Mechanical Fragmentation of RNA using the Covaris Adaptive Focused Acoustics® (AFA®) Technology for Development of mRNA-based Vaccine and Gene Therapy Platforms

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Introduction

The emergence of Next-Generation Sequencing (NGS) technologies has greatly contributed to targeted gene discovery for the generation of new mRNA-based vaccines and therapeutics [1,2]. With the advancement in the world of NGS technologies, RNA sequencing (RNA-Seq) has rapidly become the method of choice for analyzing the transcriptomes of disease states [3], of biological processes, and across a wide range of clinical study designs. Synthetic mRNA has been considered an emerging biotherapeutic agent for decades [4]. However, the outbreak of the COVID-19 pandemic promoted the application of mRNA technologies in development of SARS-CoV-2 vaccines, and there has been a huge increase in interest in the research and development of mRNA-based vaccines [5]. Despite the tremendous improvement in RNA-based NGS technologies, the mRNA-based sequencing methods still present some unique challenges in production, characterization, and quality controls, especially when compared to their traditional protein-based counterparts. One critical challenge is obtaining products that are of homogeneous sequence. In this regard, NGS has proved to be a key tool to probe and address this challenge. This study, conducted in collaboration with GreenLight Biosciences, presents a mechanical mRNA fragmentation methodology that uses Covaris' Adaptive Focused Acoustics (AFA) Technology to generate the right-sized population pivotal for successful Illumina® library preparation and reproducible sequencing data.

Material & Methods

mRNA Sample(s)

- A synthetic messenger RNA (mRNA) sample of 1929 nt was obtained from TriLink® CA, USA

mRNA Shearing with AFA Technology

Physical shearing of mRNA was tested on the Covaris R230 Focused-ultrasonicator using AFA. AFA employs highly controlled bursts of focused high-frequency acoustic energy to efficiently and reproducibly process samples in a temperature-controlled and non-contact environment. mRNA samples were added to each well of the 96 AFA-TUBE TPX Plate (PN 520291)-placed in the R230 (PN 500620). The samples were treated using a pulsing protocol as per the Covaris R230 Quick Guide [6] following the specific settings and sample volume as specified in ***Table 1***.

Table 1. mRNA shearing on the R230 using 96 AFA-TUBE TPX plate with Sonolab 10.0.1. A time course was used to determine the optimal AFA settings for the 700 nt and 500 nt, shearing buffer and sample volumes as specified in the table.

RNA Sample	mRNA	
Instrument	R230 (PN 500620)	
Sample Volume	10 µL	
Shearing Buffer	10 mM Tris pH 8.0	
Fragment Size (nt)	700	500
Consumable	96 AFA-TUBE TPX Plate (PN 520291)	
Plate Definition	"R230_520291 96 AFA-TUBE TPX Plate +0.5 offset"	
Temperature (°C)	10	
PIP (W)	200	
Duty Factor (%)	25	30
Cycles per Burst (CPB)	50	
Dithering	3 mm y-dither at 20 mm/s	
Time (s)	10	
Delay Time (s)	10	
Repeats	6	7

mRNA Quality Assessment

After sonication, samples were analyzed by RNA electrophoresis on TapeStation® 4200 (Agilent®, CA, USA) using High Sensitivity RNA ScreenTape (prod. 5067-5576) and RNA Ladder (prod. 5067-5578) to measure the mRNA quality and fragment sizes.

KAPA RNA HyperPrep Library Preparation

mRNA samples fragmented by AFA (via mechanical fragmentation) were used as input into the KAPA RNA HyperPrep library preparation kit (KAPA Biosystems, MA, USA). Library preparation was carried out according to the manufacturer's instructions including a control sample of unfragmented mRNA (chemical fragmentation). The only modification for the AFA-treated sample was a 1-minute incubation time prior to first strand synthesis to avoid additional or further fragmentation. Five (5) µL of a unique 7 µM KAPA Dual-indexed adapter were used for each 100 ng starting input samples and 5 µL of 1.5 µM adapter for each 50 ng starting input samples at the ligation step. The final libraries were purified by magnetic beads and eluted into 10 mM Tris HCl, pH 8.0 and quantified using the TapeStation 4200.

Library Quality Controls

The quality of the libraries was assessed by DNA electrophoresis on the TapeStation 4150 using D1000 ScreenTape and DNA Ladder (Agilent, prod. 5067-5582 and 5067-5586, respectively) for size calibration. Libraries were diluted in nuclease-free water and quantified using a Qubit 4 fluorometer and a dsDNA HS Assay Kit (Thermo Fisher Scientific®, MA, USA).

NGS Sequencing

After library normalization and pooling, sequencing was performed on a MiSeq™ instrument (Illumina, CA, USA) using a MiSeq v2 Reagent Kit (Illumina, prod. MS-102-2002) paired-end 2 × 151 bp sequencing parameters and 10% of PhiX control (Illumina, prod. FC-110-3001). Data was analyzed using Geneious Prime software (version 2022.2.2).

Results

mRNA Fragmentations and Size Distribution

In this study, we successfully implemented Covaris AFA Technology to generate the tight fragment distribution (500 and 700 nt) using the settings specified in ***Table 1***. AFA offers tunable mRNA fragmentation, as shown in the representative sample of pre- and post-AFA fragmentation in ***Figure 1***. Further, as a proof of concept to monitor successful mRNA shearing and library construction, a total of 34 mRNA samples were sheared using the sample volume and AFA settings listed in ***Table 1***. The average sheared RNA fragment length (500 and 700 nt) met the KAPA library prep specifications for successful library prep with Illumina sequencing.

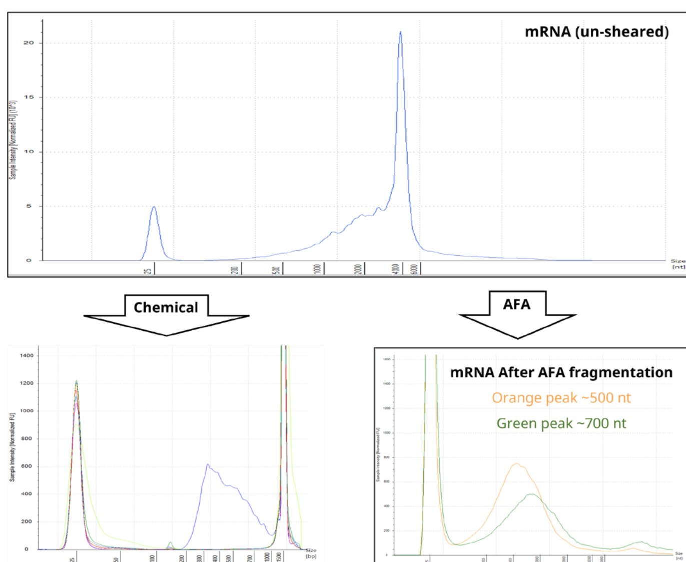


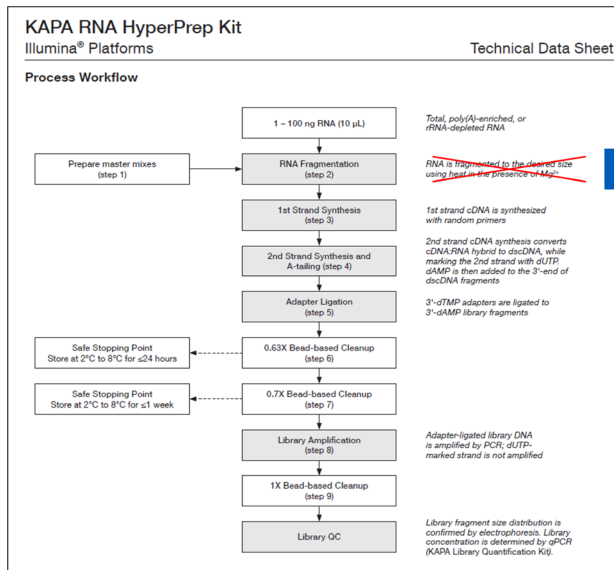
Figure 1. mRNA fragment size distributions demonstrating fragmentation tunability. RNA electropherogram prior to AFA treatment is shown on the top and the bottom electropherograms are with after chemical fragmentation (left) and AFA treatment (right) using time course.

KAPA RNA HyperPrep Library Workflow

The R230 ensures routine implementation of high-throughput mRNA fragmentation in reduced volume for further downstream library preparation. The chemical fragmentation step in the KAPA RNA HyperPrep protocol was replaced with the mechanical shearing using Covaris AFA technology (***Figure 2***). Once desired fragment size was achieved, subsequent steps in the library construction protocol were followed as per the manufacturer's instructions, while ensuring priming of the oligo for first strand synthesis (***Figure 2***). The final library size distributions were confirmed to the desired insert length as per the sequencing requirement and downstream applications. The key features and benefits of the AFA technology for the RNA library prep workflow can be summarized as:

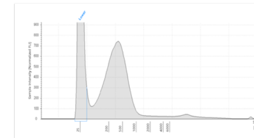
- Accommodates a wide range of sample throughput (1-384 samples), from 1-8 sample batch processing to high throughput (96/384 well plate).
- Highly accurate and precise/tunable fragmentation.
- Versatile technology to shear in vitro transcription (IVT) mRNA.
- Shearing process under controlled temperatures provides highest yields while preserving RNA sample integrity.
- Non-contact technology, hence, no need for additional clean-up protocols.
- Generates tight fragment distribution (200–1000 nt) that is pivotal for successful downstream library preparation.
- Ensures a fast turnaround time (30–45 minutes of hands-on time per library prep) by minimizing the thermocycler time to carry out fragmentation. In addition, no additional clean-up steps are required in the post-fragmentation phase.
- Less hands-on time with minimized pipetting steps.
- Better sequencing quality with reliable and reproducible data to quantify rare and fusion events in RNA sequencing.

KAPA RNA HyperPrep Library



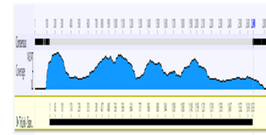
RNA Fragmentation/Physical
(Covaris AFA Technology)

Fragmentation Profiles



- tight fragmentation
- tunable size(s)
- accurate and precise fragmentation
- temperature controlled
- fast TAT

Sequencing Data

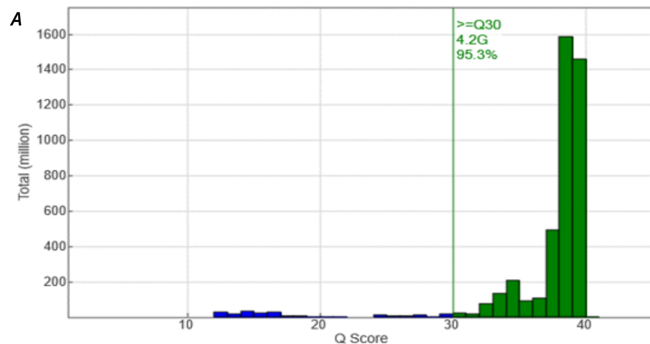


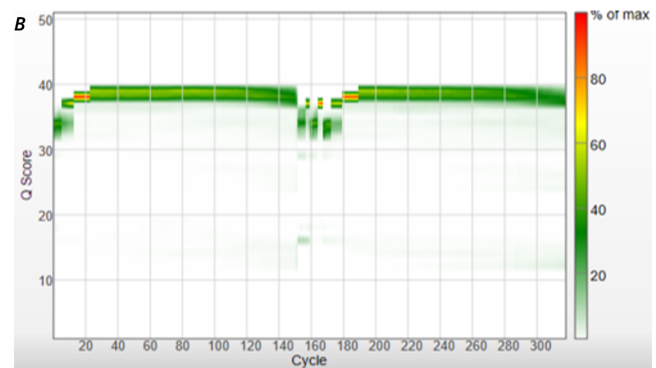
- better sequencing quality (≥99.8% mapped reads)
- extended mean coverage at contig level
- reproducible sequencing data

Figure 2. KAPA RNA HyperPrep library construction workflow. The box on the left depicts the process workflow from the manufacturer's manual, where the chemical fragmentation step (red cross) was substituted by AFA (blue arrow). On the right, in the center, an example of an mRNA fragmentation profile was verified on TapeStation and below, the reference coverage results after MiSeq sequencing.

NGS Sequencing Metrics

MiSeq sequencing metrics were optimal with 738 ± 30 K/mm^2 cluster density, 96.18 ± 22% of clusters passing the filters, 12.10% of sequences aligned to PhiX template and sequences with ≥ Q30 of 95.3% (***Figure 3***). Out of the total 14.43 million reads, 13.88 million reads passed the filters, of which 85.9% were identified (i.e., reads that were not PhiX control). These reads were evenly distributed among the i5 and i7 index combinations used in the run. We compared coverage metrics and overall, the sequencing data appeared to be equal in terms of coverage and better in terms of sequencing quality and reproducibility compared to chemical fragmentation method.





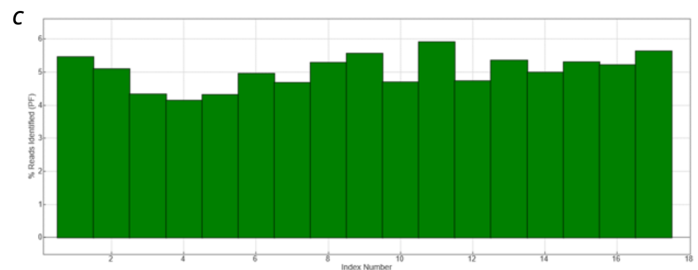


Figure 3. MiSeq sequencing analysis. Q Score distribution (***A***) and Q Score heatmap (***B***) plots. Panel (***C***) shows the percentage distribution of reads from both chemical and AFA based libraries for the various indexes used in the run.

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Discussion

Challenges in RNA Shearing

Challenges involved in reducing sample to sample variability while ensuring integrity of the RNA renders additional complexities to developing high-throughput RNA-seq methodology [7] using chemical fragmentation. This is true for a broad variety of routine chemistries including RNA-seq or depletion of ribosomal RNA (rRNA) workflows. Each of these workflows requires significant investment of time and resources to standardize the length of the RNAs that are being analyzed to a constant size through treatment with Mg++ and heat, for every sample. RNA fragmentation times can vary depending on the initial quality of the RNA and choice of fragmentation workflows. These can introduce unwanted variability and eventually, exerts a negative impact on the quality of data. Covaris' AFA-based RNA shearing offers every laboratory a robust, reliable, reproducible workflow for RNA shearing exhibiting better performance compared to cation fragmentation, regardless of the initial quality of RNA.

In summary, the R230 showcases a wealth of benefits of the AFA technology resulting in robust, reproducible RNA fragmentation, which is also agnostic to the downstream library preparation protocol. Further, the sequencing data reported in this study demonstrate high-quality libraries with sequence homogeneity produced with the Covaris-fragmented samples. Last but not the least, the wide range of scalability exhibited by the Covaris class-leading AFA technology ensures easy compatibility to every RNA shearing workflow regardless of their throughput requirements.

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