Expression Analysis of FFPE Samples

TruSeq® Targeted RNA Expression effectively profiles degraded RNA.

Introduction

The TruSeq Targeted RNA Expression (TREx) assay offers an accurate and powerful method for validating the results of gene expression and RNA-Seq studies. TREx leverages proven Illumina sequencing technology and offers multiplexed gene expression profiling for 12–1,000 targets per sample and up to 384 samples in a single MiSeq® run. TREx assays are available in either pre-designed formats for a wide variety of biological pathways or as custom panels that users configure themselves. The rapid, simple, and streamlined workflow (Figure 1), coupled with on-instrument data analysis, achieves sample-to-results time of less than 2 days.

Gene expression studies of tumor samples have gained considerable interest in recent years as researchers apply Illumina high-throughput sequencing technology to analyze large banks of formalin-fixed, paraffin-embedded (FFPE) tissue samples. However, the RNA obtained from these samples is highly variable in quality, due to the age of the samples and the selected preservation techniques. This technical note examines the application of TREx technology to FFPE RNA samples and offers guidelines for obtaining high-quality results even from degraded RNA.

Variability of FFPE Samples

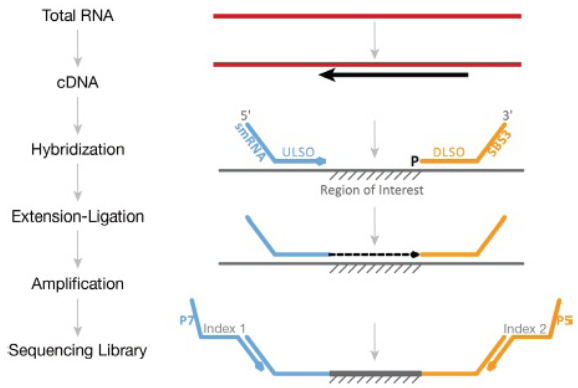
RNA isolated from FFPE samples can vary widely in quality among different specimens, or even within different samples from the same specimen. RNA undergoes substantial chemical modification when the tissue is fixed with formalin, and it is further degraded during storage[1]. Such samples can prove challenging to the methods conventionally used for expression analysis: reverse-transcription polymerase chain reaction (RT-PCR) and microarray gene expression analysis[2]. The TREx assay can yield results even with degraded RNA samples; however, highly degraded samples may contain a majority of RNA fragments that are smaller than the optimal size required for efficient hybridization of the probes. Therefore, it is important to evaluate the quality of each FFPE sample before proceeding with analysis.

Evaluating RNA Quality

Many researchers use RNA integrity number (RIN) to determine the quality of an RNA sample for gene expression analysis. However, Illumina has found that RIN is not a good predictor of the success of gene expression experiments, because the average size of RNA fragments in samples with similar RIN values can vary considerably.

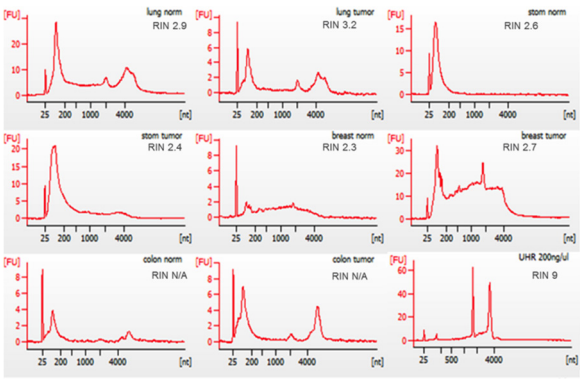
Figure 2 shows Agilent 2100 Bioanalyzer traces of RNA isolated from normal-tumor paired FFPE samples compared to the standard, Universal Human Reference (UHR) RNA. Although the RIN values for these samples were within a relatively narrow range (< 3.2), the size distribution of the RNA varied greatly among the samples. For many samples, the majority of the RNA fragments were smaller than 200 nt.

Figure 1: TruSeq Targeted RNA Expression Workflow



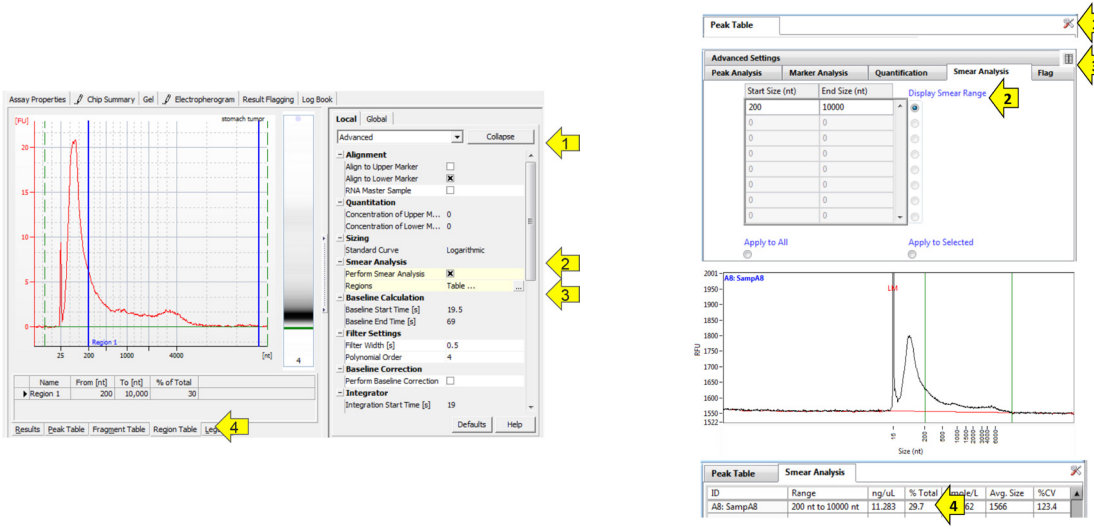
The TruSeq Targeted RNA Expression assay chemistry begins with reverse-transcribing cDNA from purified total RNA. Two custom-designed oligo-nucleotide probes hybridize to the region of interest. An extension-ligation reaction creates an amplicon that is further PCR-amplified to add indices, creating sequence-ready libraries.

Figure 2: Variability of FFPE Samples



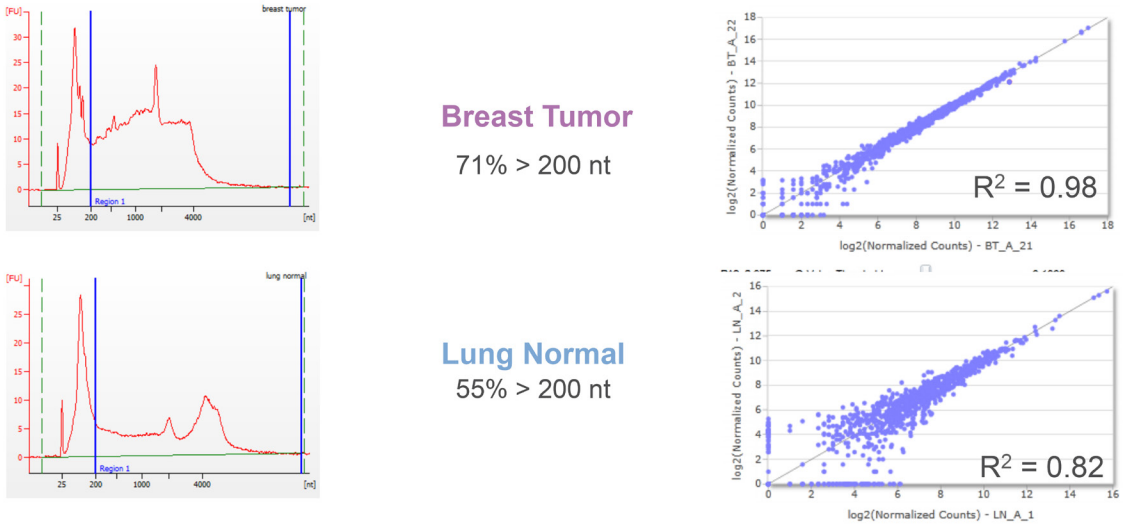
RNA isolated from FFPE samples was examined using an Agilent 2100 Bioanalyzer and the Agilent Eukaryotic Total RNA chip. RIN values were calculated from the Bioanalyzer traces.

Figure 3: Determination of RNA Quality



The percentage of RNA fragments > 200 nt (DV_{200}) can be calculated by performing a Smear Analysis as follows:
A) from the Bioanalyzer trace: 1) Under the *Local* tab, change *Normal* to *Advanced*. 2) Check box for *Smear Analysis*. 3) Click on *Table*, add a region, and enter 200–10,000 bp in the popup window. 4) Select the *Region Table* tab in the trace window to display the results.
B) from the Fragment Analyzer: 1) Click in the top right corner of the *Peak Table* window. 2) Click on the *Smear Analysis* tab and set the size range from 200-10,000 nt. 3) Click the top right corner to show results. 4) Click on the *Smear Analysis* tab to get the % Total value.

Figure 4: RNA Fragment Size Distribution Is a Reliable Predictor of Quality



Total RNA was isolated from the samples indicated and processed using the TREx assay. The DV_{200} values were calculated as described in Figure 3. Correlation analysis of normalized counts from replicates was performed for each sample.

Therefore, Illumina recommends using the percentage of RNA fragments > 200 nt (fragment distribution value; DV_{200}) as a reliable determinant of RNA quality for the TREx assay. This value can be calculated from Agilent 2100 Bioanalyzer or Advanced Analytical Technologies Fragment Analyzer readings using a Smear Analysis with a 200 nt threshold, as shown in Figure 3.

Figure 4 shows the results of replicate correlation analysis for samples with varying DV_{200} values. Excellent results ($R^2 = 0.99$) were obtained for breast FFPE tissue RNA with $DV_{200} > 77\%$. Illumina does not recommend using RNA samples with $DV_{200} < 30\%$. Table 1 gives recommended quantities of starting material for a range of DV_{200} values. This table is intended to serve as a guide. For best results, the quantity of starting material should be determined empirically for the samples being used.

Optimizing Procedures for Degraded RNA

Several parameters can be adjusted when using the TREx assay with FFPE RNA samples. These include the amount of input RNA, sample replicates, and the reverse transcription (RT) procedure.

- The standard TREx procedure requires 50 ng of input total RNA. For degraded samples, Illumina recommends increasing the total RNA input based on the DV_{200} (see Table 1).
- Assay dropouts, where the number of counts is close to zero, can occur in highly degraded FFPE samples. These dropouts can be mitigated by running samples in duplicate.
- The following modifications to the RT procedure[3] may also help to compensate for the poor quality of highly degraded FFPE RNA samples:
  a. Dilute the recommended amount of purified RNA in a volume of 3 µl.
  b. Double the concentration of ProtoScript II enzyme in the RT reaction.
  c. Add an additional 1 µl of 0.1 M dithiothreitol (DTT) to the RT reaction.
  d. Perform the reverse transcription reaction in a thermocycler as follows:

     25°C for 10 minutes

     42°C for 30 minutes

     95°C for 10 minutes

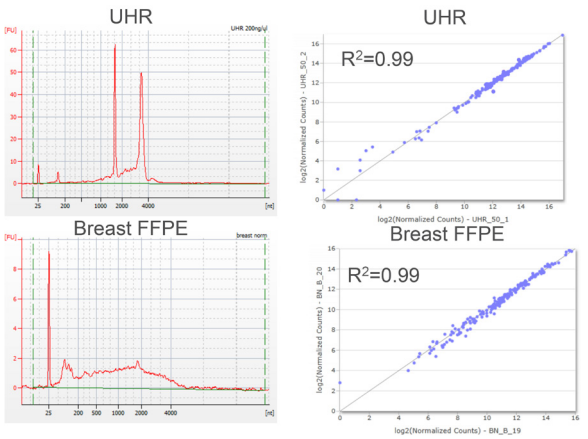
     Hold at 4°C

Using these modifications, the TREx assay can deliver high-quality results from degraded FFPE RNA (Figure 5).

Table 1: Starting Amounts of RNA

DV_{200}	Recommended Input Quantity
> 70%	200 ng
50–70%	400 ng
30–50%	> 400 ng
< 30%	Not recommended

Figure 5: TruSeq Targeted RNA Expression Results



Total RNA was isolated from a control sample (UHR) and a breast normal tissue FFPE sample, and processed using the TREx assay. Correlation analysis of normalized counts from replicates was performed for each sample.

Summary

The percentage of RNA fragments greater than 200 nt (DV_{200}), as determined by an Advanced Analytical Technologies Fragment Analyzer or an Agilent 2100 Bioanalyzer trace, is a reliable predictor for successful gene expression results using the TREx assay. Modifications to the standard RT procedure, such as those described in this technical note, can help maximize the results from degraded FFPE RNA with DV_{200} of at least 30%. Illumina also recommends increasing the amount of input RNA for these samples, as shown in Table 1.

References

1. von Ahlfen S, Missel A, Bendrat K, Schlimpberger M. (2007) Determinants of RNA quality from FFPE samples. PLoS ONE 2: e1261
2. Penland SK, Keku TO, Torrice C, He X, Krishnamurthy J, Hoadley KA et al. (2007) RNA expression analysis of formalin-fixed paraffin-embedded tumors
3. TruSeq Targeted RNA Expression Guide, Part # 15034655, Illumina Inc.

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