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Assessing the Quality of FFPE Samples for Preparation of TruSight® Tumor 15 Sequencing Libraries

A comparison of FFPE DNA extraction kits with key indicators of DNA quality for optimal next-generation sequencing results.

Introduction

Next-generation sequencing (NGS) has changed the face of oncology research by significantly increasing the breadth, sensitivity, and specificity of information obtainable within a single assay. Illumina offers simple, comprehensive solutions for producing high quality data necessary for accurate analysis, enabling an increasing number of research and clinical laboratories to embrace the power of NGS.

Traditionally, scientists have used formalin-fixed, paraffin-embedded (FFPE) tissue for morphological analysis of solid tumors. Recently scientists have developed molecular tests for these valuable samples to develop markers, and better understand the factors that drive tumorigenesis. However, the formalin-fixation and paraffin-embedding process reduces DNA quality by fragmenting, cross-linking, and introducing damage through chemical modifications. For some molecular assays, both the quality and quantity of input DNA are critical for producing high-quality data. To help with this challenge, Illumina defines several key checkpoints for DNA and library quality to ensure success with each NGS application, including the TruSight Tumor 15 assay using the MiSeq® or MiniSeq™ System.

The TruSight Tumor 15 assay[1] is a targeted sequencing solution in which library preparation and analysis steps are optimized to provide high sensitivity while avoiding FFPE-induced artifacts. The TruSight Tumor 15 Reference Guide[2] provides guidelines for sample input, including tissue requirements and DNA extraction methods. This white paper illustrates how these guidelines apply to FFPE samples. First, tumor types were assessed for variances in DNA yield. Then the quality of extracted DNA obtained using 3 commercially available kits was evaluated as it pertained to NGS performance. DNA was extracted from the ReliaPrep FFPE gDNA Miniprep System (Promega), the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN), and the AllPrep DNA/RNA FFPE Kit (QIAGEN). Then samples were evaluated for DNA amplification potential, quantity of library preparation, and quality of library preparation as determined by size analysis and sequencing metrics.

Tissue Requirements for TruSight Tumor 15

To establish guidelines for minimal tissue requirements for the TruSight Tumor 15 assay, FFPE tumor samples from 6 different tissue types were sourced from 3 commercial vendors (Table 1). FFPE blocks were sectioned using a microtome into 5 µm sections. “Cumulative area” was used to normalize yields for different tissue areas and different numbers of sections. This metric describes the sum of tissue area across multiple 5 µm sections. Following DNA extractions, a fluorometric method (Accuclear Ultra High Sensitivity dsDNA Quantitation Kit) was used to measure dsDNA concentration.

As expected, a wide distribution of yields occurred from similar tissue areas due to the highly variable nature of FFPE samples. Median values for DNA yield did not reveal a wide distribution according to tissue source (Table 1). However, when considering confidence in obtaining the minimum amount of DNA required, outlying statistical values (Table 1, bottom decile yield) are more indicative of tissue-specific behavior. The minimal tissue requirement (Table 1) indicates the amount from which 90% of samples would yield ≥ 20 ng DNA at a minimum concentration of 2 ng/µL, the amount required for the initial step of library preparation in the TruSight Tumor 15 assay. Therefore, the recommended tissue requirement for the TruSight Tumor 15 assay has been set for 5 µm sections with an area ≥ 140 mm^2 for tissues other than melanoma. Due to the physical nature of melanoma tissue, a significantly larger amount of tissue sample is required.

DNA Requirements for TruSight Tumor 15

After a preliminary screen of 11 commercially available DNA extraction kits, 3 kits were deemed suitable for further assessment of compatibility with the TruSight Tumor 15 assay (Table 2). Commercially available FFPE blocks were sectioned using a microtome into 10 µm sections, and DNA extracted according to manufacturer’s instructions. At least 20 different tumor samples from multiple tissue types were used for DNA yield and DNA amplification potential analysis (Table 3).

Table 1: DNA Yield per Volume of FFPE Sample, by Tissue Type

Parameter	Bladder	Breast	Colon	Lung	Melanoma	Ovary
No. of Samples Tested	n = 27	n = 52	n = 35	n = 37	n = 37	n = 27
Median Yield (ng/mm^2)	1.96	1.78	2.40	2.12	1.49	3.57
Bottom Decile Yield (ng/mm^2)	0.79	0.43	0.71	0.99	0.10	1.43
Minimal Tissue Requirement (mm^2)	76	139	85	61	618	42

Area calculations correspond to using 5 µm sections. Median yield: 50% of samples gave this yield or higher. Bottom decile: mean value for the lowest 10% of sample yields. Minimal tissue requirement is calculated as tissue area value for which 90% of samples give ≥ 20 ng DNA at a minimum concentration of 2 ng/µL.

Table 2: DNA Extraction Kits

Description	Vendor	Catalogue No.
ReliaPrep FFPE gDNA Miniprep System	Promega	A2352
QIAamp DSP DNA FFPE Tissue Kit	QIAGEN	60404
AllPrep DNA/RNA FFPE Kit	QIAGEN	80234

DNA Yield from Extraction Kits

Extracted DNA was characterized by DNA yield per mm^2 of tissue area. DNA concentration was measured using a fluorometric method (Qubit dsDNA HS Assay Kit). Although DNA yields varied among different tissue types and extraction kits, all 3 kits yielded sufficient DNA (≥ 20 ng) for library preparation (Figure 1).

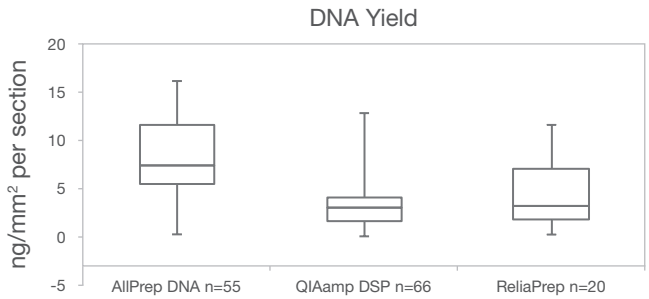

Figure 1: DNA Concentration Yield—Each DNA extraction kit was used according to the manufacturer's instructions. At least 20 samples were processed with each kit. Tissue sections were 10 µm thick.

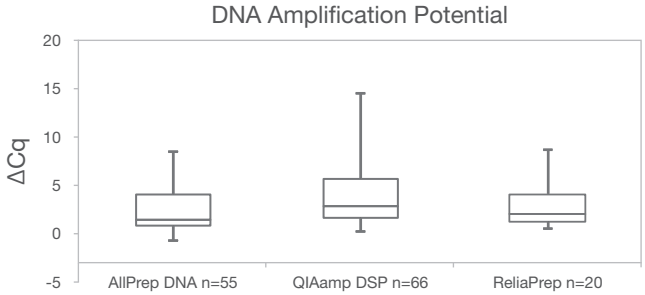

Figure 2: DNA Quality is Similar Across Extraction Kits—At least 20 samples were processed with each DNA extraction kit. DNA was qualified by qPCR. The ΔCq value indicates the difference in Ct value between each sample and that of a non-FFPE reference gDNA.

DNA Amplification Potential

Due to degradation and chemical modifications in FFPE samples, the amplifiable mass of FFPE DNA capable of generating library product will often be a fraction of the total amount extracted from FFPE tissues. The TruSight Tumor 15 multiplex PCR process relies upon the ability of a DNA polymerase to read across a DNA template. Therefore, a quantitative PCR (qPCR) approach was taken to qualify the performance of DNA extracted from FFPE tissue. By comparing the amplification potential of FFPE DNA relative to that of a non-FFPE reference gDNA, a ΔCq value can be calculated for each sample and used to predict its performance in PCR-based methodologies. Samples that amplified at later cycles than control DNA were of lower quality than samples with ΔCq close to 0. Average ΔCq values were not appreciably different among the tested kits, and all extraction kits perform well with mean ΔCq value < 3 (Figure 2).

Table 3: FFPE Tumor Samples Analyzed

Sample ID	Tumor Type	Tissue Area (mm^2)	% Tumor	Pathology Notes
1	Bladder	66	95	Transitional cell carcinoma
2	Bladder	180	70	Transitional cell carcinoma
3	Breast	135	90	Invasive papillary carcinoma
4	Breast	84	70	Infiltrating lobular carcinoma
5	Colon	198	30	Adenocarcinoma
6	Colon	100	95	Adenocarcinoma
7	Colon	160	90	Mucinous adenocarcinoma
8	Lung	180	15	Adenocarcinoma in background of lymphocytes
9	Lung	126	95	Adenocarcinoma
10	Melanoma	120	95	Metastatic melanoma to lymph node
11	Melanoma	77	95	Non-pigmented nodular melanoma epithelioid cell type
12	Melanoma	200	85	Heavily pigmented nodular melanoma epithelioid cell type

FFPE blocks were sourced from 2 commercial vendors (Proteogenex and Precision Medicine). DNA was extracted from 10 µm sections for each kit, and tissue area represents the cumulative area from 2-4 slides.

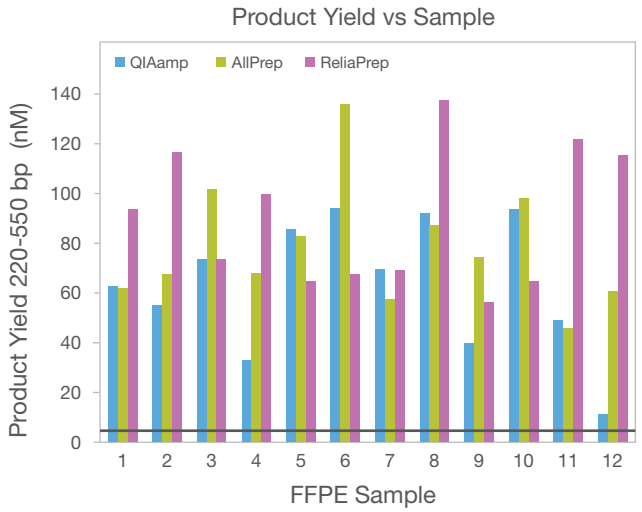

Figure 3: Product Yield After Library Prep—Following PCR purification, libraries within the 220–550 bp range were quantitated on a Fragment Analyzer. The black line indicates the threshold (4 nM) for a passing sample.

Library Product Yield

A subset of 12 samples from 5 tissue types were selected for library preparation using the TruSight Tumor 15 library prep kit (Table 3). Initial steps involved PCR amplification of the targeted regions (amplicons), and tagging the PCR products with indexed adapters. Following PCR purification, library samples were analyzed on a Fragment Analyzer to measure fragment size and yield. A passing library expected to yield high-quality NGS data will have a concentration of 4 nM in the 220–550 bp range. Although a wide range of DNA concentration was observed, all tested library samples surpassed the 4 nM threshold (Figure 3, black line).

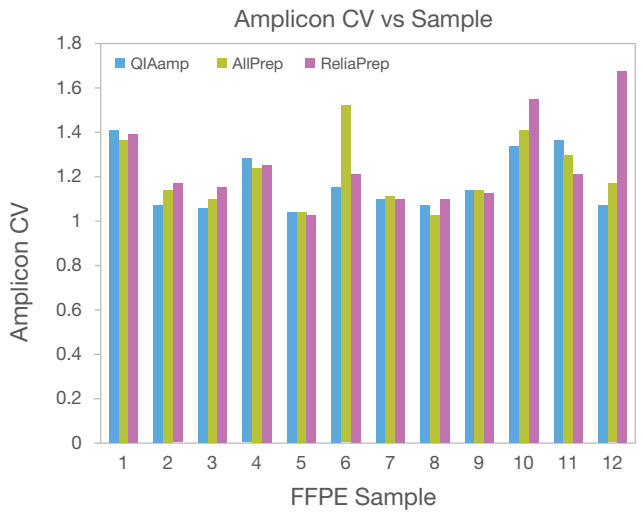

Figure 4: Amplicon CV Among Different Extraction Kits—Coefficient of variation among amplicon depths (Amplicon CV) is calculated as the standard deviation over the mean for amplicon coverage. Minimal differences between kits for each sample were detected.

Amplicon CV

Prepared TruSight Tumor 15 libraries were sequenced on the MiSeq System. Quality was assessed using the amplicon CV sequencing metric. A value for coefficient of variation among amplicon depths (Amplicon CV) assessed the uniformity of coverage over all targeted amplicons, and is calculated as the standard deviation over the mean. A comparison of amplicon CV for each sample shows minimal differences between kits, and also indicates consistency between samples for each kit (Figure 4).

Summary

The TruSight Tumor 15 assay has been used successfully with various tissue types and with DNA samples of varying quality. Focusing on FFPE sources, the pass/fail criteria of each metric in this study were used to determine current guidelines for amount of starting material and DNA extraction methods. Therefore researchers should refer to the TruSight Tumor 15 Reference Guide[2] to obtain optimal results.

Various DNA extraction kits that were tested yielded sufficient quantities of DNA; however, DNA quality from FFPE samples varied considerably when assessing QC metrics relevant to NGS data quality. Only 3 of the tested kits yielded DNA that consistently performed well at tests for DNA amplification potential, library product yield, and amplicon CV. Using DNA of sufficient quality to pass these tests, along with guidelines for minimum amounts of starting material and prepared libraries, should result in NGS data of high enough quality to make accurate analyses of FFPE tumor samples. In conclusion, the ReliaPrep FFPE gDNA Miniprep System (Promega), the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN), and the AllPrep DNA/RNA FFPE Kit (QIAGEN) consistently provided high library QC pass/fail rates. Therefore these 3 kits are recommended for DNA extraction from FFPE samples for use with the TruSight Tumor 15 assay.

Learn More

For more information about targeted sequencing with solid tumors, visit www.illumina.com/OncologyPanel.

References

1. Illumina (2015) TruSight Tumor 15. (www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/trusight-tumor-15-data-sheet-1170-2015-003.pdf).
2. Illumina (2015) TruSight Tumor 15 Reference Guide. (support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/trusight/trusight-tumor-15-reference-guide-1000000001245-03.pdf).

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