xGen™ cfDNA & FFPE Library Preparation Kit compatibility with enzymatic fragmentation of DNA from FFPE samples

Abstract

The xGen cfDNA & FFPE Library Preparation Kit offers a high conversion efficiency sample prep ideal for use with challenging sample types like cfDNA and FFPE. While cfDNA samples do not require fragmentation upstream of library preparation due to their inherently short fragment size, FFPE samples do require fragmentation. Furthermore, damage during the fixation process will create a wide range of fragment lengths undesirable for sequencing. Researchers using the xGen cfDNA & FFPE Library Prep Kit for sample prep now have the flexibility to choose a fragmentation method that best meets their needs; the data presented here shows that fragmentation of FFPE DNA with varying DIN scores can be completed via mechanical or enzymatic fragmentation with minimal impacts to data quality.

Introduction

Following extraction, the next step of library preparation is to ensure that the DNA template is an appropriate length for the desired sequencing platform. The xGen cfDNA & FFPE Library Preparation Kit recommends using mechanically fragmented (i.e., Covaris) DNA upstream of end-repair. However, there are circumstances in which mechanical fragmentation is not ideal. Researchers may not have access to a mechanical fragmentation instrument, mechanical fragmentation tends to be cumbersome for high-throughput workflows, and mechanical fragmentation can result in loss of precious sample when transferring between tubes or during post-shear quantification. In these cases, enzymatic fragmentation can also be employed for use with the xGen cfDNA & FFPE Library Prep Kit. The data presented below explores the difference in quality metrics between libraries prepared using enzymatic and mechanical fragmentation of DNA extracted from two FFPE breast cancer tissues with varying DNA integrity number (DIN) scores that were enriched using an xGen Custom Hyb panel designed for solid tumor breast cancer research.

Methods

DNA was extracted from two breast tumor FFPE samples (Discovery Life Sciences) using either the QIAGEN QIAmp® DNA FFPE Advance Kit UNG or QIAGEN AllPrep® DNA/RNA FFPE Kit. The DIN scores were determined to be 2.8 and 4.5 using Agilent TapeStation Genomic tape. NEBNext® dsDNA Fragmentase Kit was used to fragment each sample in triplicate using a 50 ng input following the vendor's protocol. A fragmentation time of 7 minutes was determined using a time-course study aiming for 300 base fragment sizes. After fragmentation, 5 µl of 0.5 M EDTA was added to stop the reaction followed by the addition of 25 µl of water. To perform a 3X bead clean-up, 150 µl of AMPure XP beads were added to each sample and mixed thoroughly. DNA was bound to the beads for 5 minutes, followed by two 80% ethanol washes on a magnet, and a 5-minute elution step in 52 µl of IDTE. Fifty microliters of cleaned sample were transferred to a new well and used directly for library preparation.

To compare to mechanical shearing, an equivalent amount of DNA from the same breast tumor samples were mechanically sheared using a Covaris ME220 ultrasonicator targeting a fragment size of 300 base pairs.

The libraries were prepared in triplicate using the xGen cfDNA & FFPE Library Prep Kit with the fragmented DNA and xGen UDI Primer Pairs, using 9 cycles of PCR. Overnight hybridization capture was performed as two 6-plexes using xGen Capture Core Reagents and an xGen Custom Hyb Panel-Accel designed to target 74 genes associated with breast cancer, resulting in a 347 kb target footprint. Captured libraries were sequenced on an Illumina® NextSeq 2000 using 2 × 150 paired-end (PE) reads. Data was subsampled to 15 M reads/sample and analyzed using Picard [**1**].

Results

Library traces across fragmentation methods

Post library preparation TapeStation traces for two low-quality FFPE samples showed consistent traces within replicates, and minimal dimers were observed regardless of which fragmentation method was used (**Figure 1A, B**). The lower quality DIN score sample (black) shows slightly smaller library fragment sizes than the higher quality sample (blue) in both approaches.

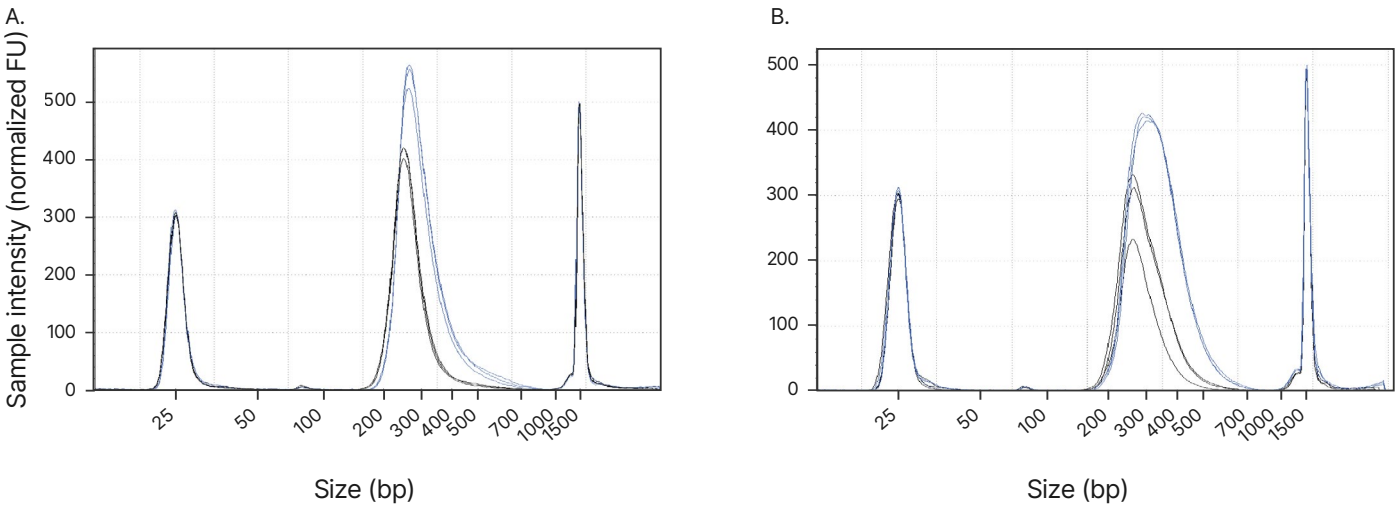

Figure 1. Library traces from two enzymatically fragmented FFPE samples show low dimer rates and consistent TapeStation. Fifty nanograms of DNA from 2 FFPE samples with a 4.5 DIN (blue) and a 2.8 DIN (black) were enzymatically fragmented (*n* = 3) using NEBNext dsDNA Fragmentase (**A**) or mechanically fragmented (**B**) prior to generating libraries using the xGen cfDNA & FFPE Library Prep Kit.

Differences in library yield and insert size per fragmentation approach

While all libraries yielded > 600 ng which allowed for downstream hybridization capture, the mechanically fragmented samples resulted in higher library yields. Overall, the samples with a lower DIN score generated lower library yields for both fragmentation approaches (**Figure 2**).

This difference in yield may be due to the addition of the bead clean-up, post enzymatic fragmentation, or due to smaller fragments being generated during enzymatic fragmentation that are then lost in downstream library preparation steps.

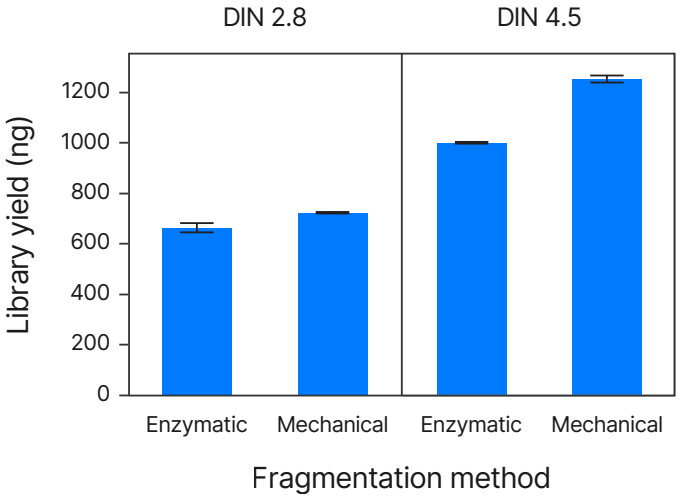

Figure 2. Mechanical fragmentation of FFPE samples results in higher library yields than enzymatic fragmentation. DNA from 2 FFPE samples were enzymatically fragmented or mechanically fragmented prior to generating libraries using 50 ng inputs into the xGen cfDNA & FFPE Library Prep Kit (*n* = 3 per condition). Error bars represent standard deviations.

Enzymatic fragmentation resulted in a shorter mean insert size (as determined by Picard [**1**]) when compared to the mechanical fragmentation samples (**Figure 3**).Each fragmentation method targeted 300 bp fragmentation length yet resulted in shorter mean insert sizes than expected. The sample with a lower DIN score resulted in shorter insert sizes for both mechanical and enzymatic fragmentation approaches. This is possibly due to the damage (nicks, gaps, uneven ends, fragmentation) inherent to extracted DNA from FFPE samples. Although the insert sizes were smaller than expected, the shorter fragments were still within useable ranges and resulted in successful libraries, hybrid captures, and sequencing. It is important to note that the mechanical shearing program on the Covaris was not adjusted based on DIN scores (program was generated for high-quality gDNA) and may have resulted in over-shearing of the FFPE samples. Additionally, the enzymatic fragmentation method introduces nicks to generate double-stranded breaks; adding nicks in already damaged FFPE DNA, may have resulted in further fragmentation during the end-repair process of library preparation. This could be alleviated by using an FFPE repair module up-stream after DNA extraction to help obtain larger mean insert sizes [**2**].

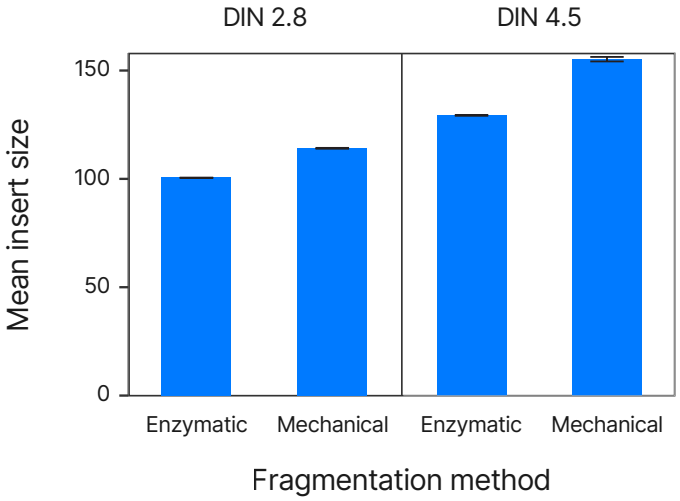

Figure 3. Enzymatic fragmentation resulted in smaller mean insert sizes than mechanical fragmentation. Libraries prepared using the xGen cfDNA & FFPE Library Prep kit were generated from 50 ng of DNA from 2 FFPE samples that were fragmented using enzymatic fragmentation or mechanical fragmentation (*n* = 3 per condition). Libraries were captured using an xGen Custom Hyb capture panel targeting genes associated with breast cancer. Captured libraries were sequenced on an Illumina® NextSeq and analyzed using Picard [**1**], error bars represent standard deviation.

Sample quality between fragmentation approaches

For the lower quality sample (DIN 2.8) mechanical fragmentation resulted in a lower HS library size (a measure of library complexity) and mean target coverage than the enzymatic fragmentation (**Table 1**). For the DIN 4.5 samples, the mechanical fragmentation had higher on-target rate and higher HS library size leading to a higher mean target coverage. Regardless of fragmentation method, complexity and coverage scaled with DIN score (higher quality samples resulted in higher complexity and coverage), as expected (**Table 1**).

Table 1. Picard metric comparison between enzymatic and mechanical fragmentation methods.

FFPE Sample	Fragmentation	Selected bases	HS library size	Mean target coverage
DIN 2.8	Enzymatic	85.2%	1.80E+06	359
	Mechanical	85.2%	1.47E+06	325
DIN 4.5	Enzymatic	84.8%	2.37E+06	554
	Mechanical	88.2%	2.54E+06	663

Conclusion

The data presented above illustrate that enzymatic fragmentation is compatible with the xGen cfDNA & FFPE Library Prep Kit. Having the choice between enzymatic and mechanical fragmentation with this library prep kit empowers researchers to tailor the workflows to the fragmentation method that best suits their lab needs and sample types. Although differences in library yield, fragmentation size, and mean insert sizes varied slightly between fragmentation approaches, both upstream methods resulted in high-quality sequencing libraries.

References

1. "Picard Toolkit." *Broad Institute*, GitHub repository: Broad Institute; 2019.
2. Steiert TA, Parra G, Gut M, *et al*. A critical spotlight on the paradigms of FFPE-DNA sequencing. *Nucleic Acids Res*. 2023;51(14):7143-7162.