

truXTRAC® FFPE RNA Extraction and Purification - Quality Metrics for Clinical Applications Powered by Adaptive Focused Acoustics® (AFA®)

Abstract

FFPE RNA quality from precious clinical samples have the potential of impacting all downstream analytical applications; therefore, performing quality tests prior to NGS, RT-qPCR, and digital PCR is highly recommended when the highest confidence in the results is required (e.g., diagnostics). In this application note, we will highlight the performance of the Covaris truXTRAC FFPE tNA Plus Kit – Mag Bead as compared to a well-established competitor kit using three commonly used RNA quality metrics: Qubit 4.0 IQ Assay, DV₂₀₀, and RT-qPCR-based Functional RNA Quantitation (FRQ) Assay. The results of these assays clearly illustrate the superior quality of FFPE RNA extracted using Covaris AFA Technology and truXTRAC kits' optimized extraction and purification reagents.

Introduction

Formalin fixation and paraffin embedding (FFPE) is the most common method of preserving and archiving solid tissues. The extraction of nucleic acids from FFPE tissue samples offers the opportunity for labs to add in the precision of modern genomic tools to enable personalized diagnostics. Unfortunately, the variability in methods used for sample preservation, the slow adoption of standardized preservation protocols, and the poor performances of most current FFPE-RNA extraction protocols, limits the full potential of FFPE-RNA samples in clinical NGS.

Industry leaders in NGS have established methods to assess RNA quality, which is used as pass/fail thresholds to increase NGS-based assay performance, maximize usable sequencing output, and develop robust NGS workflows [1].

Within the context of quantitative biology, personalized medicine, and high-throughput automation, sample preparation has become even more crucial today as workflow efficiency in clinical labs are judged by the percentage of samples that fail the quality threshold tests - Quantity Not Sufficient (QNS) than on RNA yields.

One of the methods for assessing RNA degradation is the Qubit™ RNA IQ Assay Kit. This assay uses a dye that binds specifically to large, intact and/or highly structured RNAs (mRNA, tRNA, rRNA), and a second dye that selectively binds to small and/or degraded RNAs. Based on the ratio of fluorescence emitted by these two dyes, an RNA IQ number between 1 and 10 is calculated (a score of 10 being associated with higher amounts of intact extracted RNAs) [2].

The second method introduced by Illumina takes advantage of the Agilent Bioanalyzer to assess the quality of RNA based on determining the percentage of RNA fragments, which are higher than 200 nucleotides. Illumina has proven that FFPE RNA samples with a DV₂₀₀ of greater than 30% have a higher probability of success in RNA-seq [3].

Beyond the qualitative assessment of RNA quality with RNA IQ and DV₂₀₀, it is critical to assess the quantity of amplifiable RNA extracted. Downstream NGS applications contain several enzymatic reaction steps whose performance is susceptible to the input sample quality. Thermo Fisher Scientific developed the Functional RNA Quantitation (FRQ) RT-qPCR based assay, which quantifies the concentration of amplifiable RNA and correlates it to the percentage of valid mapped reads. Thermo Fisher established that samples with FRQ score of < 0.2 ng/μL are likely of poor quality and will not reliably produce good sequencing results [4]. In this application note, we will highlight the performance of the Covaris truXTRAC FFPE kit as compared to a well-established competitor kit using these three commonly used RNA quality metrics.

Materials and Methods

Required Material

Covaris

- LE220-plus Focused-ultrasonicator ([PN 500569](#))
- truXTRAC FFPE tNA Plus Kit – Mag Beads ([PN 520255](#))
- microTUBE-500 AFA Fiber Screw-Cap ([PN 520185](#))

Other Suppliers

- Qubit 4.0 Fluorometer
- Roche LightCycler® 96 Real-Time PCR Cycler (Cat. 05815916001)
- 2100 Bioanalyzer Instrument (Cat. G2939BA)
- TaqMan™ Fast Virus 1-Step Master Mix (Cat. 4444432)
- 20X TaqMan Gene Expression Assay, GUSB (Cat. 4331182)
- Promyelocytic Leukemia (HL-60) Total RNA (Cat. AM7836)
- Qubit™ RNA IQ Assay Kit (Cat. Q33221)
- Qubit™ RNA BR Assay Kit (Cat. Q10210)
- Agilent RNA 6000 Pico Kit (Cat. 5067-1513)

Methods

Tissue Handling

FFPE Blocks were stored at 4 °C upon arrival from CHTN (Cooperative Human Tissue Network, Eastern Division University of Pennsylvania). Before sectioning, excess paraffin was trimmed from the tissue blocks. One FFPE Block was used per tissue type. All scrolls were cut by utilizing a microtome on the same day and were randomized and stored in their respective extraction tubes (microTUBE-500 AFA Fiber Screw-Cap tubes or 2 mL microcentrifuge tubes) at 4 °C prior to extraction (≤ 3 days). All extractions were performed using 2 x 10 μ m scrolls per sample tube. The comparison between kits and tissues were evaluated on the same day to ensure variability between the instrumentation was minimized.

FFPE Tissue Extractions & Storage

RNA was extracted from three different FFPE tissue types and processed in triplicates (i.e., N=9, N=3 per 3 tissues) when performing the FRQ Assay and processed once for all other metrics (N=3 per tissue) using the truXTRAC FFPE tNA Plus Kit – Mag Beads, and a competitor kit. Extractions were performed per the manufacturer's instructions. DNase treatment was used during the extraction of all RNA samples to eliminate DNA contamination. RNA samples were stored at -80 °C when not in use and on ice when in use.

Data Analysis

Cq scores and the standard calibration curve (slope & y-intercept) were recovered from the LightCycler 96 SW 1.1 software after qRT-PCR was completed and transferred to a Microsoft Excel spreadsheet for analysis. FRQ scores (concentration in ng/ μ L) were calculated using the established calibration curve [4].

Results

Qubit™ RNA IQ

The RNA IQ Assay Kit was used to measure IQ score (RNA quality) on the Qubit 4.0 [2] (**Figure 1**). RNA extracted with Covaris truXTRAC tNA Plus extraction kit generated IQ scores of greater than 8 had exceptional reproducibility for each tissue type tested. RNA Extraction with Competitor Q method produced variable results and systematically lower IQ scores of less than 8, illustrating lower quality FFPE RNA extraction from the same FFPE tissue blocks.

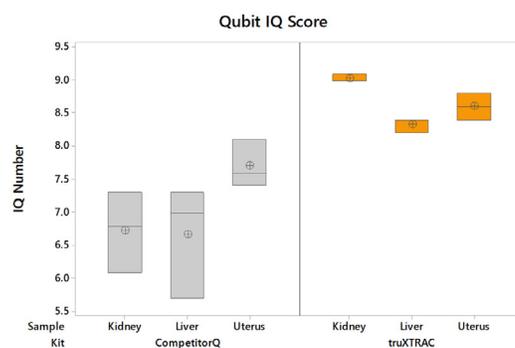


Figure 1: Qubit RNA Integrity & Quality (IQ) Score using Covaris truXTRAC tNA Plus extraction kit compared to Competitor Q's extraction kit for kidney, liver, and uterus tissues (N = 3 per tissue).

DV₂₀₀

Samples were processed on the 2100 Bioanalyzer using the RNA 6000 Pico Kit to visualize RNA profiles [3]. Smear Analysis was performed on the Bioanalyzer software (**Figure 2**). RNA extracted from the Covaris truXTRAC FFPE tNA Plus Kit – Mag Beads kit systematically obtained a DV₂₀₀ > 50%, which is far above the Illumina recommendation (30%) for all tissues and replicates tested. Meanwhile, competitor Q extracted FFPE RNA samples had consistently lower DV₂₀₀ scores with some of the replicates failing to reach the 30% DV₂₀₀ threshold.

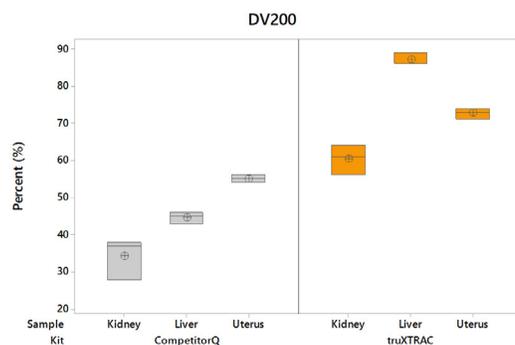


Figure 2: DV₂₀₀ scores for both Covaris truXTRAC tNA Plus extraction kit and Competitor Q's extraction kit, as measured by the Smear Analysis tool on the 2100 Bioanalyzer (200 to ~17,000 nt) for kidney, liver and uterus tissues (N=3 per tissue).

FRQ

The FRQ assay was performed following the Ion Torrent (Thermo Fisher) Application Note [4]. A Roche LightCycler was used for qRT-PCR. Calculated FRQ scores were based on a calibration curve ranging from 0.02 ng/μL to 50 ng/μL (**Figure 3**). All Covaris extracted samples displayed more than double the quality acceptance criteria of 0.2 ng/μL, both with high confidence and quality sequencing results. Competitor Q extracted samples were barely above the acceptance criteria with low confidence and quality sequencing results.

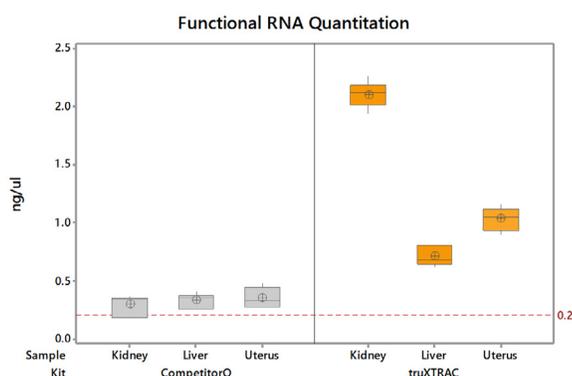


Figure 3: Functional RNA Quantitation (FRQ) Score using Covaris truXTRAC tNA Plus extraction kit compared to Competitor Q's extraction kit for kidney, liver and uterus tissues. Samples falling below the red dashed line (FRQ score of 0.2 ng/μl) are considered to be poor quality and likely to produce poor sequencing results [1].

Conclusions

The RNA extracted using Covaris AFA technology and the Covaris truXTRAC FFPE tNA Plus Kit – Mag Beads kit outperformed Competitor Q in each of the three independent quality metrics tested:

- Superior RNA integrity and functionality exhibited by the Functional RNA Quantitation Assay
- Superior quality of extracted RNA from FFPE samples shown by electrophoresis (Bioanalyzer 2100)
- Superior performance in the extraction of large, intact RNA fragments using a fluorescent measurement (Qubit IQ Assay)

Additionally, this data demonstrates that the Covaris AFA technology enables a realistic use of FFPE samples in diagnostics while previously demonstrating the scaling-up capabilities of our solutions with ease [5].

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