nCounter® GEx

nCounter®
Gene Expression

Tech Note

Using the nCounter® Analysis System with FFPE Samples for Gene Expression Analysis

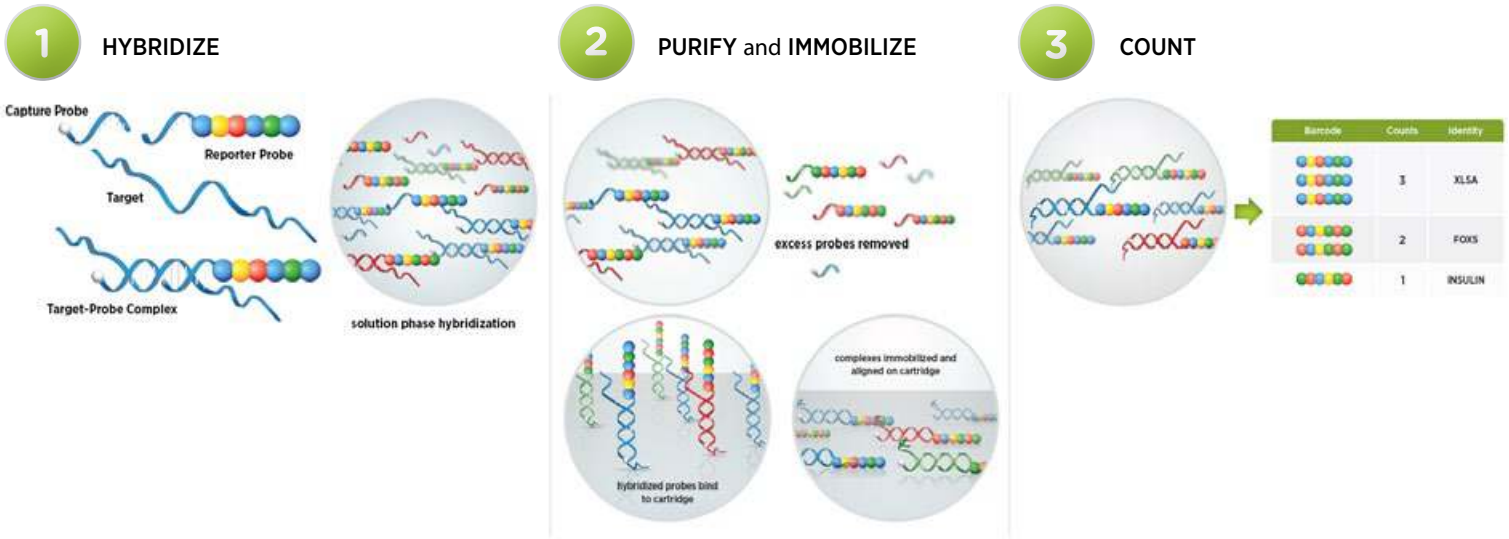
Introduction

For the past several decades, pathologists have kept samples obtained from tissue resections and biopsies. Typically, these are archived as Formalin-Fixed Paraffin Embedded (FFPE) blocks, and in some cases are sectioned and preserved on slides. These archived FFPE clinical samples are stable for years, and are valuable resources for subsequent investigations. Diagnosis, treatment, disease progression, and other clinical information are associated with many of these samples, making them extremely valuable for clinical research. Moreover, pathologists currently use FFPE blocks and slices as the preferred method to preserve samples from new patients. The ability of new molecular diagnostic tests to use FFPE samples as input material would greatly increase the usability of these tests. However, the fixation and embedding process modifies and degrades RNA, presenting challenges for gene expression studies using these samples. Other currently available gene expression profiling methods such as PCR-based techniques and microarrays have shown a significant decrease in the quality of results when using FFPE samples as input material.

In this technical note, we demonstrate that the nCounter® System accurately measures gene expression in RNA extracted from FFPE samples - even though RNA from FFPE samples is usually more degraded than RNA from other sources. Our results show high correlations of transcript levels between freshly prepared RNA and RNA extracted from matched FFPE samples across various tissues. We also show that the nCounter System can accurately measure transcript levels in crude extracts derived from FFPE samples, without RNA purification. Finally, we present guidelines for assessing RNA quality prior to running an nCounter analysis.

nCounter Analysis System Overview

The NanoString nCounter Analysis System delivers direct, multiplexed measurement of gene expression, providing digital readouts of the relative abundance of hundreds of mRNA transcripts simultaneously. The nCounter Analysis System is based on gene-specific probe pairs that are hybridized to the sample in solution. The protocol eliminates 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 six ordered fluorescent spots present on the reporter probe. The Reporter Probes on the surface of the cartridge are then counted and tabulated for each target molecule.



Methods

Frozen Tissue / Cryopreserved Sections / FFPE Sections: Matched human heart tissue samples preserved as flash-frozen tissue, cryopreserved sections, and FFPE sections were obtained from Biochain Institute Inc. Total RNA was purified from flash-frozen tissues using the QIAGEN® RNeasy® Kit according to the manufacturer's instructions. RNA was extracted from cryopreserved sections using the QIAGEN® Fibrous Tissue RNeasy® Mini-Kit according to the manufacturer's instructions. RNA was extracted from FFPE slices using the QIAGEN® RNeasy® FFPE Kit, according to the manufacturer's instructions. One hundred nanograms of RNA from each sample was hybridized in triplicate to a 96-plex human gene CodeSet and processed on the nCounter platform. The counts were averaged for each set of triplicates and normalized according to the standard protocol (Geiss et al., 2008).

Tumor / Normal; Frozen Tissue / FFPE Sections: Purified total RNA samples from frozen and FFPE biopsies of normal lung, lung tumor, normal liver, and liver tumor were provided by a customer. One hundred nanograms of RNA from each sample was hybridized to a 48-plex human gene CodeSet, processed on the nCounter platform, and normalized to beta-actin according to the standard protocol.

Crude FFPE Extracts: Crude FFPE extracts were prepared from heart and brain FFPE slices by deparaffinizing the slices and digesting with proteinase K. For comparison, total RNA was extracted from frozen heart and brain tissue and FFPE slices. The FFPE crude extracts and total RNA samples were hybrized to a 96-plex human gene CodeSet (different from that used for heart), processed on the nCounter platform, and normalized to positive controls according to the standard protocol.

RNA Degradation Studies: Controlled RNA degradation studies were conducted by incubating Universal Human Reference RNA (Stratagene®) and Human Brain Reference RNA (Ambion®) at 95°C for three minutes. One hundred nanograms of RNA from each sample was hybridized to a 96-plex human gene CodeSet, processed on the nCounter platform, and normalized according to the standard protocol.

RNA Quality: To assess RNA quality, all samples were run on an Agilent Bioanalyzer. The degree of RNA integrity was assessed using the smear analysis function in the Agilent 2100 Expert Software to measure the percentage of RNA molecules that were greater than 300bp.

Results

nCounter Analysis of FFPE Total Heart RNA

We obtained tissue samples from a single human heart preserved as flash-frozen pieces, cryropreserved slices, and FFPE slices. We first assessed the amount of degradation in RNA from each sample by smear analysis using the Agilent Bioanalyzer. In the RNA from fresh heart tissue, 94 percent of the transcripts were larger than 300bp and the 18s and 28s ribosomal RNA peaks were intact. The RNA from frozen heart sections also had intact ribosomal RNA peaks and 95 percent was greater than 300bp. In the RNA from the heart FFPE sections, only 64 percent was greater than 300bp. Also, the 18s and 28s ribosomal peaks were missing, indicating that RNA in the FFPE sample was degraded (FIGURE 2A).

Gene expression in total RNA from the frozen pieces of human heart tissue was compared to expression in RNA from cryopreserved samples and FFPE slices. The nCounter assay results for RNA from cryopreserved slices and FFPE samples were highly correlated with results for total RNA from flash-frozen tissue (FIGURE 2B).

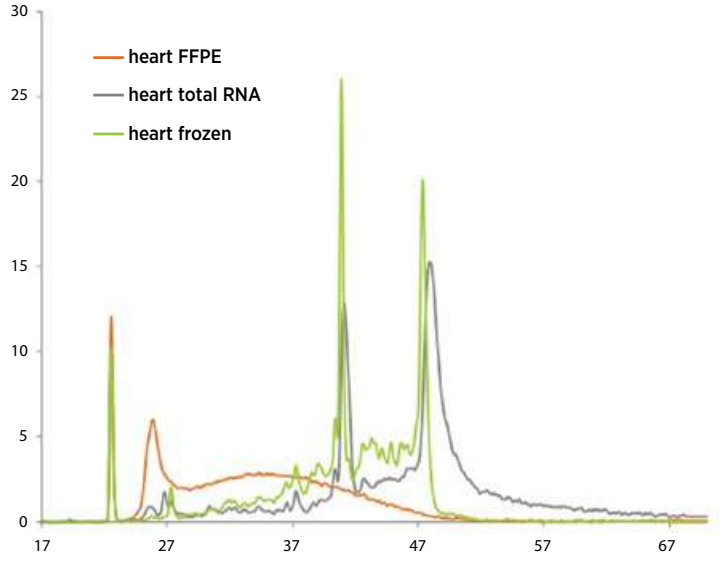


FIGURE 2A. Bioanalyzer analysis of RNA extracted from flash-frozen (grey line), cryropreserved (green line) and FFPE (orange line) archived human heart samples.

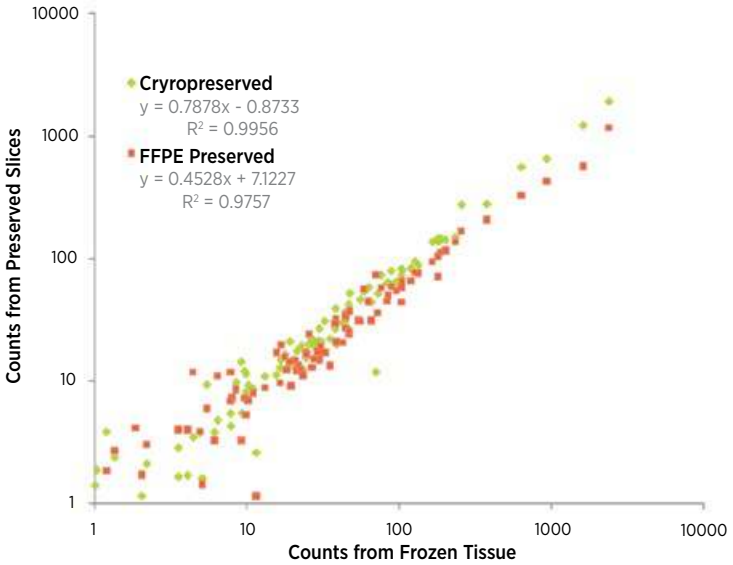


FIGURE 2B. Comparison of counts of 96 genes in 100ng of total heart RNA versus 100ng total RNA prepared from frozen heart slices (green points) and total RNA prepared from FFPE heart slices (orange points).

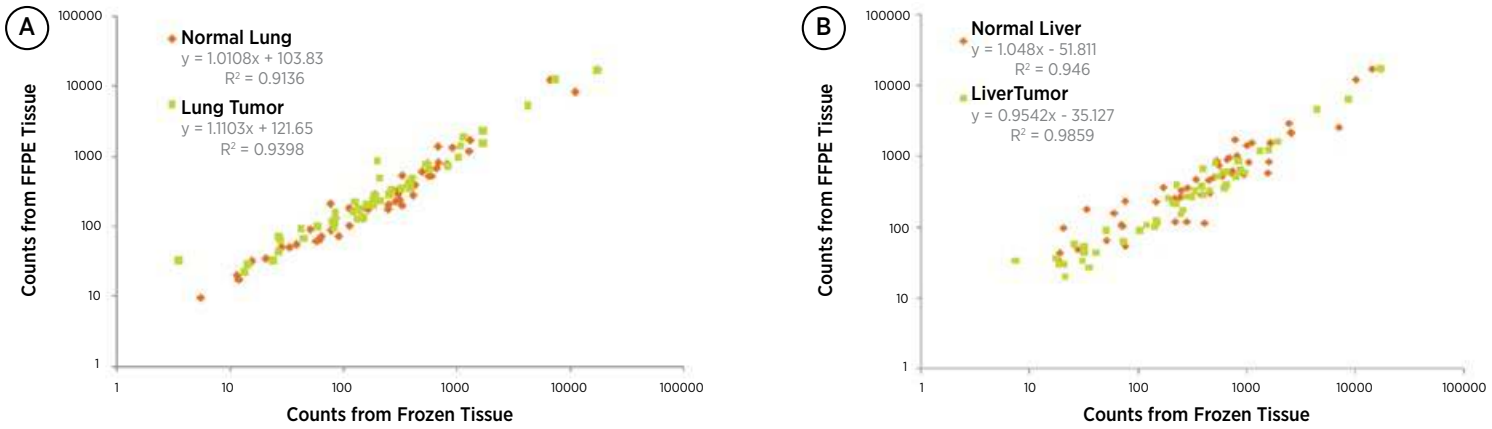


FIGURE 3. Correlations between counts for RNA samples extracted from fresh-frozen or FFPE tissues and hybridized to a 48-gene CodeSet. **A)** Counts for RNA from FFPE and fresh-frozen tissue for normal lung (orange points) and lung tumor (green points). **B)** Counts for RNA from FFPE and fresh-frozen tissue for normal liver (orange points) and liver tumor (green points).

When compared to total RNA, the expression levels of genes in the RNA from cryopreserved slices showed excellent correlation (R^2=0.995). RNA from the FFPE samples was also highly correlated to total RNA (R^2=0.976). However, there was an overall reduction in raw counts in the FFPE sample as compared with the frozen slices. This is illustrated by the reduced slope of the regression analysis for the FFPE sample and is likely due to its greater degree of RNA degradation. Since there is good correlation of expression among genes in the entire set, this reduction does not affect analyses of fold changes in expression levels. Also, the reduction in counts can be corrected by adding more input RNA (data not shown). The high correlations in counts and fold changes across these samples show that the nCounter System can accurately measure gene expression even when the target RNA is degraded.

nCounter Analysis of FFPE Total Lung and Liver RNA

We further analyzed the performance of FFPE-derived RNA on the nCounter System in collaboration with a customer. Purified total RNA samples from frozen and FFPE biopsies of normal lung, lung tumor, normal liver, and liver tumor were provided to NanoString. These samples were analyzed using a 48-gene CodeSet, normalized to beta-actin for RNA content, and the counts in the frozen- and FFPE-paired tissues were compared (FIGURE 3). In each case, the beta-actin-normalized counts were very comparable; the correlation coefficients between frozen and FFPE samples were all greater than 0.91. Because these samples were from different patients, some of the differences may also be due to biological variation. These results show that RNA from FFPE samples performs well on the nCounter system, despite the greater RNA degradation inherent in FFPE samples.

nCounter Analysis of Crude FFPE Extracts without RNA Purification

Because of the enzyme-free nature of the nCounter System, good results can be obtained with cell and tissue extracts and unpurified RNA (see **Sample Flexibility** technical note). This raised the possibility that the nCounter System could also accurately analyze FFPE samples without RNA purification. To verify this, we hybridized crude extracts from FFPE slices of human heart and brain in an nCounter assay with a different 96-gene CodeSet. For comparison, we also hybridized purified total heart and total brain RNA extracted from the frozen tissue and the FFPE slices.

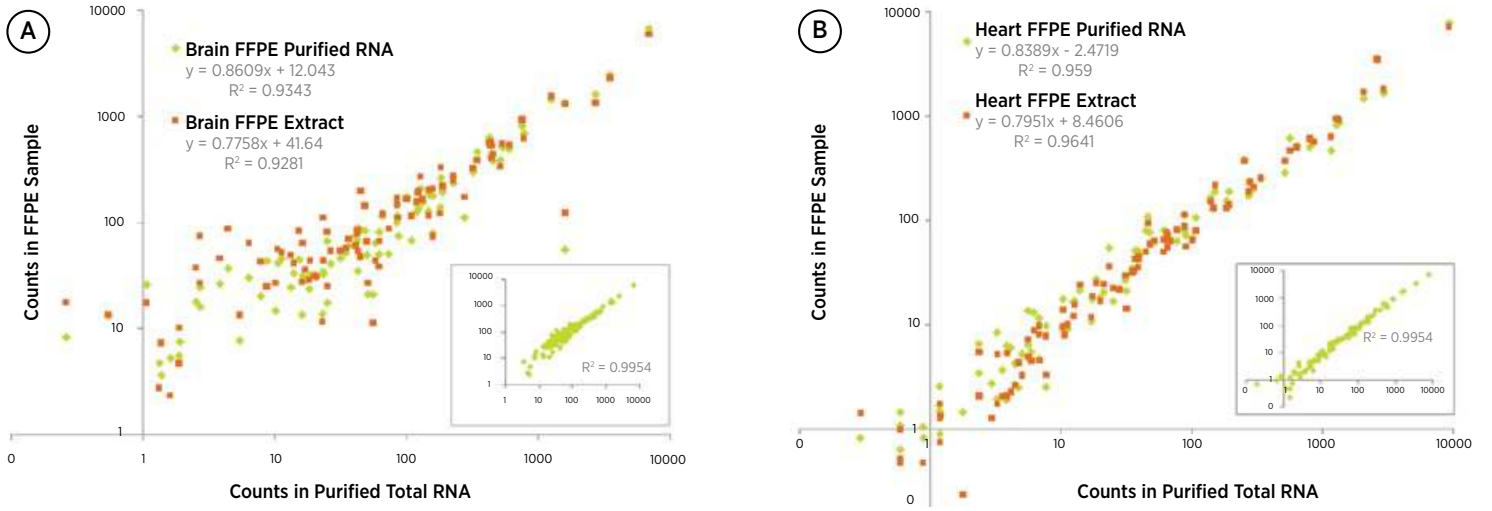


FIGURE 4. Correlation of counts from FFPE extracts to counts from purified RNA from FFPE slices and flash-frozen tissue. **A)** Correlation between brain FFPE extract (orange) and brain FFPE purified RNA (green) to purified RNA from frozen brain tissue. (A inset) Correlation between brain FFPE extract and purified RNA from brain FFPE tissue. **B)** Correlation between heart FFPE extract (orange) and heart FFPE purified RNA (green) to purifed RNA from frozen heart tissue. (B inset) Correlation between heart FFPE extract and purified RNA from heart FFPE tissue.

The nCounter System performed well when using FFPE extracts as starting material. The counts from FFPE extract were highly correlated to counts from RNA purified from FFPE slices and from flash-frozen tissue (FIGURE 4). In brain and heart, the correlation between the FFPE extract and RNA purified from FFPE was very high (R^2=0.99) (FIGURE 4A inset and 4B inset). The correlation between the brain FFPE extract and total RNA from frozen brain tissue was also high (R^2=0.93) (FIGURE 4A). In heart, the correlation between FFPE extract and total RNA from frozen heart tissue was again high (R^2=0.96) (FIGURE 4B).

Since the counts from FFPE extracts, RNA from FFPE, and total RNA from frozen tissue were all highly correlated, we expected the fold-changes derived from these counts to be highly correlated as well. We compared the ratios of transcript abundance in brain versus heart in the three sample preparation methods (FIGURE 4C). The correlation between fold changes for RNA from frozen tissue and RNA from FFPE tissue was very high (R^2=0.92); the correlation of fold changes for RNA from frozen tissue and RNA from FFPE extracts was slightly lower, but still good (R^2=0.86).

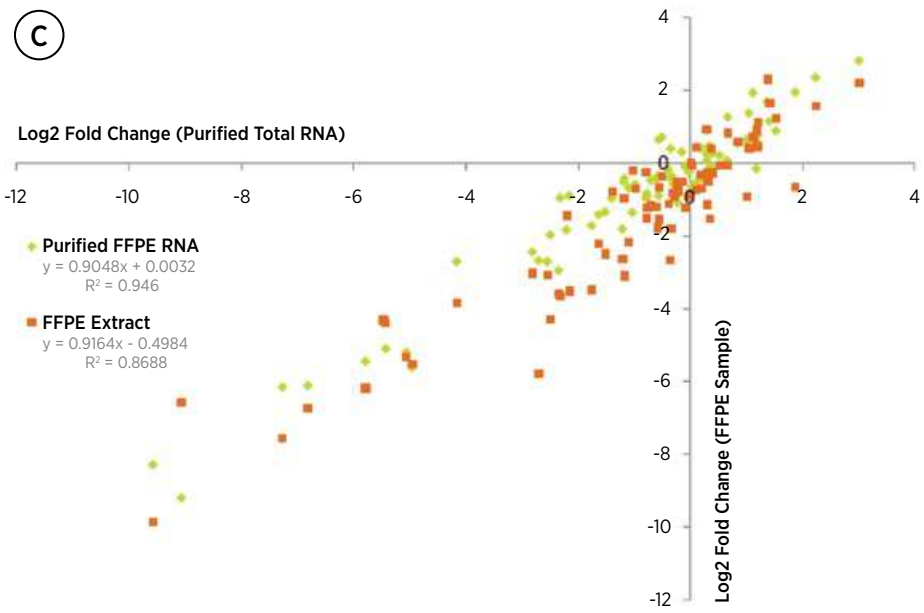


FIGURE 4. C) Fold changes of counts in heart versus brain. Fold changes for RNA purified from frozen heart and brain tissue are on the x-axis. Fold changes for FFPE extracts (orange points) or purified RNA from FFPE samples (green points) are on the y-axis.

These results suggest that crude FFPE extracts containing degraded RNA can be used to accurately quantify gene expression levels and fold changes on the nCounter platform. The results for FFPE extracts show strong correlation with intact and purified RNA samples.

Controlled RNA Degradation Study on the nCounter

Because RNA extracted from FFPE samples is usually more degraded than RNA from other sources, we conducted a controlled experiment to further characterize the effects of degraded RNA on the nCounter assay. We degraded the Universal Human Reference (UHR) and Human Brain Reference (BRN) by incubating at 95°C. After three minutes, RNA in the treated samples was substantially degraded. In the UHR sample, only 56 percent was greater than 300bp and in the BRN sample, only 57 percent was greater than 300bp. For comparison, the untreated UHR and BRN samples were intact, with 91 and 97 percent greater than 300bp, respectively.

We hybridized each of the samples with a 98-gene CodeSet, and analyzed the correlation between intact and fragmented samples for UHR and BRN. From the degraded UHR sample, we recovered approximately 72 percent of the counts of the intact UHR sample, and the counts were highly correlated (R^2 = 0.982). In the degraded BRN sample, we recovered approximately 62 percent of the counts of the intact BRN sample and the correlation was again very high (R^2=0.984)(FIGURE 5A).

Finally, we compared the fold changes for degraded UHR versus degraded BRN and intact UHR versus intact BRN (FIGURE 5B). Because the correlation of counts in the degraded and intact samples was high, we also measured excellent correlation between the fold changes of counts in the UHR and BRN samples (R^2=0.958). These experiments demonstrate that the nCounter system can accurately measure gene expression even in samples where the RNA is substantially fragmented.

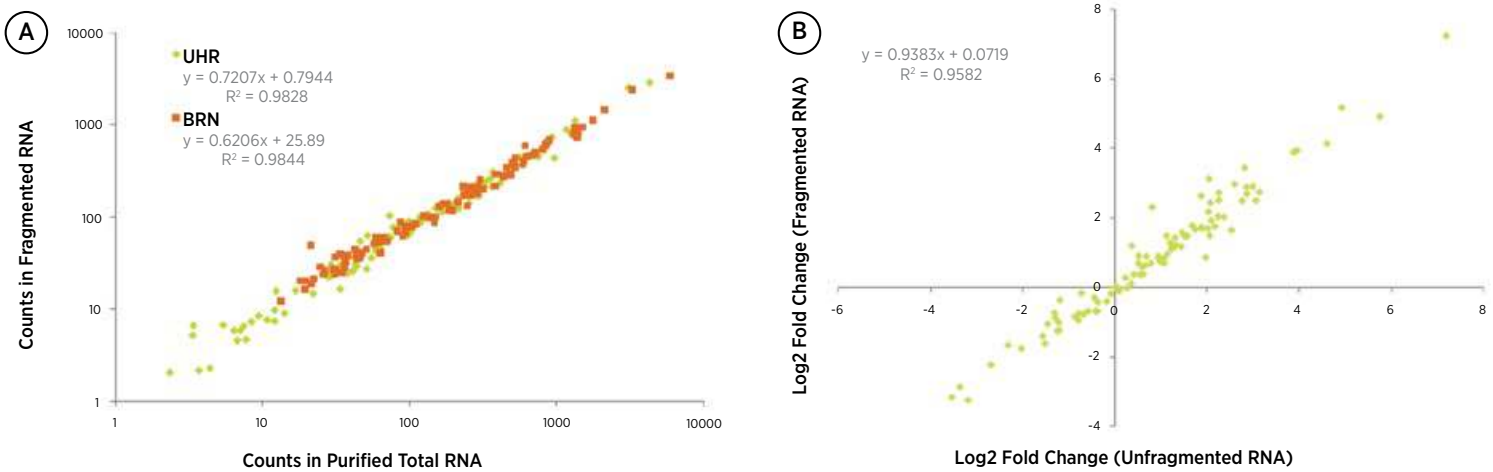


FIGURE 5. nCounter analysis of heat-fragmented Universal Reference RNA (UHR) and Human Brain (BRN) RNA using a 98-gene CodeSet. **A)** Correlation of counts between unfragmented and fragmented UHR sample (green points) and BRN sample (orange points). **B)** Fold change comparison between UHR and BRN for counts from fragmented and unfragmented samples.

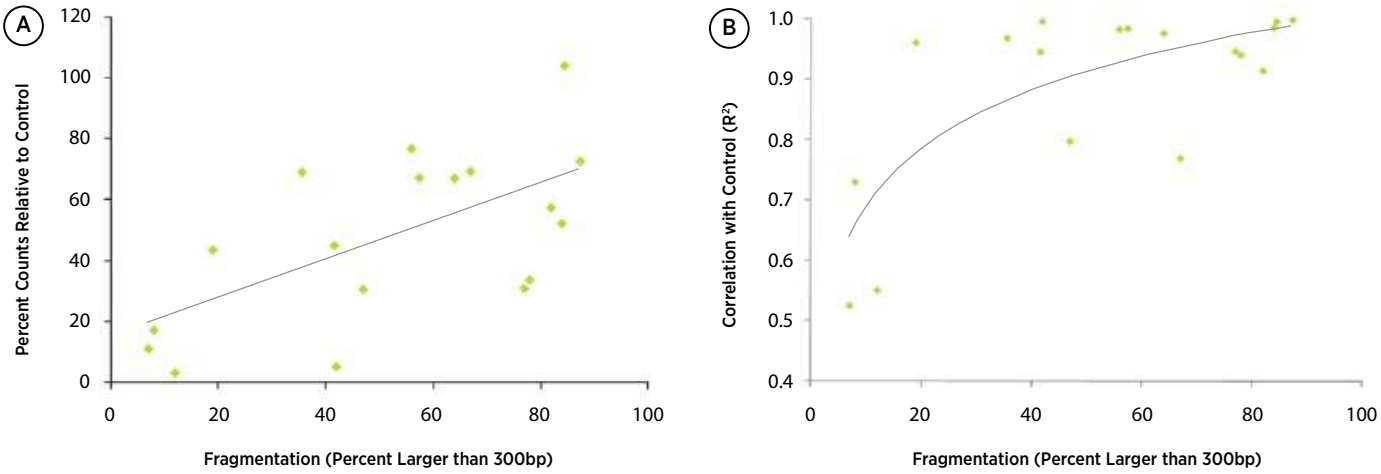


FIGURE 6. Meta-analysis of degree of RNA fragmentation and data quality in nCounter assays. **A)** Percent counts relative to intact RNA as a function of RNA fragmentation. **B)** Correlation between counts from degraded and intact RNA as a function of RNA fragmentation.

Correlating nCounter Assay Performance with RNA Degradation

Although the nCounter System generates data from fragmented RNA that correlates well with intact RNA, at some point the RNA becomes too degraded to produce good correlations. Therefore, we characterized the maximum amount of RNA degradation that still produces highly correlated data with intact RNA. We performed a meta-analysis of all our results using fragmented RNA, including some data described above and other data from degraded and FFPE-extracted samples not shown in this paper.

We first compared the percentage of counts recovered from degraded RNA relative to intact RNA to the percentage of transcripts greater than 300bp (FIGURE 6A). Although there is much variation, it is clear that as RNA becomes more degraded, fewer counts are recovered. We found that if more than 50 percent of the RNA is greater than 300bp, the correlation with intact RNA can be 0.9 or greater (FIGURE 6B). If less than 50 percent is greater than 300bp, good results can still be obtained, but the correlation with intact RNA may be lower. To obtain better results with such samples, it may be useful to increase the amount of RNA used in the hybridization. This would increase the number of fragments with intact target binding sites. Although these results are typical of what the nCounter System generates, results with different CodeSets and gene expression levels may vary from those presented here.

Conclusions

Gene expression analysis of FFPE samples is especially challenging because the RNA is usually more degraded than RNA from other sources. In this technical note, we demonstrated that the enzyme-free nature of the nCounter System enables accurate gene expression analysis of FFPE samples. On the nCounter System, expression data from RNA purified from FFPE samples is highly correlated with data from RNA from matched fresh samples. Furthermore, the nCounter System delivers accurate gene expression data directly from crude FFPE extracts, without requiring RNA purification. These capabilities make the nCounter System ideal for various types of clinical research studies and signature development, especially in cancer research.

Acknowledgements

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References

- Geiss GK et al., "Direct multiplexed measurement of gene expression with color-coded probe pairs". Nat Biotech 26(3):317- 325 (2008).
- NanoString Technologies, **"Sample Flexibility for Gene Expression Analysis with the nCounter™ Analysis System"**. (2009).

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