Tumor Tissue Evaluation with the Illumina® TruSight™ Oncology 500 (NGS) Assay using the Covaris R230 Focused-ultrasonicator for Clinical Research Studies

Authors: Dr. Angela Garding (EMEA Field Application Manager)[1], Dr. Akshay Dhingra (Field Applications Scientist)[1], BASc. Erwin van der Biezen (Senior Tech. of Dept. Moleular Pathology)[2], Dr.ir. Wendy de Leng (Clinical Molecular Biologist in Pathology and Head of Dept. Molecular Pathology)[2], and Dr. Anne Jansen (Clinical Molecular Biologist in Pathology)[2]

Affiliations: 1- Covaris, LLC, Woburn, MA 01801 and 2 - UMC Utrecht, Netherlands

Abstract

Next-generation sequencing (NGS) library preparation workflows comprise DNA fragmentation as an essential step. Covaris' Adaptive Focused Acoustics® (AFA®) Technology enables precise and high-quality data from NGS workflows by ensuring efficient, reliable, and reproducible nucleic acid fragmentation. Efficient DNA shearing is particularly important when working with precious clinical research samples for Comprehensive Cancer Profiling (CCP) using pan-cancer NGS assays such as Illumina's TruSight Oncology 500 (TSO 500) panel. The DNA fragmentation protocol for the TSO 500 panel was initially released for the Covaris Focused-ultrasonicators ME220, E220*evolution*, LE220, and ML230 (1,2).

In this application note, we present a DNA fragmentation protocol with the R230 Focused-ultrasonicator using 96 microTUBE plates to achieve the optimal fragment size for the TSO 500 panel. A comparative sequencing analysis between samples sheared with the Illumina-validated protocol on the LE220 focused-ultrasonicator and those sheared using the Covaris-developed protocol on the R230 showed equivalent or better results for samples sheared on the R230, while decreasing the sample processing time by ~30%. The results in this application note highlight that the automatable R230 enables efficient, high-throughput workflows with Covaris AFA Technology resulting in the robust, reproducible, and confident fragmentation needed for comprehensive pan-cancer panels such as Illumina´s TruSight Oncology 500 assay.

Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue specimens provide a great option for preserving precious samples for cancer research and clinical stratification. However, identifying genuine variations in DNA or RNA obtained from tissues fixed in FFPE using Next-Generation Sequencing (NGS) can be difficult due to existing fixation protocols and nucleic acid degradation. Targeted sequencing approaches allow the study of a defined set of biomarkers monitoring a specific set of genes or genomic regions based on prior knowledge. Fixed panels and custom amplicon solutions, such as Illumina's TSO 500 assay, are provided by an array of companies, allowing researchers to select the specific amplicons of interest to assist in targeted cancer sequencing studies from FFPE samples (1).

The TSO 500 assay (enrichment-based) converts DNA & RNA from FFPE tissue samples into libraries that can be sequenced on Illumina sequencing platforms. The genomic DNA (gDNA) sheared to optimized fragment sizes in the TSO workflow offers a high-fidelity determination of DNA variants across 523 cancer-relevant genes and cost-effectively screens tumor mutational burden (TMB), microsatellite instability (MSI), single nucleotide variations (SNVs), indels, copy-number/structural variations, and gene fusions. All these benefits make the TSO 500 assay an effective alternative to whole genome sequencing (WGS) for identifying clinically relevant variations while saving money, time, and valuable tissue samples. Some cancer research centers use the TSO 500 assay as a routine part of precision oncology. (1)

The success of the subsequent steps in the NGS library preparation workflow depends on the size distribution of the fragmented DNA, particularly when using nucleic acids isolated from FFPE tissue samples. For unbiased, consistent, and reproducible representation of genomic regions, Illumina recommends mechanical fragmentation of DNA for the TSO 500 assay. Covaris' Adaptive Focused Acoustics (AFA) Technology enables robust, reliable mechanical DNA fragmentation, independent of DNA concentration and pre-fragmentation steps. To this end, several Covaris instruments (ME220, E220*evolution* and LE220) have been validated for the TSO 500 workflow by Illumina (1). Recently, we validated the ML230 Focused-ultrasonicator in a collaboration with Mount Sinai Hospital, Ontario, Canada, demonstrating that the ML230 provides an ideal solution for mid-throughput, parallel 8-sample processing for the TSO 500 assay (2).

The advanced technology resulting in high-quality data encourages cancer research laboratories to develop capabilities to address more samples. Hence, for processing high-throughput samples, we present here a case study demonstrating DNA fragmentation for the TSO 500 assay on the high-throughput R230 Focused-ultrasonicator. Using a 96 microTUBE Plate, we reproducibly sheared FFPE extracted DNA to a target DNA fragment distribution of 90–250 bp, showing a similar post-sequencing output as observed for samples processed on the LE220 Focused-ultrasonicator.

Material & Methods

Materials

- Covaris R230 Focused-ultrasonicator (PN 500620)
- 96 microTUBE Plate (PN 520078)
- PSU Rack R230 TPX Plate & 130 Plate (PN 500750)
- Plate Definition for R230: R230_500750 PSU Rack 96 microTUBE Plate +0.5 offset
- Covaris LE220 Focused-ultrasonicator (PN 500219)
- 8 microTUBE Strip (PN 520053)
- Rack 12 place 8 microTUBE Strip (PN 500191)
- Plate Definition for LE220: LE220_500191 Rack 8 microTUBE Strip -4 mm offset
- Agilent® 2100 Bioanalyzer (PN G2938C)
- Agilent Bioanalyzer High Sensitivity DNA Kit (PN 5067-4626)
- Formalin-fixed paraffin-embedded tissue (FFPE) samples (various tumor tissue types) extracted using a commercially available FFPE DNA extraction kit
- TEB (TE Buffer)
- TruSight Oncology 500 Kit (PN 20028214)
- Illumina NextSeq 500/550 High Output Kit v2.5 (300 Cycles) (PN 20024908)
- NextSeq sequencing platform (PN 20028214)

Methods

Sample Source: One of the key criteria for preparing a robust and reliable library lies in the ability to efficiently shear DNA that is isolated from a variety of different tissue types to the desired fragment size distribution. To probe the robustness of this step, DNA isolated from 32 FFPE samples representing five different preserved tumor tissues were used for this study (***Table 1***). In total, 8 samples were included for each condition i.e., LE220, R230_16, R230_18, and R230_20. The tumor portion of each sample was manually scraped from multiple 4 µm thick slides of FFPE. The number of slides was dependent on the size of the tumor, aiming to extract DNA from at least 1–2 cm^2 of tumor tissue.

Sample Preparation: The extraction of DNA from different FFPE tissue specimens was performed using a commercially available FFPE DNA extraction kit with the manufacturer's instructions. FFPE extracted and purified DNA was diluted with TE Buffer to a final volume of 52 µL, containing total 80 ng (1.5 ng/µL DNA) of DNA. The DNA samples were then transferred to the Covaris Strips/Plates, i.e., 8-microTUBE Strip for shearing on LE220 and 96-microTUBE Plate for shearing on R230. Following the requirements indicated in the TSO 500 assay, appropriate AFA parameters were used on the LE220 and R230 to fragment DNA to a 90–250 bp fragment (***Table 2***). The DNA fragmentation settings for the LE220 were taken from the Illumina TSO 500 reference guide (1). On the R230, settings were taken from the Covaris R230 Quick Guide (3), where three different iterations (16, 18, & 20) were tested (***Table 2***).

Table 1. A total of 32 samples representing 5 different types of tumor tissues were tested in this validation study. *Represents number of iterations on R230.

	Number of Samples			
Tumor Tissue Type	**LE220**	**R230_16***	**R230_18***	**R230_20***
Colon	1	1	1	1
Lymph node	1	1	1	1
Thyroid	1	1	1	1
Ovarian	2	2	2	2
Brain	3	3	3	3

Table 2. Covaris AFA treatment settings used on the LE220 and R230 Focused-ultrasonicator to fragment FFPE extracted DNA (90–250 bp) for Illumina's TruSight Oncology 500 assay.

TSO 500 Settings on Covaris AFA Instrument		
Instrument	LE220	R230
Consumable	8 microTUBE Strip (PN 520053)	96 microTUBE Plate (PN 520078)
Number of Samples	8	8 (each set of Iterations)
Sample Volume (µL)	52	52
Temperature (°C)	7	10
Analytical System	Agilent Bioanalyzer High Sensitivity DNA Kit	
Repeat/Iterations (#)	N/A	16, 18, 20
Repeat Process Treatment Duration (sec)	N/A	10
Delay Duration (sec)	N/A	10
Peak Incident Power (W)	450	450
Duty Factor (%)	30	25
Cycles per Burst (#)	200	600
Dithering	N/A	1.5 mm Y at 10 mm/s
Time Per Sample (sec)	250	160, 180, 200

Sequencing and Analysis: Two DNA libraries prepared from DNA extracted from brain tissue and sheared on the LE220 and R230 (18 iterations), respectively, were sequenced on the NextSeq 500 with the Illumina NextSeq 500/550 High Output Kit v2.5 (300 Cycles) (20024908). All consumables and reagents were provided in the TruSight Oncology 500 DNA kit and were used on the NextSeq sequencing platform (Illumina, PN 20028214). Sequencing data was analyzed with an online platform called 'Franklin' by Genoox (4).

Results

DNA Fragment Size Distributions

DNA fragment size distribution was determined on the Agilent 2100 Bioanalyzer using the Agilent Bioanalyzer High Sensitivity DNA Kit (PN 5067-4626). All 32 samples were sheared to the desired fragment length of 90–250 bp (***Figure 1***). The average sheared DNA fragment length met the specifications indicated in the TruSight Oncology 500 assay.

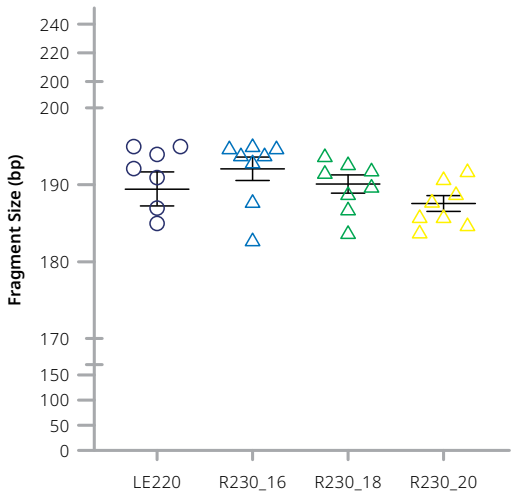

Figure 1. The distribution of average fragment size of samples sheared on LE220 and R230 (16, 18, 20 Iterations) met Illumina TSO 500 specifications. Each of the eight samples were sheared on the Covaris LE220 (circles) or R230 (triangles). For samples sheared on R230, the numbers following "R230" in the sample name represent the number of iterations (***Table 2***). The average fragment size distribution is plotted on the Y-axis with each data point representing one sample. The average of the processed samples is represented by a horizontal line, and the error bars reflect the standard error of the mean (SEM).

As can be observed in ***Figure 1***, the fragment size distribution for R230_18 was found to be comparable to that of LE220. This particular sample (extracted from brain tumor tissue) was subjected to sequencing for downstream confirmation of comparable performance.

Sequencing Metrics

The sequencing data was analysed on an online AI-based platform called Franklin by Genoox, which provides an automated workflow from the uploading of raw sequencing data (FASTQ files) to the generation of a shortlist of candidate variants to the report of coverage statistics. We compared several relevant coverage metrics in ***Table 3***, indicating slightly improved results for the sample sheared on the R230 as compared to the LE220.

Table 3. Sample metrics generated by Franklin by Genoox for samples sheared on LE220 and R230 (18 iterations).

Sample Metrics	LE220	R230_18
Average variant depth*	668	707
Percent of target covered (Depth >= 50)**	98%	98.10%
Median kit coverage***	1,034	1,108
Total reads (UMI collapsed)****	31,738,106	34,657,614

*Average Variant Depth is average depth off all variants found in the sample.
**Percent of target covered (Depth >= 50) is the percentage of the kit coverage that has a coverage of more than 50.
***Median Kit coverage is the median coverage of the TSO 500 targets (UMI collapsed).
****Total reads (UMI collapsed) are the total amount of unique reads.

Conclusion

Comprehensive pan-cancer panels such as the TSO 500 assay enable confident detection of relevant biomarkers such as TMB, MSI and HRD. Reliable and reproducible fragmentation of DNA from a variety of sample sources is key for the accuracy of such clinical research workflows. Covaris Focused-ultrasonicators enabled by AFA Technology offer unbiased, repeatable, and precise DNA fragmentation essential for Comprehensive Genomic Profiling (CGP).

Results reported in this study demonstrate and validate the Covaris R230 instrument (5) for the TSO 500 assay. The R230 enables high-throughput parallel processing of up to 96 samples and can be integrated into automated liquid handlers. As shown in the results, the protocol on the

R230 with 18 iterations was found to be significantly faster, resulting in a ~30% reduction in processing time.

Covaris instruments can support a wide range of sample throughput requirements, from 1-8 sample batch processing (ME220, E220evolution [1]) or parallel processing of 8 samples (ML230 [2]) to high-throughput, 96-sample processing capabilities (LE220 [1], R230). Each of these instruments can be used to generate robust, reliable, reproducible and confident data for comprehensive pan-cancer panels such as Illumina's TruSight Oncology 500 assay.

References

1. Illumina®. TruSight™ Oncology 500 Reference Guide (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/trusight/oncology-500/1000000067621_10_trusight-oncology-500-reference-guide.pdf) Published July 2022.
2. Pal et al. Clinical Tumor Tissue Evaluated with Illumina® TruSight™ Oncology 500 (NGS) Assay and Sheared on the Covaris ML230 Focused-ultrasonicator. Application Note published by Covaris (https://www.covaris.com/wp/wp-content/uploads/resources_pdf/m020157.pdf)
3. Covaris Quick Guide: DNA Shearing with R230 Focused-ultrasonicator (https://www.covaris.com/wp/wp-content/uploads/resources_pdf/pn_010528.pdf)
4. Genoox. Franklin by Genoox (https://franklin.genoox.com/clinical-db/home)
5. Covaris R230 Focused-ultrasonicator (https://www.covaris.com/r230-focused-ultrasonicator-500620)