mitigate some of these issues by removing a significant fraction of the 18S and 28S rRNA from a total RNA sample. During the development of the Human Exon Array and the WT Sense Target Labeling Assay, it was demonstrated that the RiboMinus step provided a significant benefit to exon-level analysis and a minimal boost to gene-level performance (see Technical Note for more information: *GeneChip® Human Exon 1.0 ST Array and GeneChip® WT Sense Target Labeling Assay for Genome-Wide, Exon-Level Expression Analysis*).

With the launch of the Human WT Gene 1.0 ST Array, we decided to revisit the benefits of removal of rRNA from total RNA for both gene and exon-level analyses. An experiment was designed to measure the array performance for targets generated with and without the RiboMinus step. One microgram of total RNA was used as starting material for RiboMinus rRNA depletion and an input range of 50-300 ng of total RNA was tested using non-depleted total RNA. Performance was evaluated by measuring the ability of the arrays to detect a change in concentration of RNA spikes that are added to the total RNA prior to rRNA reduction and target preparation in low concentration at known quantities.

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### METHODS:

#### Experimental Design

In order to measure the sensitivity and specificity produced by the 1 µg (with RiboMinus) and 100 ng (without RiboMinus) versions of the WT

assay, we employed a mini-Latin square experiment with 19 *in vitro* transcribed, unlabeled RNA spikes added to a background of HeLa total RNA. The spike transcripts were selected based on their absence of expression under normal conditions in the HeLa cell line. The lack of endogenous expression of these genes was verified by real time RT-PCR.

The spike transcripts were assembled into four groups and added to HeLa total RNA such that each group was at one of four concentrations (0, 1:200K, 1:100K, 1:50K) spread across four pools (see Table 1). The 1:200,000 dilution represents a concentration of 0.75 pM and is equivalent to approximately 1.75 copies/cell. Spikes are added to the HeLa Total RNA prior to rRNA depletion or target preparation. Target was generated from each pool in triplicate for a total of 12 labeling reactions per assay condition.

Six different input starting amounts of RNA were tested. We generated rRNA depleted RNA from 1 µg of starting material. We also carried out a mock depletion of 1 µg of total RNA following the procedure outlined for rRNA reduction but omitting the RiboMinus probe. In addition four starting amounts of non-rRNA depleted total RNA were used: 50 ng, 100 ng, 200 ng, and 300 ng.

#### Target Preparation, Hybridization, and Scanning

rRNA depletion and target preparation was carried out exactly as described in the Whole Transcript (WT) Sense Target Labeling Assay Manual (for more information see: [http://www.affymetrix.com/products/reagents/specific/wt\\_cdna\\_synthamp\\_kit.affx](http://www.affymetrix.com/products/reagents/specific/wt_cdna_synthamp_kit.affx)). 10 µg of cRNA was carried into the second cycle, first strand cDNA synthesis reaction. 5.5 µg of single-stranded cDNA was fragmented, labeled, and hybridized serially, first to the Human Exon 1.0 ST Array then the Human WT Gene 1.0 ST Array. Hybridized arrays were labeled and washed using the Hybridization, Wash and Stain Kit on a GeneChip® Fluidics Station 450 with the appropriate fluidics scripts (see manual for details). Arrays were subsequently scanned on a GCS3000 7G Scanner.

#### Data Processing and Analysis

All resulting .CEL files for each array type were processed as a single group via the Affymetrix Power Tools (APT) package using sketch normalization and the RMA algorithm to summarize probeset signal. To test the array performance, we used probeset signals for the RNA spikes and attempted to discriminate between different concentrations of the same spike. For most analyses, three concentration comparisons were used: 1:200,000 to 0, 1:100,000 to 1:200,000, and 1:50,000 to 1:100,000. As a measure of separation between the concentrations, we employed the classic t-statistic.

Table 1: RNA Spike Concentrations

Spike Group	Pool 1	Pool 2	Pool 3	Pool 4
Group 1	0	1:50,000	1:100,000	1:200,000
Group 2	1:50,000	1:100,000	1:200,000	0
Group 3	1:100,000	1:200,000	0	1:50,000
Group 4	1:200,000	0	1:50,000	1:100,000

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Calculation of the t-statistic is as follows:

$$t = \frac{\bar{X}_A - \bar{X}_B}{\sqrt{(SE_A)^2 + (SE_B)^2}}$$

Where  $\bar{X}$  is the mean of groups A and B respectively, and  $SE = \frac{SD}{\sqrt{N}}$ .

The degrees of freedom are calculated as:  $df = n_1 + n_2 - 2$ . With 3 replicates in each group, the degrees of freedom is 4 (3 + 3 - 2). The threshold value for significance of 4.604 was chosen using a table of critical values for the t-statistic with 4 degrees of freedom and  $p < 0.005$ .

As one measure of performance, we simply counted the fraction of probesets that passed the t-statistic threshold (t-stat > 4.604). Another measure of performance employs Receiver Operating Characteristic (ROC) curves. A ROC curve essentially plots the true positive rate (proportion of actual differences that are detected) versus the false positive rate (proportion of detected differences among things that did not change). ROC plots by convention graph the true positive rate (sensitivity) on the y-axis and the false positive rate (1-specificity) on the x-axis, parameterized by all possible values of  $t_0$ . Larger areas under the curve (AUC) correspond to better results. Additional information regarding use of the t-statistic can be found in the Human Gene 1.0 ST Array Performance Whitepaper

([http://www.affymetrix.com/products/arrays/specific/hugene\\_1\\_0\\_st/hugene\\_performance.affx](http://www.affymetrix.com/products/arrays/specific/hugene_1_0_st/hugene_performance.affx)).

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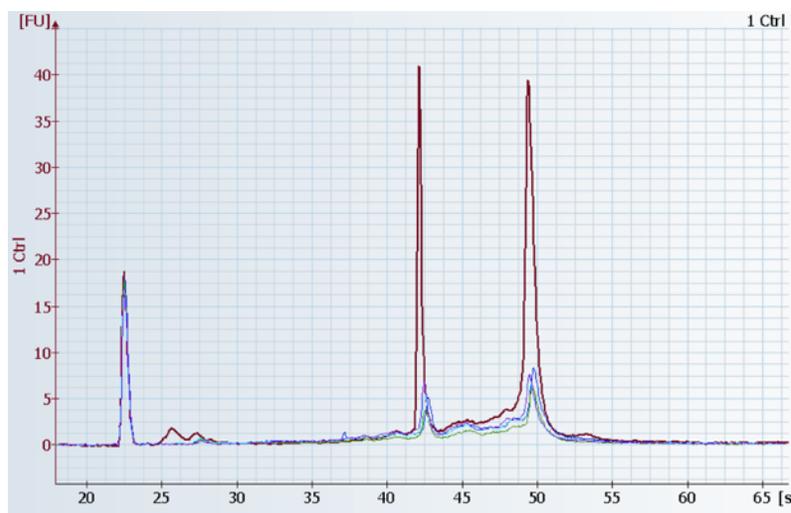
### RESULTS:

#### RiboMinus rRNA Depletion

One microgram of HeLa total RNA (+ spikes) was processed through the RiboMinus protocol to deplete the ribosomal RNAs. In addition, a mock treatment of 1 µg of the HeLa + spikes total RNA was processed through the protocol omitting the RiboMinus probe.

Depletion of 18S and 28S rRNA was verified by running 1 µL of eluted RNA on a Bioanalyzer RNA 6000 Nano Chip (Agilent). The example in Figure 1 shows a clear reduction of the rRNA peaks

relative to a control RNA. The RNA concentration following rRNA depletion (or mock rRNA depletion) was measured via UV spectrophotometry (NanoDrop). Results are shown in Table 2, along with an average total mass of RNA recovered following the RiboMinus protocol. Approximately half of the total volume was used to generate cDNA. Thus, ~200 ng of rRNA depleted RNA and ~350 ng of mock depleted RNA was carried through to the next step of the protocol.



**Figure 1: Bioanalyzer Electropherogram of control and rRNA depleted RNA**

Red line depicts a control RNA of 100 ng of non-depleted total RNA. Other colored lines represent 4 samples (one from each pool) of RiboMinus rRNA depleted RNA.

**Table 2: RNA Mass Following RiboMinus Procedure**

Input Amount	RiboMinus Probe	Spike Pool	Rep A	Rep B	Rep C	Average RNA Amount (ng)	Standard Deviation
<b>1 µg</b>	<b>+</b>	Pool 1	357.6	482.9	292.0	<b>392.0</b>	<b>69.8</b>
		Pool 2	392.6	385.8	269.5		
		Pool 3	443.3	491.7	402.8		
		Pool 4	387.6	455.0	343.0		
<b>1 µg</b>	<b>- (Mock Treatment)</b>	Pool 1	700.9	741.5	633.1	<b>702.2</b>	<b>68.6</b>
		Pool 2	742.0	590.2	711.5		
		Pool 3	642.7	683.0	736.0		
		Pool 4	721.9	665.7	858.5		

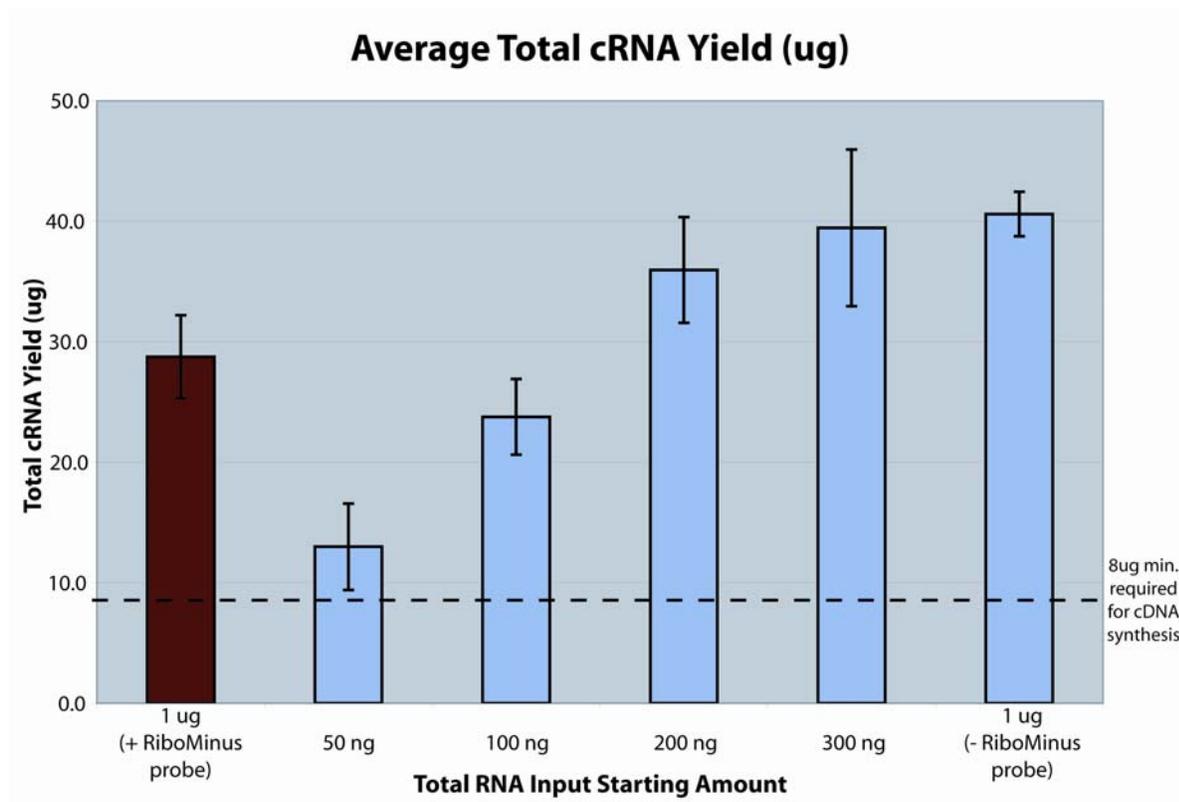
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### cRNA Yield

The cRNA concentration was measured by UV spectrophotometry (NanoDrop) following column purification. Total cRNA yield was calculated by multiplying the measured concentration by the estimated elution volume (10.5  $\mu$ L). Average total cRNA yield is shown in Figure 2. All input starting amounts generated sufficient cRNA. Ten micrograms of cRNA was used to generate second cycle, first strand cDNA. In cases where cRNA concentration was low, the volume of cRNA was reduced by SpeedVac to a maximum of 6.5  $\mu$ L.



**Figure 2: Average cRNA Yield**

Average total cRNA yield for each of the starting input amounts is shown. Each data point represents an average of 12 samples and error bars are one standard deviation.

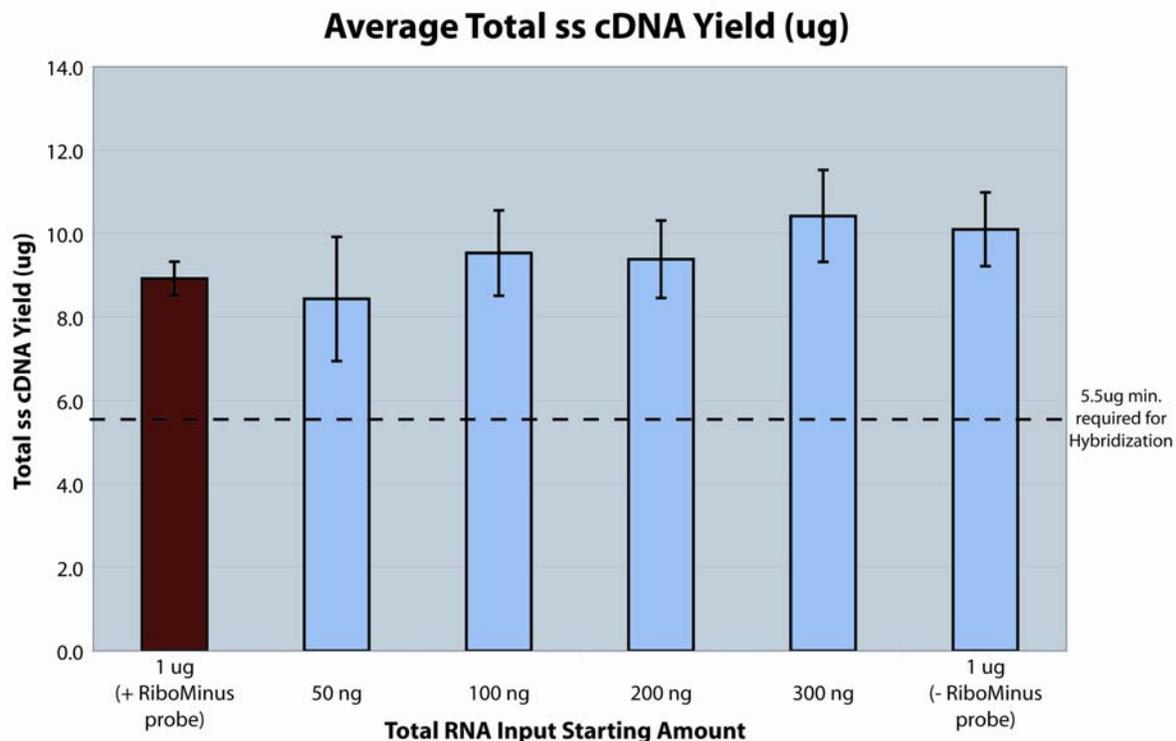
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### ss cDNA Yield

The concentration of cDNA from the second cycle was measured by UV Spectrophotometry (NanoDrop). The total yield of single stranded cDNA was calculated by multiplying the measured concentration by the estimated elution volume (28  $\mu$ L). Average single stranded cDNA yields for each input starting amount are shown in Figure 3. All RNA input starting amounts (with and without rRNA reduction) generated sufficient single stranded cDNA in the second cycle to generate a hybridization cocktail containing 5.5  $\mu$ g of labeled target. The yield of single stranded cDNA was fairly consistent across all input starting amounts. This is expected since we used a constant mass (10  $\mu$ g) of cRNA as input into this step.



**Figure 3: Average cDNA Yield**

Average total single stranded cDNA yield from the second cycle cDNA synthesis is shown. Each data point represents an average of 12 samples and error bars represent one standard deviation.

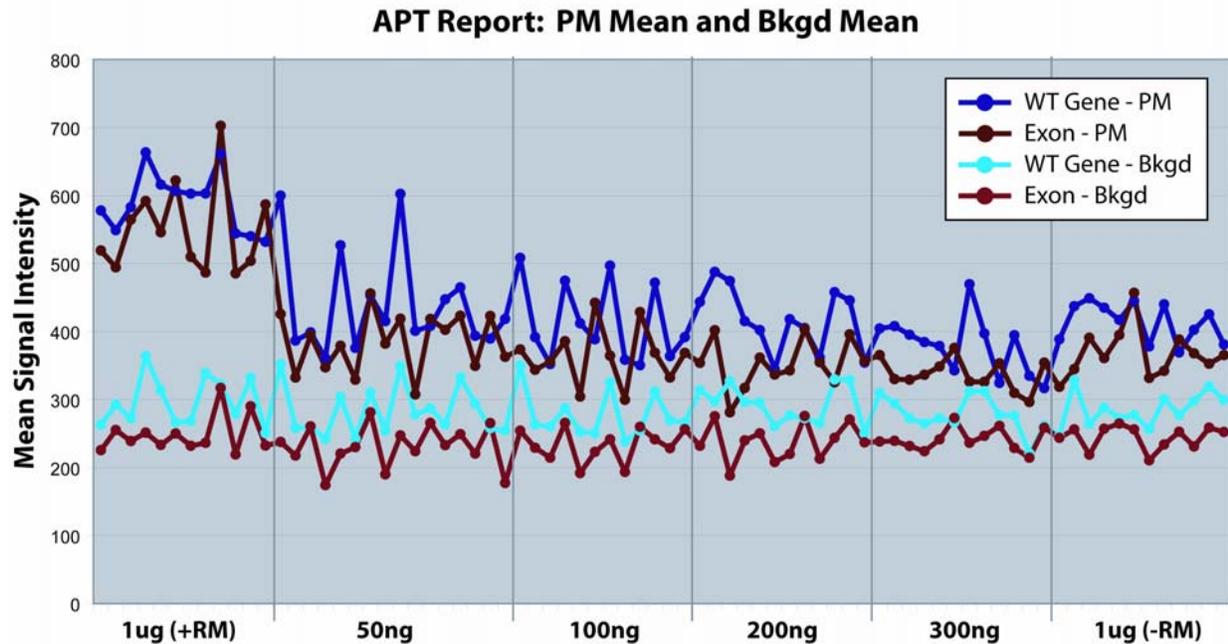
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### APT QC Metrics

To begin to examine the effects that the rRNA reduction step has on the array data, we plotted some of the QC metrics from the APT report file. In Figure 4, the mean perfect match (PM) and mean background intensity is shown for all of the samples. The graph shows a larger separation between the PM and background signals in the samples where the rRNA was depleted. This suggests that the RiboMinus procedure may increase the signal to noise ratio.



**Figure 4: Mean PM and Background Intensity**

Mean perfect match (PM) probe intensity and mean background intensity from the non-genomic background probes as reported from APT is graphed for each sample from both the Gene Array (light and dark blue lines) and Exon Array (light and dark red lines).

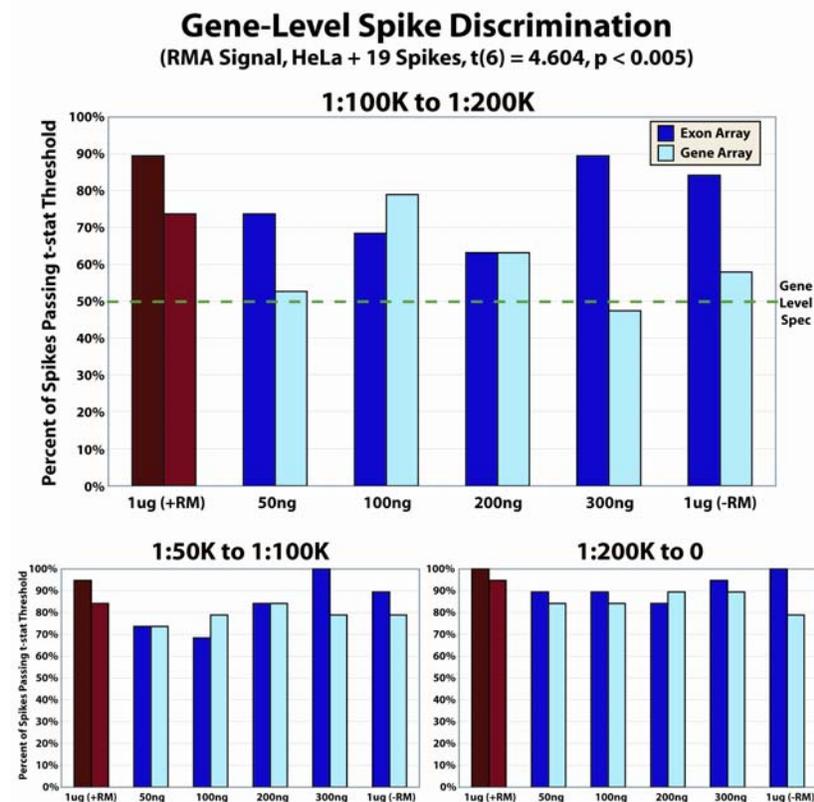
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### Gene-Level Performance

Array performance at the gene-level was measured by calculating the t-statistic for the discrimination of the same spike at different concentrations. Each concentration is represented by 3 technical replicates. The t-statistic was calculated for each of the 19 spikes in each of three concentration comparisons (1:200,000 to 0, 1:100,000 to 1:200,000, and 1:50,000 to 1:100,000). The t-statistic is a measure of the separation of signal intensities for the two concentrations relative to the variance of the 3 replicates within each concentration. The more pronounced the separation and/or the smaller the variance within the replicates, the higher the t-statistic value. The percent of spikes that had t-statistic values greater than the threshold for significance at  $p < 0.005$  is graphically represented in Figure 5. The specification for minimum performance on the Human Gene Array is 50% of the spikes having above threshold t-statistic values at the 1:100,000 to 1:200,000 comparison. As shown in Figure 5, the rRNA reduced samples are well above the specification level on both arrays. Furthermore, nearly all of the non-rRNA reduced input amounts also pass the specification on both the Gene and Exon Arrays (the 300 ng input amount on the Gene Array falls just below the 50% specification). Figure 5 also shows that the discrimination at the other two concentration comparisons was very good for both rRNA reduced and non-rRNA reduced samples on both arrays.



**Figure 5: Gene-Level Spike Discrimination**

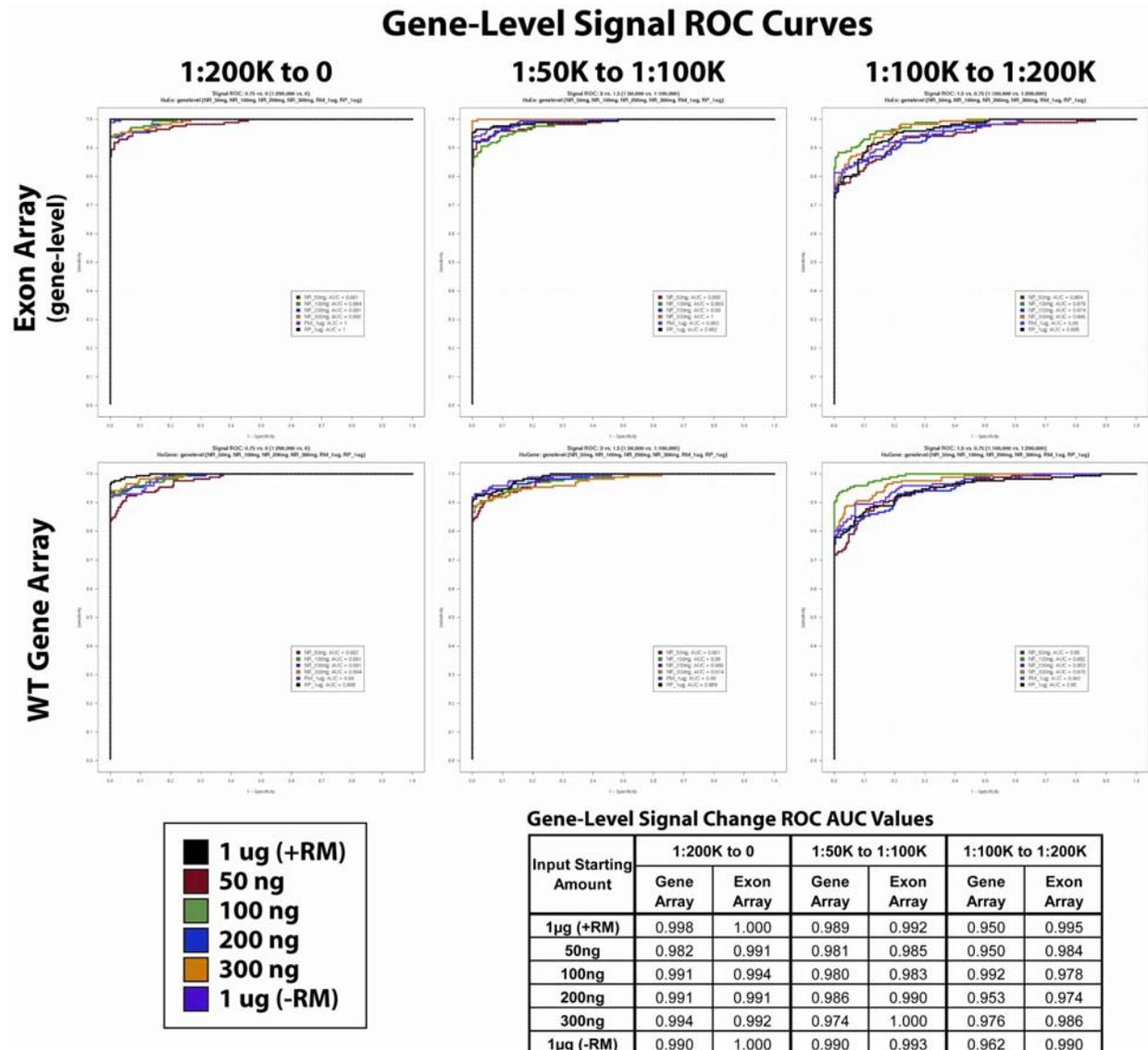
The percentage of spikes that pass the t-statistic threshold (4.604) for discrimination is plotted for each of the input amounts in each of the concentration comparisons. The samples with rRNA reduction are shown on the far left in dark red (Exon Array) and red (Gene Array). The specification for the Gene Array of 50% of the spikes passing the threshold at the 1:100,000 to 1:200,000 comparison is shown as a dashed line.

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In Figure 6, gene-level signal discrimination for each of the concentration comparisons is displayed as Receiver Operating Characteristic (ROC) curves. The area under each of the curves (AUC) is a measure of the ability to discriminate between the two concentrations (see methods). The samples that underwent rRNA reduction do not show any significant benefit in sensitivity in any of the concentration comparisons.



**Figure 6: Gene-Level Spike Discrimination ROC Curves**

Receiver Operating Characteristics (ROC) Curves are plotted for spike discrimination for all of the input starting amounts at each of the three concentration comparisons on the Exon (top row) and Gene Arrays (bottom row). Area under each of the curves (AUC) is displayed in table form at the bottom. Perfect discrimination has an AUC of 1.0.

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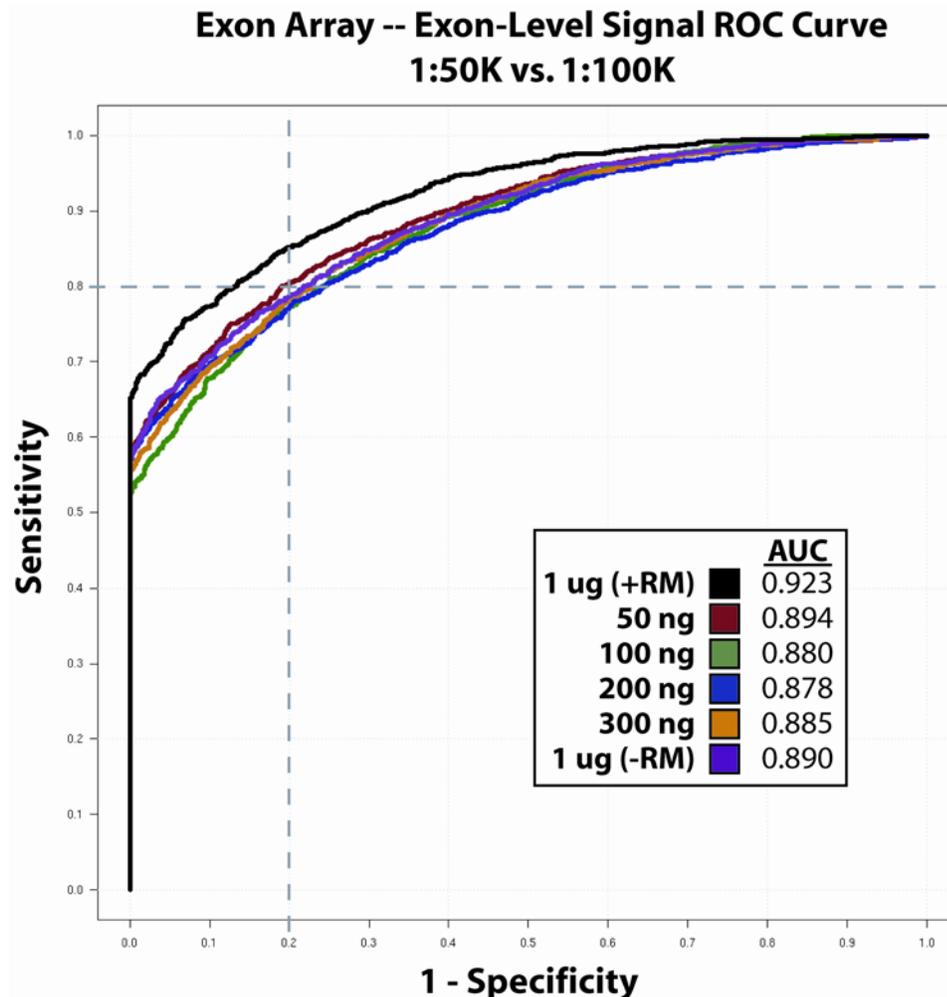
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### Exon-Level Performance

The exon-level performance was measured for samples hybridized to the Exon Array. Discrimination for the three concentration comparisons was determined using 173 exon-level probesets that map to the RNA spikes. Shown in Figure 7 is the ROC plot for the 1:50,000 to 1:100,000 comparison. For exon-level analysis, the RiboMinus samples show significantly better performance. The specification for minimum exon-level performance on the Exon Array is greater than 80% sensitivity (true positives) at the 20% 1-specificity (false positives) mark (shown as dashed lines on Figure 7). The samples that underwent rRNA reduction (black line) have performance that is clearly above the specification, while the performance for the non-rRNA reduced samples is right at or just below the specification.



**Figure 7: Exon-Level Spike Discrimination ROC Curve**

Spike discrimination for 173 exon probesets is displayed in a Receiver Operating Characteristic (ROC) curve for the 1:50,000 to 1:100,000 concentration comparison. The dashed line represents the specification of exon-level performance on the Exon Array. Area under the curve (AUC) for each of the input starting amounts is also shown.

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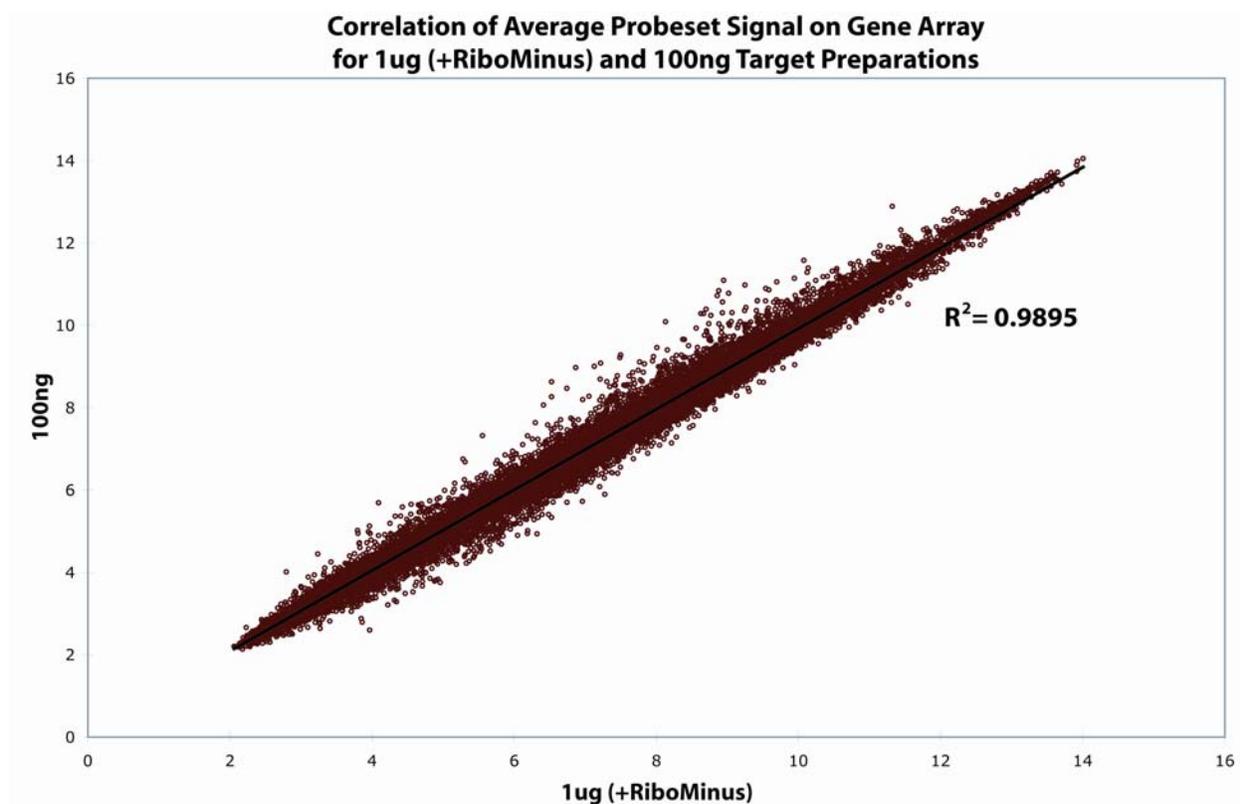
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### Correlation

To ensure that the RiboMinus procedure does not significantly bias the overall data or a subset of genes, we plotted the average Gene Array probeset signal for the 1  $\mu\text{g}$  (+ RiboMinus) input amount relative to the 100 ng input amounts. Each data point represents the average gene-level probeset signal of the 12 samples for each condition. The probesets mapping to the spiked-in transcripts were removed from the analysis. The correlation between the average probeset signal of the two protocols was very high with an  $R^2$  of 0.9895. Thus samples prepared with and without the rRNA reduction step produce highly comparable array data.



**Figure 8: Correlation of Average Probeset Signal on Gene Array**

Scatter plot of average probeset RMA signal of 1  $\mu\text{g}$  rRNA reduced samples (x-axis) and 100 ng non-rRNA reduced samples (y-axis) from the Gene Array. Each data point is the mean probeset signal from the 12 replicate samples of each input amount. Probesets mapping to the spike-in genes were removed from the analysis.  $R^2$  correlation value is also shown.

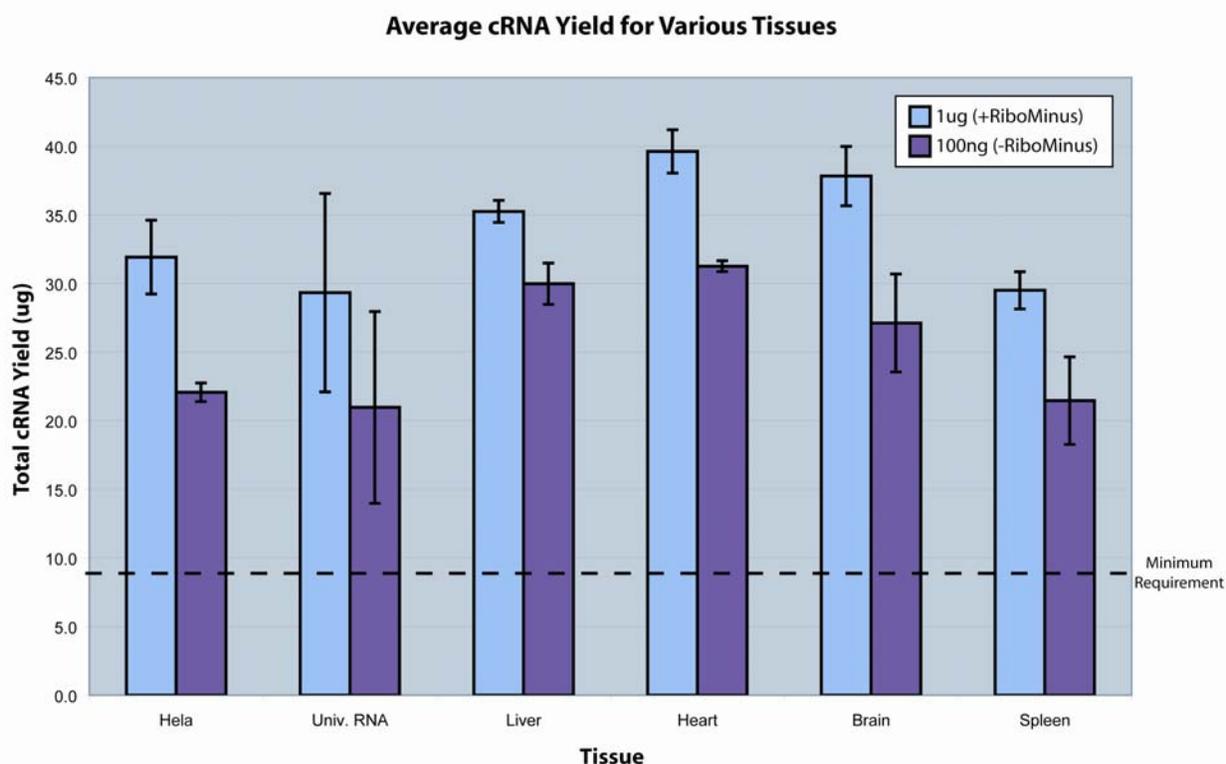
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### cRNA Yields for Various Human RNA Samples

We generated cRNA using RNAs from diverse sources to demonstrate that sufficient cRNA yields can be obtained. Commercially obtained human total RNAs from the HeLa cell line, Stratagene Universal RNA (includes a mixture of RNAs from a variety of Human cell lines), and RNAs from normal human liver, heart, brain, and spleen tissues were used to generate cRNA using both the 1  $\mu$ g (+RiboMinus) and 100 ng (-RiboMinus) protocols. For additional information on the samples used, see the table in the Supplemental Material. Figure 9 shows the average total cRNA yields from triplicate preparations. As expected with RNAs from different sources, the average total yield of cRNA varies. However, as can be seen in Figure 9, RNAs from these diverse sources produced well above the minimum amount of cRNA (~8  $\mu$ g) that is required to reliably generate at least 5  $\mu$ g of single stranded cDNA in the second cycle. This was true with and without the rRNA reduction step.



**Figure 9: cRNA Yields for RNAs from Various Tissues**

Average total cRNA yield for six diverse RNA sources for both the 1  $\mu$ g (+RiboMinus) and 100ng (-RiboMinus) samples. Averages are from 3 replicates and error bars represent one standard deviation. Minimum cRNA amount (8  $\mu$ g) generally required to generate ~5  $\mu$ g of second cycle cDNA is shown as a dashed line.

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### DISCUSSION:

In this experiment, we demonstrate that for high quality, commercially available total RNA from the HeLa cell line, sufficient cRNA can be generated from as little as 50 ng of total RNA. However, because the amount of cRNA that can be generated is likely to be variable based on the source, RNA quality, and extraction method, it may be prudent to use a higher input starting amount to ensure sufficient cRNA quantity. Using total RNAs from a variety of sources, we showed that 100 ng can reliably produce more than the minimum cRNA amount required to generate at least 5 $\mu$ g of single stranded cDNA for array hybridization. Increasing the amount of input starting material generally resulted in higher cRNA yields. However, since a maximum of 10  $\mu$ g of cRNA is used in subsequent steps, significantly higher cRNA yields are not necessary for this protocol. For the non-RiboMinus protocol, the data suggests that 100 ng of high quality total RNA may be an adequate quantity for target generation from a variety of sources.

The cRNA yield, however, is not the whole story. In order to compare the array performance of target generated from different input amounts, we carried out a mini-Latin square experiment by adding RNA spikes at four different known concentrations. The array sensitivity and specificity were measured by the ability to distinguish probeset intensities for spikes at different concentrations near the limit of detection. Likely due to the decrease in rRNA, the samples that underwent RiboMinus procedure showed increased signal to noise ratios. However, for analysis at the gene-level, we did not observe a significant benefit in terms of array performance for the rRNA reduction step on either the Gene or Exon Arrays. The specification of more than 50% of spikes having t-statistic values greater than 4.604 ( $p < 0.005$ ) was met for nearly all of the input starting amounts. Overall, the array performance of both the Exon and Gene Arrays at the gene-level was quite good. The stringency of the test to measure the ability to discriminate between spikes at two different low concentrations was increased in order to amplify the potential differences in performance of the various input starting amounts. As shown in Supplementary Figure 1, the percent of spikes that are clearly distinguishable in the 1:100,000 to 1:200,000 concentration comparison is 95-100% for all input starting amounts on both arrays.

The gene-level performance results were comparable for samples with and without the rRNA reduction step. Because the value of the t-statistic is dependent on a combination of separation of the data and the standard deviation of the concentration replicates, the exact number of spikes that meet the stringent threshold tends to fluctuate somewhat based on the variance of the samples within each input amount group. Thus, it is difficult to determine a clear winner based on that representation of the data. The ROC plots for gene-level analysis clearly show that the RiboMinus step does not provide a significant performance advantage on either array. Especially considering the added protocol complexity of the rRNA reduction step, time, cost, and potential for introduction of variability, the 100ng protocol may be the suitable choice for studies that focus on analysis primarily at the gene-level.

Conversely, analysis at the exon-level did demonstrate a significant benefit for the rRNA reduction step for Exon Arrays. The samples that underwent the RiboMinus procedure had noticeably better sensitivity for detecting change at the exon-level than

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those that did not. The difference in performance at the exon and gene level is likely due to several factors. First, exon probesets have a much smaller number of probes. The typical exon probeset consists of four probes compared to gene-level probesets that on average have 20 or more probes. Having more probes in a probeset is known to enhance performance. Second, the selection of sequences for exon probesets can be limited by the size of the probe selection region (PSR). Third, the smaller size of exons may also have an impact on the ability to select independent (non-overlapping) probes. The combination of these factors make exon-level analyses more demanding. Thus, analysis at the exon level necessitates the advantage provided by the increased signal to noise of the rRNA reduction step. Exon-level results are still quite good without the RiboMinus procedure, suggesting that the 100 ng protocol could be considered a viable option in situations where RNA quantities are limiting. However, based on the results from this experiment, the 1 µg (+ RiboMinus) protocol is recommended for exon-level analysis on Exon Arrays to achieve optimal sensitivity.

The correlation of average signal from target prepared with and without the RiboMinus procedure is very high suggesting that the data generated from the two different input starting amounts is highly comparable. However, in other experiments using HeLa plus spike-ins we have seen that assays done with no RiboMinus probe compared with assays done with RiboMinus result in subtle but consistent perturbation of signal. This perturbation is not enough to greatly affect detection of differences in spike-ins at the level used in this experiment, but will result in increasing false positive rates as group sizes increase when using t-tests to compare between the two assay conditions. In other words signals from the background HeLa probesets are more different between assay type than within assay type. This effect is minimized when comparing non-RiboMinus signal to RiboMinus signal by processing all samples together as a single group if using RMA in APT. Nevertheless, doing an analysis that includes some samples prepared with the RiboMinus procedure and others prepared without RiboMinus is not recommended. Additional information is contained within the Human Gene 1.0 ST Array Performance whitepaper

([http://www.affymetrix.com/products/arrays/specific/hugene\\_1\\_0\\_st/hugene\\_performance.affx](http://www.affymetrix.com/products/arrays/specific/hugene_1_0_st/hugene_performance.affx))

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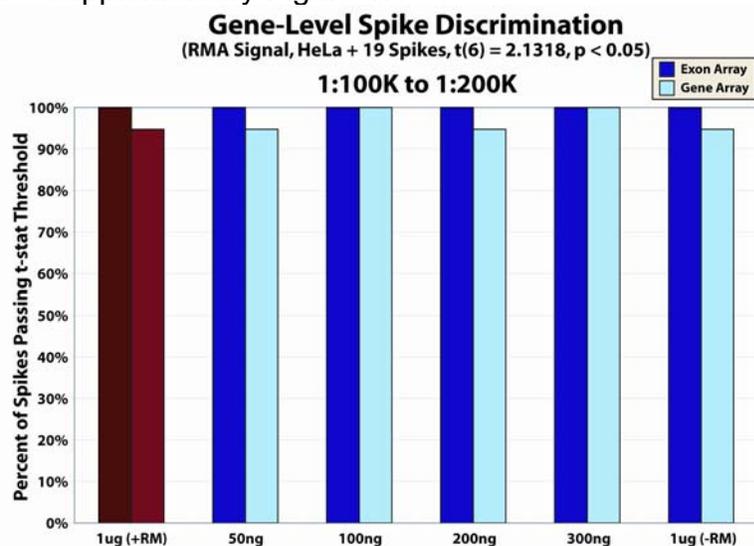
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## SUPPLEMENTARY MATERIAL:

### 1. Table of RNA Sample Information:

RNA Name	Organism	Source	Supplier	Cat #	Lot #	Replicate
HeLa	Human	HeLa-S3 Cervical Adenocarcinoma cell line	Ambion	7852	105P053754A	n/a
Universal RNA	Human	Mixture of total RNA from 10 human cell lines	Stratagene	740000	1130623	n/a
Liver_A	Human	Liver Tissue	Ambion	7960	102P0110010001A	A
Liver_B	Human	Liver Tissue	Ambion	7960	034P021001003B	B
Liver_C	Human	Liver Tissue	Ambion	7960	026P010204001P	C
Heart_A	Human	Heart Tissue	Ambion	7966	054P010201001A	A
Heart_B	Human	Heart Tissue	Ambion	7966	082P0503A	B
Heart_C	Human	Heart Tissue	Ambion	7966	024P011000001A	C
Brain_A	Human	Brain Tissue	Ambion	7962	092P030402001A	A
Brain_B	Human	Brain Tissue	Ambion	7962	034P020403079A	B
Brain_C	Human	Brain Tissue	Ambion	7962	016P040305030A	C
Spleen_A	Human	Spleen Tissue	Ambion	7970	082P0616A	A
Spleen_B	Human	Spleen Tissue	Ambion	7970	044P010303011B	B
Spleen_C	Human	Spleen Tissue	Ambion	7970	023P010802002A	C

### 2. Supplementary Figure 1:



#### Supplementary Figure 1: Gene-Level Spike Discrimination Using 95% Confidence

The percentage of spikes that pass the less stringent 95% ( $p < 0.05$ ) t-statistic threshold (2.1318) for discrimination in the 1:100,00 to 1:200,000 concentration comparison is plotted for each of the input amounts. The samples with rRNA reduction are shown on the far left in dark red (Exon Array) and red (Gene Array). Using a typical 95% confidence cut-off, 95-100% of the spikes with each input amount showed discrimination of the two concentrations.