Illumina FFPE DNA Prep with Exome 2.5 Enrichment supports tumor-only workflow for WES

Evaluation of variant detection and genomic biomarkers without matched normal samples

Perform enriched exome sequencing

Evaluate coding variants and copy number alterations from FFPE tumor-only samples

Detect low-frequency variants

Identify SNVs, insertions, deletions, and CNVs from 40 ng input with consistency

Assess genomic biomarkers

Measure TMB, MSI, and HRD with results concordant with TruSight™ Oncology 500

Introduction

Genomic profiling has become central to oncology research for characterizing disease-associated genomic alterations across tumor types.[1] Whole-exome sequencing (WES) enables broad assessment of coding regions, supporting analysis of single nucleotide variants (SNVs), insertions, deletions, copy number variations (CNVs), tumor mutational burden (TMB), microsatellite instability (MSI), and homologous recombination deficiency (HRD) within a single assay.[2-4] Advances in library preparation and enrichment methods have enabled WES from limited DNA input, expanding the applicability of exome approaches for challenging sample types such as formalin-fixed, paraffin-embedded (FFPE) tissue, where formalin fixation introduces crosslinking and fragmentation that can reduce DNA integrity and yield.[5]

Illumina FFPE DNA Prep with Exome 2.5 Enrichment supports WES from low-input FFPE DNA.[6] The assay integrates library preparation, exome enrichment, sequencing, and secondary analysis within a single workflow. The enrichment targets coding regions across the exome, enabling comprehensive evaluation of coding variants and genomic biomarkers.

While the assay is designed to interrogate tumor–normal samples, matched normal samples are not always available; for example, archival material often includes only tumor tissue. These challenges are particularly relevant in cancers such as non-small cell lung cancer (NSCLC), where patients are frequently diagnosed at advanced stages and tissue acquisition relies on small biopsies.[7] In these settings, limited material is prioritized for diagnostic evaluation and adjacent normal tissue is often not collected or retained for research use.

This technical note presents performance data for tumor-only WES conducted with Illumina FFPE DNA Prep with Exome 2.5 Enrichment. Variant detection performance, CNV analysis, and evaluation of genomic biomarkers, including TMB, MSI, and HRD, were assessed. Results were also compared with TruSight Oncology 500, a targeted oncology gene panel assay, to evaluate concordance of biomarker estimates in tumor-only samples.

Methods

Samples

FFPE human tumor tissue samples representing multiple tissue types were sourced commercially. Tissue types included bladder (Amsbio), colon (BioOptions, DLS, and Cutting Edge Biomed), liver (Amsbio), lung (BioOptions), ovary (Avaden BioSciences), stomach (Tissue Solutions), thyroid (BioOptions), and uterus (BioOptions) (Table 1).

Genomic DNA (gDNA) was extracted from FFPE samples using the QIAGEN AllPrep DNA/RNA FFPE kit (QIAGEN, Catalog no. 80234).

FFPE DNA quality was evaluated with the Illumina TruSight FFPE QC kit (Illumina, Catalog no. 20139070), and samples included a range of delta QC (ΔQC) values, from -0.8 to 4.6, representing high- to low-quality FFPE DNA, respectively. In addition, commercially available reference materials were used for evaluation of small DNA variant detection, CNV analysis, TMB, and HRD (Table 1).

Seraseq reference materials included Tumor Mutation DNA Mix v2 AF10 HC (SeraCare, Catalog no. 0710-0094), Solid Tumor CNV Mix, +3 copies (SeraCare, Catalog no. 0710-2866), gDNA TMB Mix Score 7 (SeraCare, Catalog no. 0710-1326), SeraCare, Catalog no. gDNA TMB Mix Score 13 (0710-1586); FFPE TMB reference material Score 9 (SeraCare, Catalog no. 0710-1308), HRD gDNA High-Positive Mix (SeraCare, Catalog no. 0710-2879), HRD gDNA Low-Positive Mix (SeraCare, Catalog no. 0710-2880), and HRD gDNA Negative Mix (SeraCare, Catalog no. 0710-2881).

Library preparation and sequencing

Libraries were prepared using 40 ng input of FFPE DNA per sample according to the Illumina FFPE DNA Prep with Exome 2.5 Enrichment protocol. Preparation steps included DNA processing, adapter ligation with unique molecular identifiers, hybrid–capture enrichment using the Exome 2.5 panel, and postenrichment amplification. Libraries were quantified, normalized, and pooled for sequencing according to the assay recommendations.

Table 1: Samples used for evaluation of tumor-only WES using Illumina FFPE DNA Prep with Exome 2.5 Enrichment

Sample type	Description	No. of samples	Quality (ΔCq)
FFPE tumor tissue[a]	Tissue types includied bladder, colon, liver, lung, ovary, stomach, thyroid, and uterus.	24	-0.8–4.6
Variant reference material	Seraseq Tumor Mutation DNA Mix v2 AF10 HC	1	N/A
Copy number reference material	Seraseq Solid Tumor CNV Mix, +3 copies	1	N/A
gDNA TMB reference material	Seraseq gDNA TMB Mix Score 7 Seraseq gDNA TMB Mix Score 13	2	N/A
FFPE TMB reference material[a]	Seraseq FFPE TMB RM Score 9	1	N/A
HRD reference material	Seraseq HRD gDNA High-Positive Mix Seraseq HRD gDNA Low-Positive Mix Seraseq HRD gDNA Negative Mix	3	N/A

a. Requires extraction.

FFPE, formalin-fixed, paraffin-embedded; gDNA, genomic DNA; HRD, homologous recombination deficiency; N/A, not applicable; TMB, tumor mutational burden; WES, whole-exome sequencing.

Sequencing was performed on the NovaSeq™ 6000 Sequencing System using paired-end 2 × 151 bp reads. Runs were configured to target at least 100M single-end reads per tumor sample.

Data analysis

Primary analysis, including base calling and demultiplexing, was performed using Illumina sequencing system software. Secondary analysis was conducted using the DRAGEN™ somatic pipeline, consistent with the Illumina FFPE DNA Prep with Exome 2.5 Enrichment protocol.

Reads were aligned to the human reference genome. Quality control metrics, including mean target coverage and percentage of targets achieving specified coverage thresholds, were assessed using standard DRAGEN secondary analysis output metrics.

Duplicate marking was performed prior to variant calling. Variants (SNVs, insertions, and deletions), CNVs, TMB, MSI, and HRD metrics were generated as part of DRAGEN secondary analysis.

Comparison with TruSight Oncology 500

Results from FFPE tumor-only WES performed using Illumina FFPE DNA Prep with Exome 2.5 Enrichment were compared with results obtained using TruSight Oncology 500.

FFPE tumor DNA samples were processed using the TruSight Oncology 500 Reference Guide. Sequencing data were analyzed using DRAGEN secondary analysis for TruSight Oncology 500 or the TruSight Oncology 500 local application software, as applicable.

Concordance analysis for the two assays included variant detection as well as TMB and MSI estimates.

Results

Performance of tumor-only WES using Illumina FFPE DNA Prep with Exome 2.5 Enrichment was evaluated across multiple variant classes and genomic biomarkers using FFPE tumor samples and reference materials.

Detection of low-frequency variants

Variant detection performance was evaluated using reference materials containing variants at a range of variant allele frequency (VAF) levels. Illumina FFPE DNA Prep with Exome 2.5 Enrichment detected approximately 90% of deletions, insertions, and SNVs at a 5% VAF from 40 ng of FFPE DNA input when sequenced to 100M single-end reads (Figure 1). Sensitivity reached 100% at 10% VAF. Detection performance was consistent across mutation classes.

Tumor-only WES identified variants that were not detected using the TruSight Oncology 500 targeted panel workflow in the same sample. Representative variants detected in a liver tumor FFPE sample are shown (Table 2).

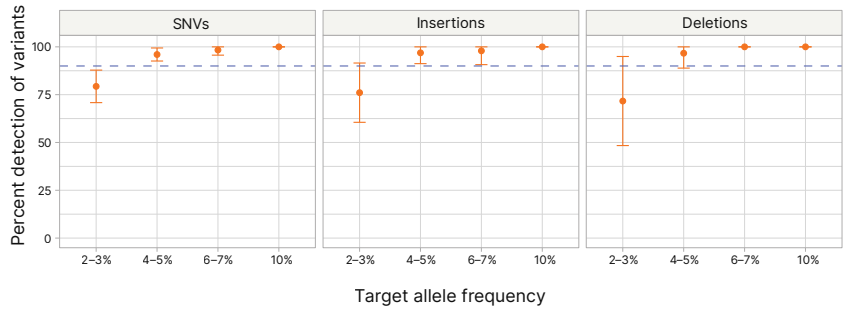

Figure 1: Detection of low-frequency variants with tumor-only WES

Detection rates for SNVs, insertions, and deletions for Illumina FFPE DNA Prep with Exome 2.5 using 40 ng input. The dashed line indicates the 90% detection threshold.

FFPE, formalin-fixed, paraffin-embedded; SNV, single nucleotide variant; WES, whole-exome sequencing.

Table 2: Variants detected using tumor-only WES that were undetected using TruSight Oncology 500 in a liver tumor FFPE sample

Chromosome	Genomic position	Gene/gene ID	VAF (%)	COSMIC ID	COSMIC reported tumor site	COSMIC confirmed somatic
Chr12	11546742	*PRB2*	11.1	COSV66982301	Large intestine (rectum)	True
Chr9	66457027	ENSG00000288838	14.7	COSV63402916	Large intestine; liver	True
Chr9	66457051	ENSG00000288838	13.9	COSV63402360	Large intestine	True
Chr9	66457076	ENSG00000288838	14.2	COSV63403156	Large intestine; prostate	True
Chr9	66457259	ENSG00000288838	8.5	COSV63401697	Large intestine	True
Chr9	136065941	N/A	23.4	COSV63004546	Skin	True
Chr9	136065942	N/A	24.3	COSV63004548	Skin	True

FFPE, formalin-fixed, paraffin-embedded; VAF, variant allele frequency; WES, whole-exome sequencing.

Gene amplification detection

Copy number analysis was evaluated using tumor-only WES with a control sample containing three additional copies per gene. Amplifications were detected across all 12 evaluated genes. Reported values represent the mean and standard deviation from eight replicate libraries generated from the copy number reference material. Observed fold changes were compared with expected values reported by the manufacturer (Figure 2)

For several genes, including *BRAF*, *EGFR*, and *MET*, amplification values appeared elevated because the reference material contains two synthetic constructs with partially overlapping regions. Copy number events spanning these regions may therefore have produced increased amplification estimates.

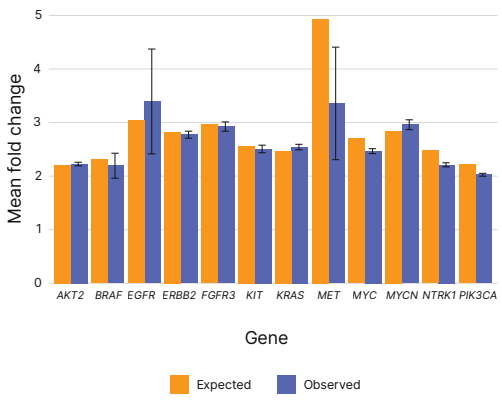

Figure 2: Gene amplification detection from copy number reference material with tumor-only WES

Copy number analysis using Illumina FFPE DNA Prep with Exome 2.5 was performed on Seraseq Solid Tumor CNV Mix, which contains three additional copies per gene. The observed amplification values represent the mean and standard deviation across eight replicate libraries and are compared against the expected fold change reported by SeraCare.

FFPE, formalin-fixed, paraffin-embedded; WES, whole-exome sequencing.

TMB evaluation

TMB per Mb of gDNA was evaluated using gDNA and FFPE reference materials with known TMB scores. Observed values were consistent with expected scores for reference materials with nominal TMB scores of 7, 9, and 13 mutations per megabase (Table 3).

TMB estimates generated using tumor-only WES in 23 FFPE samples were highly concordant with values obtained using TruSight Oncology 500. Concordance analysis produced a coefficient of determination (R^2) of 0.999 across evaluated samples (Figure 3).

Table 3: TMB evaluation with tumor-only WES using Illumina FFPE DNA Prep with Exome 2.5 Enrichment

Sample	Tumor-only WES[a,b]	Reported TMB score[c]
TMB Mix Score 7	7.52 ± 0.21	7.16
TMB Mix Score 9	8.91 ± 0.43	6.07[d]
TMB Mix Score 13	13.14 ± 0.16	12.57 ± 0.02[e]

a. Nonsynonymous TMB scores were determined using DRAGEN secondary analysis for nonsynonymous somatic variants.

b. Nonsynonymous TMB scores are reported to match the variant criteria used by SeraCare and represent the average and standard deviation of four replicates for TMB Mix Score 7 and TMB Mix Score 13, and of two replicates for TMB Mix Score 9.

c. As reported by SeraCare.

d. Although designated as TMB Mix Score 9, SeraCare reports the TMB score as 6.07[8]

e. The reported score for TMB Mix Score 13 represents the average and standard deviation of three replicates.

FFPE, formalin-fixed, paraffin-embedded; TMB, tumor mutational burden; WES, whole-exome sequencing.

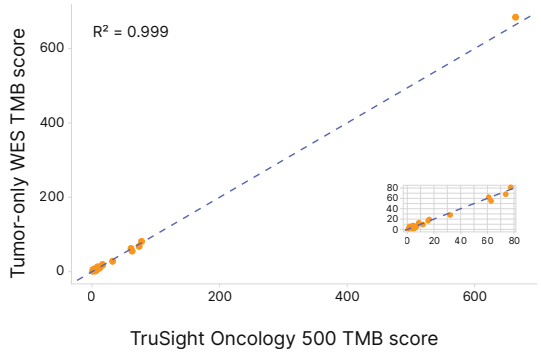

Figure 3: Concordance of TMB scores from tumor-only WES and TruSight Oncology 500

TMB scores obtained using Illumina FFPE DNA Prep with Exome 2.5 Enrichment were highly concordant with values obtained using TruSight Oncology 500. The inset shows TMB scores ≤ 80 TMB per Mb.

FFPE, formalin-fixed, paraffin-embedded; TMB, tumor mutational burden; WES, whole-exome sequencing.

MSI evaluation

MSI scores generated using tumor-only WES were compared with scores obtained using TruSight Oncology 500 in FFPE samples. Scores > 20 are classified as MSI-high in the TruSight Oncology 500 workflow. The MSI scores showed strong concordance between methods with a Pearson correlation coefficient of 0.984, although some individual samples showed differences in MSI score values between assays while preserving overall low-to-high sample ranking. (Table 4).

Table 4: MSI evaluation with tumor-only WES using Illumina FFPE DNA Prep with Exome 2.5 Enrichment[a,b]

Quality (ΔCq)	FFPE sample type	Tumor-only WES	TruSight Oncology 500
-0.8	Ovary	0.29	3.2
1	Colon	1.37	4.92
1.1	Colon	38.96	55.65
1.4	Thyroid	1.48	4.96
1.5	Bladder	1.34	4.84
1.6	Uterus	0.25	2.42
1.8	Colon	1.18	5.61
1.9	Colon	56	68.03
2	Lung	0.2	0.8
2.5	Liver	0.91	3.2
2.7	Lung	0.73	4.07
2.7	Thyroid	0.73	2.42
2.9	Liver	0.22	0.8
2.9	Lung	0.18	2.5
3.1	Uterus	12.39	14.05
3.4	Lung	1.16	4.07
3.4	Lung	0.39	2.54
3.9	Lung	0.92	2.44
4	Lung	0.22	4.8
4	Lung	0.26	2.59
4.1	Colon	30.68	39.52
4.1	Stomach	0.25	1.64
4.6	Colon	38.69	60

a. MSI JDsum score is shown. TruSight Oncology 500 classifies MSI scores > 20 as high.
b. Pearson correlation coefficient = 0.984.
MSI, microsatellite instability; WES, whole-exome sequencing.

HRD evaluation

HRD scores were evaluated using tumor-only WES on reference materials representing high-positive, low-positive, and negative HRD states and compared with the manufacturer-reported HRD scores obtained using Illumina TruSight Oncology 500 HRD and an additional orthogonal method (Table 5).[9]

For HRD-positive samples, scores generated using tumor-only WES were slightly higher than those reported for TruSight Oncology 500 HRD; however, the scores were consistent with the results obtained using the additional orthogonal method. Results for the HRD-negative sample were consistent across all methods.

Table 5: HRD evaluation with tumor-only WES using Illumina FFPE DNA Prep with Exome 2.5 Enrichment

Sample	Tumor-only WES	Orthogonal method	
		TruSight Oncology 500 HRD[a]	OncoScan[a]
High positive mix	82.5 ± 3.5	72 ± 3	81
Low positive mix	77.5 ± 2.1	54 ± 2	77
Negative mix	28.5 ± 2.1	31 ± 2	30

a. HRD scores obtained by Illumina TruSight Oncology 500 HRD and Thermo Fisher OncoScan are as reported in the Seraseq FFPE HRD Reference Material data sheet.[9]
FFPE, formalin-fixed, paraffin-embedded; HRD, homologous recombination deficiency; WES, whole-exome sequencing.

Summary

Performance of Illumina FFPE DNA Prep with Exome 2.5 Enrichment for tumor-only WES was evaluated using FFPE tumor samples and reference materials representing multiple variant classes and genomic biomarkers. Using reference materials with known variants, the assay detected approximately 90% of insertions, deletions, and SNVs at 5% VAF from 40 ng of FFPE DNA input. Gene amplification detection was consistent with expected values across evaluated targets. Estimates of TMB, MSI, and HRD were also consistent with expected values and highly concordant with results obtained using TruSight Oncology 500. These results support the use of Illumina FFPE DNA Prep with Exome 2.5 Enrichment for WES analysis of FFPE tumor samples when matched normal samples are not available.

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Illumina FFPE DNA Prep with Exome 2.5 Enrichment

DRAGEN secondary analysis

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