nCounter® Gene Expression Profiling from Low Sample Input

Introduction

Gene expression analysis from tissue biopsies and formalin-fixed paraffin-embedded (FFPE) samples are an industry standard and invaluable to scientists who seek to answer biological questions in the fields of translational and biomarker research. However, one of the major challenges faced by scientists and clinicians is one of sample conservation. Researchers working with samples with low RNA content, limited biopsy tissue, or rare cell types are faced with the difficult choice of how much (or little) of their irreplaceable samples to process, while ensuring sufficient sample remains for other analytical testing necessary to obtain the most biologically meaningful insights possible. Furthermore, samples such as FFPE are often degraded or cross-linked due to fixatives used in the tissue archival process. For this reason, it is important for molecular technologies to not only be generate quality data while consuming the least amount of sample possible. NanoString's nCounter System enables counting of unique transcripts within a sample using digital molecular barcoding. The standard nCounter protocol recommends RNA inputs of 100 ng for successful expression profiling. Here we introduce a new method for generating high quality gene expression data using a pre-amplification approach upfront of the nCounter workflow that significantly reduces RNA input requirements 10 to 100-fold depending on sample type.

Hallmarks of Cancer Gene Expression Analysis

The nCounter Hallmarks of Cancer Panel collection offers a family of gene expression assays focused on translational research into cancer pathway dysregulation, immune profiling and cancer metastasis. The PanCancer Pathways, PanCancer Immune Profiling and PanCancer Progression panels deliver > 1,850 unique gene targets, with the ability to customize up to 30 additional genes for each panel. Data for all three panels can be analyzed in one experiment using nSolver™ Analysis Software.

Low RNA Input Kit

The Low RNA Input Kit enables researchers and laboratories with limited or low expressing samples to generate the same high-quality gene expression data of a typical NanoString assay using a fraction of the sample input. The Low RNA Input Kit provides an ultra-sensitive, reproducible method that utilizes single-tube, limited cycle PCR amplification to perform multiplexed target enrichment (MTE) of samples prior to nCounter hybridization. The workflow for 12 samples requires less than 30 minutes of hands-on time and the entire protocol completed in less than 2 hours. MTE primer pools are available for all NanoString Gene Expression Panels and the Low RNA Input Kit contains all other reagents necessary for pre-amplification of your sample upfront of the standard nCounter protocol.

Here we demonstrate performance of the Low RNA Input kit with breast (FF) and endometrial tumor samples (FFPE). For FFPE samples, 10 ng and 5 ng RNA were processed using the Low RNA Input kit to generate pre-amplified cDNA for each of the three Hallmarks of Cancer Panels. For the FF samples, RNA inputs of 10 ng and 1 ng were processed in the same manner. Following reverse transcription, each sample underwent 10 cycles of amplification using MTE primer pools matched against each of the three gene expression panels. cDNA was denatured briefly at 95°C and snap cooled on ice, and hybridizations were carried out following the standard nCounter protocol. As a control, 100 ng RNA from each sample was hybridized following the standard nCounter protocol without pre-amplification to establish a baseline for gene expression comparisons. Following data collection, raw data (RCC) files were imported into nSolver Analysis Software for normalization and analysis with the Advanced Analysis module. Using the Multi-RLF analysis feature, all three gene expression panels were analyzed at once, enabling data analysis for over 1800 genes in the same experiment.

Sensitivity and Genes Detected

We compared the total number of genes detected in samples prepared with the Low RNA Input Kit vs 100 ng RNA control samples. As seen in Figure 1, for both FF and FFPE samples comparable numbers of genes are detectable above the background count threshold, defined as the average number of negative control probes present + 2 standard deviations. For samples hybridized with 100 ng total RNA, an average of 1614 and 1608 genes were detected above background in FFPE and FF samples, respectively.

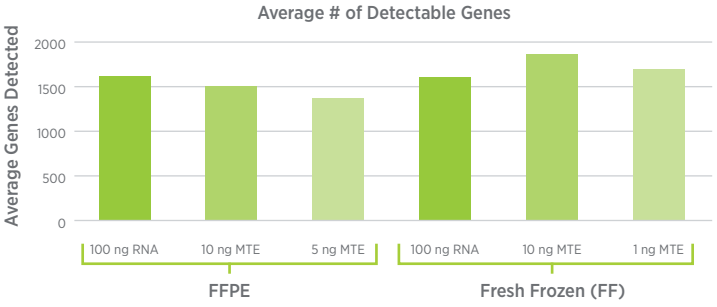

FIGURE 1 Average number of detectable genes for 4 FFPE and 4 fresh frozen tumor samples at indicated input amounts.

When 10 ng RNA was used as the input, an average of 1510 and 1873 genes were detected from FFPE and FF respectively (94% and 116% relative to 100 ng RNA input). At the lowest input amount for FFPE (5 ng), an average of 1372 genes were detected (85%) and for FF (1 ng), 1696 were detected (105%).

When working with small tissue amounts of heterogeneous cell composition, an important feature of any gene expression profiling platform is sensitivity to low-abundance transcripts. Table 1 shows the detection of the lowest expressed genes in samples prepared with the Low RNA Input Kit relative to results achieved from 100 ng RNA. Here, low expressed genes are defined as those having counts between the background threshold of 15 and 100 counts. The average number of detectable low expressing genes in FFPE samples is 605.

FFPE		
100 ng RNA	10 ng prepared with Low RNA Input Kit	5 ng prepared with Low RNA Input Kit
605	521 (86%)	436 (72%)

Fresh frozen (FF)		
100 ng RNA	10 ng prepared with Low RNA Input Kit	1 ng prepared with Low RNA Input Kit
581	572 (98%)	512 (88%)

TABLE 1 Total number of low-expressed genes (<100 counts) detected in FFPE and FF samples at the indicated input amounts. Parentheses indicate percentage of genes relative to 100 ng input amount.

Of these genes, 521 (86%) are also detectable in 10 ng MTE-prepped samples, and 436 (72%) are detectable in 5 ng MTE-prepped samples. For FF samples, the average number of detectable low expressing genes is 581, and of this set 572 (98%) are detectable in the 10 ng MTE-prepped samples and 512 (88%) detectable in 1 ng MTE-prepped samples. The observation that fewer genes are detectable in FFPE samples after low input processing can be explained by the higher degree of RNA degradation present in FFPE material, leading to less efficient amplification of target genes. Figure 2 shows representative Bioanalyzer traces of FFPE and fresh frozen samples used in this study.

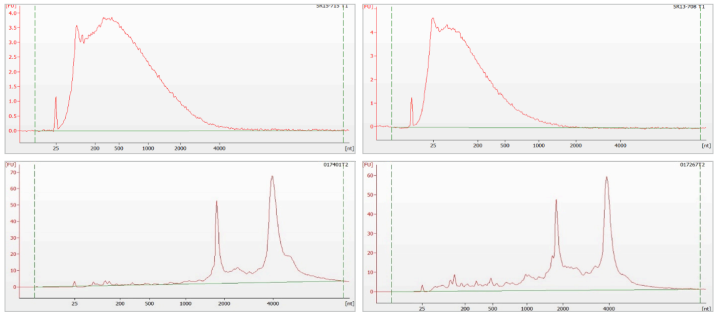

FIGURE 2 Examples of Bioanalyzer traces of FFPE (top) and fresh frozen (bottom) tumor specimens used as starting material in low RNA input kit. Fresh frozen RNA contains intact 28S and 18S rRNA peaks. FFPE RNA shows significant fragmentation.

100 ng RNA vs. Low RNA Input Hybridizations

Differential expression analysis relies on consistent performance of probes between replicate samples, independent of sample input amount. To demonstrate the utility of the Low RNA Input Kit in differential expression analysis, we compared expression profiles obtained from 100 ng RNA input to RNA processed with the Low RNA Input Kit using the same FFPE and FF starting material.

In two FF samples, a total of 1068 genes from combined analysis of the Hallmarks of Cancer Panels were detected above background in both 100 ng RNA and 1 ng MTE-processed sample hybridizations. Fold-change values of log2 counts are plotted in Figure 3. Fold-change values are highly correlated (R2>0.83).

In two FFPE samples, a total of 966 genes are detectable above background in both 100 ng RNA and 5 ng MTE sample hybridizations. As with the FF samples, the fold-change values are highly correlated at R2>0.88 (Figure 4).

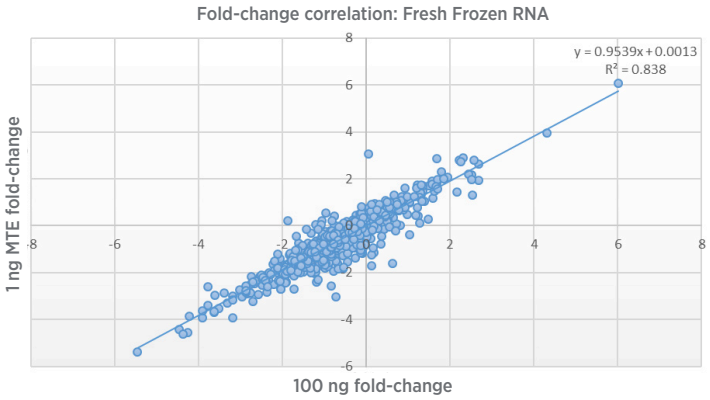

FIGURE 3 Scatter plot of fold-change values (log2) of 1068 genes between two FF samples hybridized with either 100 ng total RNA, or 1 ng MTE-processed sample.

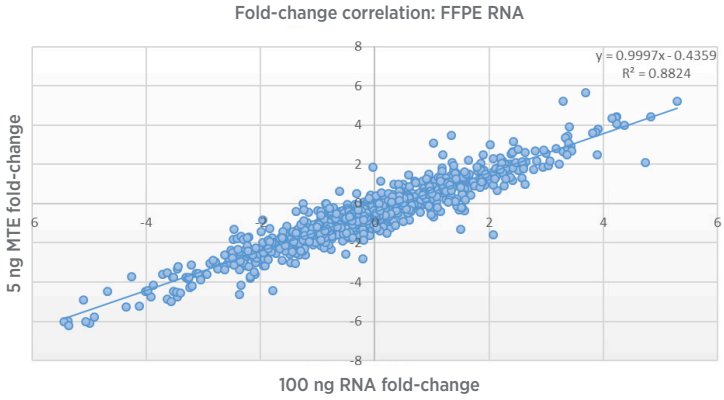

FIGURE 4 Scatter plot of fold-change values (log2) of 966 genes between FFPE samples hybridized using 100 ng total RNA and 5 ng RNA prepared with Low RNA Input Kit

Conclusions

The nCounter platform utilizes an enzyme-free, hybridization technology enabling accurate and sensitive analysis of challenging sample types such as FFPE, and is the gold standard for gene expression profiling in the field of cancer research. Here we introduce an alternative workflow for researchers looking to achieve the same accuracy and sensitivity of the NanoString assay but from less sample input. In this technical note, we demonstrate that by utilizing a reverse-transcription and pre-amplification step (MTE), up to 100-fold less RNA is sufficient to produce quality gene expression data that correlates highly to the standard 100 ng RNA input protocol while simultaneously increasing the sensitivity of detection of low expressing genes. Combining the nCounter Gene Expression Panels with the Low RNA Input kit provides a powerful new tool for researchers to maximize data from precious specimens.

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