Tumor Tissue Evaluated with Illumina® TruSight™ Oncology 500 (NGS) Assay and Sheared on the Covaris ML230 Focused-ultrasonicator

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Abstract

DNA fragmentation is a critical step in the preparation of high-quality next generation sequencing (NGS) libraries. Covaris Adaptive Focused Acoustics® (AFA®) technology guarantees highly reproducible fragmentation of nucleic acids, which is especially relevant while working with valuable clinical research samples for pan-cancer NGS assays such as the Illumina TruSight Oncology 500 (TSO 500) panel. This panel was developed jointly by Illumina and Covaris to provide high-quality sequencing results. The DNA fragmentation protocol was previously released for the Covaris E220*evolution*, LE220-plus, and ME220 Focused-ultrasonicators [1]. In this application note, we present the ML230 and microTUBE-50 protocol to shear DNA to a fragment distribution optimized for the TSO 500 panel.

Introduction

Next Generation Sequencing (NGS) is an essential tool for clinical genomics research and the ability to address higher throughput demands of DNA fragmentation by maintaining the quality of the final sequencing data is imperative. All steps in the workflow, from nucleic acid fragmentation through library preparation and sequencing, must be performed in an efficient and scalable process; single step failures along this workflow can accumulate, impacting sequencing data and rendering them unusable for analysis. The nucleic acid fragmentation step is a key component in this complex process and therefore one of the most critical steps. Quality of the fragmented DNA determines the success of the downstream steps in the NGS library prep workflow, especially when working with nucleic acids isolated from FFPE tissue samples. Covaris AFA DNA fragmentation is independent of DNA concentration and starting molecular weight, ensuring a tight fragment size distribution, minimal sample loss, and most importantly, performance reproducibility.

The TSO 500 (Illumina, San Diego, CA) protocol describes an enrichment-based approach to convert DNA and RNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples into libraries enriched for cancer-related genes that can be sequenced on Illumina sequencing systems. The TSO 500 assay prepares libraries from genomic DNA (gDNA) that is sheared to optimized fragment sizes and offers high fidelity determination of DNA variants across 523 cancer-relevant genes [1].

The TSO 500 protocol offers cost-effective, accurate, integrated pan-cancer assays to measure tumor mutational burden (TMB), microsatellite instability (MSI), single nucleotide variants (SNVs), indels, copy-number/structural variation and gene fusions. In contrast to WGS and orthogonal technologies, the TSO 500 assay is a powerful tool to identify targetable alterations, eliminating the need to spend precious tissue samples, cost, and time on iterative testing. Thus, the TSO 500 assay is the assay of choice across most cancer research centers using a standardized pipeline and therefore is suitable for routine use in precision oncology research as a comprehensive genomic profiling solution [1,2]. The assay utilizes both DNA and RNA from tumor samples to identify key somatic variants underlying tumor progression. Mechanical fragmentation is only required for the DNA library prep workflow of the TSO 500 assay. We present here a case study demonstrating DNA fragmentation on the medium throughput, parallel 8-sample processing ML230 Focused-ultrasonicator to a target DNA fragment distribution of 90 to 250 bp, and present TSO 500 panel sequencing data.

Materials & Methods

Materials

- Covaris ML230 Focused-ultrasonicator (PN 500656)
- 8 microTUBE-50 AFA Fiber H Slit Strip V2 (PN 520240)
- ML230 Rack 8 microTUBE Strip 50 (PN 500661)
- Plate Definition: ML230_500661 Rack 8 microTUBE Strip 50 +1.8 mm offset
- SonoLab 10.1.0
- Agilent® 2100 Bioanalyzer (PN G2939BA)
- Agilent Bioanalyzer High Sensitivity DNA Kit (PN 5067-4626)
- Formalin-fixed paraffin-embedded tissue (FFPE) samples (various tumor tissue types) extracted using QIAsymphony DSP DNA Mini Kit (Qiagen®; Catalog # 937236)

Methods

Sample Source: One of the main criteria for a robust, reliable library preparation is the ability to shear DNA 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 different FFPE preserved tumor tissue samples have been used for this study (***Table 1***). DNA isolation started with scrapings from 6 slides (10 µm thick) each.

Sample IDs	Tumor Tissue Type
1	Endometrial
2,12	Pelvic
3,19	Small bowel
4,6,16	Skin
5,25,32	Stomach
7,14,15,20,24,26	Colon
8,9,10,22,29	Omentum
11	Liver
13	Appendix
17	Purchased-FFPE DNA positive control
18	Thyroid
21	Pancreas
23	Bilateral ovaries and fallopian tubes
27	Cecum
28	Retroperitoneal Aortic Mass
30	Peritoneal fluid
31	Omentectomy

Table 1. Tumor sample types tested during TSO 500 validation study. In this validation work, 17 different sample types were tested, confirming the versatility of the TSO 500 assay.

Sample Preparation: The extraction of DNA was performed using the QIAsymphony DSP DNA Mini Kit (Qiagen). Purified DNA was diluted to 3.3 ng/µL in TE buffer to a total volume of 15 µL. Forty (40) µL of TE buffer was added to all diluted samples to a final volume of 55 µL. Each sample was transferred to the Covaris 8 microTUBE-50 AFA Fiber H Slit Strip V2 consumable. AFA parameters used on the Covaris ML230 Focused-ultrasonicator to fragment DNA to a 90 to 250 bp fragment as required for TruSight Oncology 500 are shown in ***Table 2***. After AFA-based DNA fragmentation on the ML230, 50 µL of sheared sample was directly used for library preparation (total of 40 ng DNA).

8 microTUBE-50 AFA Fiber H Slit Strip V2 (PN 520240)	
Sample Volume (µL)	55
Temperature (°C)	12
Analytical System	Agilent Bioanalyzer High Sensitivity DNA Kit
Repeats	36
Treatment Time (sec)	10
Delay Duration (sec)	10
Peak Power (W)	350
Duty Factor (%)	25
Cycles per Burst	1000
Dithering	3 mm Y @ 20 mm/s
Time Per Sample (sec)	360
Total Treatment Time (sec)	720

Table 2. AFA treatment settings used on the ML230 Focused-ultrasonicator for FFPE DNA fragmentation (90 - 250 bp) for the Illumina TruSight Oncology 500 assay.

Sequencing and Analysis

Thirty-two (32) total DNA libraries were sequenced on NovaSeq SP Reagent kit, v1.5 at 2x101 read lengths. Novaseq software v1.7.0 was used and analyzed with TruSight Oncology 500 Local App v2.2. The TruSight Oncology 500 Analysis Module includes several quality control analyses including Run QC and DNA Sample QC.

Results

DNA Fragment 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 to 250bp (***Figure 1***). The average sheared DNA fragment length met the specifications of the TruSight Oncology 500 assay.

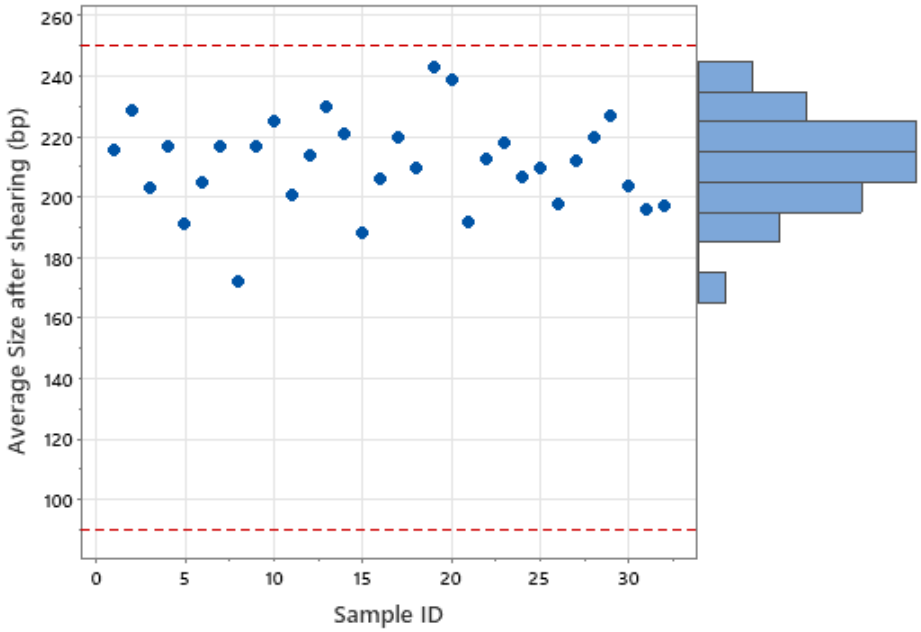

Figure 1. DNA fragment size distribution profiles of 32 validation samples using settings (***Table 2***) determined by Agilent Bioanalyzer.

NovaSeq Sequencing Run Metrics

The run metrics report provides suggested values to determine if run quality results are within an acceptable range using InterOp files from the sequencing run folder. For Reads 1 and 2, the report provides the average percentage of bases ≥ Q30 (quality score) measurement. The Q-score predicts the probability of an incorrect base call (here >30 = 1 error probable per 1,000). The metrics report is a final combined metrics report with key analysis metrics, and metadata in a .tsv file. Sample metrics within the report indicate guideline lower limits (LSL) and upper limits (USL) for each sample in the run. The metrics report also displays guideline run quality thresholds [4].

All Run QC Metrics passed for the two validation runs on NovaSeq. The run QC metrics with passing threshold values are listed below, and the actual Run QC metrics for sequencing runs have been shown in ***Table 3***.

- ***PCT_PF_READS (%)***: Total percentage of reads passing filter ≥55.0
- ***PCT_Q30_R1 (%)***: Percent of base calls with a quality score of Q30 or higher for Read 1 (threshold ≥80.0)
- ***PCT_Q30_R2 (%)***: Percent of base calls with a quality score of Q30 or higher for Read 2 (threshold ≥80.0)

[Run QC Metrics]			Run1	Run2
Metric (UOM)	LSL Guideline (*)	USL Guideline (*)	Value	Value
PCT_PF_READS (%)	55	NA	86	82
PCT_Q30_R1 (%)	80	NA	94.3	93.9
PCT_Q30_R2 (%)	80	NA	94.2	92.9

Table 3. NovaSeq Run QC Metrics.

DNA Sample QC

The three main DNA sample QC metrics that have specific threshold criteria to be met for TSO 500 assay includes Median Insert Size, Median Exon Coverage, and PCT_Exon_50X (4). These three DNA sample QC metrics that reflect library quality are defined as follows:

- ***Median Insert Size***: The median fragment length in the sample; threshold is ≥ 70 bp
- ***Median Exon Coverage***: Median exon fragment coverage across all exon bases; threshold is ≥ 150X
- ***PCT_Exon_50X (%)***: Percent exon bases with 50X fragment coverage, threshold is ≥ 90.0

DNA Sample QC Results

1. All 32 samples generated Median Insert Size ≥ 70 bp, as shown in ***Figure 2***.
2. Median Exon Coverage Count is a measurement of sequencing coverage; 26 out of 32 passed Median Exon Coverage of ≥ 150X (***Figure 3***). Six (6) samples had Median Exon Coverage < 150X, which is likely due to a sample specific issue. Smaller median exon coverage correlates with smaller insert size, but not average size after shearing.
3. PCT_Exon_50X (%) metrics is the percent exon bases with 50X coverage and reflects coverage uniformity. The threshold for this metric is ≥ 70% and all samples passed this metric (***Figure 4***).

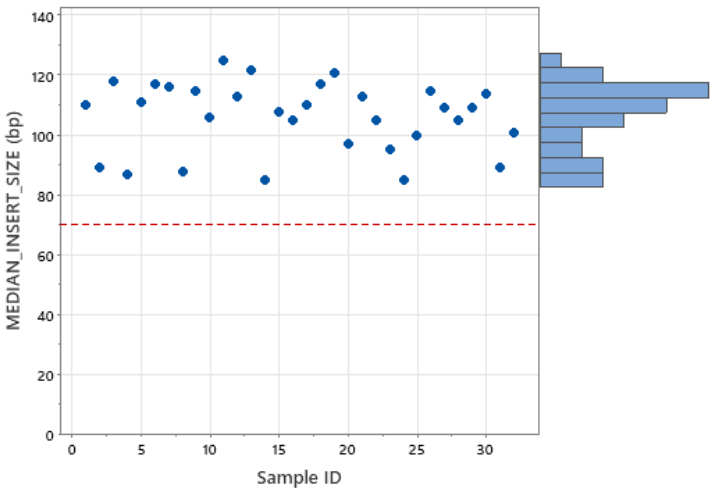

Figure 2. Marginal Plot of Median Insert Size. All samples (n=32) have Median Insert Size greater than threshold of ≥ 70.0 bp.

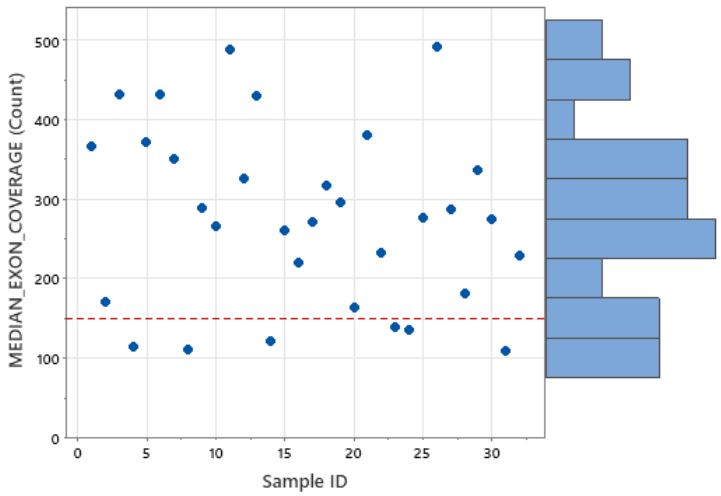

Figure 3. Marginal Plot of Median Exon Coverage Count; 26 out of 32 samples have Median Exon Coverage count greater than threshold of ≥ 150X. The 6 samples that gave Median Exon Coverage <150X was attributed to sample specific issue.

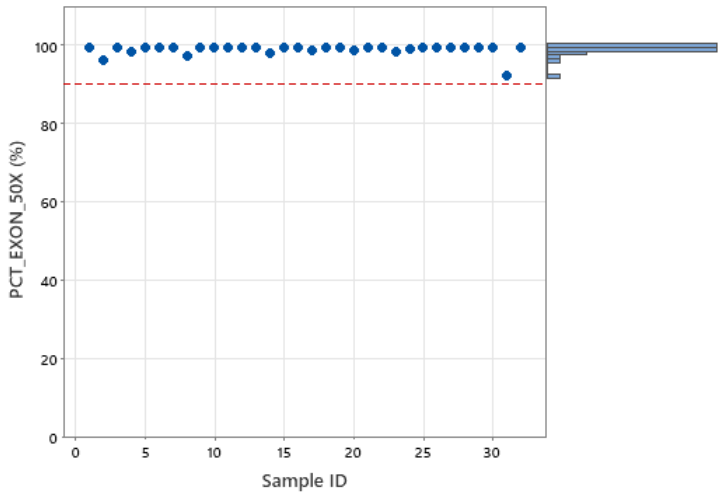

Figure 4. Marginal Plot of PCT_EXON_50X Count (%). All samples (n=32) have PCT_EXON_50X Count greater than threshold of ≥ 90%.

Discussion

For the six samples that gave Median Exon Coverage <150X, we recommended a QC check with Illumina FFPE QC Kit for input FFPE DNA to make sure that delta Cq <5 to confirm the sample quality [1].

a. For optimal performance, assess DNA and RNA sample quality before using the TruSight Oncology 500 assay
b. DNA samples can be assessed using the Illumina FFPE QC Kit
c. Use DNA samples that result in a delta Cq value ≤ 5, Samples with a delta Cq > 5 may result in decreased assay performance

The six samples that gave Median Exon Coverage <150X were attributed to a sample-specific issue. The smaller insert size despite normal size post-shearing DNA size (~200 bp) was likely indicative of highly degraded/fragmented DNA in the original FFPE sample. Highly degraded DNA has been known to cause issues with exon coverage because of possible dropouts and poorly covered regions.

Conclusion

A robust, reliable, and extensive NGS library paves the path for fast, confident detection of oncogenes. DNA fragmentation is a critical step in the preparation of good quality libraries for NGS. Enabled by AFA Technology, Covaris instruments can ensure reliable and reproducible fragmentation of DNA. In conclusion, the ML230 features all the benefits of Covaris AFA technology for robust, reproducible, and confident fragmentation required for comprehensive pan-cancer panels such as the Illumina TruSight Oncology 500 assay.

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