Purification, amplification, and labeling of RNA from FFPE tissues for reliable GeneChip® array analysis

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The RNeasy® FFPE Kit was used in combination with NuGEN's WT-Ovation™ FFPE RNA Amplification System and FL-Ovation™ cDNA Biotin Module V2 to purify, amplify, and label RNA from FFPE tissues. The resulting cDNA targets provided informative global gene expression analysis with GeneChip arrays from Affymetrix.

Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissue samples represent the single largest source of untapped biological material for basic and clinical research. However, while formalin fixation and paraffin embedding efficiently preserve tissues for morphological analysis, the effects of the fixation process on nucleic acids make molecular analyses difficult. Nucleic acids in FFPE tissues are cross-linked and often irreversibly damaged, becoming increasingly fragmented with prolonged storage. To achieve meaningful global gene expression analysis on microarray platforms, it is critical to use purification, amplification, and labeling methods that overcome these limitations of FFPE samples.

The RNeasy FFPE Kit provides special lysis and incubation conditions to reverse cross-linking of nucleic acids in FFPE tissues and to efficiently release RNA while avoiding further degradation. Highly pure RNA is then purified using proven RNeasy spin-column technology. Reliable amplification of the entire transcriptome is achieved with the WT-Ovation FFPE RNA Amplification System, which is based on unique WT-Ovation technology and adapted for use with RNA from FFPE tissues. This technology does not require intact poly-A tails, unlike double-round T7-based systems from most suppliers which inconsistently amplify RNA from FFPE samples, yielding marginal or poor-quality microarray data. Rapid fragmentation and labeling of the amplified cDNA is accomplished with the FL-Ovation cDNA Biotin Module V2, providing targets highly suited for hybridization to GeneChip arrays.

In this application note, we evaluated the performance of the combination of RNeasy, WT-Ovation, and FL-Ovation technologies. cDNA targets were prepared from rat tissues that were either stabilized in RNA*later*® RNA Stabilization Reagent or fixed in formalin and embedded in paraffin, and their performance in GeneChip array analysis was compared.

Materials and methods

Tissue preparation: Organs were excised from adult Wistar rats and cut into approx. 3–5 mm thick pieces. The tissues were immediately submerged in either 10% neutral buffered formalin (for fixation and paraffin embedding) or RNA*later* RNA Stabilization Reagent (to provide reference samples ▶

containing optimally preserved RNA). Formalin fixation was performed for 20–24 hours at room temperature. Tissues were dehydrated in 70%, 90%, and 100% ethanol followed by xylene (2 x 1 hour each solution), and then embedded in Paraplast Plus® tissue-embedding medium for 2–3 hours at 60°C.

RNA preparation: RNA was purified from the tissue samples after 1 week or 1 year of storage. For the FFPE tissues, RNA was purified from 1–4 sections of 10 µm thickness using the RNeasy FFPE Kit. Sections were cut on a standard microtome and the first 2 sections were discarded to exclude the effects of air exposure. For the RNA*later* stabilized tissues, RNA was purified using the RNeasy Lipid Tissue Mini Kit. RNA was quantitated by measuring absorbance at 260 nm on a NanoDrop® spectrophotometer. RNA quality was assessed on an Agilent® bioanalyzer in "Eukaryotic Total RNA Nano" mode.

RNA amplification and labeling: 25 ng of total RNA from each tissue sample was amplified using the WT-Ovation FFPE RNA Amplification System and purified according to manufacturer's recommendations. The FL-Ovation cDNA Biotin Module V2 was then used to prepare fragmented and labeled cDNA targets for GeneChip array analysis.

GeneChip array analysis: For each sample, the cDNA targets were added to a hybridization cocktail and hybridized to a GeneChip Rat Genome 230 2.0 Array as described in the *FL-Ovation cDNA Biotin Module V2 User Guide*. Arrays were scanned on a GeneChip scanner, and signal values were processed and normalized initially using the MAS5 algorithm on the GCOS Server.

Results

RNA was successfully purified from both the 1-week-old and 1-year-old FFPE tissues, including partially degraded small RNA fragments (Figure 1). Significant degradation of RNA in the FFPE tissues occurred after 1 year of storage, as demonstrated by the reduced size of the RNA and disappearance of the rRNA double peaks in the Agilent bioanalyzer traces. RNA purified from all tissues yielded A_{260}/A_{280} ratios* of around 2.0, an indication of high purity.

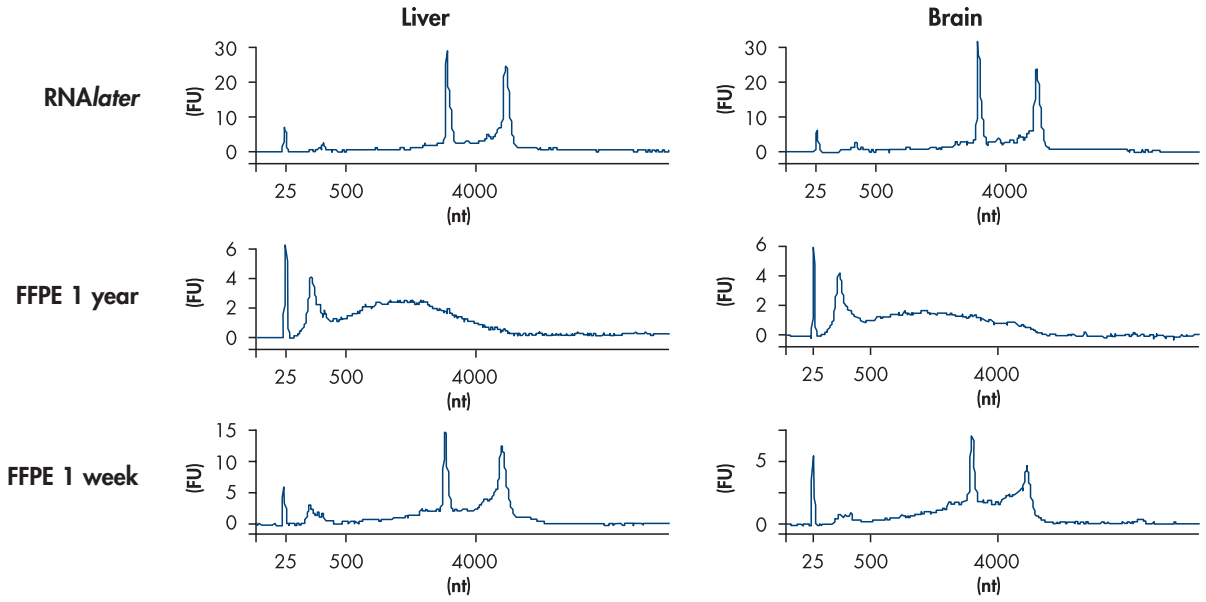

Figure 1. Recovery of small RNA fragments from FFPE tissues. Agilent bioanalyzer analysis was carried out on RNA purifed from RNA*later* stabilized tissues (**RNA*later***), FFPE tissues stored for 1 year (**FFPE 1 year**), or FFPE tissues stored for 1 week (**FFPE 1 week**).

* Ratio of absorbance readings at 260 nm and 280 nm.

Global array metrics for the FFPE tissues and the RNA*later* stabilized tissues showed a high degree of concordance in Present calls (Table 1). While the FFPE tissues yielded fewer Present calls than the RNA*later* stabilized tissues, the Present calls for FFPE liver overlapped 96% with the Present calls for RNA*later* stabilized liver. Similarly, 97% of the Present calls for FFPE brain were also Present calls for RNA*later* stabilized brain.

Table 1. Global array metrics

Sample	Storage time	SF*	BG Avg†	%P‡	3'/5' ratio (GAPDH)§	3'/5' ratio (β-actin)§
Liver (RNA*later*)	1 year	3.95	35.73	61.27	2.43	7.16
Liver (RNA*later*)	1 week	3.18	35.51	63.25	3.16	6.19
Brain (RNA*later*)	1 year	2.46	37.62	73.57	1.94	5.99
Brain (RNA*later*)	1 week	3.15	38.25	69.27	2.40	8.85
Heart (RNA*later*)	1 year	23.30	36.39	47.80	1.20	5.59
Liver (FFPE)	1 year	11.02	39.13	46.22	1.30	5.92
Liver (FFPE)	1 week	20.88	36.20	39.38	1.01	4.13
Brain (FFPE)	1 year	9.99	34.26	54.16	1.22	4.12
Brain (FFPE)	1 week	8.71	35.80	56.87	1.17	4.68
Heart (FFPE)	1 year	11.50	33.80	49.69	1.05	2.91

* SG: scale factor (an indication of signal intensity: the higher the value, the weaker the signal).

† BG Avg: background average (the lower the value, the less background there is).

‡ %P: percentage of genes called present.

§ 3'/5' ratio: ratio of signal intensities from probes corresponding to the 3' and 5' ends of the mRNA for GAPDH or β-actin (an indication of RNA integrity: the lower the value, the better the RNA integrity).

Signal values were converted to $\log_2$ values and the log ratios of genes concordantly present in both liver and brain were calculated for all the FFPE tissues and for all the RNA*later* stabilized tissues (i.e., calculations were made from tissues stored for 1 year and 1 week). A high degree of correlation (R^2 = 0.93) of differential gene abundance was observed between the FFPE tissues and the RNA*later* stabilized tissues (Figure 2), demonstrating that the FFPE tissues yielded biologically similar global gene expression results to those obtained with the RNA*later* stabilized tissues.

To check whether the integrity of the gene expression analysis data from the FFPE tissues was influenced by RNA degradation, signal value files (*.cel) were imported into GeneSpring® software and RMA normalized for further analysis using clustering and PCA (principal components analysis) (Figure 3). For each tissue type (brain, heart, or liver), data from FFPE and RNA*later* stabilized samples were clustered together, indicating that the gene expression profile in the FFPE tissues was preserved. ▶

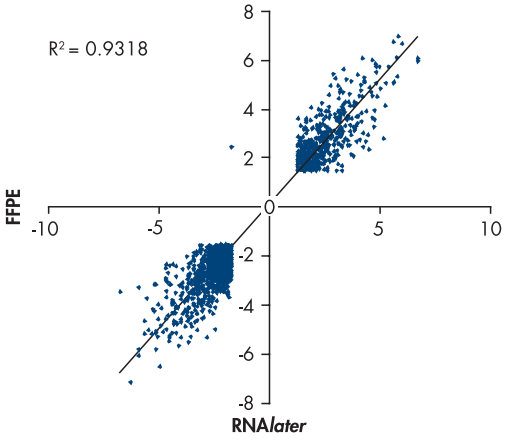

Figure 2. High correlation. The log ratios of concordantly present genes in liver and brain targets from all RNA*later* stabilized and FFPE tissues were compared. Data were filtered to exclude concordantly present genes that were less than 1.5-fold different between liver and brain. The log ratios for an overlapping set of 1351 genes are shown.

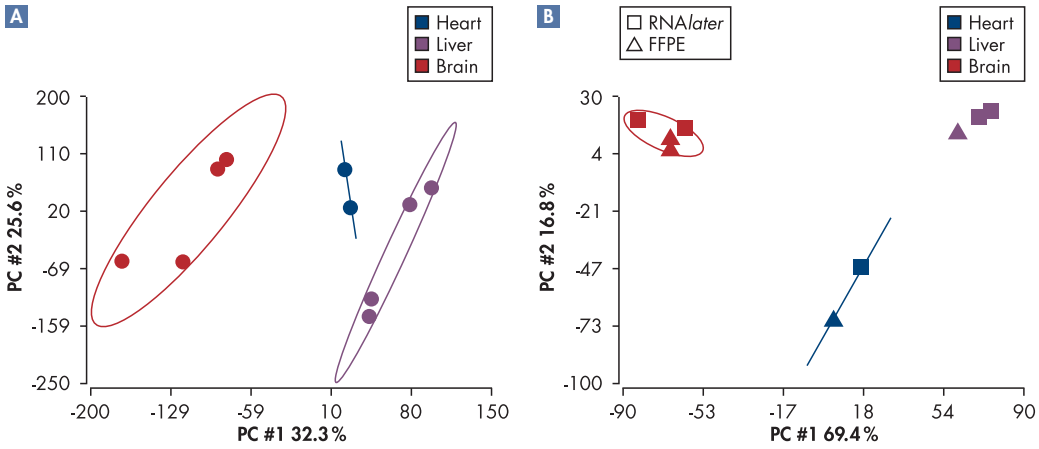

Figure 3. Preservation of the gene expression profile. PCA analysis was performed using A all genes and B 6114 genes defined by 1-way ANOVA as significant (P<0.01) in tissue stratification.

Conclusions

- The combination of the RNeasy FFPE Kit and the WT-Ovation FFPE RNA Amplification System allows the preparation of cDNA targets from FFPE tissues that deliver informative global gene expression analysis on GeneChip arrays.
- High sensitivity is achieved with RNA purified from FFPE tissues, allowing appropriate stratification of tissue samples.
- Storage of FFPE tissues for 1 year after embedding did not have a significant effect on microarray data, despite the much more fragmented state of the RNA recovered from the aged samples.

Ordering Information

Product	Contents	Cat. no.
RNeasy FFPE Kit (50)	For 50 preps	74404
RNA*later* RNA Stabilization Reagent (50 ml)	For stabilization of RNA in 25 x 200 mg tissue samples	76104

To order NuGEN's WT-Ovation FFPE RNA Amplification System (cat. no. 3400-12) and FL-Ovation cDNA Biotin Module V2 (cat. no. 4200-12), visit www.nugeninc.com or contact NuGEN customer support at custserv@nugeninc.com .

The RNeasy FFPE Kit, RNeasy Lipid Tissue Kits, and RNA*later* RNA Stabilization Reagent are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

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