Evaluating DNA Quality from FFPE Samples

Guidelines for obtaining high-quality DNA sequencing results from FFPE samples using the TruSeq® Exome Library Preparation Kit.

Introduction

Formalin-fixed paraffin-embedded (FFPE) archival tissue samples provide an invaluable repository of information for disease research, especially cancer. However, FFPE samples can be difficult to process in many molecular biology assays because the fixation and subsequent tissue storage methods often cause nucleic acid degradation, resulting in fragmentation of the DNA.[1,2] The TruSeq Exome Library Preparation Kit overcomes this challenge to prepare sequencing-ready libraries from FFPE samples for next-generation sequencing (NGS) analyses. Using proven Illumina TruSeq chemistry, coding regions are captured directly, rather than by PCR amplification with site-specific primers (Figure 1). Enrichment-based library preparation offers excellent coverage of areas that are traditionally difficult to sequence such as high GC-rich regions, promoters, and repetitive content. Also, direct capture of target regions reduces library bias and gaps. The TruSeq Exome Library Prep Kit enables exceptional variant detection and high data quality.

Obtaining reliable, reproducible results from degraded FFPE DNA samples is possible if the DNA quality is accurately assessed. Effectively evaluating DNA quality is a critical step in successful FFPE DNA sequencing. To enable successful exome sequencing of FFPE samples, this technical note:

- Provides guidance for evaluating FFPE DNA samples before library preparation
- Outlines changes to the TruSeq Exome workflow needed to accommodate FFPE samples
- Sets expectations regarding the quality of sequencing data based on results from the DNA evaluation

Variability of FFPE Samples

The quality of DNA isolated from FFPE samples can vary widely among different specimens, or with different samples from the same specimen (Figure 2). DNA undergoes substantial chemical modifications during formalin fixation: nucleic acids are crosslinked to proteins and DNA is degraded into smaller fragments. Differences in fixation methods and age of archival tissue samples add further variation to DNA quality.[1,2] The TruSeq Exome Library Prep Kit is optimized to ensure high-quality DNA sequencing data from degraded FFPE samples. This kit allows researchers to take full advantage of the high reproducibility and high quality of Illumina sequencing. Evaluation of the quality of FFPE samples before library preparation enables successful implementation of the TruSeq Exome Library Prep Kit. Although most FFPE samples perform well with the exome enrichment probe set, some highly degraded samples contain fragments that are smaller than the optimal size range needed for efficient library preparation and probe hybridization.

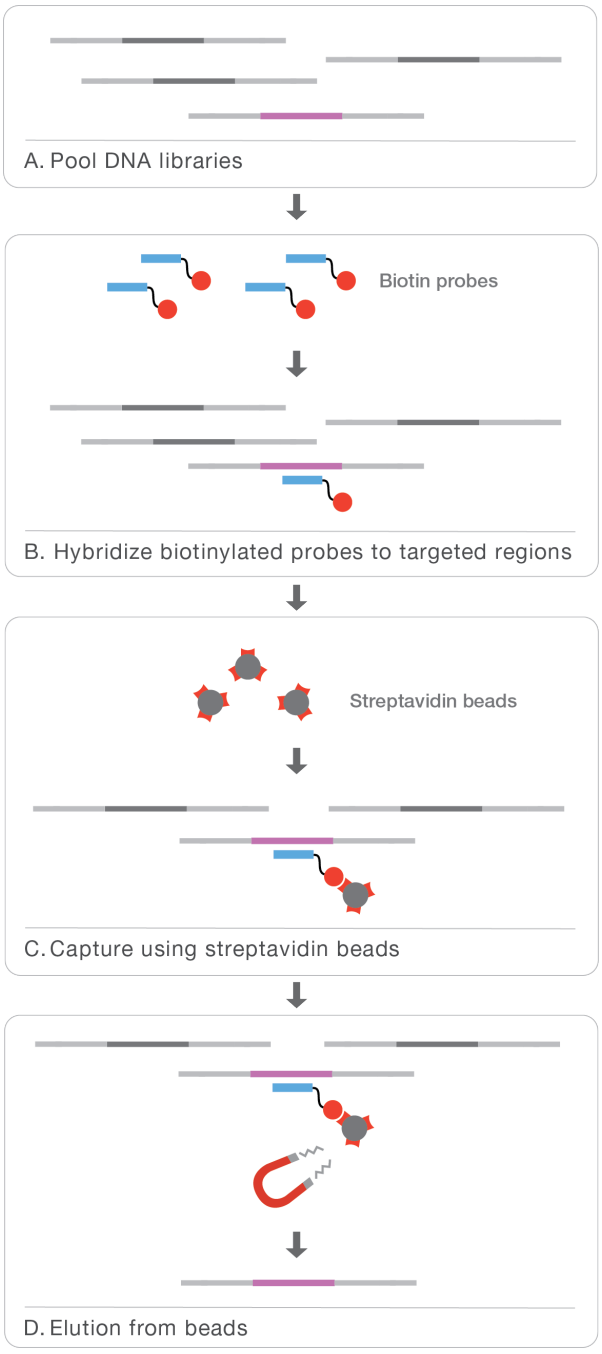
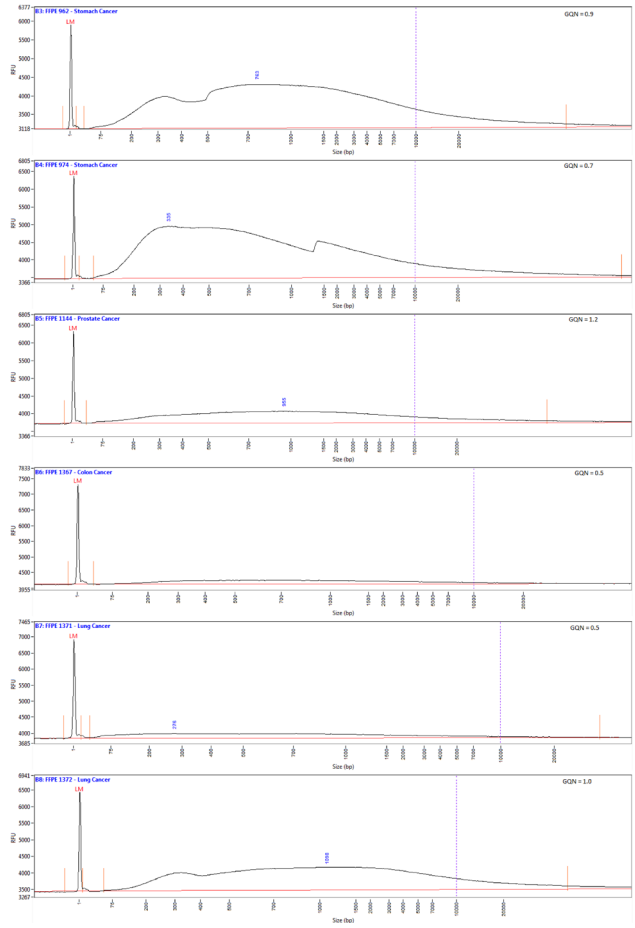


Figure 1: TruSeq Exome Capture Chemistry—The TruSeq Exome Library Prep Kit provides a simple and streamlined method for isolating targeted regions from challenging DNA samples.

A



B

Sample	Fragment Analyzer	
	Total Concentration (ng/µl)	GQN
FFPE 962 - Stomach Cancer	15.0022	0.9
FFPE 974 - Stomach Cancer	16.9694	0.7
FFPE 1144 - Prostate Cancer	5.0036	1.2
FFPE 1367 - Colon Cancer	1.2414	0.5
FFPE 1371 - Lung Cancer	1.7381	0.5
FFPE 1372 - Lung Cancer	9.1459	1.0

Figure 2: Variation in FFPE Sample DNA Quality—(A) DNA isolated from FFPE samples was analyzed with The Fragment Analyzer. A user-defined threshold of 10,000 bp was set (vertical purple line). (B) Genomic Quality Numbers (GQNs) were calculated for each sample.

Table 1: Recommended Changes to TruSeq Exome Workflow

FFPE Quality	GQN*	Input into Library Prep	No. of PCR Cycles	Input into Enrichment
High	> 0.7	100–300 ng	12	500 ng
Medium	0.3–0.7	300 ng	12	500 ng
Low	< 0.3	Not Recommended		

*Values reported from The Fragment Analyzer.

Evaluating DNA Quality

Many researchers use qPCR to determine the quality of DNA extracted from FFPE samples. When using the TruSeq Exome Kit, fragment analysis is a simpler and more reliable determinant of DNA quality.

Fragment analysis can be performed using The Fragment Analyzer, a highly sensitive, automated capillary electrophoresis system that reads fragments of 10–40,000 bp manufactured by Advanced Analytical Technologies. The system can resolve fragments down to a difference of 2 bp and accurately measure sample concentrations as low as 50 pg/µl. The Fragment Analyzer assesses DNA quality by calculating a Genomic Quality Number (GQN) for every sample. The GQN is a threshold set by the user that is defined as an application-specific base pair value considered to be good quality DNA. Fragment Analyzer software calculates the percentage of DNA above the threshold, producing a GQN value of 0–10. A low GQN value (< 2.5) represents sheared or degraded DNA. A high GQN (> 9) represents undegraded DNA. Based on the GQN values reported by The Fragment Analyzer (Figure 2), users can determine the appropriate changes needed for optimized use of the FFPE sample DNA in the TruSeq Exome workflow.

Recommendations for FFPE Samples

Based on results from The Fragment Analyzer, 3 changes are recommended to the standard TruSeq Exome workflow to accommodate FFPE samples (Figure 3). The first change is the amount of DNA input into library preparation (Step A). The second is the number of PCR cycles used in the first amplification (Step C). The third is the amount of library used in enrichment (Step D). The remaining steps of the workflow (Steps B, E-G) remain unchanged.

DNA input of 100–300 ng is recommended for high-quality samples with GQN values > 0.7, and 300 ng for medium quality samples with GQN values of 0.3–0.7 (Table 1). Although 300 ng is ideal, less input can be used in library preparation with the caveat that sequencing results may be affected. Due to their excessively degraded nature, low quality samples with GQN values < 0.3 are not recommended for use with the TruSeq Exome workflow (Table 1). Increasing the number of PCR cycles in the first amplification (Figure 3, Step C) to 12 for all samples with GQN values > 0.3 is required to increase library prep yield for a proper enrichment and to increase library complexity for improved coverage and variant calls (Table 1). Regardless of the initial input amount, due to the fragmented nature of FFPE DNA, increasing the amount for enrichment (Figure 3, Step D) to 500 ng ensures good on-target percentages from sequencing (Table 1).

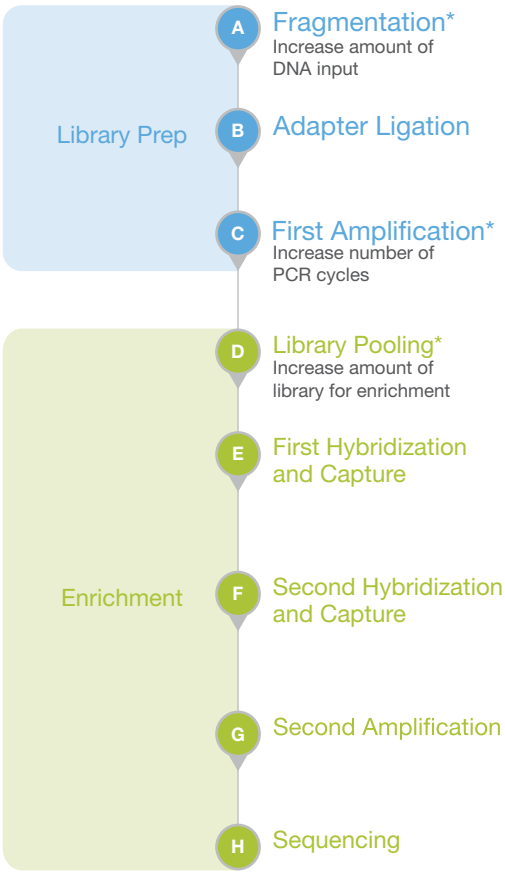


Figure 3: Recommended Changes to TruSeq Exome Workflow for FFPE DNA—Changes to the TruSeq Exome workflow to accommodate FFPE DNA are indicated. Remaining steps are unchanged.

Expected Results for FFPE Exome Sequencing

A set of FFPE and control DNA samples of varying sources and quality were taken through the TruSeq Exome sequencing workflow (Figure 4).

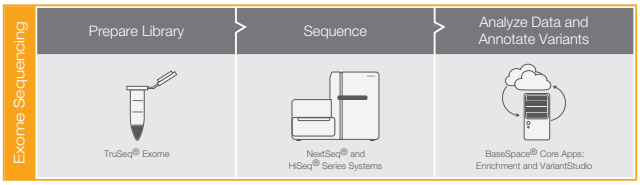


Figure 4: TruSeq Exome Sequencing Workflow—The TruSeq Exome Library Prep Kit is part of an integrated exome sequencing workflow that includes library preparation, sequencing, and data analysis.

Library Preparation

Eight FFPE DNA samples, 2 sets of vendor Tumor-Normal FFPE DNAs, and Coriell Cell Repositories NA12878 samples were run on The Fragment Analyzer. The DNF-488 High Sensitivity Genomic DNA Analysis Kit was used. A user-defined threshold of 10,000 bp was set for GQN calculations. All FFPE libraries were prepared for sequencing with the TruSeq Exome Library Prep Kit with changes to the workflow recommended for medium quality samples. The 8 FFPE DNA and NA12878 samples were run through the TruSeq Exome kit in duplicate, with 100 ng of input each. The vendor FFPE samples were run through the TruSeq Exome kit (Tumors in duplicate and Normal with a single rep) at input amounts of 100 ng and 300 ng.

These FFPE samples showed a positive correlation between the prelibrary prep GQN value and postlibrary prep yield. Samples with GQNs > 0.3 resulted in significantly higher yields from library preparation (Figure 5). Of note, FFPE samples with GQNs of 0.4-1.2 (purple and blue dots, Figure 5) resulted in similar library yields as control samples with high GQN values (blue dots with GQN = 8, Figure 5).

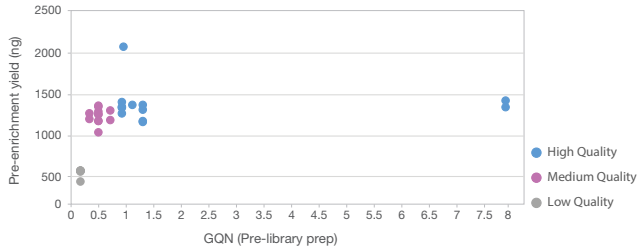


Figure 5: GQN Value Correlates to Postlibrary Prep Yield—GQN values are plotted against respective postlibrary prep yields (ng) for each sample. FFPE samples with GQNs < 0.3 (low quality, grey dots) resulted in significantly lower yields than medium and high-quality samples.

Sequencing

6-Plex hybridizations were performed with like input amounts and sequenced on the HiSeq® 2500 System in rapid-run mode with V2 chemistry and 2 × 76 bp sequencing read length. Data analysis was done in the BaseSpace® Sequence Hub using the FASTQ Toolkit to subsample the data to 10M and 50M reads and run through BWA Enrichment Application with Picard.

FFPE samples showed correlation of prelibrary prep GQN values and 2 different enrichment metrics. Low quality FFPE samples (GQNs < 0.3) failed to meet the standard metric requirement of 80% for padded read enrichment (Figure 6A). Also, they resulted in significantly lower target coverage at 20× (Figure 6B), as compared to medium and high-quality samples. Despite a range of GQN values of 0.4–1.2, medium and high-quality FFPE samples displayed similar padded read enrichment efficiencies and target coverage at 20×.

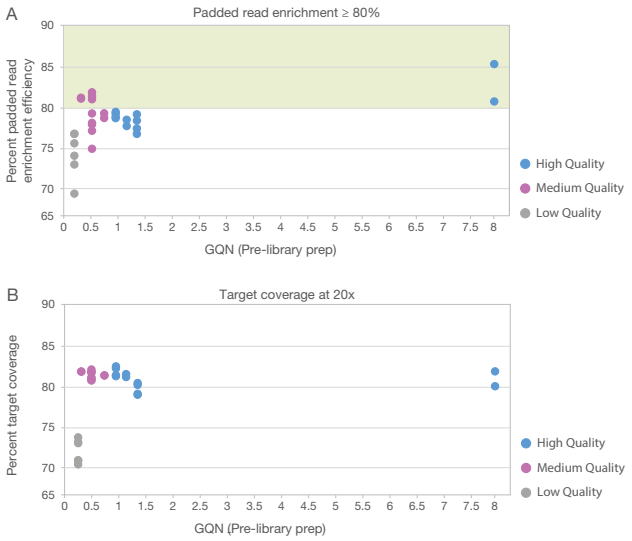


Figure 6: GQN Values Correlate to Enrichment Metrics—GQN values are plotted against respective padded read enrichment (A) and target coverage at 20× (B) from FFPE sample sequencing data. No low quality FFPE samples (GQNs < 0.3) met the standard sample metric requirement (shaded green area) and had significantly lower target coverage than medium and high-quality samples.

Collectively, these data indicate that the TruSeq Exome Kit is a robust workflow that can accommodate multiple sample types of varying quality with minimal impact on enrichment metrics. The TruSeq Exome Library Preparation Kit enables exome sequencing with low input (100–300 ng DNA) and fewer reads (≤ 50 M), allowing more samples per run for high-quality, cost-effective exome analysis of precious FFPE samples.

Summary

NGS approaches applied to FFPE tissue samples, along with their associated clinical data, offer an invaluable resource for translational research. The TruSeq Exome Library Prep Kit allows researchers to use FFPE and other challenging samples in their NGS studies. For these studies, fragment analysis of the samples, as determined by a Fragment Analyzer trace, is a reliable predictor for successful DNA sequencing results using the TruSeq Exome Library Prep Kit.

Learn More

To learn more about the TruSeq Exome Library Prep Kit, visit www.illumina.com/products/truseq-exome.html

For more FFPE solutions, visit www.illumina.com/science/education/ffpe-sample-analysis.html

References

1. Wang F, Wang L, Briggs C, et al. DNA degradation test predicts success in whole-genome amplification from diverse clinical samples. J Mol Diagn. 2007;9(4):441–451.
2. Srinivasan M, Sedmak D, and Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol. 2002;161(6):1961–1971.

Illumina • 1.800.809.4566 toll-free (US) • +1.858.202.4566 tel • techsupport@illumina.com • www.illumina.com

illumina®