TECH NOTE

Analyzing FFPE Specimens with the nCounter® Copy Number Variation (CNV) Assay

Introduction

For decades, pathologists have preserved tissue samples as Formalin-Fixed, Paraffin-Embedded (FFPE) blocks. Archives of well characterized FFPE samples offer great potential for advancing biomedical research. However, the fixation and embedding process modifies and degrades nucleic acids, making genomic analysis challenging. Contemporary genomic methods such as PCR, microarrays, and NGS show a significant decrease in the quality of results when using FFPE samples.

The nCounter Analysis System from NanoString has gained a strong reputation for delivering high-quality data for gene expression and miRNA analysis. The nCounter system uses molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction and does not require any amplification steps that might introduce bias to the results. In addition, the relatively small amount of nucleotides (100 base pairs) used to hybridize barcodes to the target means that even challenging sample types like FFPE with degraded nucleic acids can be robustly profiled with nCounter technology. (for more information, please visit **www.nanostring.com**).

In this technical note, we summarize a protocol for analyzing FFPE specimens with the nCounter CNV Assay and describe the key factors that are critical to obtaining accurate results from DNA extracted from FFPE tissue. Experiments with matched frozen and FFPE tissue from tumors reveal excellent correlations across 85 cancer-related genes including some that are amplified more than 20 times (Figure 1). The nCounter CNV assay allows researchers to screen or validate copy number variations in FFPE samples quickly and accurately.

Multiple parameters were evaluated in order to optimize the protocol. Four were found to have a large impact on the accuracy of the assay: sample quality, number of probes per region, method of fragmentation, and reference sample selection. Given the importance of these parameters, we provide a brief outline of key criteria in the following sections.

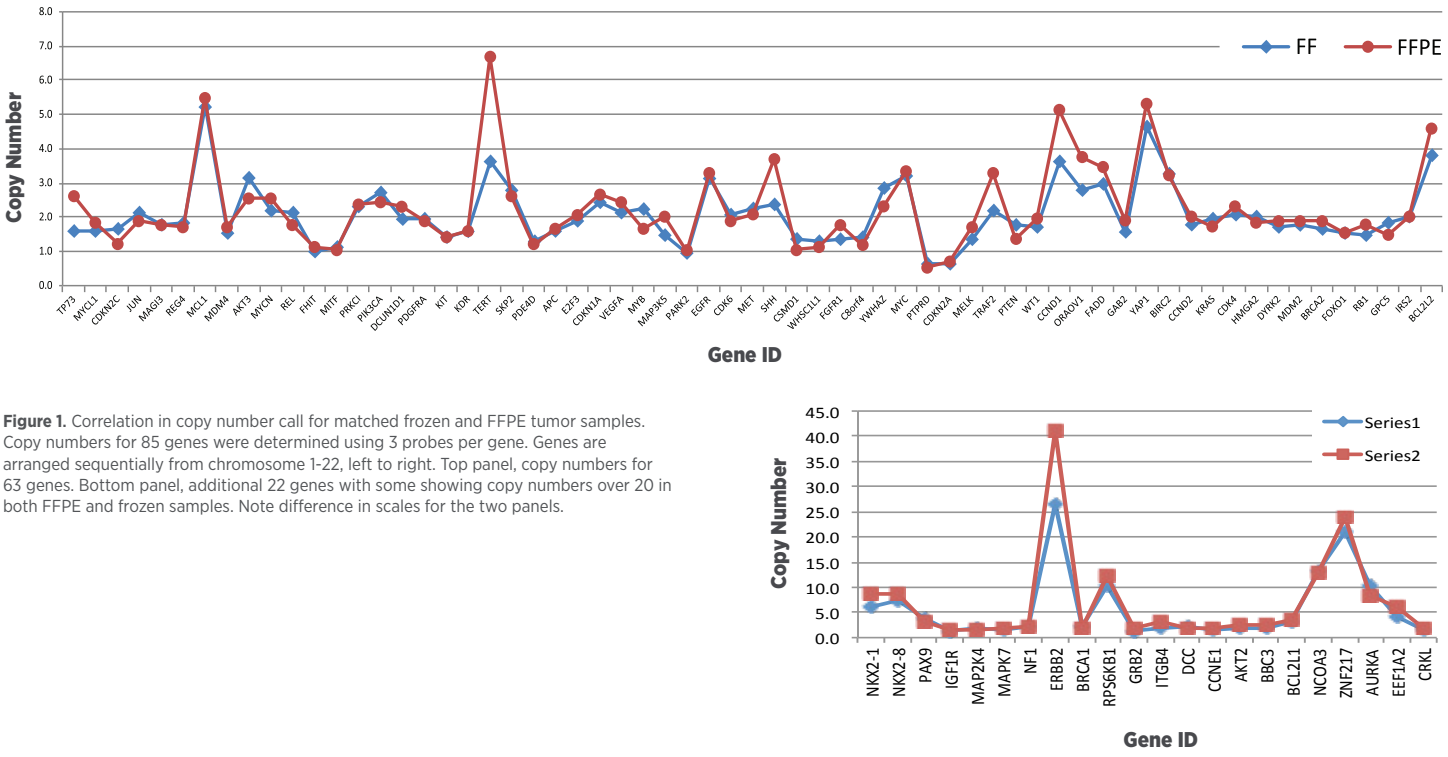

Figure 1. Correlation in copy number call for matched frozen and FFPE tumor samples. Copy numbers for 85 genes were determined using 3 probes per gene. Genes are arranged sequentially from chromosome 1-22, left to right. Top panel, copy numbers for 63 genes. Bottom panel, additional 22 genes with some showing copy numbers over 20 in both FFPE and frozen samples. Note difference in scales for the two panels.

FFPE Sample Quality

FFPE samples present a unique challenge to molecular-based assays. Formalin-fixation, paraffin-embedment, and long-term storage at room temperature leads to varying degrees of nucleic acid degradation. The variation in quality between FFPE samples can also be dramatic, with some samples exhibiting relatively low level degradation and others that are almost completely degraded. The amount of degradation can impact the quality of results.

The samples used in our optimization experiments were obtained commercially and represent a variety of tissue types and different vendors (Figure 2). In our accuracy analysis, we assumed that these normal samples were diploid for all 85 genes of interest, and thus should have a copy number of 2. The DNA from most of the 11 FFPE samples runs between 10kb and 1kb (Figure 2). However, 3 of the 11 samples exhibit a more extensive degradation profile (Samples 3, 6 and 7), with many DNA fragments being below 1kb in size. Not surprisingly, the accuracy rates for these three samples are lower than those of the other samples (Figure 3). Thus, it is important that the quality of the extracted FFPE DNA be taken into consideration when interpreting the results of the assay, as well as when selecting the number of probes per region and a copy number reference sample.

Sample Number	Sample Type	Tissue Type
1	FFPE-Normal	Kidney
2	FFPE-Normal	Spleen
3	FFPE-Normal	Small Intestine
4	FFPE-Normal	Colon
5	FFPE-Normal	Liver
6	FFPE-Normal	Colon
7	FFPE-Normal	Kidney
8	FFPE-Normal	Liver
9	FFPE-Normal	Lung
10	FFPE-Normal	Liver
11	FFPE-Normal	Colon

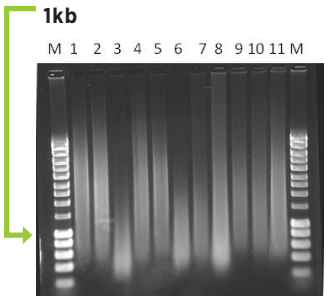

Figure 2. Samples used in optimization experiments and analysis of genomic DNA size from FFPE tissues. Eleven non-tumor FFPE samples were sourced commercially (left). DNA was extracted from three 10µm FFPE tissue sections using the QIAamp DNA FFPE Tissue Kit (Qiagen®). To assess DNA size, 150ng of extracted DNA was run on a 0.8% agarose gel (right). Hyperladder 1 was used as a marker. Samples were run in order from left-to-right according to order in the table.

Methods of Fragmentation

The nCounter CNV assay requires that the genomic DNA be fragmented and denatured prior to running the assay. Our standard procedure involves fragmenting via digestion with the restriction endonuclease AluI. While this method has many advantages (Table 1), one disadvantage for FFPE samples is that the fragment length after digestion varies depending on the initial degradation state of the sample. Since the nCounter technology is measuring DNA targets directly it is important that the the size of each target is approximately the same in all samples tested. For example, smaller fragments will exhibit faster hybridization kinetics and may result in more counts even if the number of molecules is the same. An alternative to AluI is to use a random fragmentation method, such as the Covaris® AFA technology. The accuracy rates for the 11 FFPE samples processed by both fragmentation methods are shown in Figure 3. There is a small but measureable increase in accuracy in the majority of samples when using the Covaris method.

Parameter	Covaris AHA	AluI Digestion
Availability/Cost	Covaris Sonicator Required	Simple Restriction Digestion
Predictability of fragment length in degraded samples	Predictable	Less Predictable
Direct addition to nCounter Hyb	No – Sample must be concentrated after	Yes
Average Percent Accuracy for nCounter CNV Analysis (3 probes)	95%	93%

Table 1. Comparison of recommended fragmentation methods

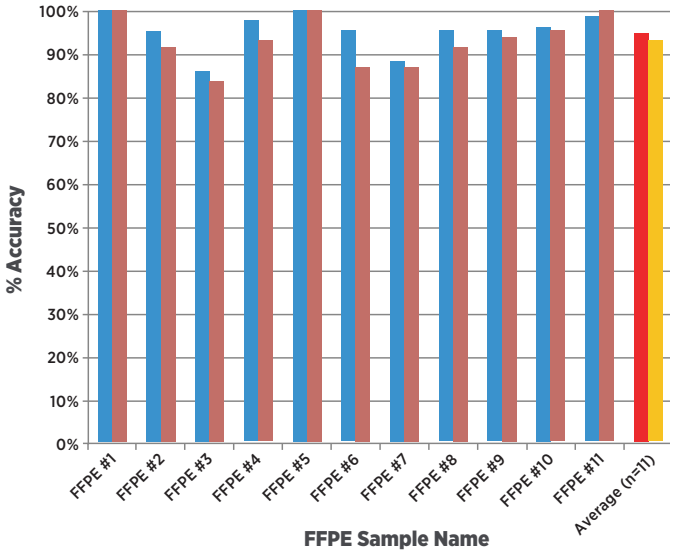

Figure 3. Accuracy rates for all 11 FFPE samples using AluI and Covaris fragmentation. Copy numbers were determined based on 3 probes per gene, using the average signal for the 11 FFPE samples as the reference. Blue bars, Covaris fragmentation; red bars, AluI fragmentation. The average accuracy for all 11 samples is shown in red and orange for Covaris and AluI respectively.

Number of Probes per Region

The number of probes per region can impact the accuracy of the CNV assay in FFPE tissue. Data from the 11 FFPE samples shown in Figure 1 show an increase in accuracy when the number of probes per region is increased (Figure 4). The improvement in accuracy is most pronounced on poor quality FFPE samples. In situations where specimens are known to be of low or variable quality, a higher number of probes per region is recommended.

Reference Sample Selection and Considerations

The nCounter CNV assay calculates copy number gains and losses relative to a reference sample or set of samples. The ideal reference samples are normal (*i.e.*, diploid) and closely match the sample type of the test samples (fresh, frozen, FFPE, cell line, etc.). For example, if the test samples are genomic DNA from non-FFPE tissues (*e.g.*, frozen or fresh tissue) then the reference DNA sample should also be from fresh or frozen tissue. Genomic DNA from cell lines can also be used as the reference sample in this situation; however, the genetic instability of some cell lines can have an impact on copy number calls if all chromosomal regions are no longer diploid. Similarly, the best reference sample(s) for FFPE tissues is DNA from normal, diploid FFPE tissue.

When using a reference type that is not matched to the experimental sample type, the method of fragmentation plays a larger role in the accuracy of the assay. Figure 5 shows the accuracy of the 11 FFPE samples using different reference controls. For example, the average accuracy of the 11 FFPE samples is 89% with Covaris and 75% with AluI when using genomic DNA from a cell line as the reference, whereas the accuracy using the average of all 11 FFPE samples as the reference results in 95% accuracy rate with Covaris and 93% accuracy rate with AluI.

Another important aspect of reference sample selection for FFPE samples is the quality of the genomic DNA from the reference sample(s). FFPE reference samples with poor DNA size distributions prior to fragmentation result in lower accuracy rates for all samples when compared to accuracy rates using a reference with good DNA size distributions (Figure 5). For example, if one chooses FFPE sample 3 as the reference, the accuracy in copy number calls for the remaining 10 samples is below 80% regardless of fragmentation method. In contrast, if FFPE sample 4 is used as the reference, accuracy increases to 89% and 85% for Covaris and AluI respectively. However, the best reference results from a pool of samples (see FFPE#1-11, FFPE#1-6 and FFPE#7-11, Figure 5). It can be difficult in practice to match the quality of FFPE samples across a project, and the use of the average of multiple samples generates a reference that is representative of a broad range of sample quality.

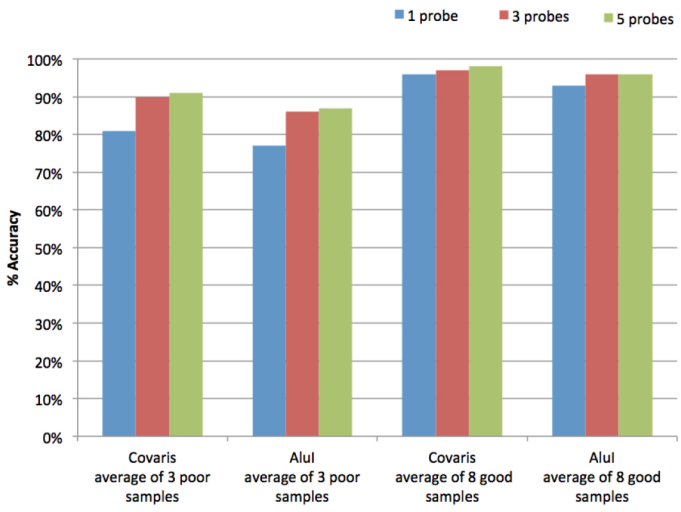

Figure 4. Average accuracy of copy number for 85 genes in normal FFPE tissue. Copy numbers for the 11 FFPE samples in Figure 1 were determined relative to the average signal for all 11 FFPE samples. For the purposes of this analysis, samples 3, 6, and 7 were considered "poor", and samples 1-2, 4-5, and 8-11 were considered "good" based on the length of the DNA (< 1kb) prior to fragmentation, Two different methods of DNA fragmentation are shown (see above for more detail).

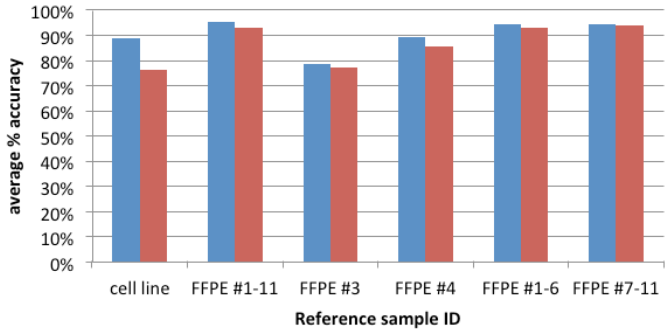

Figure 5. Comparison of average copy number accuracy using different samples as a reference, either individually or averaged as a group. The copy number accuracy is based on 3 probes per gene. Samples were processed using either Covaris-AFA technology (blue bars) or AluI restriction digest (red bars). Using a group of samples as the reference results in more accurate results.

Conclusions

The nCounter CNV Assay can be utilized for analysis of FFPE specimens and > 90% accuracy can be expected when appropriate experimental design considerations are accounted for. FFPE sample quality has a large impact on data quality, but even poor quality samples are expected to generate reasonable quality data with the right combination of fragmentation method, number of probes per region, and reference sample selection. Random fragmentation with Covaris AHA technology or AluI restriction digest may be used for fragmentation of FFPE samples. Designing a custom CNV codeset with multiple probes per region enables high quality data to be generated from FFPE samples with a broad range of sample quality. Selecting multiple diploid FFPE reference samples is the best way to ensure accurate results from FFPE samples of varying quality. For more information on how to design an nCounter CNV study for FFPE samples, please contact **support@nanostring.com**.

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