Development of an optimized workflow for sensitive variant detection in FFPE-damaged samples

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Abstract

Background/Objectives: Next-Generation Sequencing (NGS) of formalin-fixed and paraffin-embedded (FFPE) samples allows for detailed characterization of cancer from routinely collected clinical specimens. However, the preservation process often leads to significant damage and degradation of genetic material, complicating library preparation. Furthermore, because sample availability is typically limited, there is a high demand for robust workflows that can efficiently convert more molecules into sequenceable libraries. Given the high variability in damage and yield across FFPE samples, generating high-quality NGS data for variant and indel identification requires a unified, high-efficiency workflow.

Methods: Presented here is a workflow leveraging the Twist TrueAmp library preparation kit and target enrichment workflow to maximize the conversion of damaged and low mass FFPE samples for NGS.

Results: We demonstrate library conversion of severely damaged (DIN<2) FFPE with as little as 5ng input mass. Compared to other workflows, an increase in library complexity is observed. Importantly, the enzymatic fragmentation of input DNA can be tuned to provide larger insert sizes that are appropriate for sequencing with longer read length. Coupled with target enrichment, more target sites are captured and the uniformity is significantly improved. These improvements are driven by the incorporation of Twist TrueAmp polymerase, an engineered polymerase with a low error rate capable of amplifying through GC extremes to recover challenging genomic regions.

Conclusion: In summary, our optimized library preparation kit built on engineered enzymes emerges as a valuable asset for the deployment of NGS-based FFPE assays.

TrueAmp Library Preparation Kit Workflow

- Enzymatic Fragmentation Library Preparation workflow built on Twist Engineered Enzymes

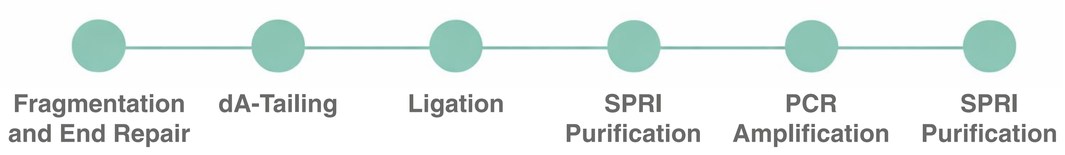


Figure 1. Method Outline for Twist TrueAmp Library Preparation Kit Library preparation relies on optimized enzymes: a tunable enzymatic fragmentation module, an efficient ligation module utilizing Twist engineered T4 DNA ligase, and a faithful amplification module with Twist TrueAmp Polymerase.

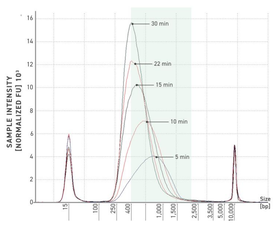


Figure 2. Tunable Enzymatic Fragmentation Fragmentation time titration demonstrates how different insert sizes can be obtained depending on downstream application needs.

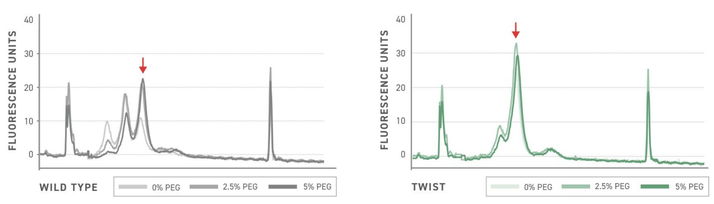


Figure 3. Improved Ligation Efficiency with Twist T4 DNA ligase Compared to WT T4 DNA ligase (left), Twist T4 DNA ligase (right) more efficiently converts insert molecules to double ligated products. Twist T4 DNA ligase does not display the dependence on crowding agents that are observed for the WT enzyme.

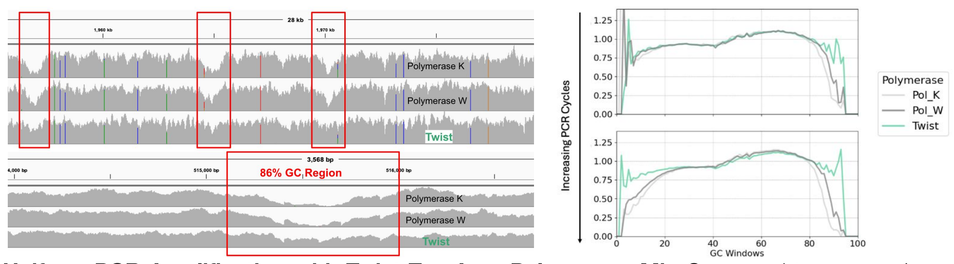


Figure 4. Uniform PCR Amplification with Twist TrueAmp Polymerase Mix Compared to commonly used NGS polymerase master mixes (polymerase K and polymerase W), Twist TrueAmp Polymerase Mix is able to amplify GC extremes up to 90% where others give significantly reduced amplification, and is able to efficiently amplify as low as 5% GC fragments.

FFPE Sample Input

- Fragment analysis depicting variable integrity of FFPE samples used in library preparation

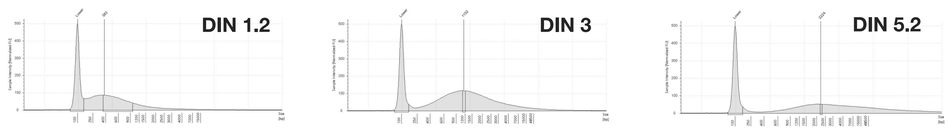
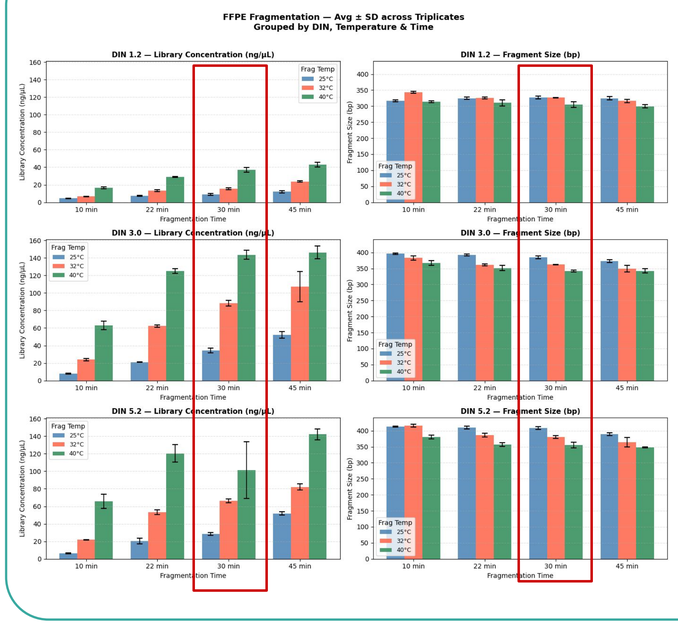


Figure 5. Fragment Analyzer Traces of Input FFPE Material Extracted FFPE DNA from Reference Medicine was analyzed on an Agilent 4200 Tapestation gDNA ScreenTape. DNA Integrity Numbers (DINs) ranging from 1.2 to 5.2 were observed.

Fragment Size Optimization



- Optimization of fragmentation conditions to achieve high yield and comparable fragment sizes for samples with varying DIN
- 40°C, 30min fragmentation identified as robust condition for FFPE samples

Figure 6. Impact on fragmentation time and temperature on library prep yield and library size Titration of time (10min, 22min, 30min, 45min) and temperature (25°C, 32°C, 40°C) shows a balance of insert size and yield.

Target Enrichment Performance

- Key Picard metrics are plotted, showing that FFPE libraries have similar quality to libraries prepared from gDNA (NA12878).
- The most extreme DIN (1.2) sample has degraded performance yet remains informative.
- Notably chimera rate, a widespread challenge for FFPE samples, stays below 5% for all samples.

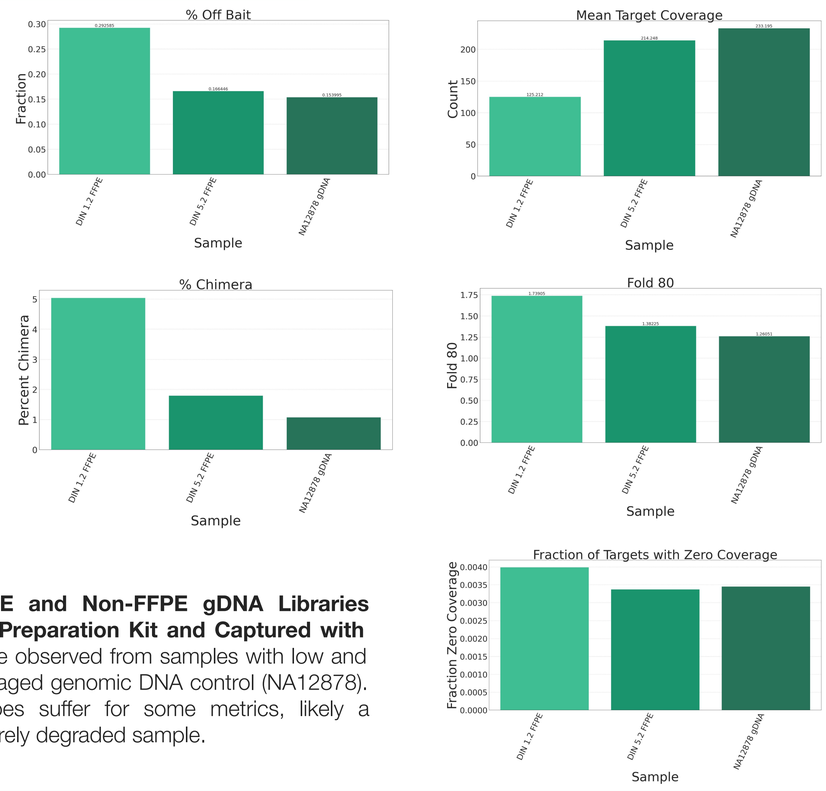


Figure 7. Picard TE Metrics of FFPE and Non-FFPE gDNA Libraries Prepared with Twist TrueAmp Library Preparation Kit and Captured with Twist CGP Panel. Consistent performance observed from samples with low and moderate DIN scores compared to undamaged genomic DNA control (NA12878). Sample with lowest DIN score (1.2) does suffer for some metrics, likely a consequence of lower insert size from severely degraded sample.

Variant Calling

- Detected VAF% for both TrueAmp (green) and Mechanical Fragmentation (blue) are similar to Horizon's expected VAF% values (dotted line).
- Accurate VAF% are able to be calculated from mild, moderate,and severe FFPE control samples, even for VAF below 5% .
- Unified enzymatic fragmentation protocol for all DIN scores used. Single fragmentation conditions allow for easier sample processing and do not require stratification of samples by DIN for library preparation.

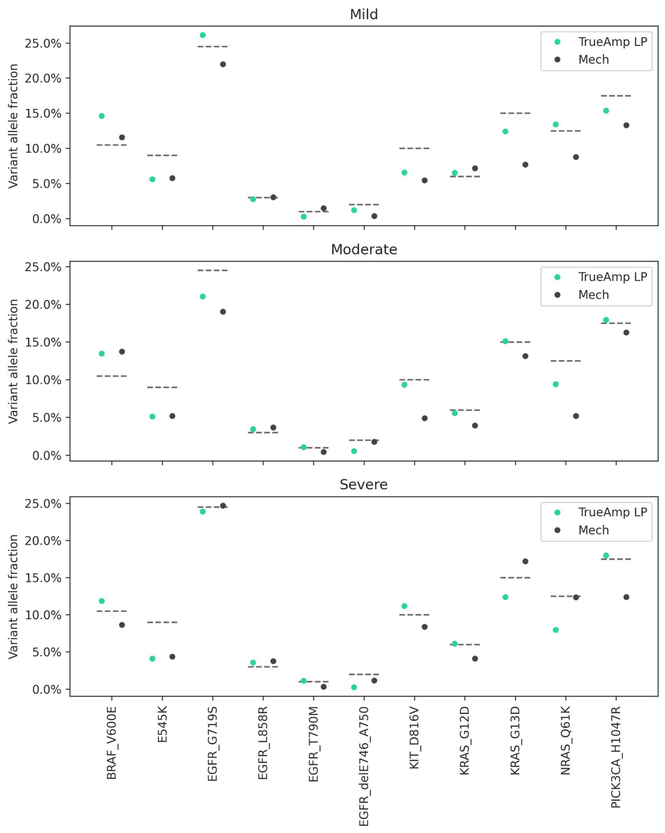


Figure 8. Variant Calling on Horizon FFPE Reference Standards Libraries. Libraries were fragmented using both Twist TrueAmp Library Preparation Kit (abbreviated as EF) for enzymatic fragmentation and Covaris Shearing (abbreviated as MF) into the Twist cfDNA Library Prep Kit with TrueAmp Polymerase Mix (cat # 116612). Samples processed are Horizon Discovery's FFPE controls that contain known variant allele frequencies (VAF) shown as dotted line. Calculated VAF for specific sites from EF and MF kits displayed as dots.

Materials and Methods

FFPE-derived DNA spanning a range of DNA Integrity Numbers (DIN) was obtained from clinical specimens sourced through Reference Medicine (CLIA ID# 03D2269440, Phoenix, AZ). Upon receipt, all samples underwent quality assessment using an Agilent 4200 TapeStation (cat. no. G2991BA) to confirm fragment size distribution and input DNA integrity prior to downstream processing.
Sequencing libraries were constructed from 15 ng of input DNA using the Twist TrueAmp Library Prep Kit (cat. no. 116481), applied to both clinical FFPE specimens and commercial FFPE reference standards (Horizon Discovery; cat. nos. HD798, HD799, HD803). The fragmentation step was systematically optimized across a matrix of reaction temperatures and incubation times to maximize library yield and improve fragment size homogeneity, with all conditions evaluated in technical triplicates.
Following library construction, target enrichment was performed using the Twist Oncology - DNA CGP Panel (cat. no. 116353) according to the Twist Standard Hybridization Solution v2 protocol. Enriched libraries were sequenced on an Illumina NextSeq 550 platform using a mid-output flow cell, targeting at least 10M reads as PE75. Sequencing data was subsequently down-sampled to a uniform depth of 500× raw coverage for comparative analysis across all conditions.
Variant calling was performed on the sequencing data. After adapter trimming, reads were aligned to the human genome (build hg38) using bwa-mem (Li and Durbin 2009). Variant calls/allele fraction estimates were obtained from raw allele pileups obtained with samtools mpileup on the aligned reads. Standard python data science libraries were used to transform and plot the data.

Conclusions

- Twist TrueAmp Library Preparation Kit is a enzymatic fragmentation based protocol that utilizes engineered enzymes for ligation and amplification.
- Through protocol optimization of fragmentation conditions a single protocol condition was found that produces high library yield and consistent insert size across a wide DNA integrity range.
- Samples processed using Twist TrueAmp Library Preparation Kit and captured with Twist CGP panel produce high quality TE data suitable for accurate variant calling from highly degraded FFPE samples.

References

Li H, Durbin R, Fast and accurate short read alignment with Burrows–Wheeler transform, Bioinformatics, Volume 25, Issue 14, 15 July 2009, Pages 1754–1760,

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