TECHNICAL REPORT

Comparison of Matched Lung FFPE and Fresh Frozen Expression Profiles Using the WT-Ovation® FFPE System

Introduction

Generation of expression signatures from FFPE-derived RNA has been historically challenging due to the extreme conditions involved in specimen handling and the tissue fixation process, which typically result in cross-linking and degradation of nucleic acids isolated from these tissues. The NuGEN WT-Ovation FFPE System overcomes these challenges by allowing researchers to amplify from limited amounts of significantly degraded RNA. This system addresses the technical gap that has persisted for global gene expression analysis of these valuable sample sources, enabling researchers to take advantage of large FFPE archived sample sets and their associated clinical data for research studies.

An important consideration regarding the value of gene expression data generated from compromised FFPE RNA is whether these results yield informative biological information. In order to address this question, NuGEN has compared expression results obtained from FFPE-derived RNA to RNA collected from matched fresh frozen samples. This Technical Report describes analysis of RNA samples extracted from human lung tumor and normal adjacent tissue (NAT), from both FFPE and matched fresh frozen samples.

Materials and Methods

RNA Samples

Fresh frozen and FFPE total tumor and matched normal adjacent tissue RNA were isolated using either Formapure™ Kits (Agencourt, Cat. # A33341), or the Qiagen RNeasy Mini Kit (QIAGEN, Cat. # 74106) according to manufacturers' instructions.

TABLE 1. **Affymetrix HG-U133A 2.0 array performance metrics.**

Sample Type	N	Avg. SF (sd)	Avg. Bkgd (sd)	Avg. %P (sd)
Frozen Normal	3	4.2 (0.6)	30.1 (0.6)	66.2 (1.1)
Frozen Tumor	3	4.9 (1)	30.7 (1)	62.6 (2.8)
FFPE Normal	4	12.2 (0.6)	29.6 (0.7)	49.9 (1.3)
FFPE Tumor	4	13 (0.6)	29.5 (0.5)	48.0 (1)

The number of arrays (N), Scaling Factor (SF), Background (Bkgd), and Percent of Present calls (%P) are listed, with standard deviation (sd) in parentheses for each value.

Amplification and Array Hybridization

The WT-Ovation FFPE System was used for all amplifications (note: the WT-Ovation FFPE System has been replaced by the Ovation FFPE WTA System, Part No. 3403), according to manufacturer's instructions. Starting total RNA amounts were 10 ng for fresh frozen samples and 50 ng for RNA isolated from FFPE samples. Target preparation for gene expression analysis using GeneChip® arrays was performed using 5 µg of the amplified cDNA as input for the Encore Biotin Module (Part No. 4200), as described in the user guide. The fragmented and biotin labeled cDNA targets were hybridized to HG-U133A 2.0 Affymetrix GeneChip arrays, stained with streptavidin-phycoerythrin with antibody amplification, and scanned following manufacturers' protocols. Array data were analyzed using Expression Console (Affymetrix), BioConductor 1.9 package and Partek Genomics Suite. Pathway and functional analysis was performed using Ingenuity Pathway Analysis software.

Data analysis

Three individual technical replicates were analyzed for frozen tissues and 4 replicates were analyzed for FFPE tissues. RMA intensity values and MAS5 PMA calls for GeneChip arrays were obtained and p-values associated with the pairwise tumor-normal Student t-test were determined in Bioconductor 1.9.

Probe sets with at least 3 out of 4 or 2 out of 3 present calls were included in the present call overlap analysis. Genes were called differentially expressed between tumor and normal in both the FFPE and frozen tissues if they met the following criteria: 2X or greater RMA expression value difference and p-values lower than 0.001 from a pair wise t-test. Differentially expressed genes (see legend for **Figure 2**) were uploaded into Ingenuity Pathway Analysis.

Results

Array performance metrics for samples extracted from FFPE tissues are shown in **Table 1**. General sensitivity can be assessed using the percent of probe

sets called present for each sample. Although the number of present calls for FFPE samples is moderately lower than for their fresh frozen counterparts, the amplification assay performs well, approaching the 50% present mark, demonstrating the ability to detect a large number of transcripts across a wide range of abundances. Array reproducibility metrics can be found in **Table 2**.

Most probe sets called present in the FFPE samples are also detected in their fresh frozen counterparts, however, there are significantly more probe sets present in fresh frozen samples. This is not unexpected considering the degree of RNA degradation in the FFPE samples and the greater percentage of present calls in the fresh frozen subset. (**Figure 1**).

Relatively few transcripts are unique to FFPE samples, indicating that differential degradation is not contributing significantly to the overall expression pattern.

A more rigorous differential expression analysis is presented in **Figure 2**. The Venn diagram represents transcripts that were significantly differentially expressed between tumor and normal tissues. Over 50% of transcripts called differentially expressed in the fresh frozen sample were also detected as differentially expressed in the FFPE samples. Stringent selection criteria were used, with p-value cutoff of <0.001 and 2-fold differential expression, so this level of concordance among differentially expressed genes detected in quadruplicate amplifications of the normal and tumor samples is very high.

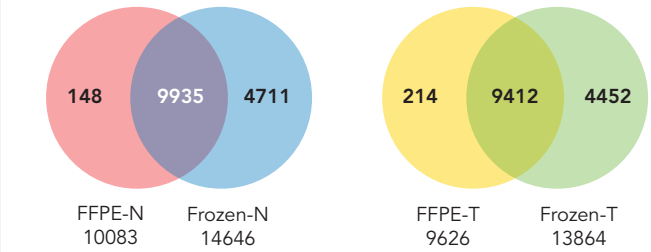
In order to understand this concordance in terms of functional expression, Ingenuity Pathway Analysis was run on the set of these differentially expressed genes. Paired differentially expressed pools of genes: FFPE T>N and FFPE N>T, as well as fresh frozen N>T and fresh frozen T>N were subjected to independent pathway

TABLE 2. **Affymetrix HG-U133A 2.0 array average Pearson correlation matrix.**

Sample Type	Frozen N	Frozen T	FFPE N	FFPE T
Frozen N	0.99			
Frozen T	0.85	0.97		
FFPE N	0.80	0.69	0.99	
FFPE T	0.77	0.82	0.89	0.99

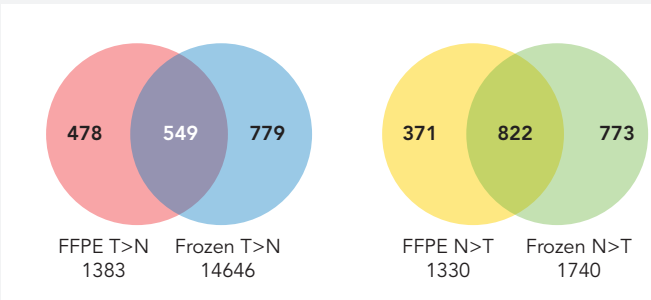
The correlation matrix demostrates the high level of reproducibility between technical replicates.

FIGURE 1. **Venn diagram of concordance of present calls between FFPE and frozen samples for both tumor (T) and normal (N) sample sets.**



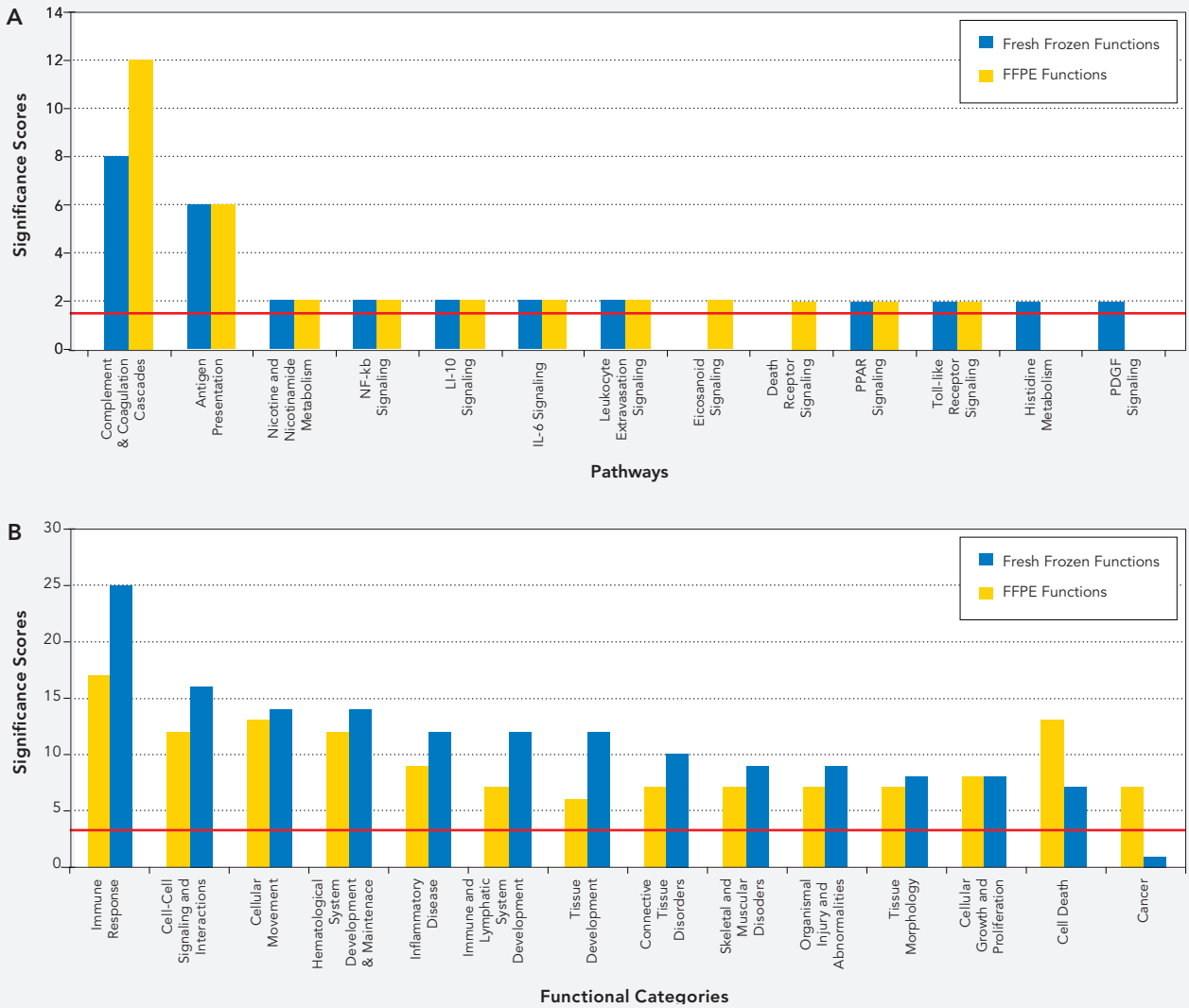
Total number of present calls is on the bottom, the middle number in the circle overlap corresponds to concordant calls, and present calls unique to each subgroup are in the circles above its respective name.

FIGURE 2. **Venn diagram of concordance of differentially expressed genes between FFPE and frozen samples.**



Genes are called differentially expressed on Affymetrix HG-U133A 2.0 between tumor and normal in the FFPE and frozen samples if they meet the following criteria: 2x or greater RMA expression value difference and p-values lower than 0.001 from a pair wise t-test. There were only 4 "false positives" in the FFPE comparisons (1 in T>N, 3 in N>T).

FIGURE 3. **Ingenuity pathway analysis of differentially regulated canonical pathways (A) and cellular functions (B) in tumor and normal tissues in frozen and FFPE datasets.**



Pathways and cellular functions are listed along the X-axis. The Y-axis represents the –log(significance score), which is defined by Ingenuity and depends on the number of genes (components) involved in the pathway/process and their relative weight in the process. The red line represents the significance threshold. Nine of eleven canonical pathways represented in fresh frozen samples were detected in the FFPE samples.

analyses. Canonical pathway analysis identified 11 pathways in fresh frozen as statistically represented in the differentially expressed transcripts. In FFPE samples, 9 of the 11 pathways were also detected at a similar statistical confidence. Two pathways were identified uniquely in the FFPE samples at a fairly low, but statistically relevant confidence, shown in **Figure 3A**.

Common biological process analysis, which is a much broader way of grouping genes by their annotations, also identified 13 out of 14 cellular processes upregulated in normal tissue versus tumor tissue, in both the fresh frozen and FFPE data. Only one functional group was detected uniquely in the FFPE samples, shown in **Figure 3B.**

Conclusions

The WT-Ovation FFPE System, in conjunction with the Encore Biotin Module, enables utilization of significantly degraded RNA isolated from

FFPE tissue sources for global gene expression profiling and microarray analysis. This technical note explores the value of the gene expression information that can be generated from FFPE samples as compared to using high quality fresh frozen RNA sources. Despite observing the expected loss in sensitivity when using FFPE RNA, the differential expression results generated with FFPE and fresh frozen samples are highly concordant. There is a high degree of similarity in:

- Transcripts detected on the arrays
- Transcripts identified as differentially regulated
- The biology (pathways and functions) identified when comparing the FFPE and the fresh frozen results.

Given the number of archived FFPE tissue samples available for retrospective and prospective studies, and the value these samples bring to linking gene expression profiles to clinical outcomes, advances in the ability of researchers to perform expression profiling on these particularly challenging RNA sources are extremely valuable.

The WT-Ovation FFPE System is one such advance that promises to accelerate the development and validation of clinically relevant gene expression signatures.

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