Matched fresh frozen and FFPE patient tissues reveal the enhanced sensitivity and data quality of a novel DNA library prep method

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Introduction

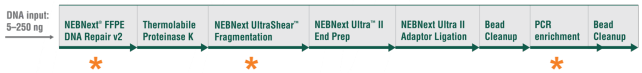
FFPE-derived DNA poses many notable challenges for preparing NGS libraries, including low input amounts and highly variable damage from fixation, storage, and extraction methods. It is difficult to obtain libraries with sufficient coverage and the sequencing artifacts arising from damaged DNA bases confound somatic variant detection. Additionally, many laboratories process FFPE tumor samples alongside matched, high quality, normal DNA and many library prep workflows are not readily compatible with both sample types.

We developed a novel NGS library prep method compatible with both high quality and very low quality FFPE DNA samples, employing three new enzyme mixes designed specifically for compatibility with FFPE samples including a DNA repair mix, an enzymatic fragmentation mix, and a high-yield PCR master mix. To validate this workflow on clinically relevant human samples, we obtained DNA extracted from matched tumor and normal tissue of various tissue types preserved by both fresh frozen and FFPE methods, with fresh frozen-extracted DNA providing the gold standard for library quality and mutation content. The FFPE DNA samples ranged in quality from DNA integrity number (DIN) 1.5 to 6.8. We prepared libraries using this method and compared with other library prep workflows and sequenced by WGS and target capture. This new enzymatic fragmentation-based library prep workflow not only reduced the false positive rate in somatic variant detection by repairing damage-derived mutations in FFPE DNA samples, but also improved the library yield, library quality metrics (including mapping, chimeras, and properly paired reads), library complexity, coverage depth and uniformity, as well as hybrid capture library quality metrics. Comparing variant calls from matched FFPE and frozen tissues revealed an improved sensitivity and accuracy using this library prep method compared to mechanical shearing and other enzymatic fragmentation library prep approaches.

This new suite of enzyme mixes improves the overall library prep success rate from challenging FFPE samples, allowing even highly damaged FFPE samples to achieve high quality libraries with a greater sensitivity for somatic variant identification. The workflow is robust and flexible, compatible with both FFPE DNA and matched high quality DNA samples as well as being automation-friendly for convenience in sample processing.

Methods

The NEBNext UltraShear® FFPE DNA Library Prep Kit Workflow



* New enzyme mixes optimized for FFPE library prep

- New and more efficient enzymatic DNA repair using NEBNext® FFPE DNA Repair v2
- New NEBNext UltraShear® enzymatic fragmentation mix optimized for use with FFPE DNA
- New NEBNext MSTC™ FFPE PCR Master Mix achieves high yields for target enrichment
- 5 – 250 ng input of FFPE DNA required, validated on FFPE DNA DIN 1.5-6
- Compatible with high quality DNA for convenience in processing with matched samples

Protocol A: Standard

- Fragmentation: 5 min
- Post-ligation cleanup: std 0.9X
- Optimal for complexity and recovery for samples DIN 1.5-5

Protocol B: Larger insert size

- Fragmentation: 30 min
- Post-ligation cleanup: modified 0.6X
- Better yield from samples DIN >5 and improved insert size for all samples
- Reduced yield and complexity from DIN <5

Results

NEBNext UltraShear FFPE Library Prep Kit enables higher yields and more on-target coverage from low quality FFPE DNA

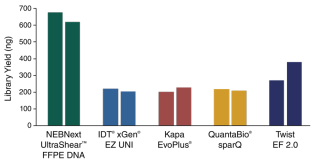


Figure 1. The NEBNext UltraShear FFPE DNA Library Prep Kit enables higher library yields than competitor library prep kits. Libraries were prepared in duplicate from 100 ng of low quality, normal tissue FFPE DNA (DIN 1.8) and 9 PCR cycles, using the NEBNext UltraShear FFPE DNA Library Prep Kit (Protocol A). Results were compared to other enzymatic fragmentation-based library prep kits that have been validated for use with FFPE samples, using each vendor's own recommended adaptors (IDT® xGen® EZ UNI, Kapa EvoPlus® Library Prep Kit, QuantaBio® sparQ DNA Library Prep Kit, and Twist Library Preparation EF 2.0 kit). Library yields (total ng) were quantified using the Qubit® High-Sensitivity dsDNA assay (Thermo Fisher Scientific®).

NEBNext UltraShear FFPE Library Prep Kit improves library quality and sequencing accuracy

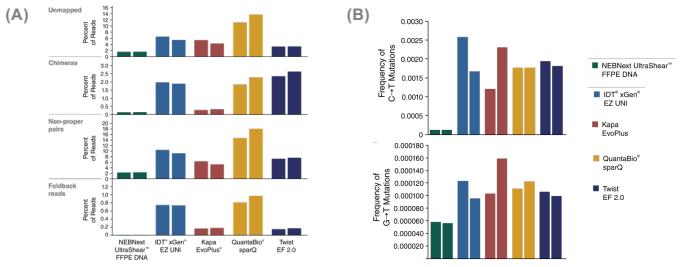


Figure 2. The NEBNext UltraShear FFPE DNA Library Prep Kit improves library quality and sequencing accuracy compared to competitor library prep kits. Libraries were prepared in duplicate from 100 ng of low quality, normal tissue FFPE DNA (DIN 1.8) and 9 PCR cycles, using the NEBNext UltraShear FFPE DNA Library Prep Kit (Protocol A). Results were compared to other enzymatic fragmentation-based library prep kits that have been validated for use with FFPE samples, using each vendor's own recommended adaptors (IDT xGen EZ UNI, Kapa EvoPlus Library Prep Kit, QuantaBio sparQ DNA Library Prep Kit, and Twist Library Preparation EF 2.0 kit). Libraries were sequenced on the Illumina® NovaSeq® 6000 (2 x 100 base reads) and downsampled to 5 million paired-end reads. Reads were mapped using Bowtie2 (version 2.3.2.2) to the GRCh38 reference and duplicates marked using Picard MarkDuplicates (version 1.56.0). (A) Library quality metrics were assessed using Picard Alignment Summary Metrics (version 1.56.0). The level of foldback reads was calculated using Seq_frag_remap (version 0.2). The NEBNext UltraShear FFPE DNA Library Prep Kit improves library quality by reducing the percentage of unmapped, chimeric, non-properly paired, and foldback reads. (B) The average frequency of C→T mutations at each C position (top) and G→T mutations at each G position (bottom) in Read 1 and 2 was calculated for two technical replicates using Tasmanian (version 1.0.7). C→T mutations arising from cytosine deamination and G→T mutations arising from oxidative damage in low quality FFPE DNA are effectively repaired by the NEBNext FFPE DNA Repair v2 Mix in the NEBNext UltraShear FFPE DNA Library Prep Kit. Other kits show a high level of C→T and G→T artifacts in low quality FFPE DNA due to a lack of DNA damage repair.

Results

High quality target enrichment libraries from both FFPE and fresh frozen

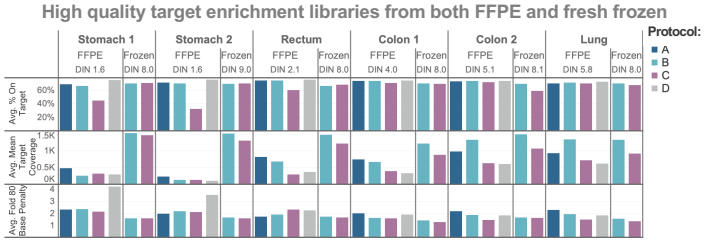


Figure 3. The NEBNext UltraShear FFPE DNA Library Prep Kit enables high quality target enrichment metrics from both FFPE and fresh frozen tissue DNA. Libraries were prepared in triplicate (1 rep shown) from 100 ng of varied quality tumor and normal from both FFPE and Fresh Frozen (only Tumor data shown), using four different protocols: A=NEBNext UltraShear FFPE DNA Library Prep Kit (Protocol A), B=NEBNext UltraShear FFPE DNA Library Prep Kit (Protocol B), C=NEBNext Ultra™ II DNA (Covaris® shearing), and D=NEBNext UltraShear module with Ultra II DNA. Libraries were captured with a custom panel from Twist Bioscience, sequenced on an Illumina NovaSeq6000 2x100, downsampled to 50M PE reads, reads were mapped using bwa-mem (v 0.7.17) to the GRCh38 reference, duplicates were marked using Picard MarkDuplicate and UMI (version 2.20.6), and capture metrics assessed using Picard HS metrics (v 2.18.29). Protocols A and B outperformed C and D for all sample qualities, due to the combined effect of FFPE DNA Repair v2 and UltraShear, with Protocol B outperforming Protocol A for samples with DIN >5. The UltraShear FFPE Library Prep Kit with Protocol B showed similar quality metrics to standard Covaris-sheared libraries for high quality fresh frozen DNA, indicating that this kit can be used for both FFPE and matched high- quality DNA in parallel.

Using matched patient samples to evaluate the NEBNext UltraShear FFPE DNA Library Prep in somatic variant calling

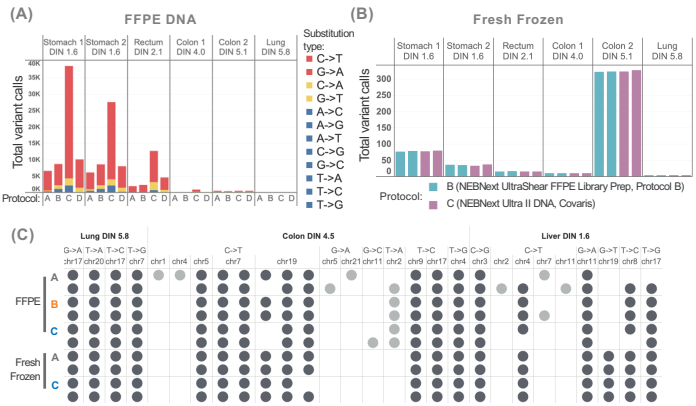


Figure 4. The NEBNext UltraShear FFPE DNA Library Prep Kit reduces variant calling artifacts and identifies more true variants than Covaris-sheared libraries. Libraries were prepared according to protocols A-D, captured using the custom Twist panel, sequenced, and analyzed according to Figure legend 4. The final bam files with UMI-based consensus reads were used for somatic variant calling with strelka2 (2.9.10). (A) The total number of variant calls (no VAF threshold) including both true and false positives are plotted for each FFPE tumor sample (1 rep shown) and colored according to substitution type: cytosine deamination (red), oxidative damage (yellow), and other (blue). (B) The total number of variant calls for each patient fresh frozen tumor sample is plotted, with the same number of variants identified using both NEBNext Ultra II DNA (Covaris) and the NEBNext UltraShear FFPE DNA Library Prep Kit (Protocol B) indicating the latter can be used for both FFPE and high quality DNA. (C) Using fresh frozen variant calls as a truth set for each patient tumor, the NEBNext UltraShear FFPE DNA Library Prep Kit identifies all true variants (AF≥2%) in FFPE DNA while Covaris misses variants and maintains more false positives. Three representative samples were selected across the range of FFPE DNA quality tested (DIN 1.5- DIN 5.8).

The UltraShear FFPE Library Prep Kit improves coverage of truth set variants in FFPE samples

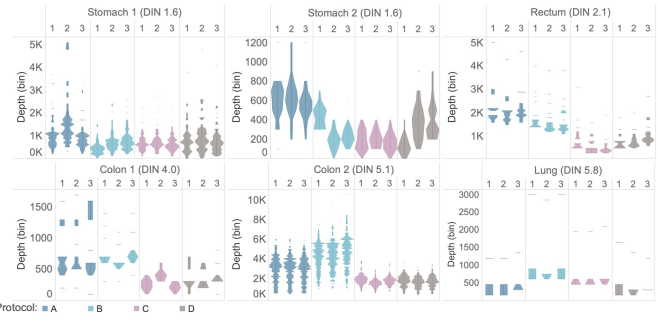


Figure 5. The UltraShear FFPE Library Prep Kit improves coverage of truth set variants in FFPE samples. Target capture libraries were prepared in triplicate from matched FFPE tumor and normal and sequenced on the NovaSeq6000 according to figure legend 2 with 50M PE reads for colon 1 and lung, and 160M PE reads for stomach 1, stomach 2, rectum, and colon 2. The final bam files with UMI-based consensus reads were used to curate all expected variants based on the matched fresh frozen variant truth set list. The total number of variants in each coverage depth bin (bin size=100), is plotted for each sample and each of the four workflows evaluated. The UltraShear FFPE Library Prep Kit Protocol A showed more variants detected in higher coverage bins for lower quality samples Stomach 1-Colon 1 (DIN <4) while UltraShear FFPE Library Prep Kit Protocol B performs better for higher quality FFPE samples Colon 2 and Lung (DIN >5) as longer fragmentation time enabled higher library conversion.

Conclusions

- The NEBNext UltraShear FFPE DNA Library Prep Kit produces higher yield, more usable data, and higher on-target coverage than other vendor workflows, enabling more sensitive variant detection
- A new protocol with longer fragmentation time and modified cleanup enhances performance from high quality FFPE DNA and matched high quality DNA samples