Using Low Input of Poly (A)$^+$ RNA and Total RNA for Oligonucleotide Microarrays

Application

Gene Expression

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Abstract

With the advancement of DNA microarray technology, there has been an increase in demand for using small amounts of RNA samples for array analysis, mainly due to the limited availability of biological supplies. The low input RNA samples typically need to be amplified and labeled to produce sufficient amounts of cRNA or cDNA targets for hybridization to microarrays. To help microarray researchers who are sample limited, Agilent Technologies has developed a Low RNA Input Fluorescent Linear Amplification kit for RNA amplification and labeling. The kit uses T7 RNA polymerase for a single round of linear amplification to produce high quality, labeled cRNA targets for oligonucleotide microarrays, or cDNA targets for cDNA microarrays. This application note describes the application of this kit for the use of poly (A)$^+$ RNA or total RNA samples. It shows that micrograms of cRNA targets can be generated within 6 hours, using as little as 10 ng of poly (A)$^+$ RNA, or 50 ng of total RNA sample input. The resulting cRNA targets were of high quality and performed reproducibly and robustly on Agilent's oligonucleotide microarrays. Furthermore, the log ratios of array results from poly (A)$^+$ RNA sample input correlated with that from total RNA input. The easy-to-use Low RNA Input Fluorescent Linear Amplification kit can be applied to a wide range of samples for gene expression studies.

Introduction

DNA microarray technology has dramatically advanced in the past few years and has become a powerful and frequently used tool to study differential gene expression. In comparison to quantitative PCR, one of the significant challenges of using DNA microarrays is having sufficient amounts of high quality RNA sample that can be labeled and subsequently hybridized onto microarrays to generate robust results. Improved sensitivity and specificity of the array technology help to ease the problem. Nevertheless, for those scientists who are working with small amounts of biopsy tissue samples, rare primary cells, or laser-captured single cells, the difficult question remains as, how to use the little amount of RNA (either poly (A)$^+$ RNA or total RNA) to generate biologically meaningful and reproducible microarray data?

As a leading supplier of DNA microarrays, Agilent Technologies has developed a series of DNA microarray platforms to meet the researchers' needs. In order to optimize array performance and provide users with the whole product solution, Agilent also provides a series of reagent products that can be used together with the arrays. One of the products launched recently addresses the researchers' sample limitation issue. This product is the Low RNA Input Fluorescent Linear Amplification kit (LRIFLA). The kit is based on the linear amplification capability of T7 polymerase and requires only one round of amplification. The resulting fluorescently labeled cRNA or cDNA targets can be hybridized to oligonucleotide microarrays or cDNA microarrays respectively.

This application note demonstrates that amplified and fluorescently labeled cRNA targets of high quality can be produced from 10 to 200 ng of poly $(A)^+$ RNA, or 50 to 5000 ng of total RNA using either HeLa or human spleen as sample sources. The cRNA targets generated from such a range of RNA input exhibited similar sensitivity on Agilent's oligonucleotide arrays in detecting differential gene expression. Furthermore, we show that the log ratios of array results using cRNA targets generated from both poly $(A)^+$ RNA and total RNA samples (HeLa and spleen) were well correlated on our human oligonucleotide microarrays.

Materials and Methods

Reagents

HeLa and spleen (human) poly $(A)^+$ RNA and total RNA samples were purchased from Ambion (part numbers 7852, 7853, 7970, 7971). The quality of these RNA samples was examined using the Agilent 2100 Bioanalyzer (part number G2940BA) to ensure the integrity of RNA samples before use. Fluorescently labeled cRNA targets were generated from various RNA input (0.1 ng to 200 ng for poly $(A)^+$ RNA, and 1 ng to 5000 ng for total RNA), using the LRIFLA kit (part number 5184-3523) following the protocol discussed in the user's manual [1]. Both Cyanine 3-labeled CTP and 5-labeled CTP (10.0 mM) were purchased from Perkin-Elmer/NEN Life Science (part numbers NEL580, NEL581).

Linear Amplification

RNA amplification and labeling was performed following the LRIFLA protocol [1]. The quantity of T7 promoter primer added to poly $(A)^+$ RNA or total RNA depends on RNA input: add 1.2 µL of primer if input less than 10 ng of poly $(A)^+$ RNA or 500 ng of total RNA; add 5 µL primer if input greater than 10 ng of $(A)^+$ RNA or 500 ng of RNA. Final volume is brought to 11.5 µL with nuclease-free water. The primer and the RNA template were denatured at 65 °C in a heating block for 10 min and cooled on ice for 5 min. A volume of 8.5 µL of cDNA mix was added to each reaction tube. The cDNA synthesis reaction mix contains 4 µL of 5X First Strand Buffer, 2 µL of 0.1 M DTT, 1 µL of 10 mM dNTP, 1 µL of MMLV RT, and 0.5 µL of RNaseOUT. Samples were incubated at 40 °C in a circulating water bath for 2 h, heated at 65 °C for 15 min (to inactivate MMLV-RT), and cooled on ice for 5 min.

A volume of 2.4 µL of 10-mM cyanine 3-CTP or cyanine 5-CTP was added to each sample tube, followed by the addition of 57.6 µL of transcription master mix. The transcription master mix contains 15.3 µL of nuclease-free water, 20 µL of 4X transcription buffer, 6 µL of 0.1 M DTT, 8 µL of NTP mix, 6.4 µL of 50% PEG, 0.5 µL of RNaseOUT, 0.6 µL of inorganic pyrophosphatase, and 0.8 µL of T7 RNA polymerase. The reaction mix was then incubated at 40 °C for 2 h. Labeled cRNA was purified using Qiagen's RNeasy mini-spin columns following the LRIFLA protocol. The cRNA product was eluted with nuclease-free water from the column in 2 × 30 µL of final volume. Yields of cRNA were determined by UV spectrophotometry.

Hybridization

Hybridization was performed following Agilent's oligonucleotide microarray hybridization user's manual [2] and Agilent's In Situ Hybridization Plus kit (part number 5184-3568). 0.50 µg of labeled cRNA (from total RNA sample), or 0.75 µg of labeled cRNA (from poly $(A)^+$ RNA sample) per channel were mixed with 50 µL of 10X control targets and nuclease-free water to a final volume of 250 µL. Then 10 µL of 25X fragmentation buffer was added to each sample tube and the reactions were incubated at 60 °C in the dark for 30 min. The addition of 250 µL of 2X hybridization buffer terminated the reaction. A 500 µL volume of the hybridization mix was applied to each of 22K 60-mer Human 1A Oligonucleotide Microarray (part number G4110A) and hybridized in a hybridization oven (part number G2505-80081) at 60 °C for 17 h. The slides were disassembled in 6X SSC, 0.005% Triton X-102. They were washed first with 6X SSC, 0.005% Triton X-102 for 10 min at room temperature, then with 0.1X SSC, 0.005% Triton X-102 for 5 min on ice, and dried using a nitrogen-filled air gun.

Analysis

The arrays were scanned by the Agilent dual-laser DNA microarray scanner (part number G2565AA) using SureScan technology, extracted by Feature Extraction software (part number G2566AA), and analyzed by Rosetta Resolver® software. An average of three replicate samples was used for each experiment.

Results and Discussion

A series of experiments were designed to define the range of poly (A)$^+$ RNA and total RNA input for oligonucleotide microarray study using Agilent's LRIFLA kit. RNA samples from a HeLa cell line and human spleen were used for this study. The resulting amplified and fluorescently labeled cRNA targets from HeLa and spleen were evaluated by co-hybridizing to human oligonucleotide microarrays and examined for sensitivity and reproducibility. The array performance of cRNA targets generated from poly (A)$^+$ RNA sample input was also compared with that from total RNA input and reported in the article.

Poly (A)$^+$ RNA results

In order to define the poly (A)$^+$ RNA sample input range and particularly the minimal input of RNA for the LRIFLA kit, poly (A)$^+$ RNA samples from HeLa or spleen were titrated from 200 ng to 0.1 ng to assess the performance across a large dynamic range. The RNA samples were reverse transcribed to produce double-stranded cDNA templates, followed by T7 polymerase-driven *in vitro* transcriptions to synthesize cRNA targets. Cyanine 3-labeled or cyanine 5-labeled CTPs were incorporated into the cRNA targets during the synthesis.

Figures 1A (HeLa) and 1B (spleen) show the amounts of labeled cRNA targets being produced from 0.1 to 200 ng of poly (A)$^+$ RNA. When the amount of input poly (A)$^+$ RNA was 0.1 ng or 1 ng, low yields of cRNA products were observed, ranging from 0.8 to 1.7 µg. As the amount of input RNA was increased, a significant increase (up to 67 µg in HeLa and 66 µg in spleen with 200 ng of poly (A)$^+$ RNA input) in labeled cRNA targets was observed, indicating robustness in amplification up to 600-fold. As expected, the observation is sample-type independent, since similar yields were obtained with both HeLa and spleen RNA.

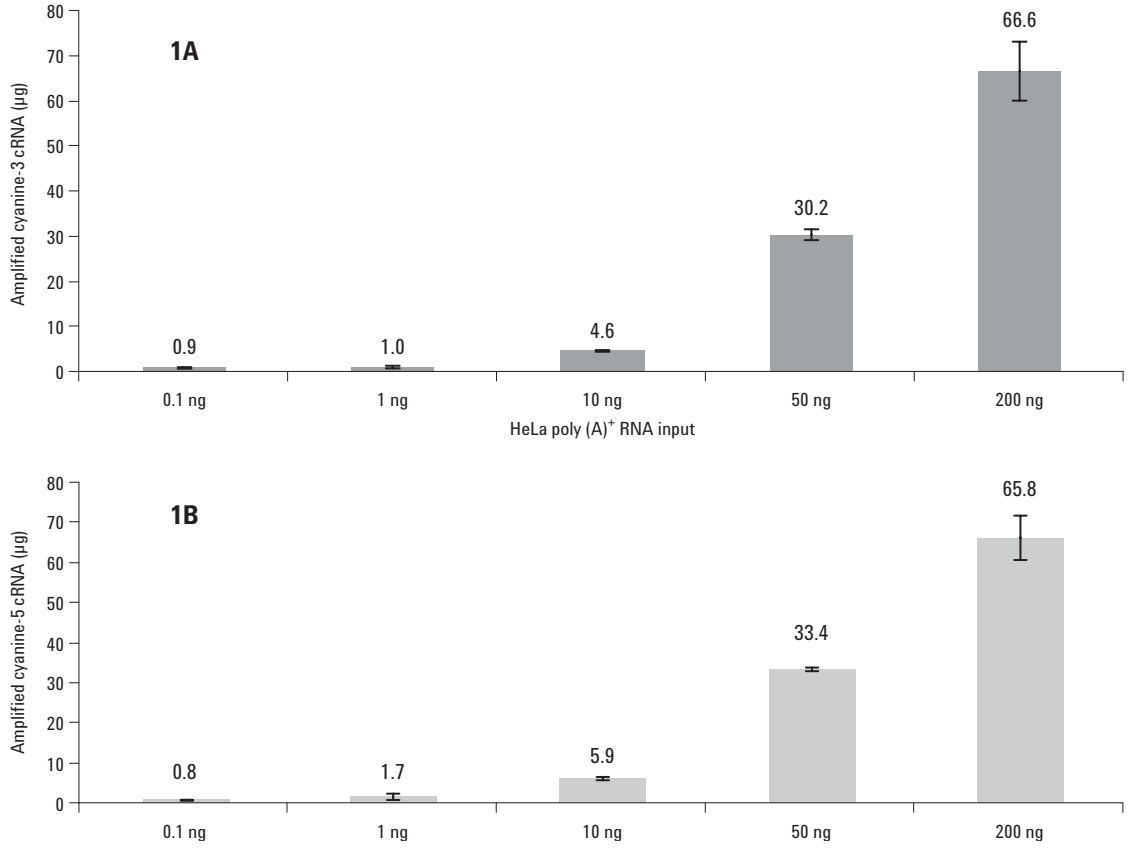

Figure 1. Producing micrograms of labeled cRNA from poly (A)$^+$ RNA after a one-round of amplification. Amplified and labeled cyanine-3 or cyanine-5 cRNA was generated from 0.1 ng to 200 ng of poly (A)$^+$ RNA from HeLa cells (1A) and human spleen tissues (1B), respectively, using the LRIFLA kit. The cRNA yields were determined by UV spectrophotometry and represent an average of three replicates. The error bars represent the standard deviation of the mean derived from replicates.

Next, we examined the performance of these cRNA targets on arrays by mixing equal amounts of cyanine-3 labeled targets (HeLa) with cyanine-5 labeled targets (spleen), and co-hybridizing them to Agilent's human oligonucleotide arrays. These arrays were scanned on an Agilent scanner, extracted using feature extraction software, and analyzed using Resolver software.

We compared the correlation coefficient (R^2) of log ratios for signature genes using 200 ng poly $(A)^+$ RNA input as a standard, since the performance of this input was previously validated (data not shown). As shown in Figure 2, the R^2 value of log ratios was excellent (above 0.97), when the input RNA was between 10 ng and 200 ng. The correlation of log ratios remained good (R^2 = 0.91) with 1 ng of input RNA. However, this number dropped drastically to 0.59 when 0.1 ng of poly $(A)^+$ RNA was used. Since the amounts of cRNA targets were the same in the hybridization reactions, a significant decrease in the R^2 value indicates that poor quality cRNA targets, containing nonspecific products, were generated at the 0.1 ng level, leading to the decreased sensitivity of array detection.

Differential gene expression was further analyzed using Resolver software, by comparing the number of genes that were up-regulated, down-regulated or unchanged in HeLa versus spleen samples as a function of RNA input. As shown in Figure 3, the number of genes that were up-regulated, down-regulated, or unchanged were similar when 10 ng, 50 ng, or 200 ng of input poly $(A)^+$ RNA was used. By contrast, when using only 1 ng or lower of poly $(A)^+$ RNA, the number changed significantly. More than 2000 genes failed to be detected as being differentiated, in comparison to results from 10 ng of poly $(A)^+$ RNA. In addition, an increase in the number of anticorrelated genes was observed,

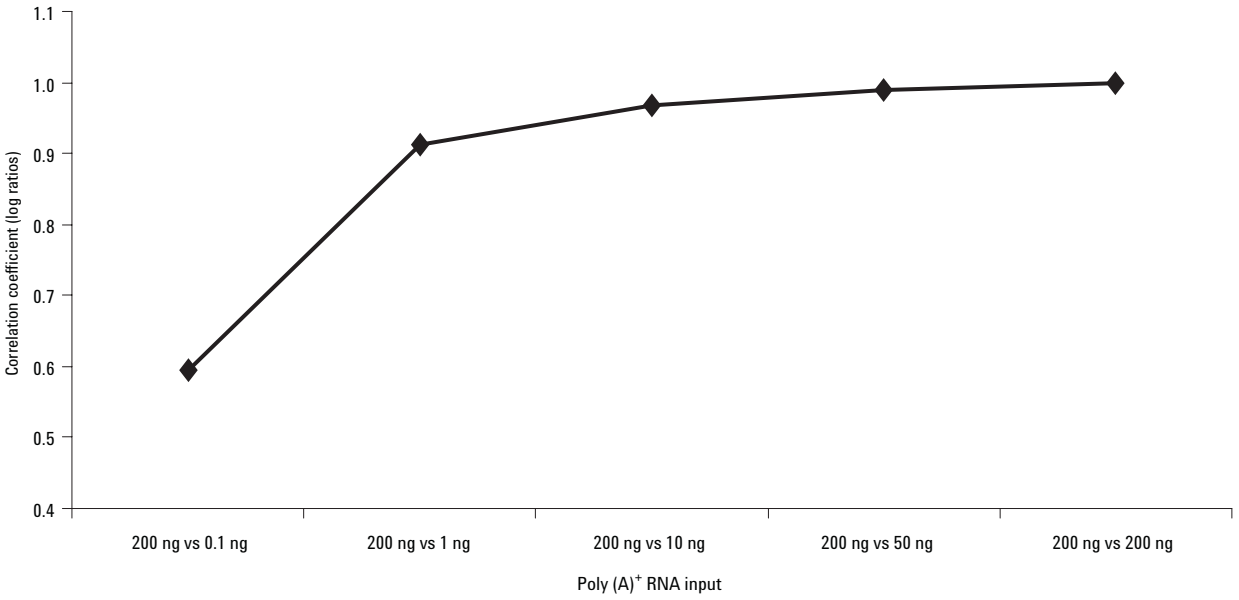

Figure 2. Log ratio correlation comparison between 200-ng and less poly $(A)^+$ RNA input in the amplification and labeling reactions. Three replicates of 0.1 ng, 1 ng, 10 ng, 50 ng, and 200 ng of poly $(A)^+$ RNA from HeLa and spleen were amplified and labeled. They were then hybridized to Human 1A Oligo Microarray, extracted by Feature Extraction software, and analyzed by Resolver software. The correlation coefficients of log ratios (nonweighted) were compared with that from 200 ng of poly $(A)^+$ RNA input.

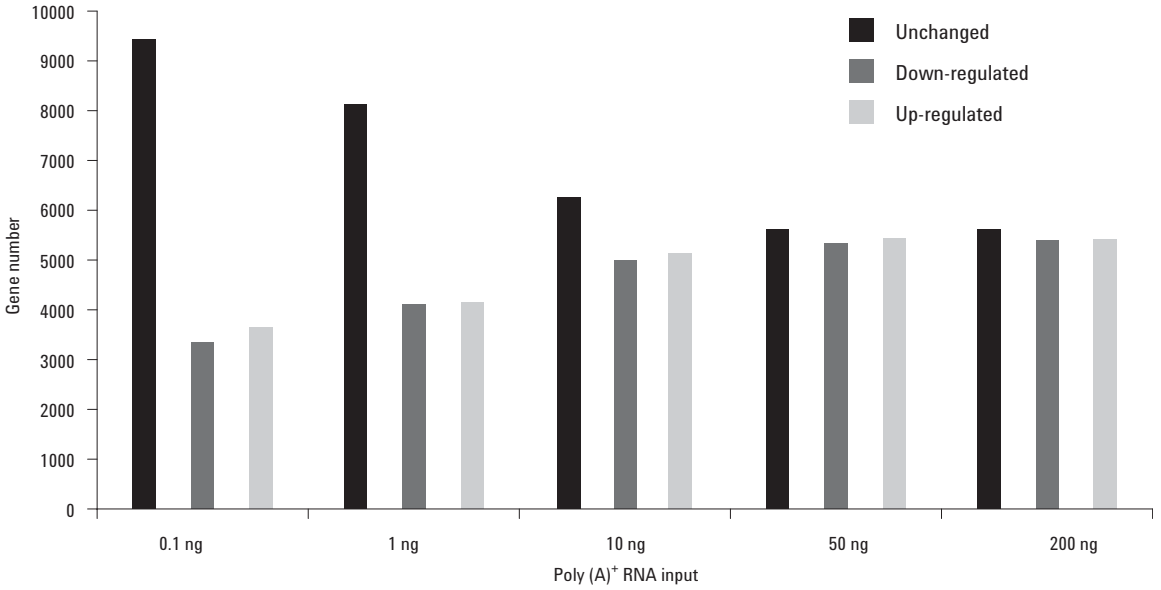

Figure 3. Differential gene expression comparison using different amounts of poly (A)$^+$ RNA for amplification and labeling. Amplified and labeled RNA products from 0.1 ng, 1 ng, 10 ng, 50 ng, and 200 ng of poly (A)$^+$ RNA input were hybridized to Agilent Human 1A Oligo Microarray. Differential gene expression was analyzed by Resolver software and the number of differentially expressed genes between HeLa and spleen are shown: unchanged, down-regulated, or up-regulated.

from 2 (50 ng) to 827 (0.1 ng) (Table 1). Nevertheless, at 1 ng of poly (A)$^+$ RNA input level or higher level, the percentage of anticorrelated genes was less than 0.6% of total genes (17,986) examined on the array, suggesting the consistency of the Agilent kit performance on arrays.

Total RNA results

Table 1. Anticorrelation of Signature Genes Using Poly (A)$^+$ RNA

Poly (A)$^+$ RNA input	Anticorrelated genes*	Percentage of total
200 ng vs 50 ng	2	0.01
200 ng vs 10 ng	25	0.14
200 ng vs 1 ng	96	0.53
200 ng vs 0.1 ng	827	4.60

*Anticorrelated genes refer to those that are up-regulated in one experiment but down-regulated in another. The percentage was calulated based on the number of probes printed on the array (17,986).

For most researchers who have a limited amount of samples, total RNA is a preferred choice for gene expression work. Some data on the use of total RNA with LIFLA kits was reported in a recent poster brochure [3]. A further examination of the functionality of the kit was made across a large dynamic range of total RNA input. Amplified and labeled cyanine-3 or cyanine-5 cRNA products were generated from 1 ng to 5000 ng total RNA from HeLa and spleen, mixed and hybridized to human oligonucleotide arrays. The log ratios of correlation coefficients were compared.

As shown in Figure 4, when 50 ng or above total RNA was used, the correlation of cRNA performance on arrays was excellent, as indicated by the correlation coefficient (R^2) above 0.9 for all signature genes as defined by Resolver software. The R^2 was reduced to 0.8 with 25 ng of total RNA, and further reduction was observed with 10 ng or 1 ng of total RNA input. This result is consistent with the findings by Carter et al [4]. Furthermore, the number of up-regulated, down-regulated, and unchanged genes were similar when using 50 ng, 500 ng, or 2000 ng of total RNA input (Figure 5). In contrast, there was an approximate 2-fold decrease in the number of differentially expressed genes when 1 ng of total RNA was used for amplification and subsequent labeling, in parallel with a 1.9-fold increase in the number of undifferentiated genes. This result suggests a decrease in sensitivity in detecting differential gene expression at the 1-ng level.

Taken together with the poly (A)$^+$ RNA data, we recommend the use of a minimum of 50 ng of total RNA or a minimum of 10 ng of poly (A)$^+$ RNA input for the LRIFLA kit in order to ensure the consistent and robust performance of labeled cRNA targets on the oligonucleotide arrays.

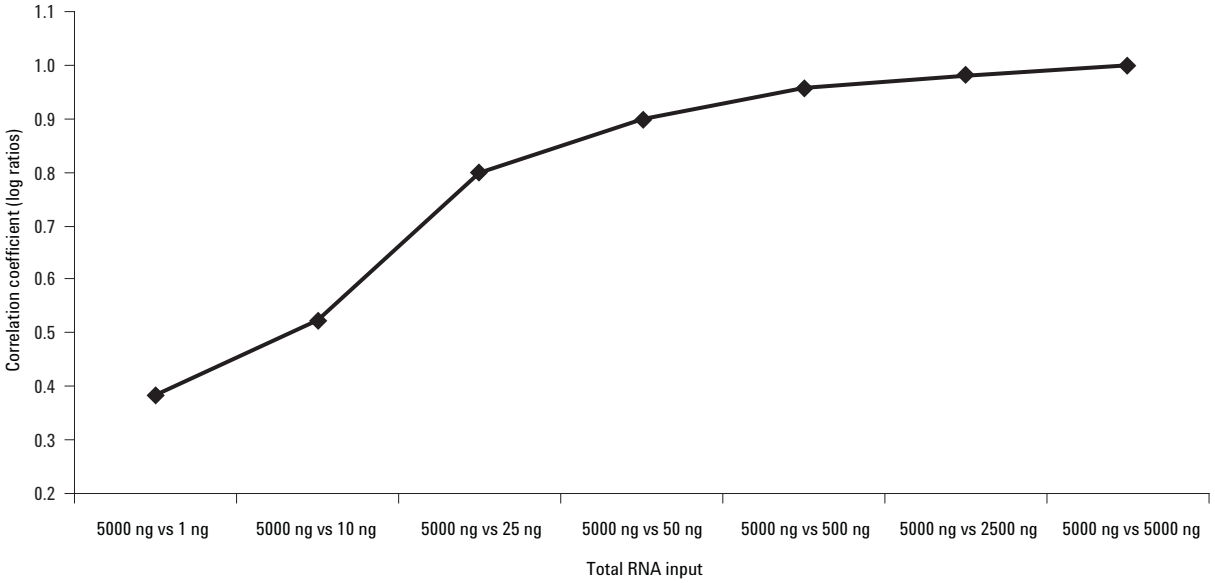

Figure 4. Log ratio correlation comparison using a broad range of total RNA input.
Three replicates of 1 ng, 10 ng, 25 ng, 50 ng, 500 ng, 2500 ng, and 5000 ng of total RNA samples from HeLa and spleen were amplified, labeled, hybridized to Human 1A Oligo Microarray, extracted by Feature Extraction software, and analyzed by Resolver software. The correlation coefficient of log ratios (nonweighted) was compared with that from 5000 ng of total RNA input.

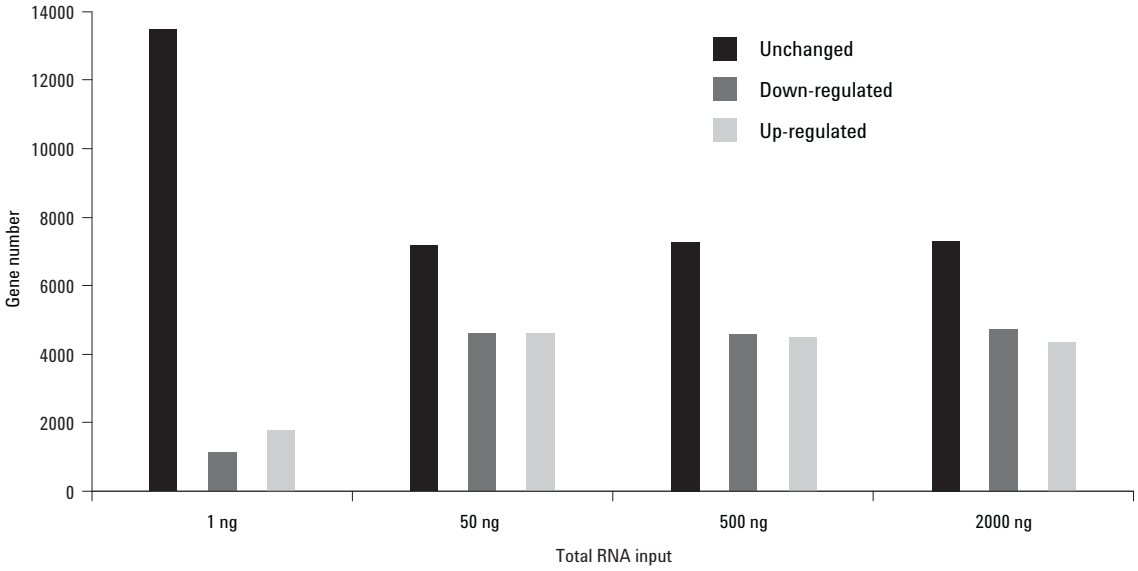

Figure 5. Differential gene expression comparison using 1 ng to 2000 ng of total RNA input in the amplification and labeling reactions.
Gene expression using 1 ng, 50 ng, 500 ng, and 2000 ng of total RNA as sample input was analyzed by Resolver. The number of differentially expressed genes between HeLa and spleen are shown: unchanged, down-regulated, or up-regulated.

Comparative results between poly (A)$^+$ RNA and total RNA

As shown previously both total RNA and poly (A)$^+$ RNA produced comparable microarray data in side-by-side experiments when using the Agilent Flourescent Linear Amplification Kit [5]. To determine whether the cRNA products generated from total RNA samples were comparable to those generated from poly (A)$^+$ RNA samples using the LRIFLA kit, we examined the microarray data of total RNA (2000 ng) with that of the similar level of poly (A)$^+$ RNA (50 ng) input. As shown in Figure 6, the overall correlations of log ratios between poly (A) RNA and total RNA were excellent (above 0.9). Similar results were obtained by comparing other total RNA input with the similar levels of poly (A)$^+$ RNA input (data not shown). This suggests that while the type of RNA input (poly (A)$^+$ RNA versus total RNA) may differ in amplification reactions, it does not significantly affect the gene expression data, as indicated by the log ratio comparison.

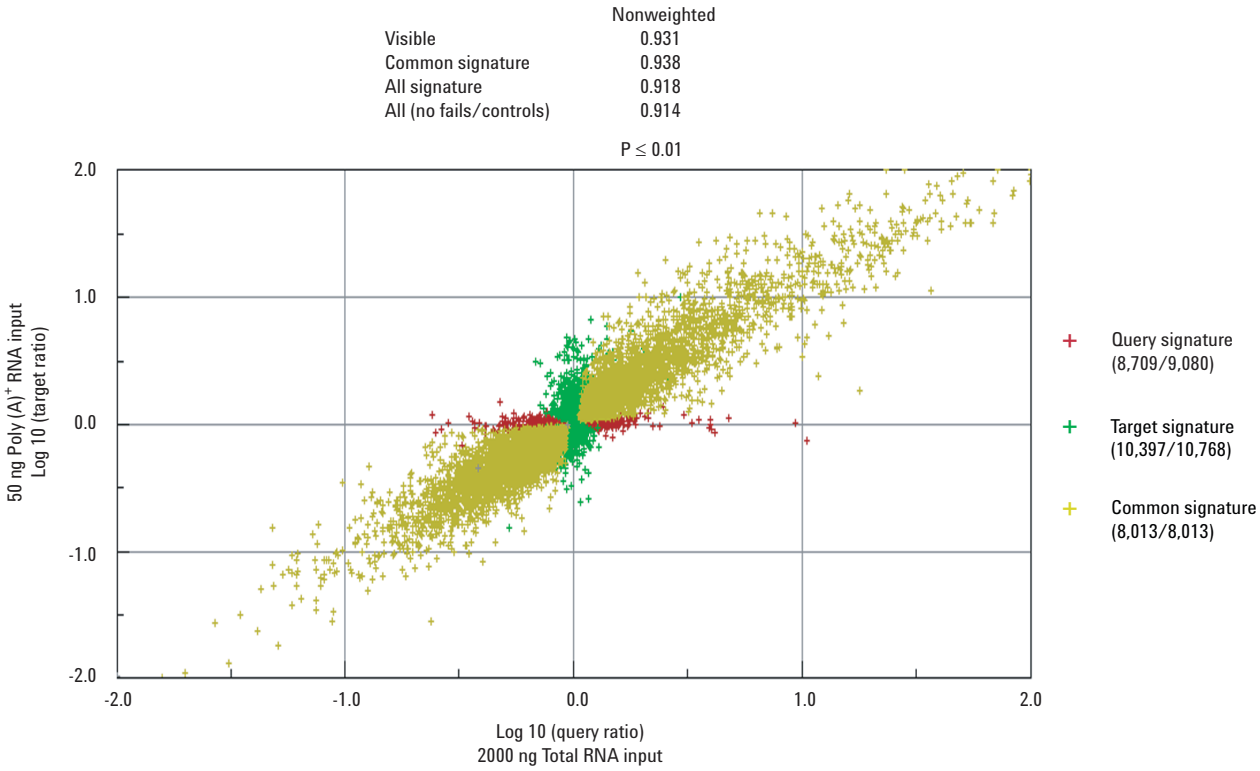

Figure 6. Microarray data comparison of poly (A)$^+$ RNA input and total RNA input in the LRIFLA kit.
The array result of 2000 ng of total RNA input (HeLa and spleen) was compared with that of 50 ng of poly (A)$^+$ RNA input (HeLa and spleen). Common signature represents genes that were found to be differentially expressed in both the 2000 ng of total RNA and 50 ng of poly (A)$^+$ RNA sets. The correlation coefficients of the log ratio comparison are shown above the chart.

Conclusions

The Agilent LRIFLA kit allows users to produce sufficient amounts of labeled cRNA targets from minimal sample input in a single round of linear amplification. The reaction procedure is simple and easy to follow. The resulting cRNA targets perform consistently and reproducibly on our oligonucleotide DNA microarrays over a large dynamic range of sample input (10 ng to 200 ng for poly $(A)^+$ RNA and 50 ng to 5000 ng for total RNA). Furthermore, the array performance of cRNA targets generated from total RNA input is comparable with that generated from poly $(A)^+$ RNA. The LRIFLA kit provides the feasibility and flexibility for researchers to choose the type and the amount of RNA samples in their gene expression studies with consistency and high performance.

References

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