

FFPE Library Construction Workflows Using Twist TrueAmp™ Library Preparation Kit

INTRODUCTION

Formalin-fixed, paraffin-embedded (FFPE) tissue samples represent the most abundant source of clinically archived biological material in pathology repositories worldwide. While the FFPE preservation process has been the histopathology standard for over a century (offering excellent morphological stability and ambient-temperature storage), the same formaldehyde chemistry that ensures long-term tissue integrity severely compromises the nucleic acids within. Cross-linking, depurination, and cytosine deamination collectively yield highly fragmented DNA prone to artifactual C>T and G>A transition mutations that can be difficult to distinguish from true somatic variants, which affects downstream sequencing applications.¹

Library preparation for FFPE-derived DNA must accommodate damaged input material (where quality may vary greatly between different FFPE samples) and preserve sequence fidelity to generate meaningful genomic data. While acoustic shearing delivers highly reproducible fragmentation, it requires specialized equipment, consumables, maintenance, and instrument-specific optimization that can bottleneck throughput and limit accessibility in clinical or high-volume settings.²

The Twist TrueAmp Library Preparation Kit relies on an enzymatic module to fragment, end-repair, and A-tail double-stranded DNA.³ Enzymatic fragmentation allows for library preparation without the need for specialized shearing instrumentation while achieving equivalent, if not improved, performance. This application note details the performance of the Twist TrueAmp Library Preparation Kit when preparing FFPE-derived DNA samples for target enrichment and other downstream workflows. Depending on laboratory and analysis priorities, this application note details two practical workflows for processing FFPE samples using the Twist TrueAmp Library Preparation Kit. We evaluate the performance and trade-offs of a standardized, high-throughput workflow that offers a "one-protocol-fits-all" approach independent of sample integrity, versus an optimized, stratified workflow that maximizes insert size by separating samples into two tracks (DIN < 2.5, referred to as low DIN score, and DIN > 2.5 referred to as high DIN hereinafter) (**Figure 1**). The tradeoffs between these two approaches will allow the user to choose their preferred protocol.

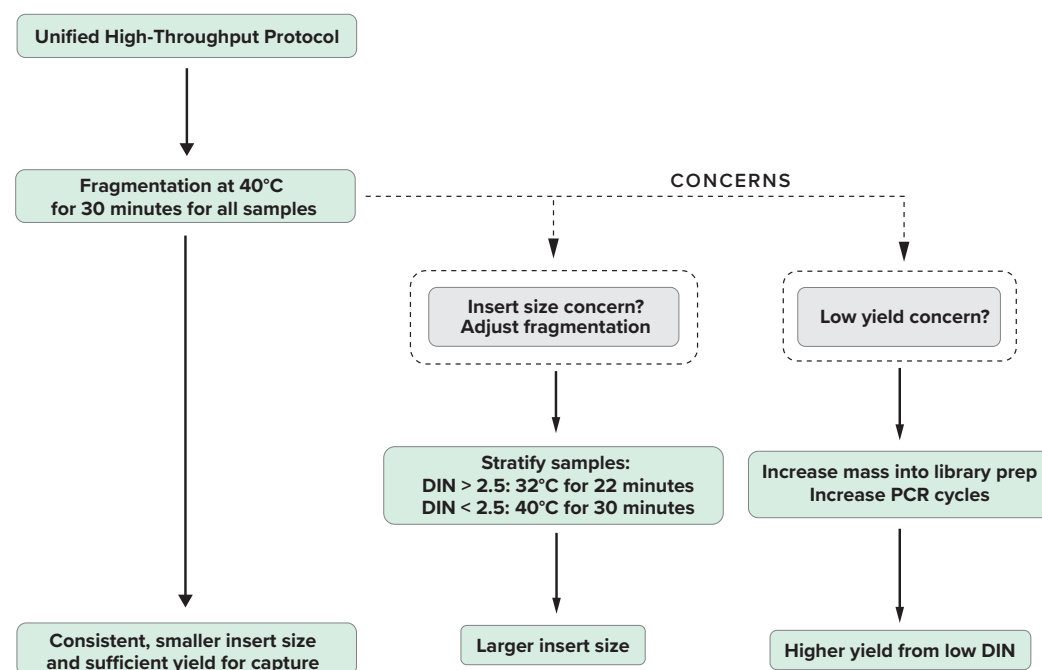


Figure 1. Twist TrueAmp Library Preparation workflows for FFPE. A comparison of a unified high-throughput protocol versus optimized workflows tailored to maximize insert size (stratified by DIN) or improve yield based on specific laboratory concerns.

MATERIALS & METHODS

FFPE-derived DNA from clinical specimens spanning a range of DIN was sourced through Reference Medicine (CLIA ID# 03D2269440, Phoenix, AZ), with fragment size and integrity confirmed by Agilent TapeStation 4200 prior to processing (**Figure 2**). Commercial FFPE reference standards (Horizon Discovery; HD798, HD799, HD803) were included as controls. Libraries were prepared from 15 ng input DNA under the following conditions: mechanical shearing (Covaris ML230) followed by library preparation with Twist cDNA Library Preparation Kit with TrueAmp Polymerase Mix (cat# 116612), or the Twist TrueAmp Library Preparation Kit (cat# 116481) with fragmentation at 40°C for 30 minutes or 32°C for 22 minutes. Libraries from both kits were adapter ligated with Twist Universal Adapter System (cat# 101308) prior to index PCR amplification. Mechanically sheared samples were processed using a Covaris ML230 instrument using the following sonication settings: dithering: Y=3, speed: 20 mm/s, iterations: 20, duration: 10 secs, PIP: 210 W, DF: 25%, and CPB: 50. Final libraries were quality-assessed by ThermoFisher Qubit 4 fluorometer and TapeStation 4200 on a DNA ScreenTape D5000 with the range set up for a 100 bp to 1000 bp region (cat# 5067-5588).

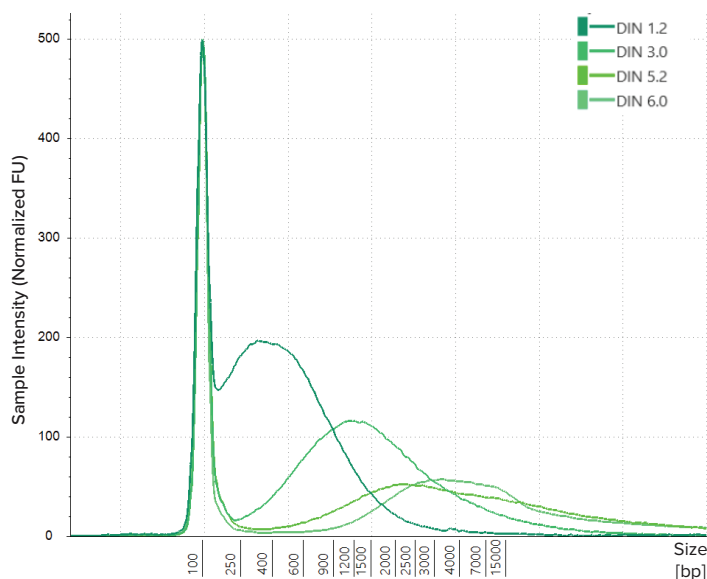


Figure 2. Agilent TapeStation traces of FFPE DNA with varying DIN scores. Electropherograms of FFPE-derived genomic DNA exhibiting a range of DNA Integrity Numbers (DIN). Lower DIN scores (e.g., 1.2) correlate with highly fragmented, degraded DNA (peak size 372 bp), while higher DIN scores (e.g., 6.0) indicate higher molecular weight fragments (peak size 3,095 bp). The internal lower sizing marker is visible at 100 bp in all traces.

Following library construction, all libraries were captured following the Twist Target Enrichment Standard Hybridization v2 Protocol with the Twist Oncology DNA CGP Panel (cat# 116353), a 2.4 Mb panel targeting 562 genes and covering SNVs, indels, CNVs, fusions, TMB, and MSIs. Sequencing was performed on an Illumina NextSeq 2000 (P3 flowcell, PE150, targeting 18M reads), and data was downsampled to a uniform 500× raw coverage for cross-condition comparison. Files were aligned to the human hg38 genome with BWA-MEM. Target enrichment metrics were calculated with Picard tools CalculateHsMetrics, and variant fractions were assessed from pileups using samtools.

RESULTS

Fragmentation Optimization Across DIN Scores

Critical to many variant-calling assays is the library yield following library preparation, as many target enrichment workflows have a minimal threshold depending on the application. Insert size is also an important consideration for maximizing usage of sequencer read length. Importantly, input DNA quality and fragmentation conditions will dictate library yield and insert size. We performed time and temperature titration of the fragmentation step to identify conditions that are suitable for FFPE samples from a wide range of DIN scores (**Figure 3**). Table 1 provides a detailed overview of final library yield and average library size to guide optimization. We observe trade-offs that should be considered when choosing fragmentation conditions. For the Twist TrueAmp Library Preparation Kit library yields are typically highest when fragmentation is performed using higher temperatures and longer times because fragmentation and end-repair enzymes are more active under these conditions. Higher yields are also obtained from higher-quality input DNA as expected. High DIN scores, indicating intact input DNA, produce sufficient yield for target enrichment with standard fragmentation conditions (32°C for 22 minutes). However, with this condition, low-DIN-score samples do not reach library yield sufficient for downstream target enrichment. Fragment size stays within the range of ~300-400 bp for all conditions tested, and high-temperature fragmentation provides the most uniform insert sizes from FFPE samples across the range of DIN tested. In general, unless the DIN score is low, lower temperatures and less fragmentation time lead to larger fragment sizes, allowing for fragment size modulation.

Based on this data, the optimal condition for FFPE samples with a wide range of DINs is 40°C and 30 minutes. Across all DIN scores tested, this condition results in high and robust library yields appropriate for many downstream target enrichment workflows. This single fragmentation condition simplifies the library preparation workflow by avoiding upfront sample sorting.

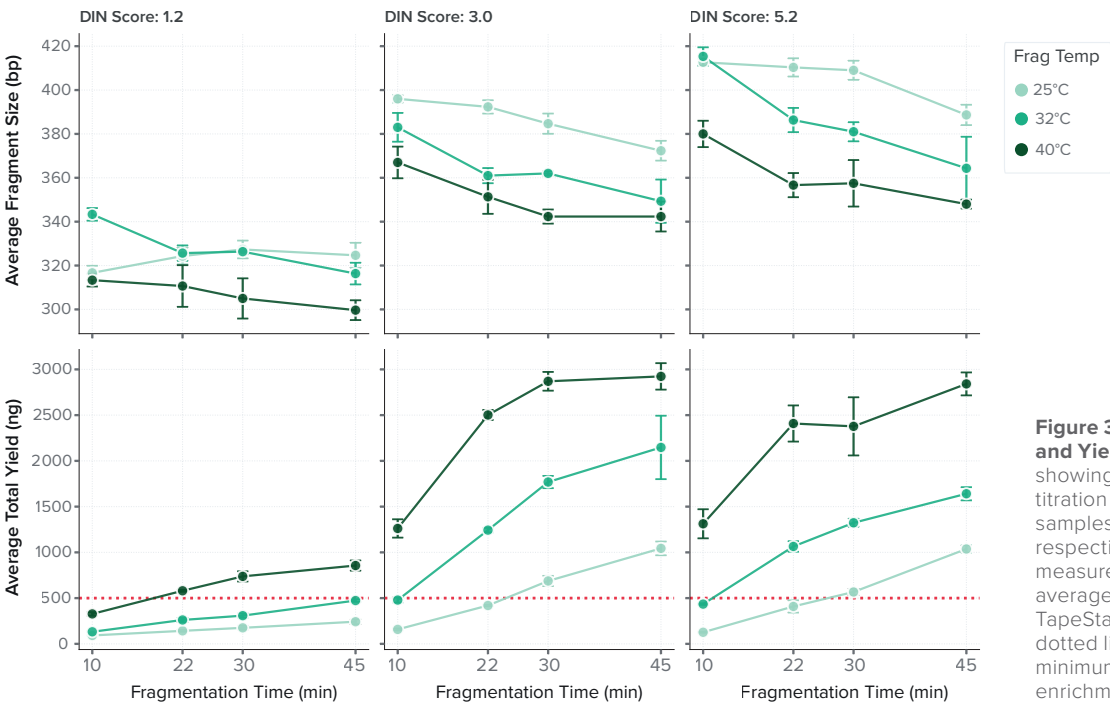


Figure 3. Fragmentation Kinetics: Size and Yield over Time. Triplicate data showing fragmentation and temperature titration of three FFPE-derived DNA samples of different quality, with their respective effects on final library yield as measured by Qubit BR dsDNA (bottom) and average library size as measured by Agilent TapeStation DNA D5000 assay (top). Red dotted line in bottom panel represents minimum library yield requirement for target enrichment.

DNA Integrity	Temperature	Fragmentation Time (min)	Final Yield (ng)	Average Library Size (bp)
DIN 1.2	25°C	10	92 ± 5	317 ± 3
		22	142 ± 11	324 ± 4
		30	175 ± 22	327 ± 4
		45	242 ± 26	325 ± 6
	32°C	10	132 ± 9	343 ± 3
		22	261 ± 22	326 ± 4
		30	308 ± 24	326 ± 1
		45	473 ± 15	316 ± 5
	40°C	10	327 ± 20	313 ± 3
		22	580 ± 11	311 ± 10
		30	738 ± 56	305 ± 9
		45	856 ± 55	300 ± 5
DIN 3.0	25°C	10	158 ± 10	396 ± 2
		22	419 ± 6	392 ± 3
		30	687 ± 56	385 ± 5
		45	1044 ± 75	372 ± 5
	32°C	10	479 ± 27	383 ± 7
		22	1243 ± 24	361 ± 3
		30	1769 ± 67	362 ± 1
		45	2147 ± 126	349 ± 10
	40°C	10	1261 ± 100	367 ± 7
		22	2502 ± 55	351 ± 8
		30	2870 ± 102	342 ± 3
		45	2924 ± 145	342 ± 7
DIN 5.2	25°C	10	127 ± 6	413 ± 2
		22	409 ± 67	410 ± 4
		30	567 ± 33	409 ± 4
		45	1037 ± 41	389 ± 5
	32°C	10	434 ± 9	415 ± 4
		22	1065 ± 58	386 ± 6
		30	1323 ± 44	381 ± 4
		45	1641 ± 72	364 ± 14
	40°C	10	1313 ± 159	380 ± 6
		22	2409 ± 198	357 ± 6
		30	2332 ± 238	356 ± 8
		45	2841 ± 125	348 ± 2

Table 1. Summary table for optimization of yield and size based on fragmentation condition. Higher temperatures and longer fragmentation times generate more library yield but decrease the average library size. Overall, to generate sufficient library yield for target enrichment, fragmenting at 40°C for 30 minutes will allow for processing of all samples regardless of DIN score.

Standardized Workflow Performance Provides Equivalent Performance to Mechanical Fragmentation

In order to evaluate the target enrichment performance of the Twist TrueAmp Library Preparation Kit with a fragmentation at 40°C for 30 minutes, we compare this condition with mechanical shearing and library preparation with the Twist cfDNA Library Preparation Kit and TrueAmp Polymerase Mix. Mechanical shearing serves as a critical reference as it is a common technique to process FFPE samples with minimal bias. However, mechanical shearing can lead to wide differences in insert size between FFPE samples with varying DIN. Using a fragmentation at 40°C for 30 minutes condition, all samples, independent of starting DIN, are sheared to comparable insert sizes (**Figure 4**, top left). It is notable that for median insert size, at DIN 4 and 6, the Twist TrueAmp Library Preparation Kit produces fragments with slightly smaller sizes than those produced by mechanical shearing. Small insert sizes may impact median target coverage, a critical metric for variant calling (**Figure 4**, top right). However, for the high-DIN sample, this condition gives higher complexity than the mechanical shearing control (**Figure 4**, top middle). These two conditions both have comparable chimera rates

(**Figure 4**, bottom middle) and similar uniformity (**Figure 4**, bottom right). This high-temperature enzymatic fragmentation using the Twist TrueAmp Library Preparation Kit has consistent performance across the range of DINs tested and provides comparable results to mechanical-shearing-based library preparation.

Despite the smaller insert size compared to mechanically sheared libraries, the Twist TrueAmp Library Preparation Kit produces accurate variant calling performance with a fragmentation at 40°C for 30 minutes (**Figure 5**). Using Horizon FFPE controls with known variant allele frequency (% VAF), the Twist TrueAmp Library Preparation Kit performed similarly to the conventional mechanical-shearing method. Consistent variant frequency is observed between the two methods, including for low-frequency variants in all three controls tested (representing varying states of intactness). This data shows that variant calling workflows are accurate when using the Twist TrueAmp Library Preparation Kit with the unified fragmentation protocol.



Figure 4. Picard QC Metrics - Twist TrueAmp Library Prep (40°C for 30 minutes) against Mechanical Shearing. Triplicate data summary of Picard Metrics from target enrichment using the Twist Oncology DNA CGP Panel (cat# 116353). Comparing FFPE DNA with distinct DIN scores fragmented at 40°C for 30 minutes to mechanically sheared libraries.

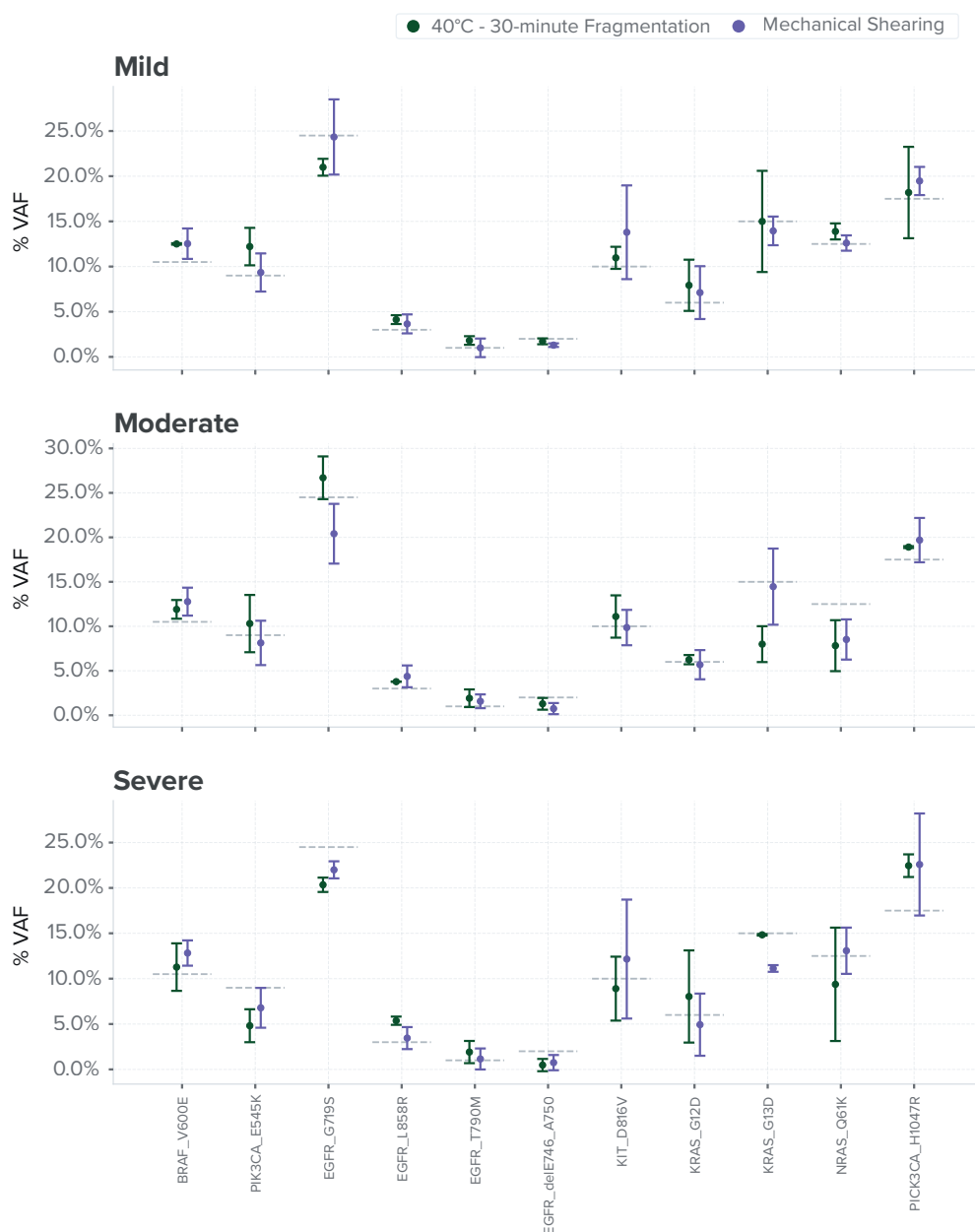


Figure 5. %VAF by Severity, comparing fragmentation at 40°C for 30 minutes vs Mechanical Shearing. Replicate data showing observed %VAF for various mutations (dotted lines show expected %VAF) based on the mechanical shearing workflow in purple or the Twist TrueAmp Library Preparation Kit in dark green using a fragmentation at 40°C for 30 minutes for different FFPE reference standards. For these standards, mild is DIN 5, moderate is DIN 3.5, and severe is DIN <2.3.

Stratified Approach for Increased Insert Size

In some applications, insert size is a critical metric that must be maximized. Decreasing fragmentation time and temperature (to 32°C for 22 minutes) can help achieve larger insert sizes and potentially better coverage for more intact samples, but this approach comes with significant trade-offs. Namely, the modified conditions cannot be used with lower-DIN samples because library yield is insufficient for target enrichment (**Figure 3, Table 1**). Users wishing to maximize insert size will need to stratify samples and run different fragmentation protocols. The 32°C for 22 minutes fragmentation condition results in a larger insert size for medium- and high-DIN samples (**Figure 6**). For samples

with lower DIN, the 40°C for 30 minutes condition can still be used. Both fragmentation conditions generate accurate and comparable variant calling with the FFPE reference standards (**Figure 7**). Users of the Twist TrueAmp Library Preparation Kit can adjust the fragmentation conditions when using FFPE samples to emphasize processing all samples, including those with low DIN (40°C for 30 minutes), or can choose to stratify samples and prioritize larger insert sizes for higher-DIN samples (32°C for 22 minutes).

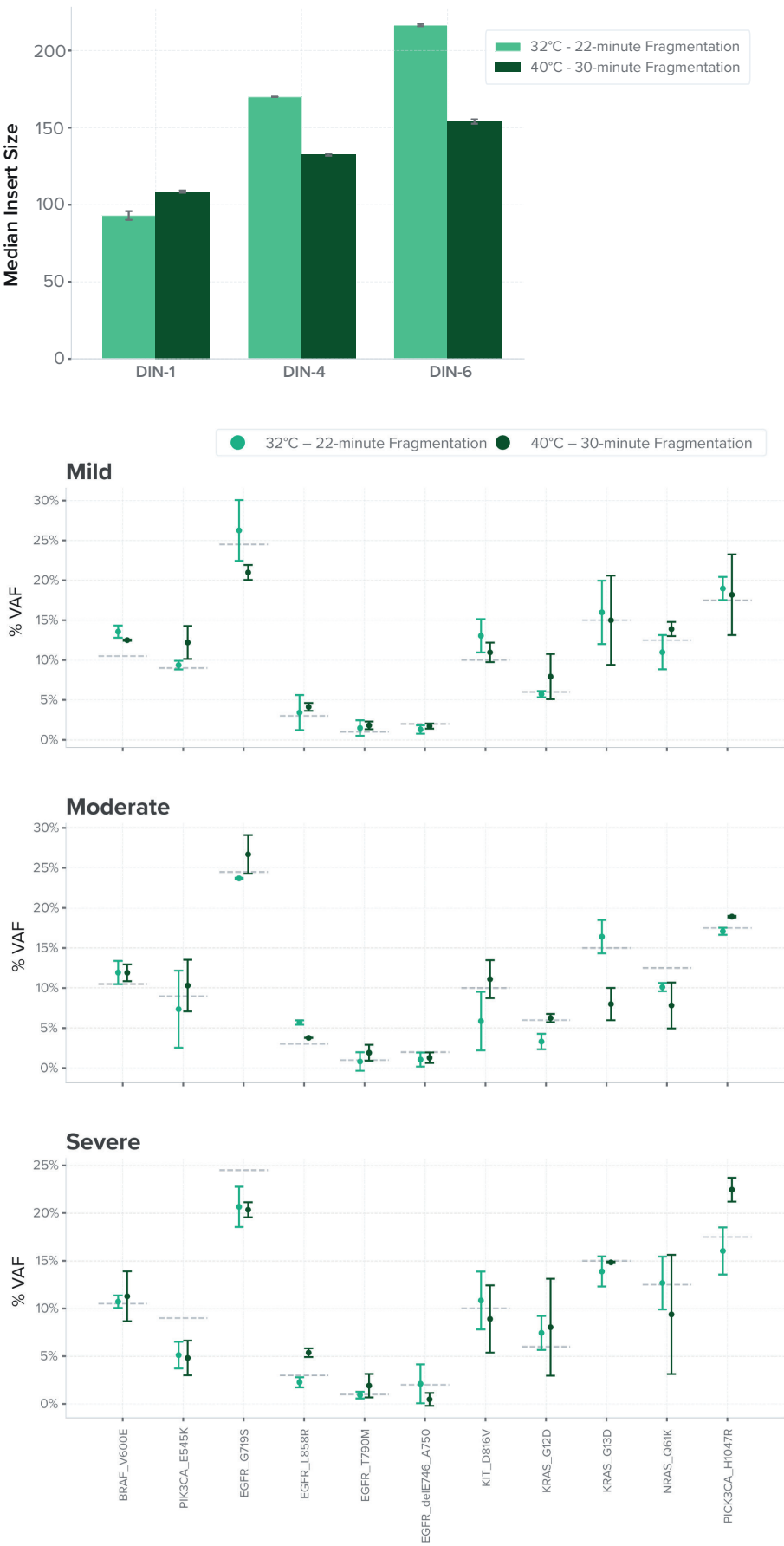


Figure 6. Insert Size Difference between fragmentation at 40°C for 30 minutes and 32°C for 22 minutes. Triplicate data of median insert size from target enrichment using the Twist Oncology DNA CGP Panel (cat# 116353). Comparing FFPE DNA with distinct DIN scores fragmented with either 40°C for 30 minutes or 32°C for 22 minutes. Shorter fragmentation time allows for a larger median insert size.

Figure 7. %VAF by severity, comparing fragmentation at 40°C for 30 minutes against 32°C for 22 minutes. Replicate data showing observed %VAF for various loci mutations across FFPE standards with differing degrees of DIN using Twist TrueAmp Library Preparation Kit when fragmented at 32°C for 22 minutes (light green) and 40°C for 30 minutes (dark green). Expected %VAF shown with dotted line.

Rescue of Low-DIN FFPE Samples

In our testing with FFPE samples, we have observed sample-to-sample variability in conversion. This can be expected due to differences in sample processing, storage conditions, and tissue source, among others. Variability in conversion presents the largest challenge for low-DIN FFPE samples, and some samples may not yield enough library for downstream processing. In order to address this variability, users can either increase the mass input into library preparation or increase the PCR cycles used (**Figure 8**). Increasing mass input above the standard 15 ng will increase yield and allow more consistent processing of low-DIN samples. However, higher mass is not always available. When mass inputs must remain the standard 15 ng or less, increasing PCR cycles by two will allow recovery of these samples, but concomitant higher duplication rates are expected. These two options will allow for the recovery of low-DIN samples for users who experience unwanted yield dropouts. These solutions can be applied to both the unified and stratified fragmentation protocols.

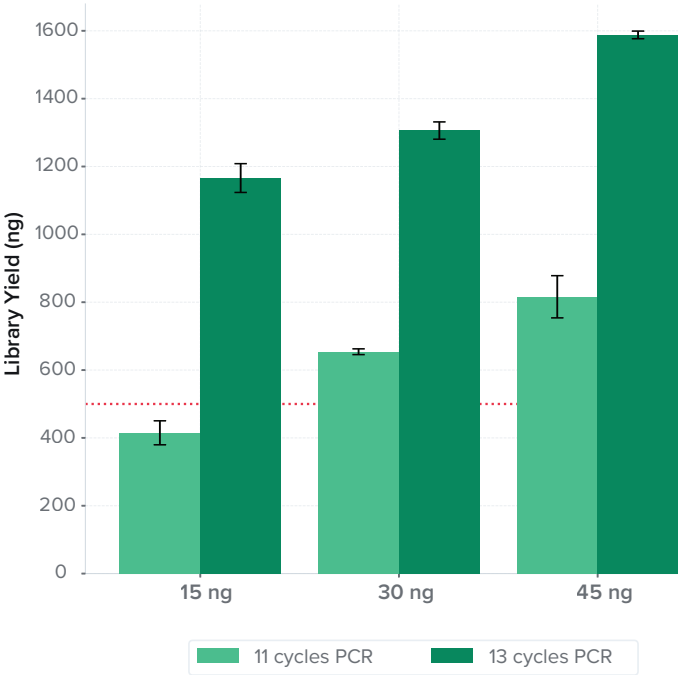


Figure 8. Final library yield from optimization of mass input into library preparation. Final library yield from optimization of mass input and PCR cycles for samples with DIN of 1.2 fragmented at 40°C for 30 minutes. The red dotted line shows the minimum library yield needed for single-plex target-enrichment capture.

CONCLUSION

We demonstrate that enzymatic fragmentation with the Twist TrueAmp Library Preparation Kit is a robust and accessible alternative to shearing-based library preparation. Testing across a broad spectrum of FFPE sample quality (DIN 1.2–6) and against established reference standards confirms the workflow's reliability even with the most challenging clinical inputs.

A robust protocol for processing all samples, including severely damaged FFPE samples, is suggested for convenience and efficiency. For this protocol, all samples, independent of DIN, have similar insert sizes, but this size is shorter than observed for mechanical shearing. Larger insert sizes can be obtained with FFPE samples using lower time and temperature during the fragmentation step, but severely damaged samples will have low yield when using this condition and must be processed separately with the higher-temperature protocol. We share these options to enable users to identify the best condition for their specific use. For challenging low-DIN FFPE samples, we provide suggestions for increasing library yield and producing more consistent results.

Performing mechanical shearing followed by a non-fragmenting library preparation kit, such as Twist cfDNA Library Prep Kit with TrueAmp Polymerase Mix (cat # 116612), remains a viable option. However, the Twist TrueAmp Library Preparation Kit allows researchers to replace mechanical shearing with a simple thermal cycler-compatible enzymatic step, substantially simplifying the library preparation workflow without compromising performance.

The Twist TrueAmp Library Preparation Kit addresses a practical barrier that has long limited the adoption of FFPE-based NGS in settings where Covaris instrumentation is unavailable or cost-prohibitive. Its compatibility with both CGP and custom target enrichment panels positions it as a versatile solution for genomics research using FFPE material.

REFERENCES

1. Steiert, T. A. et al. A critical spotlight on the paradigms of FFPE-DNA sequencing. *Nucleic Acids Res.* 51, 7143-7162 (2023).
2. Gregory, T. et al. Characterization and mitigation of fragmentation enzyme-induced dual stranded artifacts. *NAR Genom. Bioinform.* 2, lqaa070 (2020).
3. Twist TrueAmp and PCR-Free WGS Library Preparation Kit Product Sheet. Twist Bioscience <https://www.twistbioscience.com/resources/product-sheet/twist-trueamp-and-pcr-free-wgs-library-prep>

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