TECHNICAL REPORT

The WT-Ovation® FFPE System Performance

Introduction

Gene expression analysis of clinical samples has long presented challenges to researchers based on sample availability, amount, and integrity. These challenges are most significant for small and typically severely degraded formalin-fixed, paraffin-embedded (FFPE) samples widely used in cancer research. FFPE is the most common method of collection and storage of solid tumor and adjacent normal tissue, therefore these samples comprise the major percentage of all archived specimens in the world. These archives are of significant value to today's discovery efforts since they are often collected before and after clinical trials and have in-depth retrospective records associated with them regarding response to treatment and outcome.

Working with nucleic acids isolated from FFPE samples has been challenging, since such samples exhibit marked degradation and change in response to long-term storage and fixation methods. Until now, these sample sets have been viewed as unlikely candidates for global gene expression analysis using microarrays.

NuGEN's whole transcriptome approach used in the WT-Ovation FFPE System enables amplification and target preparation of FFPE samples through a simple, robust, and easily automatable solution that allows RNA target preparation from as little as 50 ng of total RNA for analysis on Affymetrix GeneChip® arrays in one day (note: the WT-Ovation FFPE System has been replaced by the Ovation® FFPE WTA System, Part No. 3403). This approach employs the Ribo-SPIA® technology and provides both 3'-initiated and random-primed amplification. It offers the benefit of whole transcriptome amplification for degraded samples and flexibility in qPCR assay design. The system yields sufficient cDNA for analysis of at least one GeneChip array as well as qPCR and QC analysis, in about six hours. The amplified cDNA can then be fragmented and labeled using NuGEN's Encore® Biotin Module (Part No. 4200), allowing the amplification and hybridization to be accomplished in the same day.

This amplification system can also be used in archiving strategies where the amplified cDNA serves as a more stable source of experimental samples, for use with various analytical platforms and for sharing among collaborators. This solution's low requirement for input RNA allows the preservation and archiving of the original FFPE RNA for future applications and studies.

Due to the expected wide range of quality and integrity for FFPE-derived RNA, there is a pressing need for tools that allow assessment of sample quality so researchers can determine the level of performance expected with the samples under study.

The WT-Ovation FFPE System sample assessment tool includes guidelines for sample qualification and recommendations to enable the assessment of sample quality and to closely predict the performance range of various sample sets. For information on the sample assessment tool and guidelines refer to the RNA Sample Quality Assessment Test Technical Report or contact the NuGEN Technical Support team.

This report demonstrates the performance of this system across a wide range of samples varying in age, showing reproducibly and sensitivity of the system both with qPCR and array performance.

Materials and Methods

RNA Preparations

FFPE total RNAs used in studies presented here have been obtained through collaborations and partnerships, including NuGEN's collaboration with Expression Analysis, Inc. Most of the amplifications and experimental procedures have been completed at NuGEN. A variety of RNA isolation methods were used for the FFPE samples, depending on sample source, and where applicable have been stated in the data graphs and results sections. Some of the RNA isolation procedures and products successfully used are Formapure™ Kits (Agencourt, Cat. # A33341), and the High Pure FFPE RNA Micro Kit (Roche, Cat. # 04 823 125 001).

Total HeLa cell line RNA and total RNA from a human Colon Tumor and Normal Adjacent Tissue (NAT) were purchased from Ambion, Inc (Cat. #7852, #7236, respectively, Austin TX).

RNA Amplification, Labeling and GeneChip® Array Hybridization

Amplifications were performed with 50 ng of FFPE total RNA input (unless otherwise noted) following procedures described in the WT-Ovation FFPE System user guide. Amplified cDNA product was quantitated using a NanoDrop ND-1000 (NanoDrop Tech, Wilmington, DE). Target preparation for Affymetrix GeneChip arrays was performed using 5 µg of amplified cDNA and the NuGEN Encore Biotin Module following the product's user guide. Fragmented and biotin-labeled target was hybridized to Affymetrix HG-U133A 2.0 or HG-U133A Plus 2.0 GeneChip arrays.

Hybridization, washing and staining (with GeneChip® Fluidics Station 450) and scanning (with GeneChip® Scanner 3000) were performed following protocols outlined in the Encore Biotin Module user guide and the Affymetrix Expression Analysis Technical Manual.

qPCR Analysis

Real time qPCR assays were designed using the Universal ProbeLibrary™ and primer design software (Exiqon/Roche). Primers were ordered from Integrated DNA Technologies (Coralville, IA). Multiple assays were designed for a number of gene spanning the 5′, middle, and the 3′ regions. Assays were screened for good efficiency as close to 100% as possible with a slope of 1 ± 0.1. qPCR reactions were set up using the purified amplified cDNA at 100 ng/reaction into TaqMan® Fast Universal PCR master mix with 500 nM each of the forward and reverse primers and 100 nM ProbeLibrary™ probe or with ABI's Assays-on- Demand™ primer and probe mix, following vendor's instructions, in 20 µL final volume. Further primer and probe information and accession numbers are provided on request.

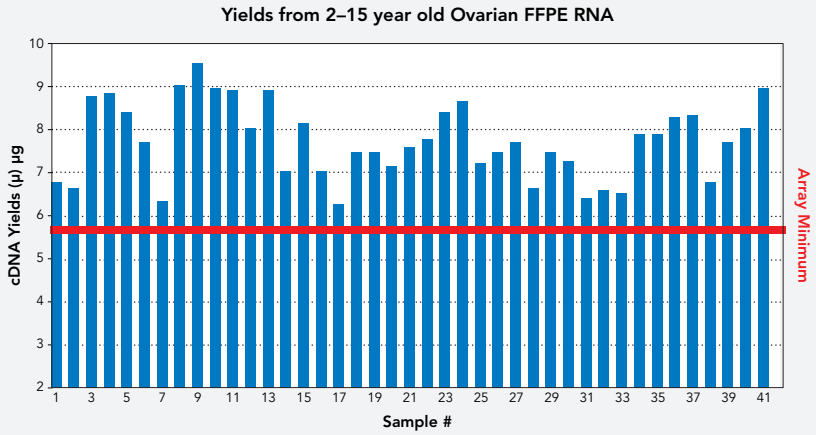
Results

A large number of studies are represented here to demonstrate the basic performance of the WT-Ovation FFPE System for cDNA yield, qPCR, and subsequent array performance in terms of sensitivity and reproducibility.

In a collabortive study with samples obtained from the Moffitt Cancer Center & Research Institute, the amplification of a large set of ovarian tumor samples yielded sufficient cDNA for array analysis for all samples, shown in **Figure 1**. The amount of amplified cDNA required for one array analysis is 5.8 µg, denoted by a red bar on the histogram.

Array peformance averages across all individuals in this study were also robust and consistent despite the wide range of sample ages and the

FIGURE 1. **cDNA amplification yields.**



Forty-one FFPE-derived ovarian tumor samples are shown. A minimum of 5.8 µg is required for analysis on GeneChip arrays (shown by the red bar) a criteria met by all amplifications in this study.

TABLE 1. **Array metrics.**

No. of Arrays	%P	Background Signal	Scaling Factor	cDNA Yield (µg)
40	36.3 ± 8.5	28.8 ± 3.1	26 ± 14	7.8 ± 0.88

Array metrics for the same set of ovarian FFPE samples shown in Figure 1, obtained from analysis on the HG-U133A Plus 2.0 GeneChip arrays. % Present calls ranged from 18–50% for this set of 2–15 year old samples.

TABLE 2. **Multiple tissue sources.**

FFPE Tissue	Age (Years)	Scaling Factor	%P
Colon	5	5.7	40.4%
Colon	4	5.9	42.5%
Colon	1	9.3	39.1%
Liver	1	18.4	21.4%
Lung	5	53.7	9.1%
Lung	1.5	6.5	47.9%
Ovary	4	25.9	49.8%
Ovary	11	30.9	35.5%
Breast	3	7.0	52.8%
Breast	7	25.3	23.2%

Shown here are a random selection of FFPE samples chosen from sample sets representing a variety of ages, tissue sources, collection sites, and RNA isolation methods. Even with this wide range of variables, the amplification system enables quality analysis to be performed on most samples. Sample age is normally expected to inversely correlate with performance, yet the lung sample highlighted in gold is less successful than the 11 year old ovarian sample. This observation highlights the importance of a robust QC system to assess probability of success for FFPE-derived RNA.

TABLE 3. **Reproducibility and array performance.**

A

Signal R^2	1	2	3	4
2	0.985			
3	0.983	0.986		
4	0.981	0.987	0.991	
5	0.987	0.989	0.987	0.986

B

% Call Concordance	1	2	3	4
2	84.6			
3	85.3	84.4		
4	84.8	84.5	85.0	
5	85.7	85.0	85.5	85.4

C

Array Metrics	SF	Bkgd	%P	GAP	Act	RawQ
Average	6.8	29.0	53%	0.8	12.0	0.8
SD	0.58	1.13	1.1%	0.03	0.48	0.05

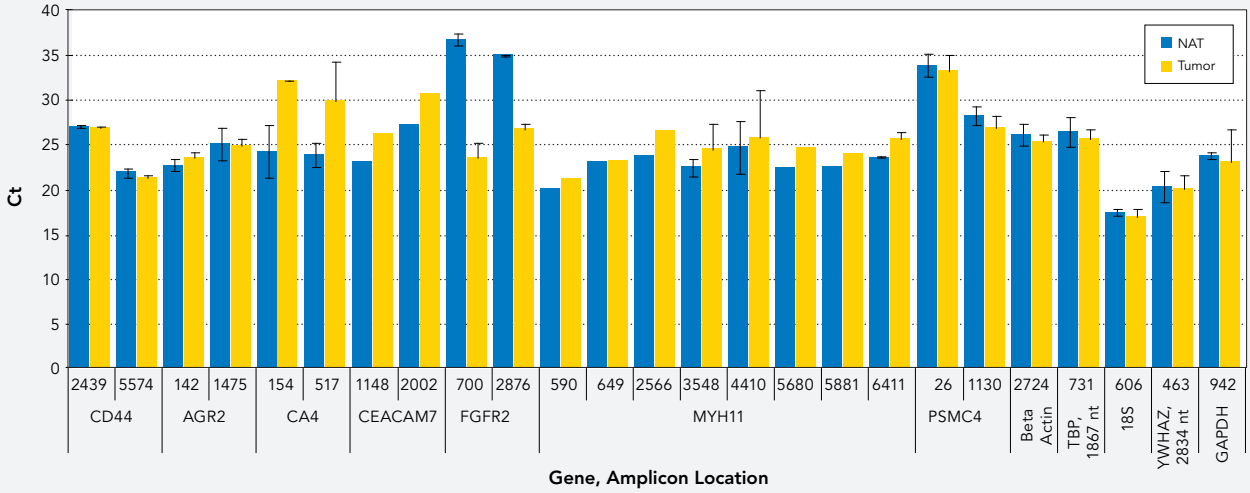
Five replicate amplifications performed on 50 ng of total RNA derived from a 3 year old breast tumor FFPE sample were analyzed on GeneChip arrays. Results show highly reproducible and robust array performance. Signal R^2 and call concordance shown in **Tables 3A** and **3B** have an average of 0.986 and 85.0 respectively. The %P shown in **Table 3C** ranges from 52.0–54.4%.

expected variablity among different individuals (**Table 1**).

The system's consistent and robust performance across diverse samples is demonstrated in **Table 2**, which lists scaling factor and %P for a number of FFPE samples from multiple sources. These sample sets represent a variety of ages, tissue sources, and collection sites, as well as different RNA isolation methods. The samples shown here are by no means the "best" performers in each sample set. Instead, they were intentionally picked at random from their respective sets to demonstrate the inherent variablity among FFPE samples, and to highlight the challenge of predicting how well any FFPE sample set would perform in gene expression studies. It is essential to employ a reliable sample qualification approach such as that developed by NuGEN and described in the RNA Sample Quality Assessment Test Technical Report for the WT-Ovation FFPE System product.

Table 3 demonstrates the high level of reproducibility, concordance, and quality of array metrics in 5 techni-

FIGURE 2. **qPCR results on amplified Colon Tumor and NAT FFPE samples.**

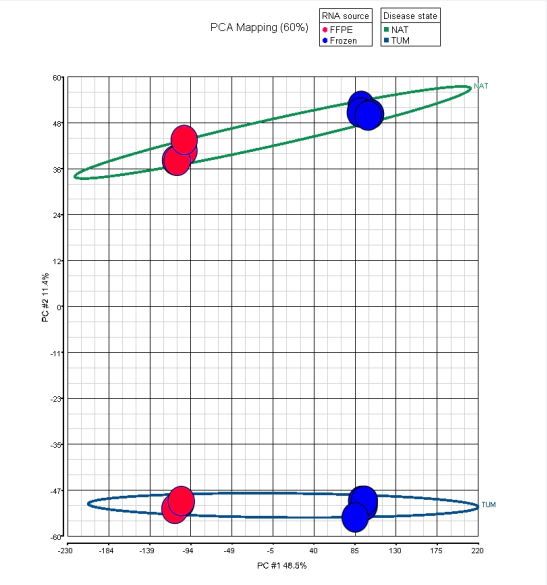


qPCR analysis of 1 year old Colon Tumor FFPE RNA and NAT was performed on amplified cDNA using a total of 35 assays for 12 genes across a range of abundance. The gene and amplicon location is listed on the x axis and the qPCR Cts are plotted on the Y axis. Cts are consistent across the length of transcripts. Tumor vs. NAT differential expression is observed for FGFR2, CA4, and the CEACAM7 transcripts and is reflected by all assays for each if the transcripts.

cal replicates from amplification of a 3 year old breast tumor FFPE sample with R^2 average of 0.986, call concordance average of 85.0, and a %P range of 52.0–54.4%. The qPCR analysis of amplified cDNA from FFPE samples was performed for 12 genes and the data are shown in **Figure 2**. FFPE samples used here were one year old colon tumor and normal adjacent tissue (NAT). qPCR was performed on the amplified cDNA products using 25 assays designed for 12 genes, across a range of abundance. For some transcripts, multiple assays were designed across the length of the transcript to show that the whole transcript has been amplified from RNA. qPCR Cts are consistent across the length of these transcripts. Tumor vs. NAT differential expression was observed for FGFR2, CA4, and the CEACAM7 transcripts and was consistently maintained with all assays for each of these transcripts.

To demonstrate that the amplification of FFPE RNA maintains the integrity of the biological data, PCA (Principle Components Analysis) was performed

FIGURE 3. **Principle Components Analysis (PCA) of colon tumor and normal adjacent tissue (NAT).**



Targets were prepared from RNA extracted from formalin-fixed, paraffin-embedded tissue (FFPE) from one donor (red) and fresh frozen tissue from a second donor (blue). Each sample was amplified in quadruplicate and hybridized to Affymetrix HG-U133A 2.0 GeneChip arrays. PCA was performed using Partek Genomics Suite software. The green and blue ellipses (NAT and Tumor, respectively) define the boundary of 2 standard deviations from the centroid of each cluster indicating a statistically significant separation of samples based on the disease state of the tissue. This demonstrates that the amplification system maintains the integrity of the biological data.

(**Figure 3**). Targets were prepared from FFPE RNA from one donor (red) and fresh frozen tissue from a second donor (blue). Each sample was amplified in quadruplicate, and hybridized to HG-U133A 2.0 GeneChip arrays. The PCA was performed using Partek Genomics Suite software. The green and blue ellipses show NAT and tumor clusters respectively and define the boundary of 2 standard deviations from the centroid of each cluster. This indicates a statistically significant separation of samples based on the tissue disease state, demonstrating the amplification system has clearly maintained the biological data integrity.

Conclusions

Gene expression analysis of limited and degraded clinical samples such as FFPE-derived RNA has been a difficult challenge, hindering clinical cancer research.

The NuGEN WT-Ovation FFPE System addresses this technology gap and enables the robust, reliable, and sensitive amplification and target preparation of these highly compromised samples. The simple, easily automatable process yields a significantly higher quality and quantity of expression profile data than previously possible and allows the vast archives of valuable FFPE samples to be accessed for clinical research projects.

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