

NUGEN'S WT-OVATION™ FFPE SYSTEM COUPLED WITH A NOVEL LABELING PROTOCOL ENABLES GENOME-WIDE GENE EXPRESSION PROFILING OF FFPE SAMPLES ON THE ILLUMINA BEADARRAY PLATFORM

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INTRODUCTION

NuGEN provides industry leading technologies for the amplification and labeling of limited and degraded RNA samples, such as those obtained by RNA isolation from archived formalin-fixed, paraffin-embedded (FFPE) tissue samples. FFPE tissue samples represent a significant source of clinical biological material for both basic and clinical research projects. However, the specimen collection process, fixation and embedding techniques, as well as long term storage are all factors that contribute to extensive degradation, cross-linking and modification of FFPE RNA. As a result the FFPE mRNA is difficult to reverse transcribe and amplify sufficiently for meaningful analysis on global expression platforms such as microarrays.

This report presents results obtained in a collaborative study conducted by NuGEN, Expression Analysis and Illumina. The report describes a simple and novel labeling and hybridization protocol for efficiently preparing cDNA targets generated by the WT-Ovation™ FFPE System (NUGEN, Cat.# 3400-12) for interrogation on Illumina's Genome-Wide Expression BeadChips. cDNA is generated from FFPE RNA samples using the WT-Ovation FFPE System. Targets are labeled using methods described below and hybridized to the Illumina HumanRef-8 v2 Expression BeadChips (Cat#: BD-25-213) generating robust, reproducible and biologically relevant global gene expression data.

MATERIALS AND METHODS

Tissue sources and RNA Isolations:

A matched set of lung tissue specimens from a tumor and the normal adjacent tissue (NAT) were obtained from a commercial vendor. For fresh-frozen tissue, the RNA was isolated using the QIAGEN RNeasy® procedure according to the manufacturer's instructions (including the on-column DNase treatment). For the FFPE samples, paraffin blocks were cut into 10 µm sections by the vendor and shipped to Expression Analysis. RNA was isolated from five sections using Agencourt Bioscience's Formapure™ isolation kit. Table 1 displays the RNA yields. As expected, the RNA from the FFPE sections is largely degraded compared to the RNA isolated from the matching fresh frozen tissue (Figure 1.)

RNA amplification:

Fifty ng of total RNA extracted from FFPE tissues was used as input into the WT-Ovation FFPE 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 lung tumor and normal adjacent tissue. The yields were very reproducible and higher in the fresh frozen RNA samples than in the FFPE RNA samples, as expected, and shown in Table 2. (see also NuGEN's WT-Ovation FFPE System Technical Report #3 (www.nugeninc.com/pdfs/ffpe_techrep3.pdf))

cDNA labeling:

Required Reagents and Equipments:

UNG buffer [10 mM Phosphate buffer pH 8.0 (KH₂PO₄, Sigma-Aldrich, Inc, Cat.# P9666), and 4 mM MgCl₂ (USB, Cat.# 78641)]

Nuclease-free water (USB, Cat.# 71783)

UNG enzyme (Epicentre, Cat.# HU5901K, 1U/µl)

Labeling buffer [0.952 M Acetic Acid, (Sigma-Aldrich, Inc, Cat.# A62830), and 28 mM MgCl₂ (Sigma-Aldrich, Inc, Cat.# 78641)]

ARP solution [11.3 mg/mL ARP (N-(aminooxyacetyl)-N'-(D-biotinoyl) hydrazine, trifluoroacetic acid salt (Molecular Probes, Cat.# A10550), in 22.4 mM Phosphate buffer pH 8.0 (KH₂PO₄, Sigma-Aldrich, Inc, Cat.# P9666)]

Clean & Concentrator 25 column (Zymo, Cat.# D4005)

Nanodrop®, (Nanodrop, model # ND-1000)

Thermal Cycler

Protocol:

The protocol overview is shown in Figure 2. After purification according to manufacturer's instructions, the amplified single-stranded cDNA was labeled with biotin as follows:

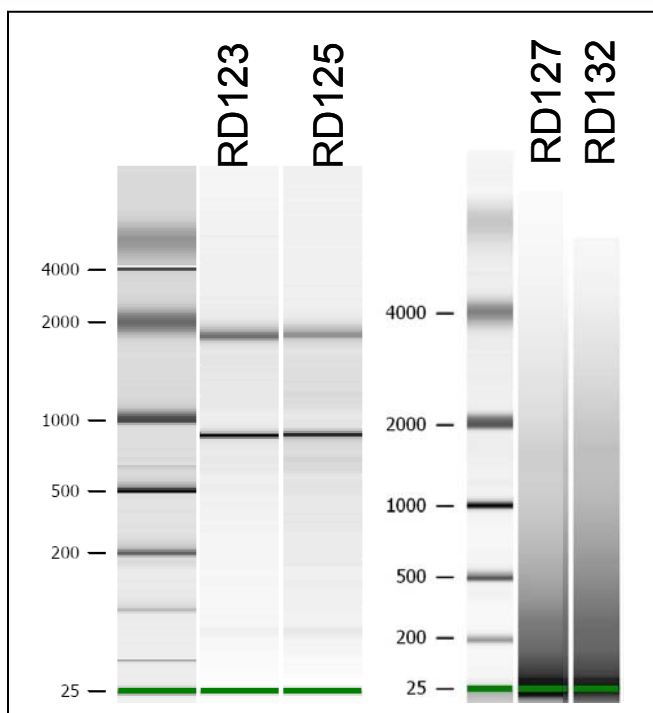
1. Place a total of 3-5 µg amplified and purified cDNA in a 0.2 mL PCR tube.
2. Bring up the volume to a final 25 µl with nuclease-free water
3. Add 5 µl of UNG buffer and 5 µl UNG enzyme to the cDNA.
4. Mix the resulting solution thoroughly by pipetting up and down several times.

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ID	Tissue	260/280	µg RNA	RIN
RD123	Frozen Lung Tumor	2.20	143.2	7.8
RD125	Frozen Lung NAT	2.21	53.6	6.8
RD127	FFPE Lung Tumor	1.73	8.3	-
RD132	FFPE Lung NAT	1.81	1.1	-

Table 1. RNA purification yields

Figure 1. Agilent Bioanalyzer traces of Frozen and FFPE lung tumor

RNA sample	Tissue Source	Total RNA input (ng)	Amplified cDNA yield µg (SD)
RD123	Frozen Lung Tumor	10	10.9 (0.2)
RD125	Frozen Lung NAT	10	10.4 (0.1)
RD127	FFPE Lung Tumor	50	5.6 (0.1)
RD132	FFPE Lung NAT	50	5.8 (0.1)

Table 2. cDNA yields and standard deviation after amplification using WT-Ovation FFPE System.

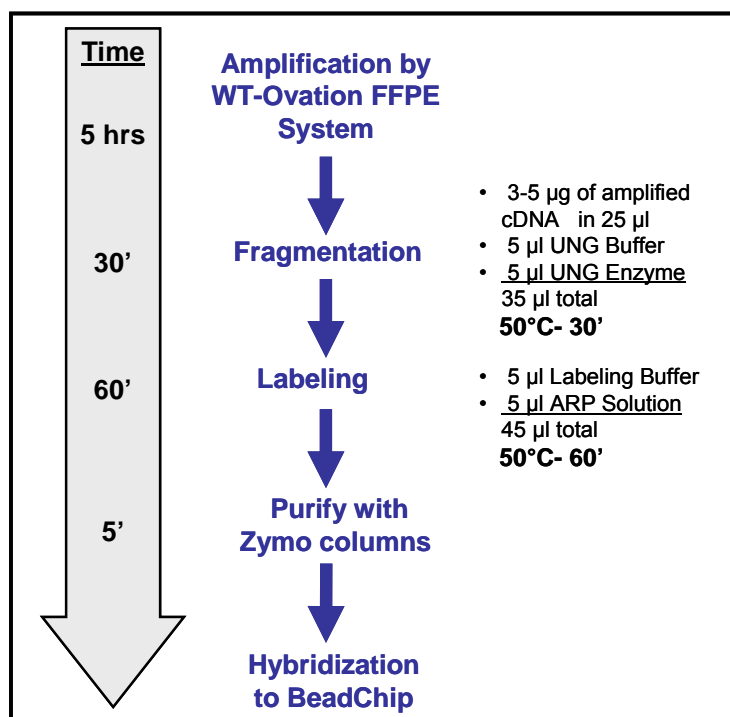


Figure 2. Protocol overview.

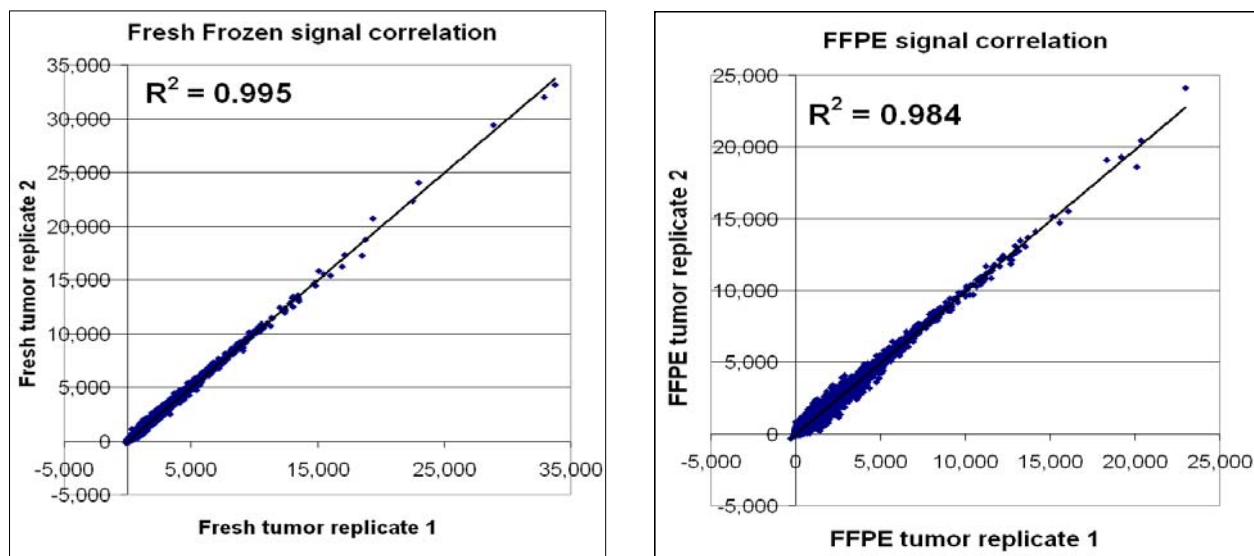


Figure 3. High reproducibility with average R^2 of 0.99 for fresh frozen and 0.98 for FFPE samples. High sensitivity with an average of ~46% of the probes detected using fresh RNAs from tumor and normal adjacent, and ~31% probes detected with FFPE RNAs (compared to cRNA targets generated from FFPE RNA using the standard IVT method which detected below 25% of on the array).

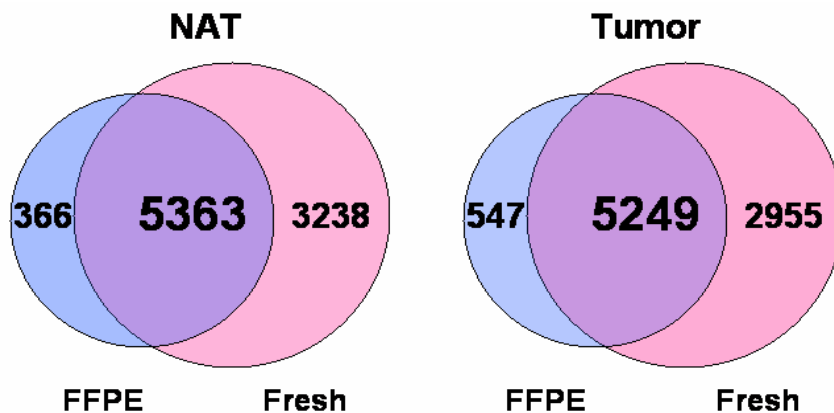


Figure 4. High degree of concordance is demonstrated in detected genes between the FFPE samples and the fresh frozen samples with >90% of the genes detected in the FFPE samples also being detected in the fresh samples, in both Tumor and Normal Adjacent Tissue.

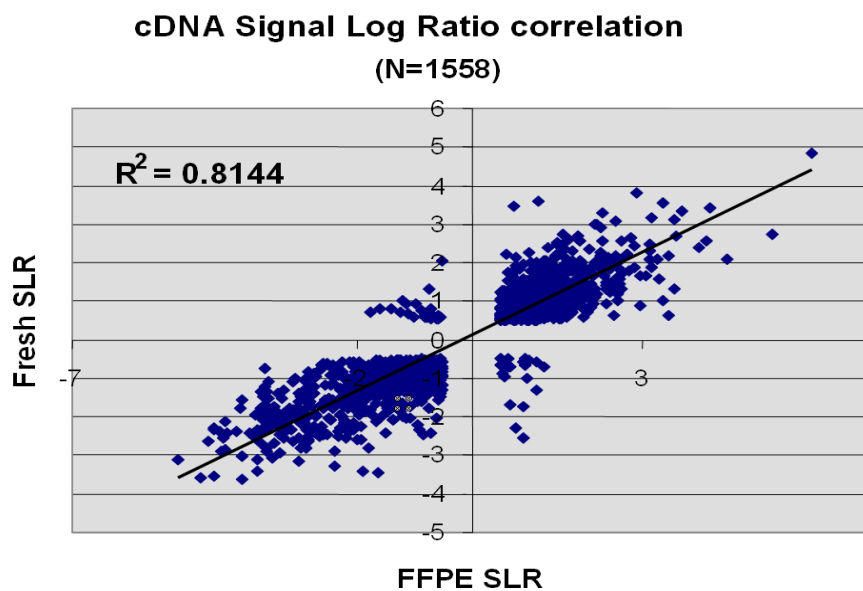


Figure 5. Accuracy of the gene expression data is shown by the high degree of correlation of log ratios ($R^2=0.81$) for a set of 1558 genes between targets derived from fresh samples and targets derived from FFPE samples

5. Incubate at 50°C for 30 minutes in a thermal cycler with heated lid
6. Following the incubation, remove the tube from thermal cycler and place on ice.
7. Add 5 µl labeling buffer and 5 µl ARP solution.
8. Mix the resulting solution thoroughly by pipetting up and down several times.
9. Incubate at 50°C for 60 minutes in a thermal cycler with heated lid.
10. After the incubation, purify the labeled cDNA using a Clean & Concentrator 25 column following manufacturer's instructions.
11. Following purification, determine the final concentration of cDNA on a Nanodrop spectrophotometer.

BeadChip hybridization:

750 ng biotin-labeled cDNA was used per array on the Illumina HumanRef v2 Expression BeadChips. The hybridization cocktail was prepared according to the BeadChip manufacturer's instructions. The arrays were hybridized, washed and scanned essentially according to the manufacturer's instructions, with the exception that the hybridization temperature was reduced to 48°C to accommodate the altered hybridization kinetics of cDNA/DNA pairs relative to cRNA/DNA pairs.

Data Analysis Results:

The reproducibility of the WT-Ovation FFPE Solution-generated targets on BeadArray 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 R^2 was 0.99 for fresh frozen samples and 0.98 for FFPE samples. An example of linear scatter plots of array signal for each is shown in Figure 2.

The sensitivity of the WT-Ovation FFPE Solution-generated targets on BeadArray was evaluated by the number of transcripts concordantly detected with a P-value of less than or equal to 0.05 in all four replicate arrays for each RNA source. Using this threshold, 45% and 47% of the probes on the array are detected using fresh RNAs from tumor and normal adjacent, respectively. The number of detected transcripts is reduced when using FFPE sample RNAs down to ~31%. A reduction in sensitivity is expected in FFPE RNA samples due to the highly degraded and cross-linked nature of the RNA. As a comparison, the sensitivity of detection for cRNA targets generated from FFPE RNA using the standard IVT method is below 25%.

There is a high degree of concordance in detected genes between the FFPE samples and the fresh frozen samples with >90% of the genes detected in the FFPE samples also being detected in the fresh samples. This demonstrates that while there may be a reduction in the depth of gene expression data available in FFPE samples, the accuracy of the data is uncompromised. (Figure 4)

As an additional demonstration of the accuracy of the gene expression data obtained from FFPE samples on BeadArray, we calculated the correlation of log ratios (tumor vs. normal) in genes concordantly

detected in FFPE and fresh samples of both tumor and normal adjacent tissue. Figure 5 is a graph illustrating the correlation of log ratios between targets derived from fresh samples and targets derived from FFPE samples. Filtering for genes present in all samples and excluding those with an absolute fold change of 1.5, there is a high degree of correlation ($R^2=0.81$) for a set of 1558 genes.

CONCLUSIONS

The novel labeling approach described above, coupled with NuGEN's WT-Ovation FFPE RNA Amplification System enables global gene expression analysis on the Illumina BeadArray platform using small and degraded RNA samples, such as those obtained from FFPE tissue. This labeling approach allows the same Ovation technology to be used as the upfront amplification method for increased consistency, providing flexibility and choice in the selection of microarray platforms.

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Frequently Asked Questions

NuGEN Illumina Solution

Q1. *What is the Illumina Solution?*

The NuGEN Illumina Solution consists of a protocol described in an Application Note. The protocol describes the cDNA labeling method, lists the specific materials and reagents required, and provides source and order information. The technical report includes results from the validation of this protocol using Ovation and WT-Ovation-generated cDNA on the Illumina BeadArray platform.

Q2. *What is the workflow for using the Illumina Solution?*

The workflow consists of the following steps:

- Define Sample type: Based on sample characteristics, choose one of NuGEN's amplification system products.
- Amplify RNA: Obtain the appropriate amplification system and amplify RNA to yield amplified cDNA.
- Label cDNA: Using the detailed protocol in Illumina Solution Application Note #2, label the amplified cDNA and proceed with hybridization to the Illumina whole genome expression arrays.

Q3. *Which NuGEN amplification systems can I use with this protocol?*

The NuGEN Illumina labeling protocol can be used with the Ovation™ RNA Amplification System V2 (Cat. #3100), the WT-Ovation™ Pico System (Cat. #3300), or the WT-Ovation™ FFPE System (Cat. #3400).

Q4. *What sample types can I use with this protocol?*

The sample type and characteristics primarily defines which amplification system is best to use. For example, for FFPE RNA the WT-Ovation FFPE System should be used while for whole blood RNA the Ovation WB solution is optimal. For typical small samples (above 500 pg) or compromised and partially degraded RNA samples the WT-Ovation™ Pico System is the standard amplification approach.

Q5. *Does NuGEN support this protocol? Who do I call if I have questions or problems?*

The labeling protocol is supported by NuGEN. In case of questions and problems you may contact the NuGEN Technical Support team.

Q6. *Can I use alternate sources of reagents other than those specified in the technical report?*

We have tested and validated the protocol using only the reagents and sources listed in the Application Note #2. If other reagents are used, NuGEN may not be able to support this protocol.

Q7. *Are the Illumina hybridization and wash conditions different with this protocol?*

The hybridization temperature is reduced to 48 degrees, but all the other conditions are exactly as outlined by Illumina for the BeadArrays.

Q8. *Are the data analysis approaches different with this protocol?*

No, we recommend you follow all data analysis procedures outlined by Illumina for BeadArrays.

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