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Assessing DNA and RNA Quality from FFPE Samples for TruSight® Tumor 170

FFPE tissue requirements and comparison of nucleic acid extraction kits with key indicators of DNA and RNA quality for optimizing next-generation sequencing.

Introduction

Next-generation sequencing (NGS) confers the ability to examine a broad array of molecular variants, and enables cancer research by significantly increasing the breadth, sensitivity, and specificity of information obtainable within a single assay. As an increasing number of research and clinical laboratories embrace the power of NGS, Illumina offers simple, comprehensive solutions for producing high-quality data necessary for accurate analysis.

Formalin-fixed, paraffin-embedded (FFPE) tissues are a valuable source of material for tumor analysis. However, the formalin fixation and paraffin embedding process impacts nucleic acid quality by fragmenting, cross-linking, and introducing damage through chemical modifications. As the assays used to evaluate DNA and RNA have evolved to obtain more information, the quality and amount of nucleic acid extracted from FFPE material has become more critical for producing high-quality data.

TruSight Tumor 170 is a comprehensive sequencing panel that detects small variants, amplifications, fusions, and splice variants known to contribute to solid tumor progression. The assay is compatible with low-quality FFPE samples, using 40 ng DNA and 40 ng RNA. To ensure sequencing success, the TruSight Tumor 170 Reference Guide[1] provides guidelines for quantity and quality of nucleic acid input. This white paper illustrates how these guidelines were established for nucleic acid extraction from FFPE sources. After assessing nucleic acid yield in different tissue sources, the quantity and quality of extracted nucleic acids from three commercially available kits were evaluated as they pertain to generation of NGS data with TruSight Tumor 170. The samples were assessed by nucleic acid quantification, DNA amplification potential, RNA fragment size, and quality of library preparation as determined by sequencing metrics.

Tissue Requirements

To establish guidelines for minimal tissue requirements for TruSight Tumor 170, FFPE tumor blocks from six different tissue types (Table 1) were sectioned using a microtome. To normalize the yields for different tissue areas and different numbers of sections, the metric of "cumulative area" was used, which describes the sum of tissue area across multiple 5-µm sections. Following extractions, a fluorometric method was used to measure nucleic acid concentration.

Nucleic acid yields from similar tissue areas were highly variable, as expected due to the variable nature of FFPE samples. The minimal tissue requirement value (Table 1) indicates the amount from which 90% of samples would yield ≥ 40 ng of nucleic acid, the minimum needed for input into TruSight Tumor 170. While melanoma samples require more sample to reach this minimum, for other tissues Illumina recommends nucleic acid extraction from a minimum of 2 mm^3 of FFPE tissue to ensure that an adequate yield is achieved.

Nucleic Acid Requirements

After a preliminary screen of 11 commercially available nucleic acid extraction kits, three kits (Table 2) were deemed suitable for further assessment of compatibility with TruSight Tumor 170. Commercially available FFPE blocks from 6 tissue types (Table 3) were sectioned by microtome and used for simultaneous DNA and RNA extraction according to manufacturer instructions.

Table 2: Nucleic Acid Extraction Kits

Description	Vendor
AllPrep DNA/RNA FFPE Kit	Qiagen
Maxwell CSC DNA FFPE Kit	Promega
VERSANT Tissue Preparation Reagents Kit	Siemens

Table 1: Tissue Requirements for FFPE Samples According to Tissue Type

DNA Extraction	Tissue Type						
	Breast	Colon	Lung	Ovary	Pancreas	Melanoma	Combined
No. of samples tested	n = 81	n = 59	n = 64	n = 52	n = 20	n = 68	n = 344
Minimum tissue requirement[a]	1.85 mm^3	0.60 mm^3	0.65 mm^3	0.55 mm^3	0.56 mm^3	5.64 mm^3	1.15 mm^3
RNA Extraction	**Tissue Type**						
	Breast	Colon	Lung	Ovary	Pancreas	Melanoma	Combined
No. of samples tested	n = 29	n = 22	n = 20	n = 25	n = 20	n = 31	n = 147
Minimum tissue requirement[a]	0.93 mm^3	2.73 mm^3	0.50 mm^3	0.48 mm^3	0.50 mm^3	1.59 mm^3	0.98 mm^3

Minimal tissue requirement is calculated as tissue area value for which 90% of samples give ≥ 40 ng DNA at a minimum concentration of 3.3 ng/µl.
a. 9th decile: mean value for the lowest 10% of sample yields.

The AllPrep DNA/RNA FFPE Kit consistently yielded the highest concentration of DNA across multiple tissue types (Figure 1A). Notably, all but one of the extractions yielded > 3.3 ng/µl,the minimal concentration required for TruSight Tumor 170.

Due to degradation and chemical modifications in FFPE tissue samples, the amplifiable mass of DNA capable of generating library product will often be a fraction of the total amount extracted from the FFPE tissues. Therefore, a quantitative PCR (qPCR) approach was taken to qualify the performance of DNA extracted from FFPE tissue. By comparing the amplification potential of 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 values close to zero. DNA extracted from the AllPrep DNA/RNA FFPE Kit yielded the lowest ΔCq levels for all but one sample (Figure 1B).

RNA yields were not consistently higher from any of the kits tested, while the minimal concentration requirement of 3.3 ng/µl was met for all samples (Figure 1C). Given its consistent yields and DNA quality, Illumina recommends using the AllPrep DNA/RNA FFPE Kit to extract nucleic acids for use with TruSight Tumor 170.

Table 3: Samples for Analysis of Nucleic Acid Extraction Kits

Original Sample ID	Description
617B	FFPE bladder cancer sample
795B	FFPE bladder cancer sample
866V	FFPE ovarian cancer sample
L305	FFPE lung cancer sample
M4921	FFPE melanoma sample
M8324	FFPE melanoma sample
PCO2	FFPE colon cancer sample
R7522	FFPE breast cancer sample
V7	FFPE ovarian cancer sample

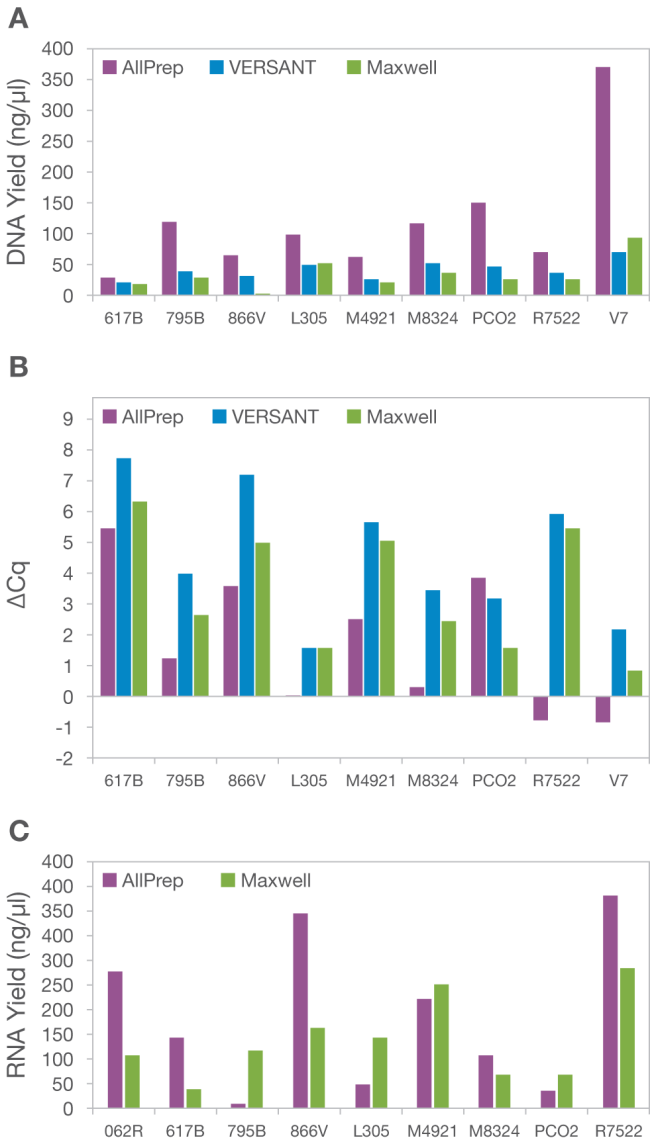

Figure 1: Comparison of yield and quality from nucleic acid extraction kits — Equal amounts of each FFPE tissue (Table 3) were processed with each kit (Table 2) according to manufacturer instructions. A. DNA quanitity is expressed as ng/µl. B. DNA quality is expressed as ΔCq, the difference in Ct value between each sample and that of a non-FFPE reference gDNA. C. The RNA quantity is expressed as ng/µl. Required concentration for both RNA and DNA input into TruSight Tumor 170 is ≥ 3.3 ng/µl.

Nucleic Acid Quality and Assay Performance

To assess the impact of nucleic acid quality and quantity on performance with TruSight Tumor 170, the remaining studies were performed with samples extracted with the AllPrep DNA/RNA FFPE Kit.

Illumina recommends an input range of 40–120 ng of DNA for TruSight Tumor 170. To assess the impact of DNA quality on performance at minimum input, DNA samples were grouped into 3 ranges of ΔCq values, and 40 ng was used for library prep and sequencing. To determine DNA variant calling accuracy, TruSight Tumor 170 uses two sample metrics: median insert size and the percent of exons exhibiting greater than 100× coverage. Using these metrics, sample success rate was near 100% for samples with ΔCq values < 5, but decreased considerably with ΔCq values > 5 (Figure 2). Therefore, Illumina recommends using samples with a ΔCq value < 5 to obtain optimal performance of TruSight Tumor 170.

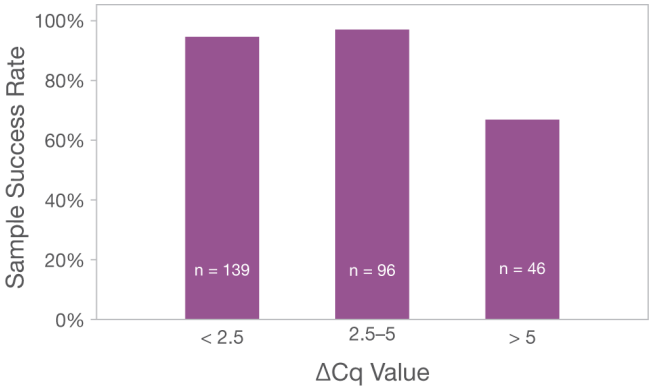

Figure 2: TruSight Tumor 170 Performance with DNA Samples of Varying Quality—DNA samples were placed into three groups according to ΔCq values, and 40 ng of each sample was sequenced with TruSight Tumor 170. The number of samples in each group is indicated. Variant calling was performed with the TruSight Tumor 170 App, and the percentage of samples successfully passing variant calling quality control metrics are shown.

FFPE materials are sometimes scarce enough that researchers choose to submit low-quality samples for sequencing. To address this problem, the impact of adding higher input amounts was assessed. Samples with increasing ΔCq values were sequenced in duplicate at low- and high-input levels using sequence coverage as a metric for sample success. At low input (40 ng), all samples with ΔCq values < 5 passed acceptance criteria while two samples with ΔCq values > 5 did not pass (Table 4). Raising the input level to 120 ng DNA increased success rates with the low-quality samples, while no impact was observed with high-quality samples. Due to these results, Illumina recommends adding > 40 ng of DNA to TruSight Tumor 170 when enough sample is available. Although ΔCq is a good indicator for sample success to be used prior to the library prep stage, TruSight Tumor 170 has quality control checks after enrichment, during sequencing, and after variant calling. These checks provide the user with additional confidence in the quality of results from each sample.

Table 4: TruSight Tumor 170 Performance with DNA Samples of Varied Quality at Low and High Input

DNA QC Metric	Sample ID	ΔCq	40 ng rep 1	40 ng rep 2	120 ng rep 1	120 ng rep 2
% of Bases ≥ 100×	1	-2.59	100%	100%	100%	100%
	2	-1.09	100%	100%	100%	100%
	3	1.25	100%	99%	100%	100%
	4	1.33	100%	98%	100%	100%
	5	2.12	100%	100%	100%	100%
	6	2.28	100%	99%	100%	100%
	7	3.13	100%	100%	100%	99%
	8	3.55	100%	100%	100%	100%
	9	5.33	27%	29%	100%	100%
	10	5.78	89%	86%	100%	82%

Samples with increasing ΔCq value were used for library preparation with TruSight Tumor 170. Each sample was prepped in duplicate at low input (40 ng) and high input (120 ng). Sequencing performance was assessed as percentage of bases covered at ≥ 100×. Acceptance criteria for this metric is ≥ 95% of bases with ≥ 100× coverage.

To assess the impact of RNA quality on sequencing performance, RNA fragment size was used as an indicator of quality. Specifically, the DV_{200} metric represents the percentage of RNA fragments > 200 nucleotides. RNA samples were grouped according to DV_{200} values, then 40 ng was used for library prep and sequencing. To determine RNA variant calling accuracy, TruSight Tumor 170 uses two sample metrics: median insert size and median coverage uniformity per transcript. Using these metrics, sample success rate was close to 100% for samples with DV_{200} values ≥ 20, and decreased considerably with DV_{200} values < 20 (Figure 3). Due to these results, Illumina recommends using samples with a DV_{200} values ≥ 20 for TruSight Tumor 170. The recommended RNA input range is 40 to 80 ng of RNA. Therefore, if optimal performance is required from samples with DV_{200} value < 20, Illumina recommends adding up to 80 ng RNA input.

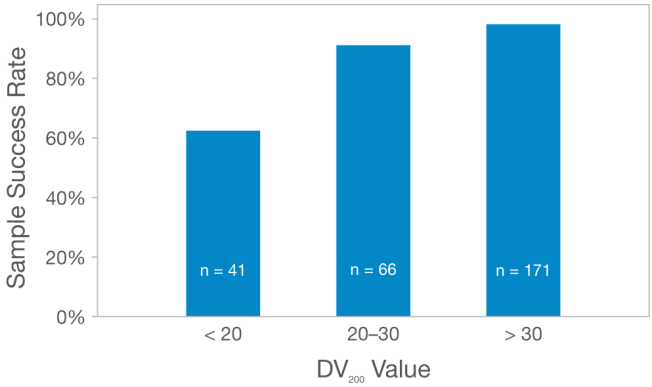

Figure 3: TruSight Tumor 170 Performance with RNA Samples of Varied Quality—RNA samples were placed into three groups according to DV_{200} values, and 40 ng of each sample was sequenced with the TruSight Tumor 170 Assay. The number of samples in each group is indicated. Variant calling was performed with the TruSight Tumor 170 App, and the percentage of samples successfully passing variant calling quality control metrics are shown.

Summary

Obtaining high-quality nucleic acid from FFPE tissue samples is a key factor in the performance of any NGS-based panel. This white paper demonstrates the impact of quantity and quality of nucleic acids on performance of TruSight Tumor 170. Results of this study indicate that 2 mm^3 of FFPE sample is the optimal amount for obtaining sufficient quantity of nucleic acids for tissues other than melanoma. The data also indicate that the AllPrep DNA/RNA FFPE Kit gives the highest quantity and quality of both DNA and RNA. To obtain optimal performance with TruSight Tumor 170, 40 ng of DNA with a ΔCq value < 5, and 40 ng of RNA with a DV_{200} value > 20 are recommended. When using lower quality nucleic acid, optimal performance can sometimes be achieved by adding more than 40 ng of DNA and/or RNA.

Learn More

To learn more about TruSight Tumor 170, visit www.illumina.com/TruSightTumor170.

References

1. Illumina (2017) TruSight Tumor 170 Reference Guide. (support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/trusight/tumor-170/trusight-tumor-170-reference-guide-1000000024091-00.pdf)

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