Efficient Sample Preparation with Covaris Adaptive Focused Acoustics® (AFA®) Technology Enhances Confidence in Illumina® TruSight™ Oncology 500 Assay

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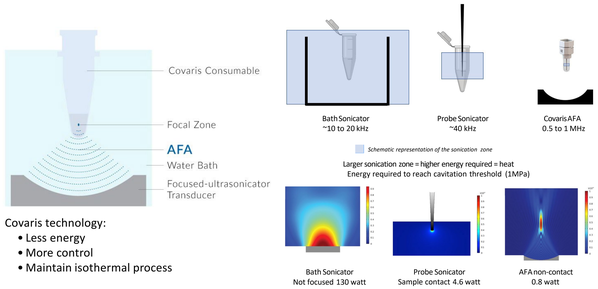
A robust, reliable, and extensive NGS library can enable the process of detection, diagnosis and survival chances of cancer. DNA fragmentation enabled by AFA Technology ensures reliable and reproducible fragmentation of DNA. The ML230 focused-ultrasonicator can be used efficiently for comprehensive pan-cancer panels such as Illumina®'s TruSight™ Oncology 500 assay.

Emerging Enabling Easy



Covaris technology:
- Less energy
- More control
- Maintain isothermal process

Key Takeaways

Covaris promotes robust, easy, and scalable sample preparation through:

- Standardized tissue/cell disruption
  - Improved consistency and reproducibility
  - Hands-free, automated
  - Processing in different formats, from single tube up to 96-well plate, with same sample-to-sample quality
- Fast, non-contact, easy, cold, and reproducible extraction for all soft and hard tissue samples*
  - Use your buffer of choice
  - Scale up from single tube to 96 well format and beyond
- Active, non-toxic deparaffinization and improved rehydration of FFPE and LCM tissue
  - Enables "fresh frozen like" protein extraction from FFPE samples, without any organic solvent

*Includes non-mammalian samples like plants, yeast and bacteria

Introduction

Next Generation Sequencing (NGS) is an essential tool for clinical genomics and addressing higher throughput demands of DNA fragmentation by maintaining the quality of the final sequencing data is imperative. DNA fragmentation using AFA 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 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.

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 for 17 different sample types.

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 to 250bp) for Illumina's TruSight™ Oncology 500 assay.

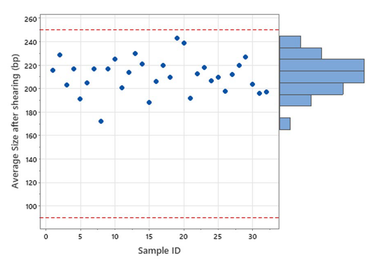


Figure 1. DNA fragment size distribution profiles of 32 validation samples using settings (***Table 2***) determined by Agilent® Bioanalyzer®.

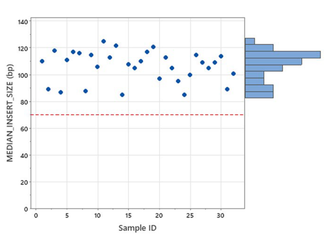


Figure 2. Marginal Plot of Median Insert Size. All samples (n=32) have median insert size greater than threshold of ≥ 70.0 bp.

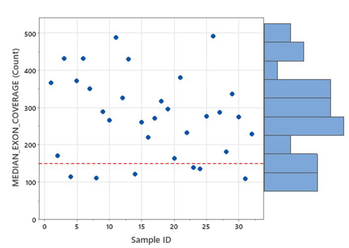


Figure 3. Marginal plot of median exon coverage Count; 26 out of 32 samples have median exon coverage count greater than threshold of ≥ 150X.

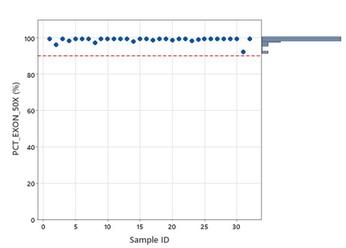


Figure 4. Marginal plot of PCT_EXON_50X Count (%). All samples (n=32) have PCT_ EXON_50X count greater than threshold of ≥ 90%.

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 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 ML230 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 Covaris ML230, 50 µL of sheared sample was directly used for library preparation (total of 40 ng DNA).

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 meets the specifications of the TruSight™ Oncology 500 assay.

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 Read 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.

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. 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, and PCT_Exon_50X (%): Percent exon bases with 50X fragment coverage, threshold is ≥ 90.0

DNA Sample QC Results: All 32 samples generated Median Insert Size ≥ 70 bp, as shown in ***Figure 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. 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***).

Discussion

For the 6 samples that gave Median Exon Coverage < 150X, we recommended QC check with Illumina® FFPE QC Kit for input FFPE DNA to make sure that delta Cq < 5 to confirm the sample quality. **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 was attributed to 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.

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