MICROARRAY Technologies

Research Report

RNA Amplification Strategies for cDNA Microarray Experiments

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ABSTRACT

The biological materials available for cDNA microarray studies are often limiting. Thus, protocols have been developed to amplify RNAs isolated from limited amounts of tissues or cells. RNA amplification by in vitro transcription is the most widely used among the available amplification protocols. Two means of generating a dsDNA template for the RNA polymerase are a combination of reverse transcription with conventional second-strand cDNA synthesis and a combination of the switch mechanism at the 5′ end of RNA templates (SMART) with reverse transcription, followed by PCR. To date, there has been no systematic comparison of the efficiency of the two amplification strategies.

In this study, we performed and analyzed a set of six microarray experiments involving the use of a "regular" (unamplified) microarray experimental protocol and two different RNA amplification protocols. Based on their ability to identify differentially expressed genes and assuming that the results from the regular protocol are correct, our analyses demonstrated that both amplification protocols achieved reproducible and reliable results. From the same amount of starting material, our results also indicated that more amplified RNA can be obtained using conventional second-strand cDNA synthesis than from the combination of SMART and PCR. When the critical issue is the amount of starting RNA, we recommend the conventional second-strand cDNA synthesis as the preferred amplification method.

INTRODUCTION

The expression of thousands of genes can be measured simultaneously using cDNA microarray technology (3,5,6). To extend this technology to investigations using limited starting amounts of RNA, it is crucial to develop protocols to detect reliable and reproducible gene expression signals from minute amounts of RNA.

Such protocols for cDNA microarray experiments amplify either the RNA materials (RNA amplification) (9,11,15) or the resulting hybridization signal (fluorescent signal amplification) (1,13). In this study, we evaluated two different RNA amplification protocols.

Both protocols combine cDNA synthesis with a template-directed in vitro transcription reaction. During the reaction, a synthetic oligonucleotide containing a bacterial RNA polymerase promoter sequence, such as the T7 or SP6 RNA polymerase promoter sequence, is incorporated into cDNA molecules. The second strand of cDNA, which serves as the template for the RNA polymerase, can be generated either by conventional second-strand cDNA synthesis (11) or by combining the switch mechanism at the 5′ end of RNA templates (SMART), followed by PCR (15). Using either method, RNA amplification is achieved by following the initial step with in vitro transcription using RNA polymerase, and the amplified RNA is labeled with Cy3- or Cy5-dCTP by reverse transcription.

Although these RNA amplification protocols have been used in many laboratories, there has been no systematic comparison of the two protocols. In this study, we performed a set of six microarray experiments using the regular (unamplified) protocol and the two different RNA amplification protocols. We evaluated the results within a statistical framework that is designed to compare how effectively each protocol identifies differentially expressed genes.

MATERIALS AND METHODS

Microarray Production

A total of 2304 known human cDNAs were prepared by PCR from the Research Genetics cDNA clone library using two primers on the vector. The sequences of the two primers were: upstream, 5′-CTGCAAGGCATTAAGTTGGGTAAC-3′ and downstream, 5′-GTGAGCGGATAACAATTTCACACAGGAAACAGC-3′. We purified the PCR products using MultiScreen® PCR plates (Millipore, Bedford, MA, USA) and carried out the sequencing in our Cancer Genomics Core Laboratory to verify the products before printing (14). We used a robotic arrayer (Genomic Solutions, Ann Arbor, MI, USA) to spot the DNA clones in 394-well plates onto poly-L-lysine-coated microscope slides.

We replicated each of the 2304 genes twice on an array that also contained 96 positive controls, 96 negative

controls, and 192 blank spots (4800 total spots/slide). After printing, the slides were dried, cross-linked by UV (650 J/cm^2), washed with water, dried again, and stored.

RNA Isolation and cDNA Synthesis

We isolated total RNA from the K562 leukemia cell line and the RKO colon cancer cell line, respectively, using TRI reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's instructions. We carried out the first-strand cDNA synthesis by reverse transcription (RT) in a solution of 20 µL volume containing 1 µg oligo-dT_{25}-T_7 (5′-AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGATT-3′), 1 µg total RNA, 4 µL first-strand reaction buffer (Invitrogen, Carlsbad, CA, USA), 2 µL 10 mM dithiothreitol (DTT; Invitrogen), 1 µL 10 mM dNTPs, 1 µL SUPERase-in™, (Ambion, Austin, TX, USA), and 200 U SuperScript® II reverse transcriptase (Invitrogen).

For the conventional second-strand cDNA synthesis protocol, we added the following reagents into the 20-µL RT reaction: 91 µL nuclease-free water, 30 µL 5× second-strand buffer (Invitrogen), 10 U *E. coli* DNA ligase (New England Biolabs, Beverly, MA, USA), 40 U *E. coli* DNA polymerase I (New England Biolabs), and 2 U RNase H (Invitrogen). The reaction was carried out in a final volume of 150 µL at 16°C for 2 h.

For the template-switching protocol, we included 1 µg of template-switching primer (primer sequence: 5′-AAGCAGTGGTAACAACGCAGGGACCGGG-3′) during the synthesis of the first strand of cDNA. The reaction was performed at 42°C for 2 h. To synthesize the second strand of cDNA, we added 1 U RNase H (Roche Applied Science, Indianapolis, IN, USA) to the 20-µL RT reaction and then incubated the solutions at 37°C for 15 min. We then added the following reagents: 57 µL nuclease-free water, 10 µL 10× PCR buffer (Roche Applied Science), 10 µL 25 mM $MgCl_2$, 1 µL 10 mM dNTPs, and 5 U AmpliTaq Gold® DNA Polymerase (Roche Applied Science). The reaction was carried out at 95°C for 10 min and then for three cycles at 95°C for 1 min, 65°C for 6 min, and up to 12 min in the final elongation cycle. We then purified the cDNA products generated from both protocols using QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA, USA).

RNA Amplification and Target Labeling

We performed antisense RNA amplification by T7 in vitro transcription using the reagents from MEGAscript T7 Kit (Ambion). The reaction was carried out in a total volume of 40 µL, including 7.5 mM NTPS, 4 µL 10× buffer, 4 µL enzyme mixture, and all the cDNA products from the cDNA synthesis. After RNA amplification, we removed the cDNA template by incubating the reaction with 4 U RNase-free DNase I (Ambion) at 37°C for 15 min and purified the RNA using the RNeasy® Mini Kit (Qiagen). We labeled the purified 5 µg amplified RNA with Cy3 or Cy5 by RT in a solution containing 2 µg of random hexamer, 4 µL first-strand reaction buffer (Invitrogen), 2 µL 10 mM DTT, 1 µL 2 mM dATP, dGTP, dTTP, and 1 mM dCTP, 1 µL SUPERase-in, 1 µL Cy3-AP3-dCTP or Cy5-AP3-dCTP (Cy3-dCTP and Cy5-dCTP; Amersham Biosciences, Piscataway, NJ, USA), and 200 U SuperScript II reverse transcriptase. The labeling was carried out at 42°C for 2 h. We then purified the labeled cDNA using Microspin™ G-50 columns (Amersham Biosciences) and reduced the volume to approximately 10 µL using a SpeedVac® System AES2010 (Savant Instruments, Holbrook, NY, USA) before hybridization.

Hybridization and Image Scanning

To hybridize the slides, we added 70 µL ExpressHyb™ solution (BD Biosciences Clontech, Palo Alto, CA, USA) to the purified and labeled cDNA targets. We also added a mixture of blocking reagents containing 8 µg poly$(dA)_{40–60}$ (Amersham Biosciences), 2 µg of yeast tRNA (Invitrogen), and 10 µg of human Cot I DNA (Invitrogen) to the labeled targets, producing a final volume of approximately 80 µL. The mixture was heated to 95°C for 10 min, applied to the microarray slide, and covered with a coverslip. Hybridization was carried out at 60°C for 14–16 h in a moisturized box in a humid incubator. We then washed the microarray slides at 37°C, once in 1× SSC, 0.01% SDS, 0.2× SSC, 0.01% SDS, and twice in 0.1× SSC sequentially for 2 min each washing. We then scanned the hybridized arrays at a resolution of 20 µm on a GeneTAC™ LSIN scanner (Genomic Solutions).

Imaging Quantification

We used ArrayVision™ (Imaging Research, St. Catherines, ON, Canada) to quantify the microarray images. The fluorescent signal intensity was determined as the volume in a fixed-size circle, and the background was estimated as the median pixel value in a diamond-shaped region between each spot. Signal-to-noise ratio was calculated by dividing the background-corrected intensity by the standard deviation of the background pixels. Quantification files were loaded into S-Plus 2000 (Insightful, Seattle, WA, USA) for data processing and analysis.

Data Processing

Many factors, such as differences in target hybridization among the arrays, differences in the Cy3 and Cy5 incorporation or degradation rates, and fluorescent intensity variations induced by differences in gain settings when producing images, can complicate the process of comparing results from different microarray experiments. To correct for these variations, we applied a global normalization method that multiplicatively normalized the background-corrected spot intensities for each channel of each array to set the 75th percentile to equal 1000. In many experiments, this method is nearly equivalent to setting the median of expressed genes to equal 1000. If one assumes that most genes are not differentially expressed and that the numbers of overexpressed and underexpressed genes are about the same, then this method is also equivalent to the common normalization method that balances the fluorescence signal intensities by setting the median ratio between the channels to equal 1. Plots of the log ratio against the mean log intensity of

every spot, also known as a M-versus-A plot (Figure 1C), suggested that this procedure adequately corrected for the difference between channels, so we did not pursue more elaborate normalization strategies (4,17).

We then replaced spots with intensity levels below 150 by the threshold value of 150. Most of the blank spots on the array had normalized signals below this level. In addition, we found that the threshold value of 150 roughly corresponded to a spot for which the signal-to-noise ratio was equal to 1 on these arrays. Any spot with a background-corrected intensity below this threshold could not be reliably distinguished from the background noise. We then log-transformed (base 2) the background-corrected intensities for data analysis.

Data Analysis

Dual channel fluorescence cDNA microarray data contains a wide range of signal intensities. Using the fold difference between the two channels to identify differentially expressed genes does not provide sufficiently accurate information because it does not account for the variability associated with the signal intensity. In particular, it is more difficult to assess differential expression for low-intensity genes because these measurements are associated with higher variation, which can be primarily ascribed to background noise. In this study, we applied a statistical approach that identified differentially expressed genes based on the studentized log ratio. We briefly describe this approach, and a detailed description can be found elsewhere (2).

Recent publications (2,4,10,12) on microarray data analysis have shown that the standard deviation of the log ratio of intensities varies as a function of the mean in log-transformed signal intensity. In our approach, we first used replicate spots to estimate both the mean log intensity and the standard deviation of the log intensity of the genes within a single channel (on our array, every spot has been printed in duplicate). We carried out this procedure by fitting a smooth (loess) curve for each channel that described the standard deviation as a function of the mean log intensity. We take the two channels to be independent, so the variance of the log ratio, var(log(A/B)) = var(log(A)) - log(B)), can be estimated as the sum of the variances of the log intensities, var(log(A)) + var(log(B)). We pooled the two smooth curves giving the within-channel estimates to obtain a common estimate of the standard deviation of the log ratio between the channels.

Figure 1 illustrates how these smooth curves are applied. In Figure 1, A and B, we examine the agreement between replicate spots within each channel. The logarithm of the ratio between duplicate spots of the same clone is plotted on the vertical axis, and the mean log intensity of the duplicates is plotted on the horizontal axis. The curves added to the graph represent three times the loess fit of the standard deviation of the log intensity within that channel; duplicate pairs whose log ratio falls outside these bounds are flagged as poor replicates. In Figure 1C, we plot the log ratio between channels vertically and the mean log intensity horizontally. In this graph, the curves superimposed on the graph represent three times the pooled estimate of the standard deviation; points falling outside these bounds represent genes that are differentially expressed.

To assign a statistical significance to the differentially expressed genes, we divide the log ratio of the two channels by the pooled standard deviation to compute a studentized log ratio for each gene by

$$\log R_{studentized} = \frac{\log_2(A) - \log_2(B)}{\sigma_{pooled}} \quad \text{[Eq. 1]}$$

where $\log_2(A)$ and $\log_2(B)$ are the log-transformed background-corrected intensity of each gene in each channel, respectively. This process produces locally studentized values and is a more robust method to assess differentially expressed genes.

RESULTS AND DISCUSSION

In a previous study, we introduced a data analysis framework to evaluate the efficiency of an amplification protocol (16). In applying the framework to this study, we focused on the following issues: (*i*) enhancement of signal intensity; (*ii*) consistency and reliability of signal intensity; (*iii*) array reproducibility; and (*iv*) ability to detect differential gene expression profile.

Enhancement of Signal Intensity

To evaluate the enhancement of signal intensity, we quantified and compared the number of genes with detectable signal intensity on the amplified versus the unamplified arrays. Amplified arrays should produce more spots with adequate signal intensity. We assessed this criterion using the signal-to-noise ratio, which requires a spot to have a signal-to-noise ratio greater than two to be measurable.

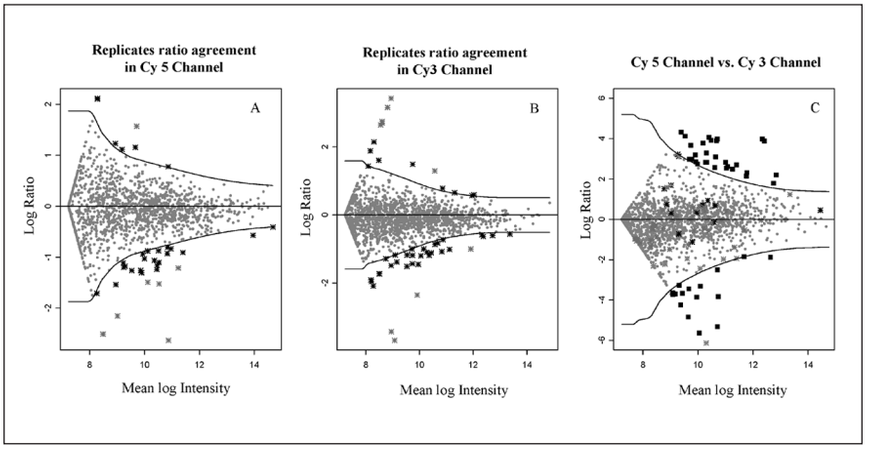

Figure 1. Plots of log intensity versus log ratio for an array. Duplicate spots produced using the regular protocol. (A and B) Average log intensity versus log ratio within each channel based on replicated genes. The asterisks represent poor replicate genes, and the bands correspond to ± 3 standard deviation. (C) Cy5 channel versus Cy3 channel; the squares outside of the bands are considered differentially expressed genes.

Table 1. Analysis of Signal Enhancement

Array ID	Cy5 (K562) Spots with S/N >2 (2304 spots total)	Cy5 (K562) %	Cy3 (RKO) Spots with S/N > 2 (2304 spots total)	Cy3 (RKO) %
R-1[a]	704	30.6	1352	58.7
R-2[a]	763	33.1	1770	76.8
A-S1[b]	1483	64.4	2029	88.0
A-S2[b]	1468	63.7	1988	86.3
A-T1[c]	1169	50.7	1877	81.5
A-T2[c]	1238	53.7	1919	83.3

S/N > 2, signal-to-noise ratio greater than two.
[a]R-1 and R-2: arrays produced by the conventional protocol.
[b]A-S1 and -S2: arrays produced by the second-strand amplification protocol.
[c]A-T1 and -T2: arrays produced by the template-switching amplification protocol.

We assessed all six arrays produced by the three different protocols (Table 1). A higher number of genes with adequate signal intensity in both channels resulted from the amplified versus the unamplified protocols. Additionally, more spots with sufficient signal intensity in both Cy5 and Cy3 channels were produced by the second-strand cDNA synthesis amplification protocol compared to the template-switching protocol.

Consistency and Reliability of Signal Intensity

To evaluate whether the amplification protocols preserved the gene signals, we first determined all the spots in each channel that consistently had a signal-to-noise ratio value greater than two on both arrays produced by the regular protocol. We then computed the percentage of those genes that also had a signal-to-noise ratio value greater than two on each set of the arrays produced by the two different amplification protocols (Table 2). More than 93% of the genes detected on the arrays produced by the regular protocol could also be detected on arrays produced by the two amplification protocols. In addition, arrays produced with the second-strand cDNA synthesis protocol had a slightly higher percentage agreement with regular protocol arrays than did the template-switching amplification arrays.

Array Reproducibility

We assessed the array reproducibility within the same protocol and between different protocols by computing the concordance correlation coefficient (r_c) between both the log ratio values and the studentized log ratio values. Although similar to the Pearson correlation coefficient, the concordance correlation coefficient specifically measures how well points follow the identity line (of perfect agreement) instead of more general linear relationships (8).

Figure 2 illustrates the reproducibility of the unscaled log ratios and the studentized log ratios within each protocol. The results of both analyses demonstrated the high reproducibility of arrays using the same protocol.

The r_c between the studentized log ratios for the regular and the second-strand cDNA synthesis amplification protocols ranged from 0.690 to 0.816 across four arrays, with a median value of 0.753. The r_c between the regular and the template-switching amplification protocols ranged from 0.656 to 0.772 across four arrays, with a median value of 0.711. The r_c between the second-strand cDNA synthesis and the template-switching amplification pro-

tocols ranged from 0.857 to 0.872 across four arrays, with a median value of 0.867. The concordances between the unscaled log ratios were similar, but slightly higher (data not shown).

Ability to Detect Differentially Expressed Genes

To identify differentially expressed genes, we computed a single studentized log ratio for each gene from duplicated microarrays produced under the same protocol among each of the three protocol types.

We considered genes to be differentially expressed if the combined studentized log ratio exceeds a significance threshold of |studentized log ratio| >3. Using this cut-off value, we found that the regular protocol identified 46 genes that were differentially expressed between K562 and RKO cell lines. The second-strand cDNA synthesis amplification protocol identified 51 differentially expressed genes, with 30 genes in common with the regular protocol. The template-switching amplification protocol identified 50 differentially expressed genes, with 27 genes in common with the regular protocol. All three protocols shared 24 genes in common. The results of this analysis are displayed in Venn Diagrams (Figure 3). For every gene identified as differentially expressed by at least one of the protocols, the sign of the studentized log ratio value (which determines whether the gene was overexpressed or underexpressed) was the same no matter which protocol was used (Figure 4).

Because a principal application of microarray technology is to identify differentially expressed genes, we feel that it is important to assess the ability of alternative protocols to accomplish this goal as well as the standard protocol. Amplification brings inherent complications into this assessment. The efficiency of amplification may well differ from gene to gene so that we cannot rely on the relative intensities of gene expression measurements within a single sample to remain fixed. Fortunately, the efficiency should be the same for a given gene across samples, so one expects the (log) ratios between samples to remain the same. However, the accuracy of microarray measurements of the log ratio is a function of the mean log intensity, which is why we use studentized log ratios and not simple fold differences to identify differentially expressed genes (2,4,10,12). Moreover, some genes that are expressed at low intensity may be more accurately measured following amplification.

If one assumes that the regular protocol identified all the differentially expressed genes, then we found agreement levels of about 65% (30/46) and

Table 2. Analysis Consistency of Signal Intensity

Second-Strand Synthesis Amplification			Template-Switching Amplification		
Array	Cy5 (K562)	Cy3 (RKO)	Array	Cy5 (K562)	Cy3 (RKO)
A-S1	98.9%	99.3%	A-T1	93.3%	96.9%
A-S2	98.6%	99.3%	A-T2	95.9%	97.4%

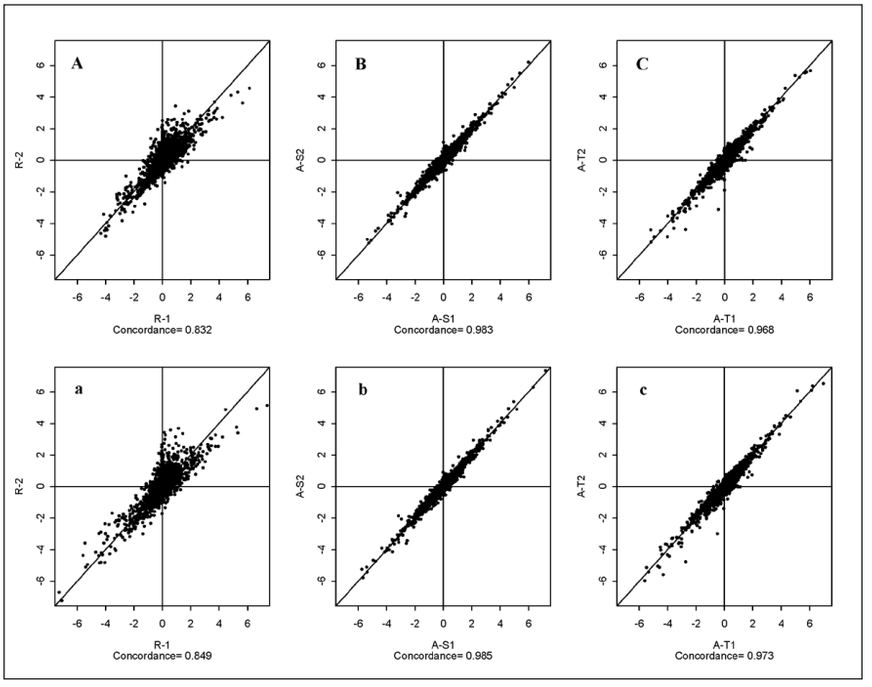

Figure 2. Reproducibility of microarray experiments. (A) Unscaled log ratios between arrays produced by the regular protocol; (B) unscaled log ratios between arrays produced by the second-strand synthesis amplification protocol; and (C) unscaled log ratios between arrays produced by the template-switching amplification protocol. Plots a, b, and c display the reproducibility using the studentized log ratios of each pair of arrays, respectively.

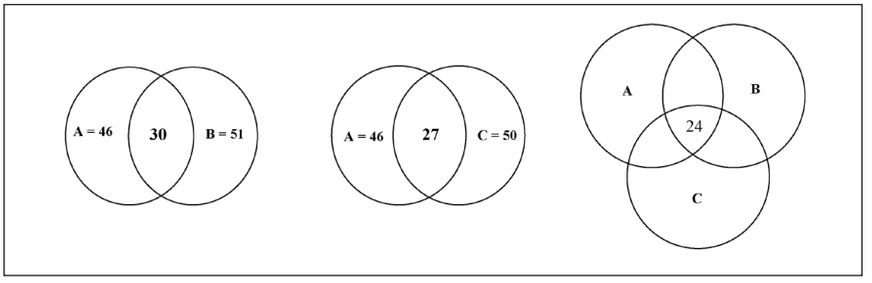

Figure 3. Venn diagrams of differentially expressed genes found on each array, commonly determined between two different protocols, and across three protocols. (A) Regular protocol; (B) second-strand synthesis amplification protocol; and (C) template-switching amplification protocol.

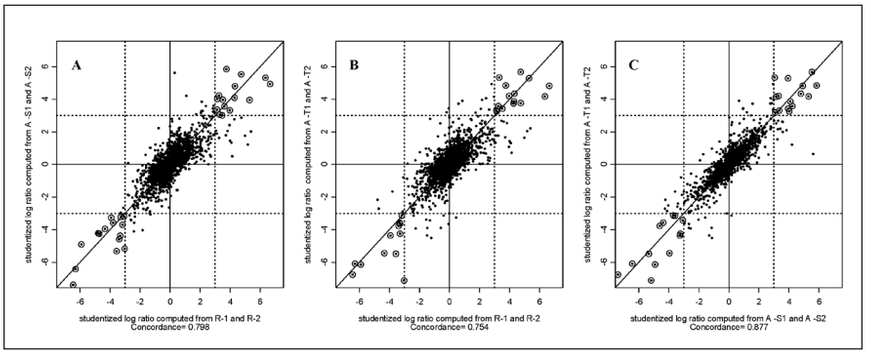

Figure 4. Illustrations of differentially expressed genes identified using two different experiment protocols. Genes identified between (A) the regular and second-strand synthesis amplification protocols; (B) the regular and template-switching amplification protocols; and (C) the second strand synthesis and template-switching amplification protocols. The differentially expressed genes in each illustration are indicated with circles. The dotted lines correspond to the studentized log ratio cut-off value of ± 3.

59% (27/46) when comparing it with the second-strand cDNA synthesis and template-switching amplification protocols, respectively. In terms of the identification of differentially expressed genes, the two amplification protocols performed similarly. However, more spots with detectable signal intensities consistently resulted in both channels when using the second-strand cDNA synthesis compared to the template-switching amplification protocol. The second-strand cDNA synthesis amplification protocol also produced more amplified RNA after the RNA amplification. This is a big advantage for studies using very limited RNA samples, and particularly so for tissue samples of rare pathologies, such as a rare tumor.

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