A Sense-RNA Amplification Method that Preserves Both 3′ and 5′ Transcript Information

Roy Sooknanan,[1] John Hitchen,[1] and Haiying Grunenwald[2]

1, RiboTx inc.; 2, EPICENTRE Biotechnologies

Introduction

Transcription profiling using microarrays has greatly aided our understanding of gene expression patterns in many different organisms, tissues, and disease states. However, as new microarray platforms—such as exon arrays that are suitable for quantification of alternatively spliced transcripts—gain popularity, there is a critical need for RNA amplification methods that yield amplified RNA with minimal or no loss of sequence information, or amplification bias. The new RiboMultiplier™ Sense-RNA Amplification Kit uses a unique terminal-tagging process that ensures production of full-length sense RNA (sRNA) from intact total RNA. A RiboMultiplier Kit reaction produces microgram amounts of sRNA from as little as 10 ng of input total RNA.

In this report, we demonstrate the high fidelity of RiboMultiplier Kit amplification by showing excellent correlation between microarray data obtained with sense RNA-derived target, and with unamplified RNA-derived target on NimbleGen Systems expression arrays.

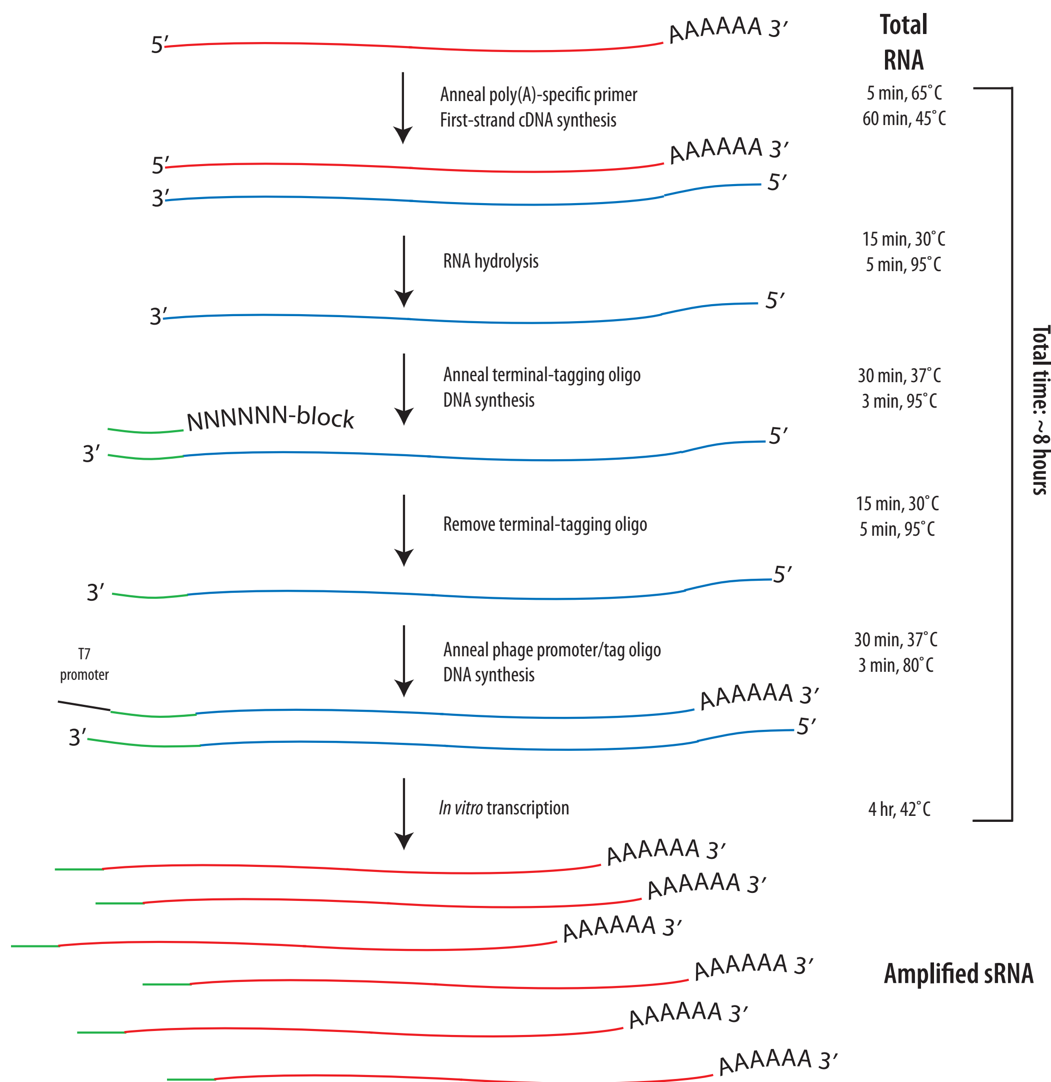

Fig. 1. Overview of the RiboMultiplier™ Sense-RNA Amplification Kit procedure.

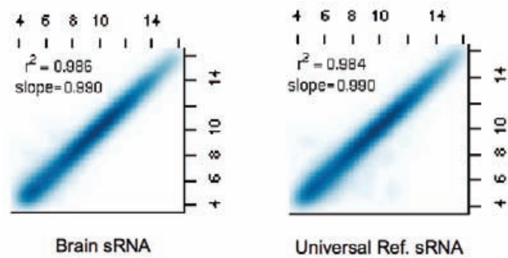

Fig. 2. sRNA produced by the RiboMultiplier™ Sense-RNA Amplification Kit yields highly reproducible microarray results. X-axis = $\log_2$ signal from replicate 1. Y-axis = $\log_2$ signal from replicate 2.

Methods and Results

The RiboMultiplier Sense-RNA Amplification Kit Procedure

An overview of the RiboMultiplier Sense-RNA Amplification Kit procedure is shown in Fig. 1. Briefly:

1. First-strand cDNA is synthesized from ≥10 ng of intact total RNA. The reverse transcription reaction uses a primer that anneals to the 3′-poly(A) tail of the mRNAs in the sample.
2. The RNA is removed by enzymatic digestion, and a "terminal-tagging" oligonucleotide is annealed to the cDNA. The tagging oligonucleotide contains i) a 5′ universal sequence, ii) a random sequence at its 3′ end, and iii) a 3′-blocking group. The terminal tagging oligonucleotide anneals to the cDNA. The 3′ end of the cDNA is then extended by a DNA polymerase, thereby producing a population of cDNAs that now all contain the complement to the 3′ tagging sequence. The 3′-blocking group present on the terminal tagging oligonucleotide prevents its extension by the DNA polymerase.
3. The terminal-tagging oligonucleotide is removed, and an oligonucleotide primer, containing a phage T7 transcription promoter at its 5′ end, is annealed to the universal tag sequence now present at the 3′ end of all the cDNAs. Second-strand cDNA is then generated by a DNA polymerase. The resulting product is a double-stranded DNA (dsDNA) containing a T7 RNA polymerase promoter in an orientation that will generate sRNA during the subsequent *in vitro* transcription reaction.
4. The dsDNA produced is transcribed into full-length sRNA in a rapid, high-yield *in vitro* transcription reaction.
5. The sRNA is purified using columns or other methods.

The entire single-tube RiboMultiplier Sense-RNA Amplification Kit procedure can be completed in 1 day, with about 1 hour of hands-on time.

Yield of sRNA from a RiboMultiplier Kit Reaction

The yield of sRNA from the standard RiboMultiplier Sense-RNA Amplification reaction is summarized in Table 1. The actual yield of sRNA may vary depending on the quality of the RNA sample and the poly(A) RNA content of the sample.

Table 1. Yields of sRNA produced by the RiboMultiplier™ Sense-RNA Amplification Kit. A control reaction with no input RNA did not yield detectable amplified product.

Input RNA	sRNA Yield from Universal Human Reference RNA	sRNA Yield from HeLa Total RNA
10 ng	8.2 µg	7.3 µg
25 ng	23.4 µg	17.5 µg
100 ng	86.7 µg	71.8 µg

Performance of sRNA Produced by the RiboMultiplier Kit in Microarray Analysis

The performance of the sRNA produced from a RiboMultiplier Sense-RNA Amplification Kit was assessed on NimbleGen Systems gene expression microarrays. One hundred nanograms of Human Brain RNA (Ambion) and Human Universal Reference RNA (Stratagene) were independently amplified using the RiboMultiplier Sense-RNA Amplification Kit, yielding 76 µg and 84 µg of sRNA, respectively. A 10-µg aliquot of each sRNA was then converted to dsDNA using the RiboMultiplier™ dsDNA Synthesis Kit. Unamplified RNA was used as a control in the experiment. Ten micrograms each of unamplified Human Brain RNA and unamplified Human Universal Reference RNA were converted to dsDNA using the NimbleGen protocol[1] for gene expression analysis.

Cy3™-labeled target DNA was produced from each dsDNA preparation by the standard NimbleGen Systems labeling procedure. The labeled target was hybridized to a NimbleGen Systems HG18 4-Plex Array. Data analysis was performed by NimbleGen Systems, using the Bioconductor package.[1]

The high r^2 values (0.985 for amplified targets, and 0.993 for unamplified targets) prove the excellent reproducibility of the RiboMultiplier Kit process.

To assess reproducibility of the RiboMultiplier Kit procedure, we plotted $\log_2$ signal values obtained with microarray targets generated from independently amplified sense-RNA batches (Fig. 2.) For comparison, we also showed reproducibility of technical replicates obtained with unamplified targets. The high r^2 values (0.985 for amplified targets, and 0.993 for unamplified targets) prove the excellent reproducibility of the RiboMultiplier Kit process.

Correlation between Amplified and Unamplified Microarray Targets

Labeled target cDNA prepared from RiboMultiplier-amplified human brain and universal human reference RNA was hybridized to microarrays by NimbleGen Systems, and the $\log_2$ values of brain RNA to universal human reference RNA signal ratios were determined for each transcript. Similar analysis was conducted with unamplified brain RNA and universal human reference RNA. Fig. 3 shows the correlation

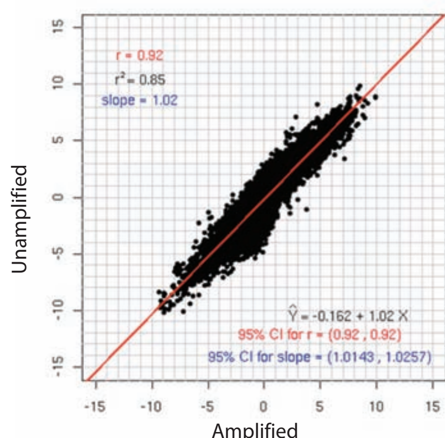

Fig. 3. Correlation of sRNA produced by the RiboMultiplier™ Sense-RNA Amplification Kit with unamplified RNA. Log_2 ratios were plotted for unamplified brain/universal human RNA (Y-axis) vs. amplified brain/universal human RNA (X-axis); r = 0.92, slope = 1.02.

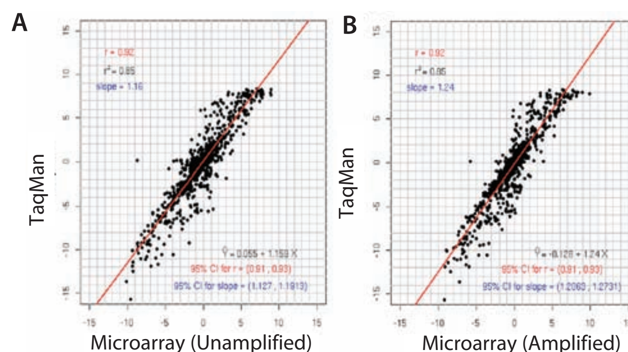

Fig. 4. Correlation of sRNA produced by the RiboMultiplier™ Sense-RNA Amplification Kit with MAQC TaqMan data. Log_2 ratios were plotted for TaqMan assay-based data for brain/universal human RNA (Y-axis) vs. corresponding microarray results (X-axis) for A) unamplified, and B) amplified RNA.

between expression ratios obtained with amplified and unamplified targets (r = 0.92), indicating that the expression profiles of the original samples are being faithfully preserved during the RiboMultiplier process.

Analysis of 3′/5′ Ratios by qPCR

Using 100 ng of Human Reference Total RNA, cDNA samples were prepared from either first-round RiboMultiplier sRNA or an oligo(dT)-primed aRNA amplification method. The samples were diluted 100-fold with distilled water and 1-µl aliquots were used in 25-µl qPCR reactions with TAQurate™ Green PCR MasterMix (EPICENTRE). Primer pairs specific to several genes were used (Table 2). The cycle threshold (C_T) values obtained with each primer pair were used to calculate the 3′/5′ ratio for each cDNA produced using the equation 3′/5′ ratio = $2^{(C_{T}5' - C_{T}3')}$. In general, 3′/5′ ratios around 1.0 were seen for the RiboMultiplier sRNA sample, which was significantly better than the corresponding aRNA sample, demonstrating that both 5′ and 3′ transcript information are preserved using the RiboMultiplier process.

Table 2. 3′/5′ ratios for RiboMultiplier sRNA compared to aRNA, as determined by qPCR.

Gene	Transcript Length (nt)	3′/5′ Ratio	
		sRNA	aRNA
GUSB	2,245	0.8	36.8
TUBA	1,706	0.9	223
ENSA	2,512	0.9	24.3
ACTB	1,792	0.3	73.5
TFRC	5,010	0.3	256
GAPDH	1,310	1.2	181
PKG1	2,338	0.4	24.3
H3F3A	1,117	1.6	121
ALB	2,215	1.5	9.8
HPRT	1,331	0.3	6.1

Correlation with TaqMan® Data from the MAQC Project

The Microarray Quality Control Consortium (MAQC) has assembled a panel of ~1,000 transcripts, and the abundance of these transcripts in Human Brain Reference RNA (Ambion), and in Universal Human Reference RNA (Stratagene) was determined by TaqMan assays. These data are publicly available from the MAQC website. In Fig. 4, we show the correlation between log_2 expression ratios from TaqMan assays and microarray data.

The correlation coefficient that we obtained (r = 0.92) is equal to the highest values published by the MAQC Consortium,[2] indicating that both sRNA-derived and unamplified RNA-derived targets produced microarray results that correlate very well with the TaqMan results.

Conclusions

The RiboMultiplier Sense-RNA Amplification Kit uses a unique terminal-tagging process to produce microgram amount of full-length sRNA in 1 day from as little as 10 ng of total RNA. The sRNA produced is readily converted to labeled target for use in microarray studies. The labeled target generated from the sRNA shows high correlation with unamplifed RNA as demonstrated using NimbleGen Systems expression microarrays. In addition, there was a high "true positive rate" of differential expression for sRNA, similar to unamplified RNA, when compared to the MAQC TaqMan assay results.

References

1. Gentleman, R. C. *et al.* (2004) *Genome Biol.* **5**:R80.
2. MAQC Consortium. (2006) *Nature Biotech.* **24**:115.

Ordering Information

RiboMultiplier™ Sense-RNA Amplification Kit

RM80510 10 reactions