TECHNICAL REPORT

Microarray Performance Comparison of the NuGEN® Ovation® FFPE WTA System and the Genisphere® Sensation™ RNA Amplification Kit

Introduction

Formalin-fixed, paraffin embedded (FFPE) tumor tissue is a valuable source of material for retrospective and prospective studies of cancer genomes and other clinical samples. Nucleic acids extracted from FFPE tissues, however, are typically severely degraded and of poor quality. As a leader in sample preparation technologies, NuGEN provides solutions to enable gene expression profiling studies of FFPE tissues using a wide range of technologies such as microarrays, qPCR and next generation sequencing.

This technical report compares two technologies for RNA amplification and target preparation for highly degraded RNA from FFPE tissue sources for global microarray gene expression studies — the NuGEN Ovation FFPE WTA System and the Genisphere Sensation RNA Amplification Kit.

Technology Comparison

The NuGEN Ovation FFPE WTA System (Part No. 3403) uses a whole transcriptome amplification approach based on SPIA® technology. The System requires as little as 50 ng of FFPE-derived total RNA as starting material to generate double-stranded cDNA (ds-cDNA) in just five hours. The ds-cDNA can be archived, analyzed by qPCR or hybridized to microarrays of both sense and antisense orientation from different vendors. Prior to microarray hybridization, ds-cDNA must be fragmented and labeled using the NuGEN Encore® Biotin Module appropriate to the array.

The Genisphere Sensation RNA Amplification Kit uses a whole transcriptome amplification approach based on *in vitro* transcription (IVT) using T7 RNA polymerase. The kit requires 50–200 ng of total RNA and the protocol includes an overnight IVT incubation step to generate sense RNA. The resulting RNA can be used for qPCR or microarray analyses after fragmentation and labeling steps. Fragmentation and labeling are specific to Affymetrix® expression arrays and may add up to 5.5 hours to the protocol time, depending on the type of array.

Comparison Study Goals

Our studies were designed to address the value of gene expression information generated from FFPE samples by answering the following questions:

- Will a comparison of cancer tissue types yield a tissue-specific set of genes?
- Do these tissue-specific gene lists overlap between assays?
- Do the non-overlapping genes add relevant biological information?
- Which assay appears more sensitive?

Materials and Methods

For this study, scientists from an independent laboratory used RNA samples (RIN scores of 2–3) from four distinct FFPE tissue types — human spleen, lung, liver and lymph node — for amplification with either NuGEN's Ovation FFPE WTA System or Genisphere's Sensation RNA Amplification Kit according to the manufacturer's instructions. Starting total RNA amounts for all tissues were 50 ng and 100 ng and amplifications were performed in triplicate. The amplified products were fragmented and labeled then hybridized to Affymetrix HG-U219

FIGURE 1. **Principal components analysis using all genes on the array.**

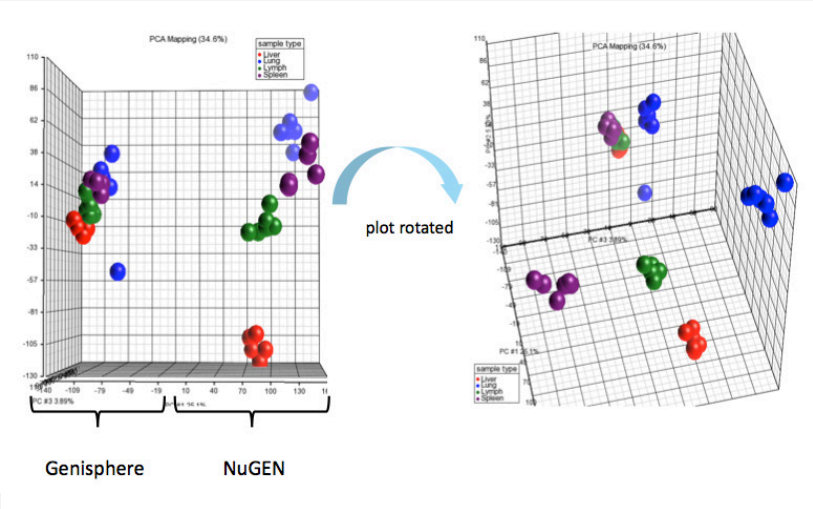

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plate arrays, according to the manufacturer's protocol.

Array data were acquired by the Affymetrix GeneTitan® System running on default settings. Data were then analyzed using Partek Genomics Suite (Partek, Inc., St. Louis, USA). We determined significantly changed genes between study groups using 1-way ANOVA ($p < 0.01$) and performed functional annotation analysis of significantly changed genes using Genomatix Software Suite v2.5 (Genomatix GmbH, Munich, Germany).

Results

An unsupervised Principal Component Analysis (PCA) using all genes was performed as shown in **Figure 1**. The NuGEN data clearly segregate into four distinct clusters representing the four tissue types, whereas the Genisphere data show much lower cluster resolution. These data indicate superior sensitivity towards tissue-specific gene expression signatures in data sets generated with NuGEN's Ovation FFPE WTA System.

For each assay type, we used a 1-way ANOVA to determine significantly changed genes between an individual tissue and a pool of the remaining three tissues. The resulting gene sets were then tested for concordance and biological plausibility based on functional annotation. Both the overlapping genes and those found specific to a single assay are summarized in **Table 1** and **Table 2**.

TABLE 1. **Comparison analysis of an individual FFPE tissue versus the pool of the other three FFPE tissue types as determined by 1-way ANOVA at a p-value threshold < 0.01.**

Comparison	# Genes Genisphere	# Genes NuGEN	# Genes Overlap	CC	# Discordant	UniGene Rank 1	UniGene Rank 2	UniGene Rank 3
Liver_Pool	2901	4379	1024	0.913	18	Liver	Liver & biliary system	Gall bladder
Lung_Pool	2836	2972	923	0.659	4	Arterial system	Aorta	Artery
Lymph_Pool	957	1049	113	0.831	3	Haemolymphoid system	Spleen	Dorsal root ganglion
Spleen_Pool	1570	2180	294	0.648	6	Spleen	Adipose tissue	Dorsal root ganglion

This table lists the total number of genes identified in the Genisphere or the NuGEN data set, the overlapping genes, the Pearson correlation coefficient (CC) and the number of discordancies.

TABLE 2. **Comparison analysis of an individual FFPE tissue versus the pool of the other three FFPE tissue types, determined by 1-way ANOVA at a p-value threshold < 0.01.**

Comparison	# Genes Genisphere Only	CC with NuGEN	# Discordant	UniGene Rank 1	UniGene Rank 2	UniGene Rank 3	# Genes NuGEN Only	CC with Genisphere	# Discordant	UniGene Rank 1	UniGene Rank 2	UniGene Rank 3
Liver_Pool	1877	0.528	540	Integumental system	Gland	Mammary gland	3355	0.437	1056	Liver	Liver & biliary system	Muscle
Lung_Pool	1913	0.568	299	Skeleton	Bone	Connective tissue	2049	0.407	569	Respiratory system	Lung	Cardiovascular system
Lymph_Pool	844	0.472	298	Hippocampus	Hippocampal region	Cerebral cortex	936	0.371	312	Leukocyte	Nervous system	Haemolymphoid system
Spleen_Pool	1276	0.448	416	Renal/urinary system	Lung	Placenta	1886	0.437	609	Gland	Mammary gland	Integumental system

This table lists genes solely identified in the Genisphere or the NuGEN data set, Pearson correlation coefficient (CC) and the number of discordancies. It also shows UniGene annotation of the top three ranks according to the tissue type category.

FIGURE 2. **Genes significantly changed in liver versus the pool of the other three FFPE tissue types as determined by 1-way ANOVA at a p-value threshold < 0.01.**

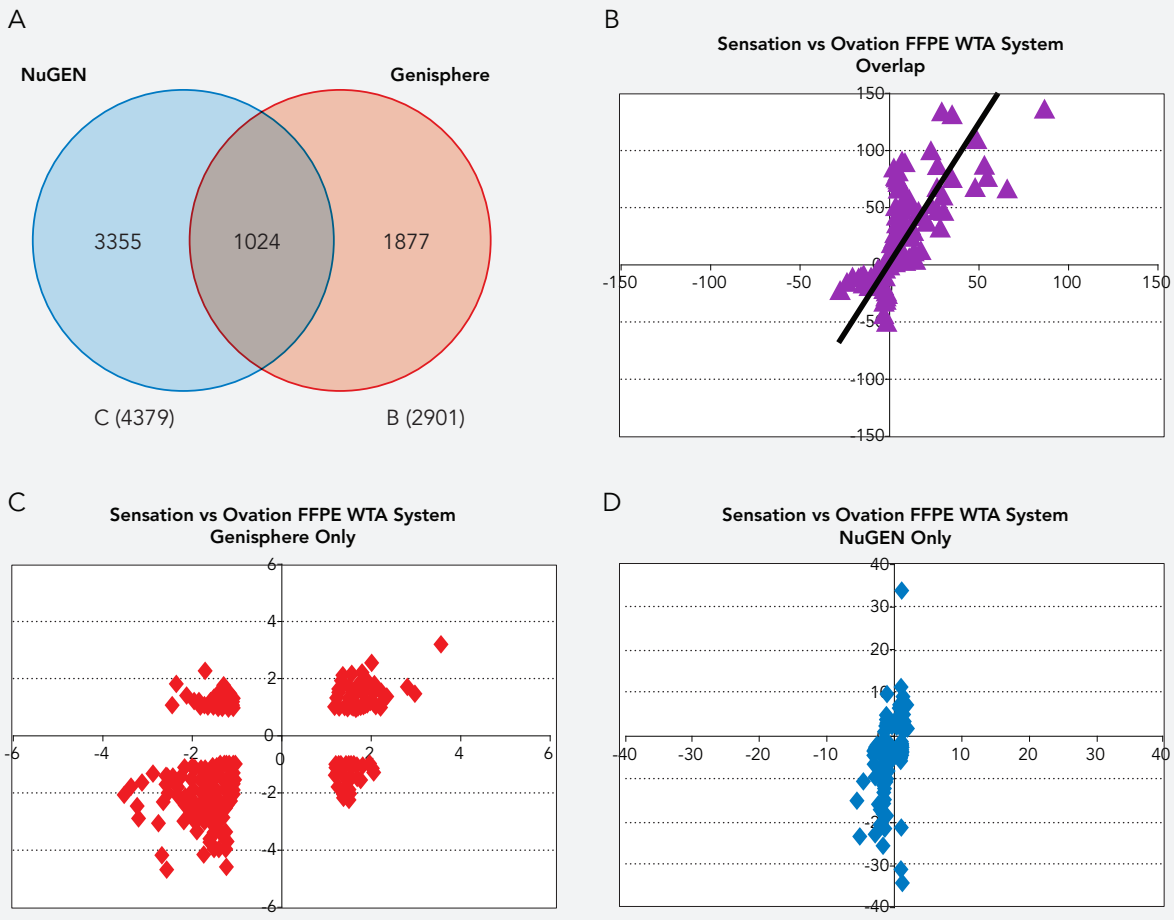

Figure 2 shows data typical of this study and analysis. In these figures, liver tissue was compared to the pool of lung, lymph and spleen samples. The analysis identified a total of 4,379 significantly changed genes in the NuGEN data set, in contrast to 2,901 genes found in the Genisphere data set. Additionally, it identified 1,024 overlapping genes (**Figure 2A**), displaying good concordance with a Pearson correlation coefficient of 0.913.

Of these overlapping genes, only 18 were found discordant between the data sets, indicating that both assays, at a first glance, detect the same robustly changed genes. In **Figure 2B**, corresponding fold changes are displayed in a scatter plot, with Genisphere data on the x-axis and NuGEN data on the y-axis. The linear regression line has a slope of 2.08 indicating a significantly compressed dynamic range in the Genisphere data.

To elucidate whether these genes have a meaningful biological context, we performed an annotation analysis based on the UniGene and the MeSH (Medical Subject Headings) databases. The three most significant annotations found through this analysis appear specific to liver tissue (rank 1: liver; rank 2: liver and biliary system; rank 3: gall bladder) and liver associated diseases (rank1: liver diseases; rank 2: carcinoma hepatocellular; rank 3: liver neoplasms), confirming that both assay types reveal valuable biological information. (see **Table 1**).

We were interested in determining whether genes found significantly changed in only one assay type provided additional biological information. In the data set produced using the Sensation RNA Amplification Kit, 1,877 signficantly changed genes were identified that were not detected by the Ovation FFPE WTA System assay (**Figure 2A**). As can be seen from the scatter plot

(**Figure 2C**), these genes generally show low-fold changes. Both data sets also show a significant level of discordance.

Following the same functional annotation analysis described above, this gene set revealed largely random annotations (e.g., integumental system, gland, nervous system (see **Table 2**), indicating that this data set includes an elevated level of noise. Similar results were found for all other comparisons of individual FFPE tissue versus the pool of the other three FFPE tissues, as summarized in **Table 2**.

These results indicate that the annotation of genes specifically identified in the data set produced using the Sensation RNA Amplification Kit could not be linked to the respective tissue in question.

In contrast, **Figures 2A** and **2D** show that the data set produced using the Ovation FFPE WTA System not only allows identification of more significantly changed genes (n = 3,355 genes), but that those genes also exhibit a much higher dynamic range than those from the data produced using the Genisphere product. Functional annotation analysis of these genes resulted in liver-tissue-specific annotations (e.g., liver, liver and biliary system, neoplasm), confirming that these genes add biologically relevant information. We found similar results for comparisons of other individual FFPE tissues versus the pool of the remaining tissues, as summarized in **Table 2**.

Conclusions

Both NuGEN's Ovation FFPE WTA System and Genisphere's Sensation RNA Amplification Kit enable gene expression studies of highly degraded RNA derived from FFPE tissue sources. This technical report compares the assay performance of these two systems using clinical FFPE samples that were prepared for analysis on Affymetrix HG-U219 microarrays.

About 30% of the significantly changed genes found by both approaches overlap and their annotation is specific to the tissue and disease studied. However, these studies demonstrate that ds-cDNA targets generated with the Ovation FFPE WTA System provide overall higher sensitivity and specificity in microarray analysis compared to the targets generated by the Genisphere Sensation RNA Amplification Kit. Data produced by the Ovation FFPE WTA System not only show a better dynamic range, but the genes detected using only the NuGEN System were found biologically relevant whereas additional genes detected using only the Sensation RNA Amplification Kit appear to be biologically non-informative based on this analysis.

FFPE tissue samples are an extremely valuable source of RNA for retrospective and prospective disease studies. The NuGEN Ovation FFPE WTA assay clearly outperforms the Genisphere Sensation assay in finding biologically relevant genes from a set of clinical samples. In addition, the NuGEN assay requires less time, is compatible with any microarray type from different platform providers and is automatable on several robotics systems.

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