

High-throughput RNA-Seq of Low Input and Clinically Derived FFPE Samples using Covaris Adaptive Focused Acoustics® (AFA®) Technology

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Background

Transcriptomic studies using next-generation (NGS)-based RNA sequencing (RNA-Seq) have significantly advanced molecular profiling for solid tumors obtained from tumor resection, biopsy, or cytological specimens that have been formalin-fixed, paraffin-embedded (FFPE) [1]. Similarly, researchers have focused on examining complex organs and tissues at single-cell resolution and capturing nascent RNA in RNA-Seq assays to uncover coordinated global transcription across the genome [2]. The presence of numerous variants of uncertain clinical significance and the challenges in interpreting noncoding variants are significant gaps in genomic analysis. These gaps can be addressed by providing functional evidence through RNA sequencing assays, which complement genomic data.

RNA-Seq data not only reveal gene expression levels but also provide insight into RNA processing patterns and allele-specific expression. However, due to the variable quality and quantity of RNA obtained from FFPE and single-cell samples, the library preparation process often presents challenges and requires significant improvements. To address these challenges, Covaris Adaptive Focused Acoustics (AFA) Technology offers a flexible solution for customers with varying throughput, batch, and automation needs. This technology enables the routine implementation of large-scale RNA sequencing for degraded samples by using a novel method of RNA fragmentation combined with reduced sample volumes for library preparation.

Challenges in RNA Quality and Fragmentation in FFPE Samples

There are several challenges associated with nucleic acid quality and fragmentation in FFPE samples. These hurdles arise from the nature of FFPE samples, and the various steps involved in the FFPE process and subsequent library prep. Some common challenges include:

- **Fragmentation:** Formalin fixation may cause fragmentation of RNA, leading to shorter fragment lengths. These shorter fragments can impact downstream applications, such as sequencing, resulting in reduced amplification efficiency or sequence coverage.
- **Low Yield:** FFPE samples typically yield lower amounts of nucleic acids compared to fresh or frozen tissue samples. The fixation and embedding processes can result in loss, making it challenging to obtain sufficient RNA for fragmentation and downstream library prep.
- **Cross-linking:** Formalin fixation can induce cross-links between DNA and proteins or other molecules, making it difficult to extract intact RNA from FFPE samples.
- **Contaminants and Impurities:** FFPE samples may contain contaminants, such as formalin residues, paraffin, or other chemicals used during the preservation process. These contaminants can affect RNA purity and interfere with downstream analysis, including cDNA conversion and PCR amplification.

- **Heterogeneity:** FFPE samples can exhibit tissue heterogeneity, where different regions of the tissue may undergo varying degrees of fixation and preservation. This heterogeneity can lead to variations in quality and yield within a single FFPE sample, potentially affecting the reliability and reproducibility of results.

Why Choose Covaris for RNA Shearing?

One promising solution to these challenges is the use of Adaptive Focused Acoustics (AFA) technology, available through Covaris Focused-ultrasonicators. This technique utilizes focused ultrasound to shear RNA with precise bursts of high-frequency acoustic energy, which can be used to process samples of varying quality.

Additionally, this workflow offers a robust and seamless solution for both manual and automated library preparation, ensuring reliable sequencing results. By employing AFA-based sample preparation and integrated shearing, this approach allows for the comprehensive characterization of the entire transcriptome, or a significant portion of it, in a single experiment.

The Advantages of AFA-based RNA Shearing:

- Covaris shearing is agnostic to sample type and quality, including highly degraded samples, and shows minimal risk of over-fragmentation.
- Integrates into a broad spectrum of RNA-Seq protocols with no observed trade-off to data fidelity.
- Provides high-throughput preparation of FFPE embedded tissue samples with significant reduction in hands-on time.
- Covaris shearing is suitable for low-input RNA (≤ 2 ng) and supports a wide range of input volumes and concentrations, providing reliable sequencing data.

Novel RNA-Seq Workflow Using AFA-based RNA Shearing

To assess the quality control metrics of AFA-based RNA shearing, RNA libraries were prepared from UHR RNA aliquots degraded to varying levels (RIN range 1-9) using heat, as well as from 69 'real-world' FFPE samples, as described by Moiso et al [3]. Briefly, 100 ng of RNA at 10 ng/ μ L from liver and uterus tumor tissue was sonicated using a pulsing protocol according to the Covaris R230 Quick Guide (PN 010528) [4], following the specific settings and sample volumes outlined in **Table 1**. The generalized RNA-Seq workflow is illustrated in **Figure 1A**. After sonication and library preparation, fragment sizes were confirmed using gel electrophoresis (**Figure 1B**).

Libraries were prepared using RNA fragmented by three different methods: Cation-based chemical processing (Manual), Covaris with heat pre-treatment at room temperature (Covaris R230 RT) and Covaris with heat pre-treatment at 70 °C (Covaris R230 70 °C). As shown by Pearson correlation analysis, no biases were observed between replicates using Covaris R230-prepared samples at room temperature and at 70 °C (**Figure 1B**). Additionally, libraries prepared with a minimum of 2 ng of sonicated RNA input were highly homogenous and well correlated (Pearson > 0.99).

A key sequencing metric to assess RNA-Seq quality is the average transcript length. Covaris sheared libraries showed equivalent average transcript length (Mean Log2 TPMs) compared to the manual method. RNA-Seq data (**Figure 1C**) clearly demonstrated that the 5' and 3' UTR coverage was similar or better to standard RNA-Seq library prep methodologies, with robustness across a range of GC content and no evident bias against any RNA species (e.g. lncRNA, protein coding genes, etc., unpublished data).

In summary, Covaris AFA technology is agnostic to sample quality, making it ideal for processing RNA from FFPE and low-input samples. This facilitates easier and more cost-effective analysis of large numbers of FFPE samples, significantly reducing hands-on time.

Table 1. RNA from 69 FFPE tumor tissues of unknown origin sheared on the R230 using the 96 AFA-TUBE TPX Plate with SonoLab 10.0.1. A time course is used to determine the optimal fragment sizes as needed for the library prep. The shearing buffer and sample volumes are used as specified in the table.

Sample Type	Instrument	Sample Volume	Sample Buffer	Fragment size (nt)	Vessel	Plate Definition
RNA from FFPE & UHR	R230 Focused-ultrasonicator	10 μ L	10 mM Tris pH 8.0	300	PN 520291	R230_520291 96 AFA-TUBE TPX plate +0.5 offset

Temp.	PIP	Duty Factor	Cycles per Burst	Dithering	Total Treatment Time	Delay Time	Repeats
10 °C	200 W	25%	50	3 mm Y at 20 mm/s	60 seconds	10 seconds	6

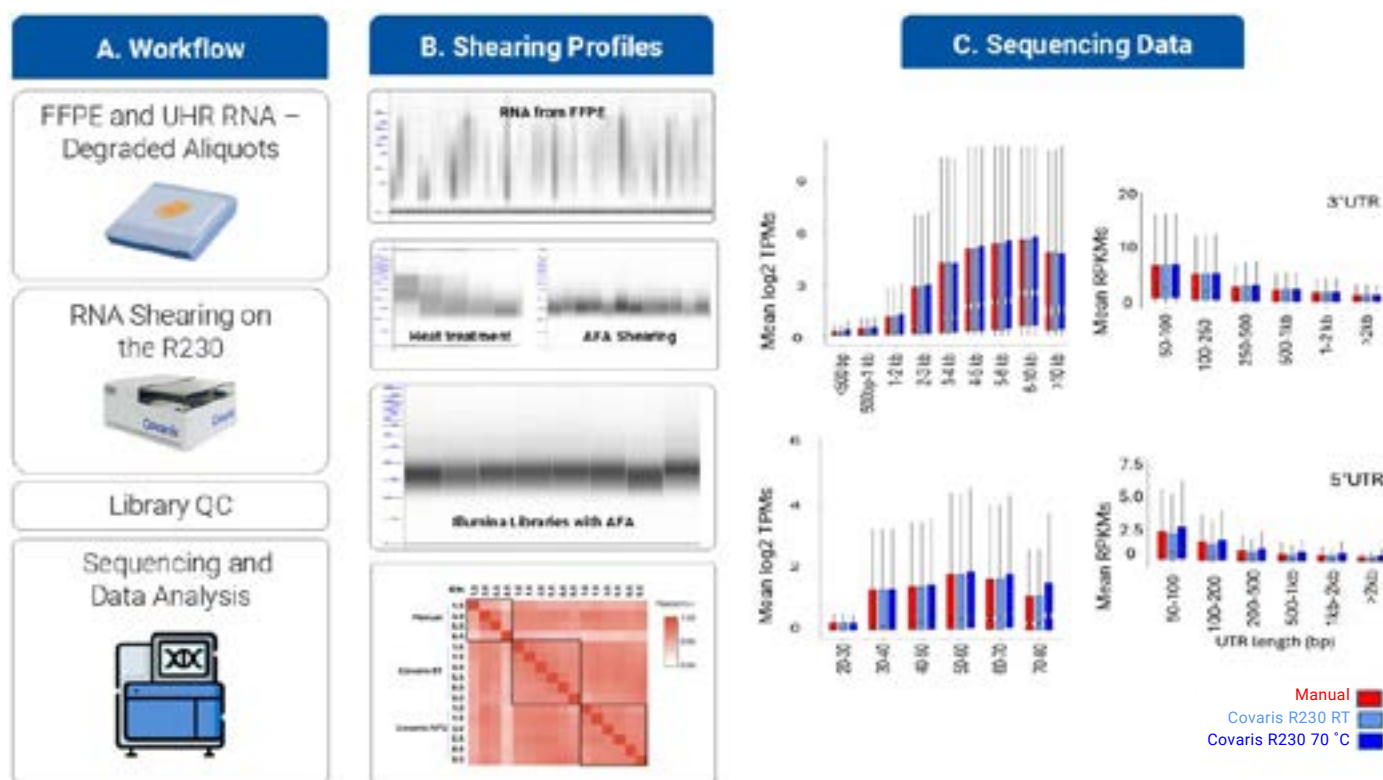


Figure 1. A novel RNA-Seq workflow using Covaris AFA-based RNA fragmentation. *Panel A* shows a generalized RNA-Seq workflow depicting various steps involved, from the source of samples to sequencing. *Panel B* shows the pre- and post-shearing (heat and AFA based) of RNA from FFPE plus library prep profiles analyzed by gel-electrophoresis; the Pearson correlation shows the high consistency among the replicates using Covaris shearing. *Panel C* shows the sequencing data bar graphs for the average transcript length and UTR coverage.

Conclusion

Covaris and the MIT BioMicro center have developed a novel approach for preparing RNA-Seq libraries from degraded FFPE samples using Adaptive Focused Acoustics (AFA) for RNA sonication to standardize fragment sizes. Shearing RNA using the Covaris R230 enables high-throughput, unbiased quantification of transcripts, whether degraded or intact, without being limited to specific species. This versatile technique can be used upstream of standard ribosomal depletion-based RNA-Seq library preparation. In contrast, other methods, such as divalent cation-mediated RNA degradation (e.g. Mg²⁺ [5] and zinc-mediated RNA cleavage [6]), rely on hydrolysis, resulting in a mix of 2' or 3' phosphate ends on mononucleotides.

Expanding chemical-based fragmentation to accommodate a wide array of real world samples requires significant time on a sample-by-sample basis or the use of probes to capture only a subset of the transcriptome. Our results demonstrate that Covaris AFA-based RNA shearing provides equivalent performance to cation-based fragmentation, without the need for sample-specific tuning. This makes Covaris shearing compatible with low-input RNA-Seq and high throughput workflows, offering less hands-on time, faster turnaround, and high-quality sequencing data.

References

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