

NUGEN'S WHOLE TRANSCRIPT RNA AMPLIFICATION SYSTEMS ENABLE ROBUST EXPRESSION PROFILING OF SMALL AND DEGRADED SAMPLES ON THE AGILENT DUAL-MODE GENE EXPRESSION ARRAY PLATFORM

INTRODUCTION

Advances in sample extraction and isolation technologies are driving efforts to further decrease the size and increase the homogeneity of samples used in gene expression biomarker discovery and validation studies. Robust and reliable target preparation has therefore become critical for processing small, often compromised RNA samples such as those from LCM, fine needle aspirates and other clinical tissue biopsies as well as from archived FFPE samples. As new technical and content advances are offered by the spectrum of gene expression platform providers, it is important for gene expression researchers to maintain platform flexibility in their choice of target preparation methodology.

This report presents results obtained using a simple labeling protocol for efficiently preparing cDNA targets generated by the WT-Ovation™ Pico and FFPE Systems (NuGEN, Cat.#s 3300 & 3400) for interrogation on Agilent's Dual-Mode Gene Expression microarrays. cDNA targets are labeled using either the Agilent Genomic DNA Enzymatic Labeling Kit (Cat. # 5190-0449) or the Invitrogen BioPrime® Total Genomic Labeling Kit (Cat.# 18097-011) according to the manufacturer's recommendations. Targets were then hybridized to Agilent 4x44K Whole Human Genome Microarrays (Cat.# G4112F) generating robust, reproducible and biologically relevant global gene expression data.

MATERIALS AND METHODS

RNA sources:

Total RNA from a matched set of fresh frozen (FF) and formalin fixed paraffin embedded (FFPE) colon tissue specimens from both tumor and the normal adjacent tissue (NAT) were obtained from Asterand. Table 1 and Figure 1 illustrate the RNA quality metrics. As evidenced by the reduced RIN scores, the RNA from the FFPE tissue is largely degraded compared to the RNA isolated from the matching fresh frozen tissue. Universal Human Reference (UHR) RNA was obtained from Stratagene (Cat. # 740000) and Human Brain Reference RNA was obtained from Ambion (Cat. # AM6050).

RNA amplification:

Fifty ng of FFPE RNA and 10 ng of fresh frozen RNA were used as input into the WT-Ovation FFPE RNA Amplification System according to manufacturer's instructions. Quadruplicate amplifications were performed on each of four samples, comprising a matched set of paired fresh-frozen and FFPE-archived colon tumor and normal adjacent tissue.

One ng and 500 pg UHR and Brain total RNA were used as input into the WT-Ovation Pico RNA Amplification System according to manufacturer's instructions. Quadruplicate amplifications were performed on each sample at each input level.

cDNA labeling:

For this study, the Agilent Genomic DNA Enzymatic Labeling Kit was used for generating Cy3/Cy5 labeled cDNA targets. Labeled targets generated for Agilent array analysis using the Invitrogen BioPrime Total Genomic Labeling System have shown equivalent quality array results (customer communication).

Two ug of purified, amplified cDNA were used as input into the Agilent Genomic DNA Enzymatic Labeling Kit according to the manufacturer's instructions. The Cy3/Cy5-labeled cDNA was quantitated and dye incorporation determined by Nanodrop. Labeled cDNA yields and dye incorporation were both within expectations for all labeled samples.

Array hybridization:

Labeled targets were hybridized to Agilent Whole Human Genome Dual-Mode Gene Expression Microarrays essentially as recommended by the manufacturer in the One-Color and Two-Color Microarray-Based Gene Expression Analysis User Manuals (v 5.7) with the following changes:

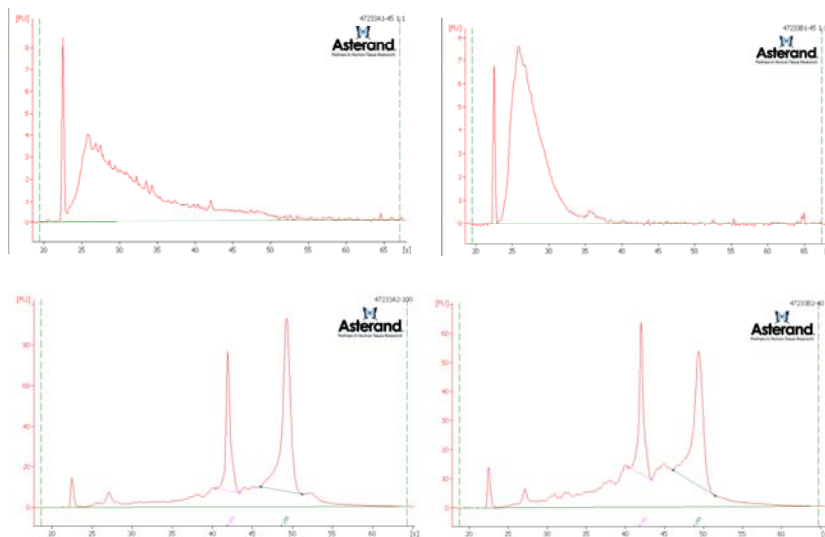
1. Labeled cDNA targets were not subjected to the fragmentation step and therefore the fragmentation buffer was not used.
2. Three ug labeled cDNA target was hybridized per 4x44K array. (Note: For highly degraded and variable RNA samples such as those from FFPE tissue, it is recommended to use up to 5 ug labeled cDNA target)



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ID	Tissue	RIN
47233A1	FFPE Colon Tumor	2.4
47233B1	FFPE Colon NAT	1.3
47233A2	Frozen Colon Tumor	9.1
47233B2	Frozen Colon NAT	7.5

Table 1. Colon Tumor/NAT FFPE/FF quad set RNA RIN scores.

Figure 1. Agilent Bioanalyzer traces of Frozen and FFPE Colon tumor and normal RNA samples. From left to right, top to bottom: FFPE Tumor, FFPE NAT, Fresh Frozen Tumor, Fresh Frozen NAT.

Hybridization cocktails were assembled as follows:

- Three ug labeled cDNA was brought up to 44 uL final volume with water.
- Eleven uL of 10X Blocking Agent was added for a final volume of 55 ul.
- Fifty-five uL 2x GEx Hybridization Buffer HI-RPM was added for a final hybridization cocktail volume of 110 uL.

Microarray processing continued according to manufacturer's instructions.

Data Analysis Results:

The reproducibility of the WT-Ovation -generated targets on Agilent DNA oligo microarrays was determined by calculating pairwise Pearson correlations using all signal values from the array among four independently amplified and labeled replicates per sample type. The average signal R^2 was 0.99 for commercial UHR and Brain RNAs at the 500 pg input level and 0.98 for the fresh frozen and FFPE colon tumor samples. An example of linear scatter plots of array signal for representative sample types is shown in Figure 2.

The sensitivity of the WT-Ovation Pico and WT-Ovation FFPE Solution-generated targets on Agilent array was evaluated by the average number of transcripts detected well above background across four replicate arrays for each RNA source (Table 2). The number of detected transcripts is reduced when using FFPE sample RNAs. This reduction in sensitivity is expected in FFPE RNA samples due to the highly degraded nature of the RNA.

The Venn diagram in Figure 3 illustrates the high degree of concordance in detected genes when targets prepared from degraded FFPE RNA samples are compared to targets prepared from matched fresh frozen tissues. Over 98% of the genes concordantly detected in 3 of 4 FFPE replicates are also detected in the matching fresh frozen sample. While the number of genes detecting in FFPE RNA sample targets is lower than the number detected in fresh frozen samples, the high concordance of detection is evidence that targets prepared from FFPE RNA samples using the WT-Ovation FFPE system yield accurate biology, despite the degraded nature of the RNA extracted from FFPE tissue samples.

CONCLUSIONS

The target preparation approach described here was demonstrated to generate reproducible and sensitive results on the Agilent microarray platform using very small and degraded RNA samples, such as those obtained from FFPE tissue. This labeling approach enables researchers using Agilent's microarray platform to interrogate small and difficult clinical samples that were previously out of reach for global gene expression studies.

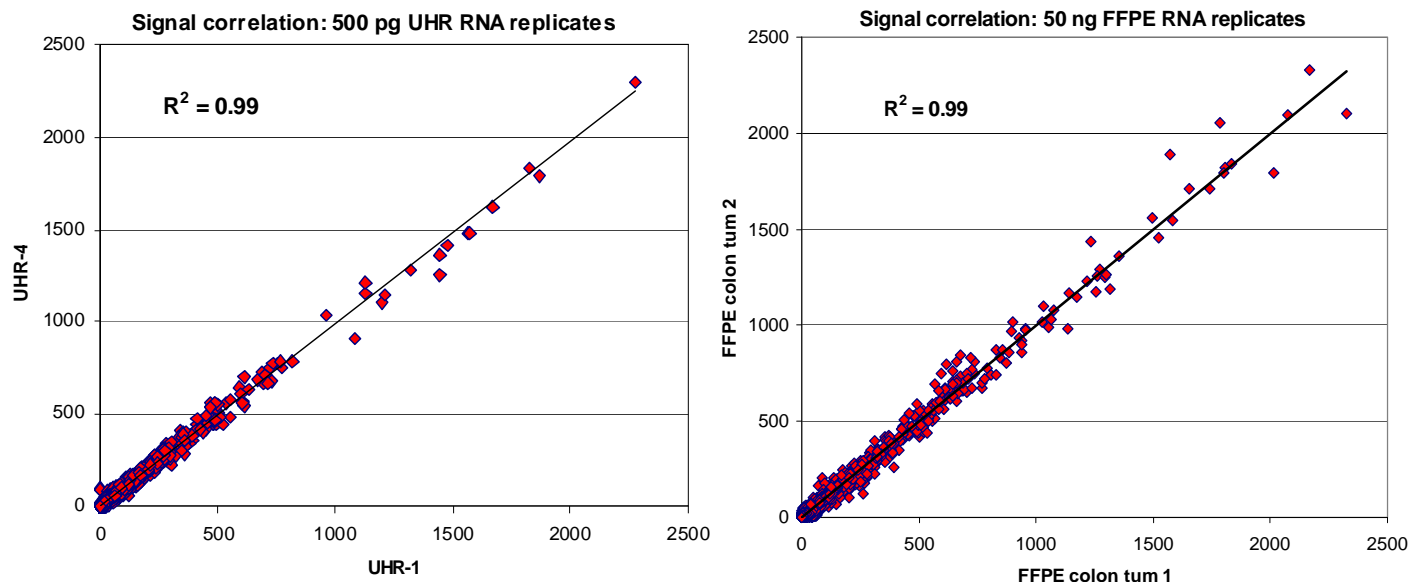


Figure 2. High amplification reproducibility with signal R^2 of 0.99 for independent UHR control replicates and independent FFPE RNA* replicates on 4x44K Agilent Whole Human Genome microarrays.

*Signals from five outlier high-abundance genes were excluded as they artificially inflate the linear correlation scores for the FFPE samples.

Table 2. Average percentage of genes detected as 'well above background' in each sample type. Note: MAQC sample data are reported as the percentage of MAQC core genes (13,621) detected.

Sample	RNA input	Avg. % genes detected
MAQC-A (UHR)	500 pg	84.2
MAQC-B (Brain)	500 pg	82.9
Fr Frozen colon tumor	10 ng	83.4
Fr Frozen colon normal	10 ng	89.3
FFPE colon tumor	50 ng	57.8
FFPE colon normal	50 ng	73.4

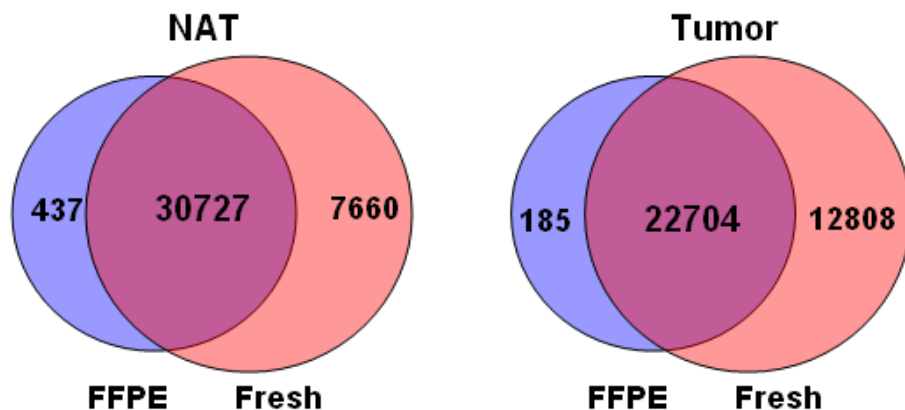


Figure 3. High degree of concordance is demonstrated in detected genes between the FFPE samples and the fresh frozen samples with >98% of the genes detected in the FFPE samples also being detected in the fresh samples, in both Tumor and Normal Adjacent Tissue.

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