nanoString TECHNOLOGIES

Amplification of RNA for Subsequent Analysis by the nCounter™ Analysis System

Using the EPICENTRE RiboMultiplier™ Sense-RNA Amplification Kit

Overview

Typically, the nCounter Analysis System requires 100 nanograms (ng) of total RNA in an assay. However, many samples of interest, such as those obtained from small biopsies, laser capture microdissection or fluorescently-activated cell sorting (FACS) protocols, can be very small and yield less than the recommended 100 ng of total RNA. In such cases, a reliable amplification method can be used to produce sufficient input RNA for analysis with the nCounter Analysis System. This technical note demonstrates that as little as 5 ng of RNA can be amplified to produce sufficient input for the nCounter Analysis System, using the RiboMultiplier™ Sense-RNA Amplification Kit from EPICENTRE Biotechnologies. Fold-change measurements performed with the amplified RNA show excellent correlation with those obtained from an unamplified sample.

nCounter™ Analysis System

The NanoString nCounter Analysis System delivers direct, multiplexed measurement of gene expression, providing digital readouts of the relative abundance of hundreds of mRNA species simultaneously. The nCounter Analysis System is based on gene-specific probe pairs that are hybridized to the sample in solution. The protocol obviates any enzymatic reactions that might introduce bias in the results (Figure 1, step 1). The Reporter Probe carries the fluorescent signal; the Capture Probe allows the complex to be immobilized for data collection. Up to 550 pairs of probes specific for a particular set of genes are combined with a series of internal controls to form a CodeSet. After hybridization of the CodeSet with target mRNA, samples are transferred to the nCounter™ Prep Station (Figure 1, step 2) where excess probes are removed and probe/target complexes are aligned and immobilized in the nCounter™ Cartridge. Cartridges are then placed in the nCounter™ Digital Analyzer for data collection (Figure 1, step 3). Each target molecule of interest is identified by the "color code" generated by the ordered fluorescent segments present on the reporter probe. The reporter probes on the surface of the cartridge are then counted and tabulated for each target molecule.

RiboMultiplier Sense-RNA Amplification System

The RiboMultiplier Sense-RNA Amplification Kit from EPICENTRE Biotechnologies provides the level of amplification required to produce sufficient input RNA for the nCounter Analysis System, while preserving measured fold-changes in gene expression across samples. The kit can generate microgram quantities of sense RNA (sRNA) from less than 10 ng of total RNA, and the amplification procedure can be completed in one day.

Using this amplification system, double-stranded cDNA with a T7 promoter on one terminus is generated from the input RNA (Figure 2). The T7 promoter is then used for an *in vitro* transcription (IVT) reaction to produce large amounts of sRNA that will be used as the input for the nCounter Analysis System.

Experimental Method

The RiboMultiplier Sense-RNA Amplification Kit was tested with 5 and 10 ng of Human Reference (Stratagene) and Brain Reference (Ambion) total RNAs. Amplifications were performed using the protocol supplied with the kit and all reactions were done in duplicate. The resultant amplified sRNA was used as input for a standard nCounter™ Gene Expression Assay, and the results were compared to those obtained with 100 ng of the unamplified reference total RNA samples. The correlations between amplified and unamplified RNA samples for both fold-change values and absolute counts were then determined.

Amplified RNA was purified from the *in vitro* transcription reactions using the

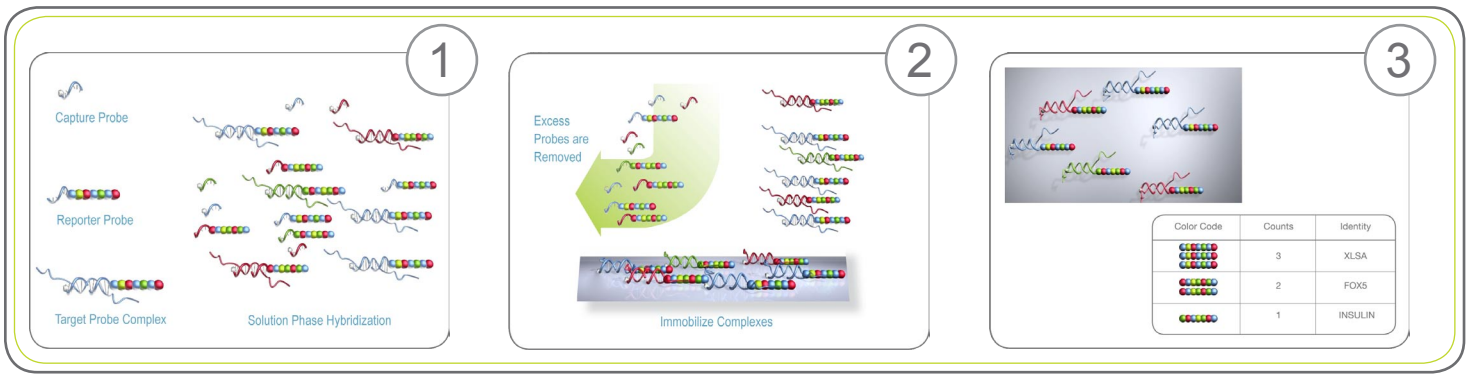

Figure 1. The Nanostring™ technology.

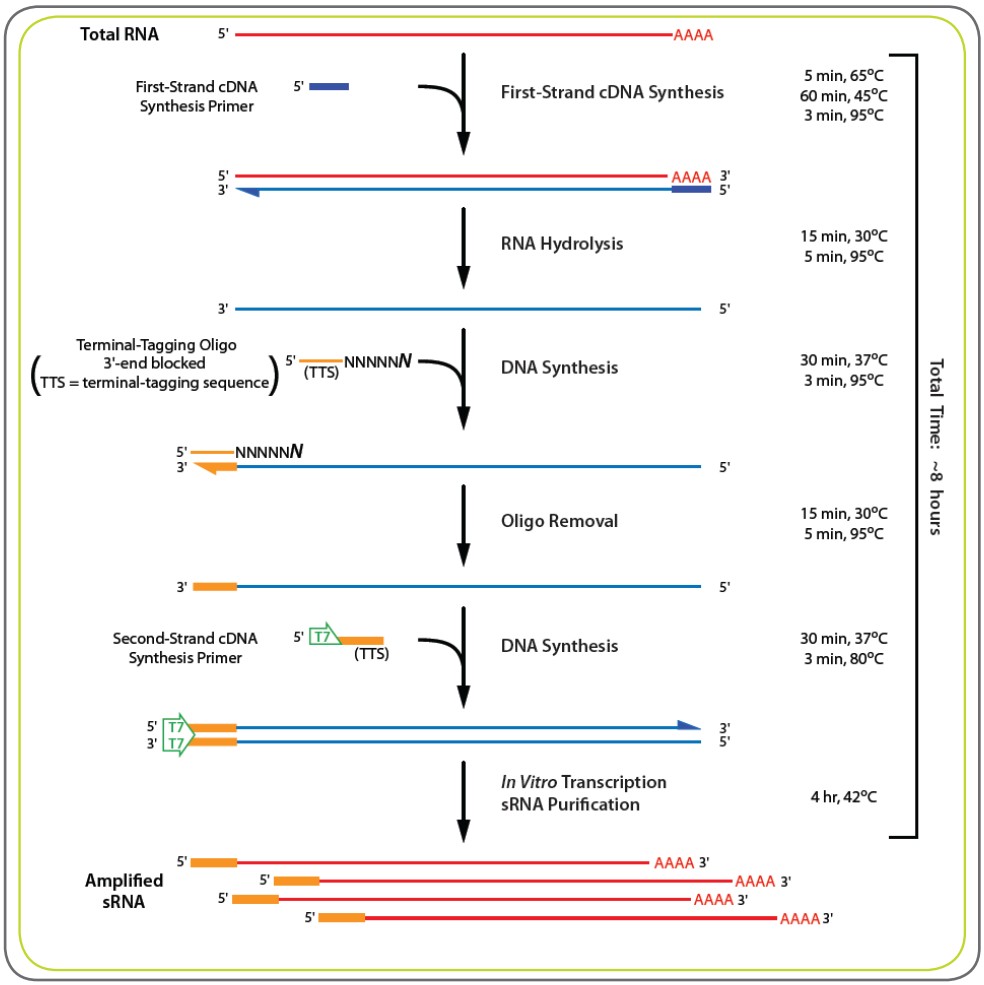

Figure 2. Schematic overview of the RiboMultiplier Sense-RNA Amplification procedure.

QIAGEN RNeasy mini kit. Quantitation of RNA yield from the amplification reactions was performed using the Nanodrop spectrophotometer, and qualitative analysis was performed on an Agilent Bioanalyzer 2100, using the RNA 6000 Nano kit.

Varying amounts of amplified RNA were analyzed on the nCounter Analysis System to determine a concentration of amplified sRNA which most closely approximated the counts obtained with 100 ng of unamplified total RNA. Hybridizations were then performed on Human Reference and Brain Reference RNA from both amplified (test) and unamplified (control) samples, using a CodeSet specific for a subset of the genes characterized by the Microarray Quality Control (MAQC) consortium. The correlation in fold-change values (Brain Reference/Human Reference RNA) was determined for both test and control samples. All hybridizations were performed following the standard protocol in 30 µL at 65°C for at least 16 hours, and processed on the nCounter Prep Station and Digital Analyzer. Each sample was run in triplicate. To account for slight differences in hybridization and purification efficiencies, raw data was normalized to internal positive spike-in controls present in every reaction. All mathematical manipulations were performed using Microsoft Excel.

Results

Approximately 1.5 µg and 6.5 µg of sense RNA were produced from 5 and 10 ng of input RNA respectively, using the RiboMultiplier kit (Table 1). The majority of the amplified transcripts were shorter than 200 bases (Figure 3). It was determined that 100 ng of this amplified sRNA gave comparable results in the nCounter Analysis System to 100 ng of total unamplified RNA (data not shown). This outcome was somewhat unexpected, given that the amplification procedure should enrich for mRNA molecules. The discrepancy is likely explained by two observations: a) approximately half of the amplified product is below the optimal target size (100 bases) for the system and, b) rRNA is detected in the amplified product, suggesting the amplification process is not 100% specific for mRNA.

Table 1: Amplification Yields of 10 ng and 5 ng Input Levels of Total RNA

	Set 1 ng/µL	Set 1 µg	Set 2 ng/µL	Set 2 µg
HR 10 ng	92.01	5.5	113.19	6.8
HR 5 ng	22.7	1.4	24.3	1.5
BR 10 ng	108.65	6.5	103.51	6.2
BR 5 ng	24.59	1.5	25.77	1.5

The hybridization results show excellent fold-change (Brain Reference/Human Reference) correlation between the unamplified and amplified samples (R2 = 0.97 and R2 = 0.98) for both replicates, using 10 ng of input RNA (Figure 4). The fold-change correlation decreases slightly (R2 = 0.95 and R2 = 0.94) when using 5 ng of input RNA, but remains very reproducible across the duplicate data set (Figure 5). The measured gene set used in every nCounter Gene Expression Assay experiment included genes expressed at levels just above background, a large number of medium-expressed genes, and some highly expressed genes as well (Figures 4 and 5). The absolute counts after amplification still show good correlation (R2=.88) when compared to absolute counts obtained from the unamplified control (Figure 6, A and B). However, since the correlation is lower than that obtained for fold-changes, comparison of absolute counts in biological samples should be performed with caution.

Discussion

The nCounter Analysis System is a powerful tool for direct measurement of the expression levels of large numbers of genes simultaneously with sensitivity better than microarrays and equivalent to qPCR (1). Gene expression studies are often performed with starting quantities of RNA much greater than the 100 ng required by the nCounter Analysis System. However, for applications such as small biopsies, laser capture microdissection or FACS, which generate much less than 100 ng, an amplification technique may be required.

The data presented here demonstrate that the EPICENTRE RiboMultiplier Sense-RNA Amplification Kit can produce enough sense RNA to use as input for nCounter Gene Expression Assay studies without introducing significant bias into the results. Gene expression fold-changes are preserved, when compared to unamplified RNA, across a wide range of expression levels (Figures 4 and 5). The combination of the RiboMultiplier amplification kit with the nCounter Analysis System thus provides a reliable solution for accurate measurement

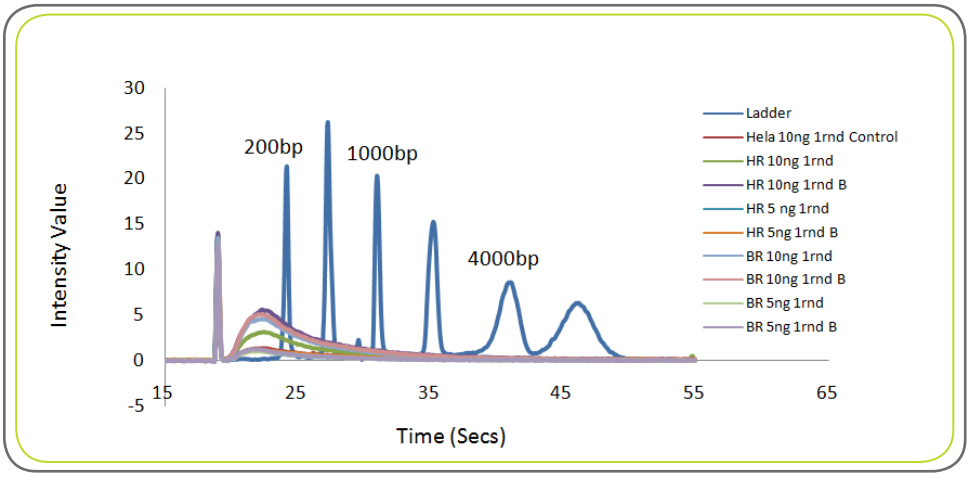

Figure 3. Analysis of amplified total RNA on the Agilent 2100 Bioanalyzer using the RNA 6000 Nano kit

of changes in gene expression in very small samples.

The levels of amplification of different transcripts in an RNA population may vary relative to one another due to sequence-dependent efficiency of reverse transcription and amplification. The amplification level of any given transcript is quite constant, regardless of the source of the sample (human reference or brain reference in this case). The end result is that while fold-changes in gene expression are preserved after amplification, the correlation of absolute counts (amount of transcript) between amplified and unamplified samples varies (Figure 6). Therefore, RiboMultiplier amplification with the nCounter Analysis System can be used to accurately determine fold-changes for a given gene across samples, but absolute count data should be analyzed carefully to assure that the comparison of the relative levels of expression of two or more genes in the same sample is valid.

It should be noted that the results shown here pertain to a specific set of genes with a wide range of expression levels. The expression of these genes varies from quite low (just detectable above background), to medium levels (a few hundred counts), to high expression levels (several thousand counts). The degree of fold-change correlation between amplified and unamplified RNA might vary with the set of genes being studied, depending upon the expression levels, nature of the sample and structures of the genes in the set. It is always best to confirm good fold-change correlation between unamplified and amplified RNA for the specific gene set being studied, before beginning a large study with amplified RNA. The gene expression fold-change correlation is quite good with 5 and 10 ng of input RNA, but decreases below 5 ng (data not shown). The fewer transcripts that are available for any gene, the less is the likelihood that those transcripts will be amplified. Thus genes expressed at low levels may not be amplified efficiently when using input levels less than 5 ng.

One key advantage to the RiboMultiplier system is that it produces sense RNA, which the nCounter Analysis System requires as input when analyzing unamplified total RNA. As a result, a CodeSet designed for use with unamplified total RNA can also be used with amplified product from the RiboMultiplier kit without any change. It is also important to note that any amplification system that produces antisense RNA cannot be used with the nCounter Analysis System, without changing the CodeSet.

It is possible to analyze gene expression from very small RNA samples using the nCounter Analysis System. Amplification of as little as 5 ng of total RNA is attainable using the EPICENTRE RiboMultiplier Sense-RNA Amplification Kit, without compromising the accuracy of fold-change measurements. Enough input sRNA is obtained to perform multiple nCounter Analysis System gene expression experiments, which provide fold-change profiles that are equivalent to those obtained with unamplified control total RNA.

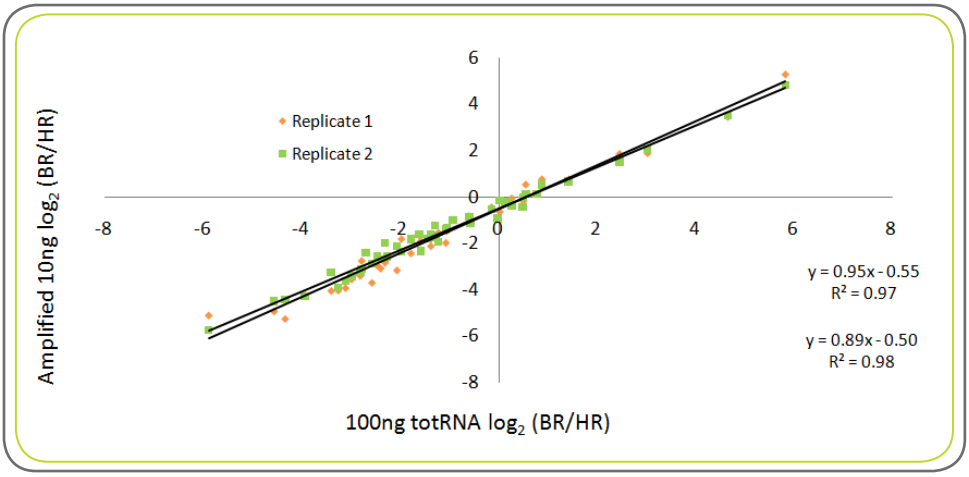

Figure 4. Correlation of Brain Reference/Human Reference fold-changes between amplified sRNA and unamplified control total RNA, using 10 ng of input RNA

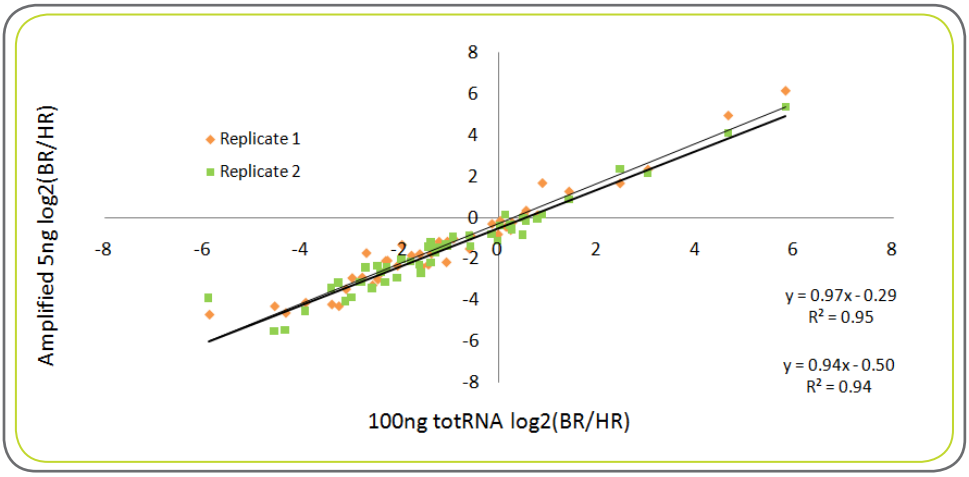

Figure 5. Correlation of Brain Reference/Human Reference fold-changes between amplified sRNA and unamplified control total RNA, using 5 ng of input RNA

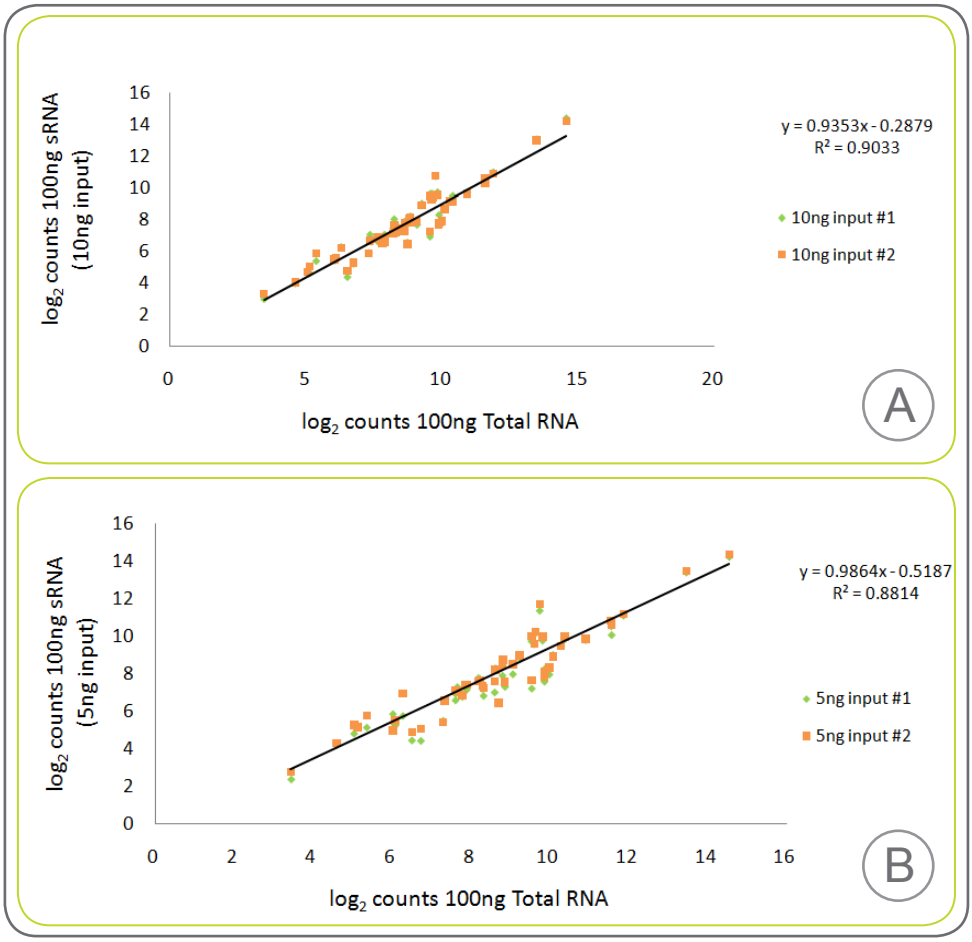

Figure 6. Correlation of Human Reference absolute counts between amplified sRNA and unamplified control total RNA, using 10 (A) or 5 (B) ng of input RNA for the amplification.

Purchasing Information

Nanostring Technologies®

Product Description	Catalog No.
nCounter™ Analysis System Includes the Prep Station and the Digital Analyzer	NCT-SYST
nCounter™ GX CodeSet Gene Expression Custom CodeSet	GXA-P1CS
nCounter™ Master Kit All reagents, sample cartridges, and consumables necessary for processing 48 Nucleic Acid Assays.	NAA-AKIT-48

EPICENTRE® Biotechnologies

Product Description	Catalog No.
EPICENTRE RiboMultiplier™ Sense-RNA Amplification Kit 10 Reactions	RM80510

References

1. Geiss GK, *et. al*. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol*. 2008 Mar;26(3):317-25.

NanoString Technologies, Inc.
530 Fairview Ave N, Suite 2000
Seattle, Washington 98109
(206) 378-NANO • (206) 378-6266
www.nanostring.com

v.20090608