APPLICATION NOTE

Detect rare somatic variants in FFPE tumor samples using Illumina Cell-Free DNA Prep with Enrichment

- Unique molecular identifiers for error correction enable increased accuracy during sequencing
- Compatible with user-supplied enrichment panels for flexible experimental design
- Scalable workflow accommodates DNA extracted from both liquid biopsy and FFPE tissue samples

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Introduction

Formalin-fixed paraffin-embedded (FFPE) tissue samples are an important source of materials available for tumor molecular characterization. Unfortunately, the process of fixation and paraffin embedding tissue samples can cause fragmentation, crosslinking, or chemical modifications of nucleic acids.[1] This leads to low or variable nucleic acid quality that can make tumor genetic analysis challenging, especially for detecting rare mutations. As a result, robust and sensitive next-generation sequencing (NGS) workflows are necessary to process input materials from FFPE samples for downstream analysis.

Combining the findings from genomic analysis of tissue biopsy with complementary liquid biopsy data provides comprehensive insights into tumor biology. Illumina Cell-Free DNA Prep with Enrichment is a versatile library preparation kit that can be used to prepare sequencing-ready libraries from both circulating cell-free DNA (cfDNA) or genomic DNA (gDNA) extracted from FFPE tissue samples (Figure 1). The workflow includes unique molecular identifiers (UMIs) for error correction and reduction of false positives, enabling accurate and sensitive detection of low-frequency mutations in FFPE tumor samples. Illumina Cell-Free DNA Prep with Enrichment is compatible with Illumina and third-party enrichment probes or panels to support flexible experimental design. This application note demonstrates the excellent performance of Illumina Cell-Free DNA Prep with Enrichment in generating high-quality NGS libraries and identifying low-frequency somatic variants from FFPE samples.

Methods

Samples

Libraries were prepared from a combination of human tissue FFPE samples and FFPE cell lines (Table 1). FFPE cell lines tested were from Horizon Discovery (Catalog nos. HD301 and HD653) and SeraCare Life Sciences* (FFPE TST Custom DNAv2). Human FFPE tissue samples were provided by the Illumina Biological Specimen Inventory System. FFPE DNA was extracted using the AllPrep DNA/RNA FFPE kit (QIAGEN, Catalog no. 80234), quantified with the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, Catalog no. Q32850), and qualified using the TruSeq Custom Amplicon Dx-FFPE QC kit (Illumina, Catalog no. 20006259).

DNA fragmentation

Mechanical shearing was used for DNA fragmentation. DNA extracted from FFPE samples was diluted in Tris-EDTA (TE) buffer to 0.23 ng/µl for 10 ng input, 0.45 ng/µl for 20 ng input, or 0.90 ng/µl for 40 ng input. Next, 52 µl of each diluted sample were sonicated in one well of an 8 microTUBE Strip (Covaris, Catalog no. 520053) using the Covaris LE220 Focused-ultrasonicator (Table 2).

* SeraCare Life Sciences is now part of LCG Diagnositics.

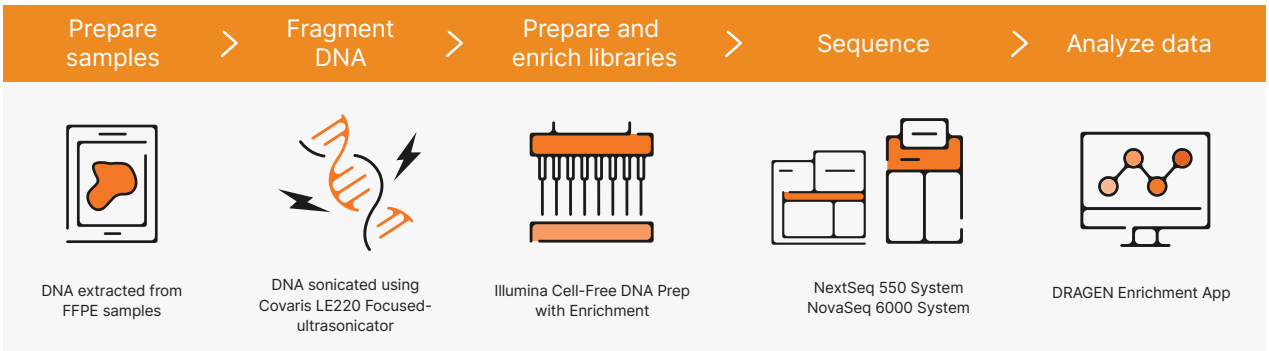


Figure 1: Illumina Cell-Free DNA Prep with Enrichment workflow for processing FFPE samples.

Table 1: Samples analyzed using Illumina Cell-Free DNA Prep with Enrichment

Sample ID	Sample type	Sample source	ΔCq value
8752	FFPE tissue (human)	Non-small cell lung cancer	5.7
315	FFPE tissue (human)	Non-small cell lung cancer	5.5
4075	FFPE tissue (human)	Colorectal cancer	4.2
679	FFPE tissue (human)	Breast cancer	4.1
11883	FFPE tissue (human)	Colorectal cancer	2.9
3952	FFPE tissue (human)	Breast cancer	2.2
448	FFPE tissue (human)	Non-small cell lung cancer	2.1
449	FFPE tissue (human)	Non-small cell lung cancer	1.5
FFPE TST Custom DNAv2	FFPE cell line	N/A	0.87
HD653	FFPE cell line	N/A	-1.23
HD301	FFPE cell line	N/A	-1.87

Table 2: Recommended shearing settings for Covaris ultrasonicators

Setting	Covaris LE220	Covaris E220	Covaris ME220
Peak incident power	450 watts	175 watts	50 watts
Duty factor	30%	10%	30%
Cycles per burst	200	200	1000
Treatment time	250 s	280 s	10 s
Temperature	7°C	7°C	12°C
Pulse repeats	N/A	N/A	20
Average power	N/A	N/A	15 watts
Other	N/A	Intensifier	Wave guide

Data for this study were generated using the Covaris LE220 Focused-ultrasonicator.

Library preparation and enrichment

After sonication, libraries were prepared from fragmented DNA according to the instructions detailed in the Illumina Cell-Free DNA Prep with Enrichment user guide. Specific enrichment protocols were used depending on the panel type and enrichment format. For this study, two enrichment panels were used; an 80-bp single-stranded DNA (ssDNA) 2000-kb custom-designed panel and a 120-bp double-stranded DNA (dsDNA) 37.5 Mb Illumina Exome 2.5 Enrichment Panel. Enrichment was performed following the 1-plex or 4-plex enrichment workflow as described in the Illumina Cell-Free DNA Prep with Enrichment user guide.

Sequencing

Libraries enriched using the Illumina Exome 2.5 Enrichment Panel were sequenced† on the NovaSeq™ 6000 System at 2 × 151 bp read length. All other libraries were sequenced on the NextSeq™ 550 System at 2 × 149 bp read length.

† Illumina Cell-Free DNA Prep with Enrichment is compatible with all Illumina mid- to high-throughput systems. However, library performance using FFPE samples has only been demonstrated on the NovaSeq 6000 and NextSeq 550 Systems.

Data analysis

For cloud-based data analysis, raw sequencing data (BCL files) were demultiplexed and converted to FASTQ files in BaseSpace™ Sequence Hub. Downstream secondary analysis was performed with the DRAGEN™ Enrichment App v4.0.3 enabling Somatic Small Variant Caller with default settings. UCSC hg 19 Alt-Aware was used as the reference human genome. UMI settings were turned ON, with UMI-aware variant calling set to 'Low Depth'. The minimum number of supporting reads for UMI was set to 1. Variant Annotation by Illumina Connected Annotation (formerly known as Nirvana) for germline tagging was enabled, with additional DRAGEN command line arguments.‡ Passing variants were filtered using bcftools 1.17 and benchmarked against ground-truth variants using rtg vcfeval 3.12.1.

Results

To demonstrate the excellent performance of Illumina Cell-Free DNA Prep with Enrichment in generating high-quality sequencing libraries from FFPE DNA, key performance metrics were evaluated for DNA extracted from FFPE cell lines and FFPE tumor samples.

Excellent library performance metrics for FFPE samples

Mean target coverage was evaluated at two sequencing depths, 10M and 20M clusters, to make sure that prepared libraries had the necessary depth of coverage across the targeted regions to enable detection of low-abundance variants. The findings demonstrate that high-quality FFPE DNA (ΔCq ≤ 2) generates libraries that achieve > 100× mean target coverage depth even at inputs as low as 10 ng DNA (Figure 2). The coverage decreases with poor sample quality (higher ΔCq) but can be improved with deeper sequencing or increased input (Figure 2B). The Illumina Cell-Free DNA Prep with Enrichment kit produces libraries with > 110-bp fragment length and delivers high-quality sequencing data, assessed as read enrichment and percent of targets with ≥ 50× coverage for FFPE samples with ΔCq ≤ 4 (Figure 2C).

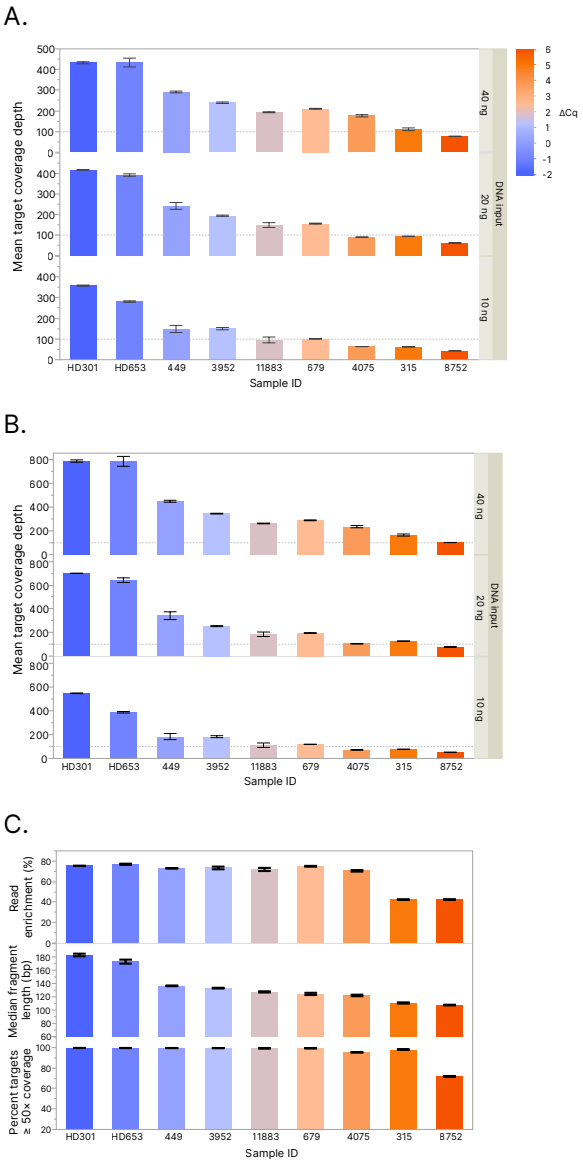


Figure 2: Performance metrics for libraries prepared from FFPE samples using Illumina Cell-Free DNA Prep with Enrichment— Triplicate libraries were prepared using DNA extracted from FFPE cell lines (HD301 and HD653) and FFPE tissues from breast, colon, and lung tumors of varying quality and input amounts (10 ng, 20 ng, and 40 ng). Samples are graphed left to right in order of decreasing quality as indicated by the ΔCq value, with blue being samples of the highest quality and red denoting those of lowest quality. Libraries were enriched with an 80-bp ssDNA 2000 kb panel and sequenced on the NextSeq 550 system at 2 × 149 bp. Mean target coverage was evaluated at two read depths, (A) 10M and (B) 20M clusters. (C) Additional library performance metrics for FFPE samples. Data shown are for 20 ng input DNA and 20M clusters.

‡ --umi-verbose-metrics true --umi-start-mask-length 1 --umi-end-mask-length 3 --germline-tagging-db-threshold 10 --tmb-enable-proxi-filter true --vc-enable-germline-tagging true

Sensitive detection of low-frequency variants

The sensitivity of the Illumina Cell-Free DNA Prep with Enrichment kit for detecting single nucleotide variants (SNVs) and insertion and deletion (indel) mutations was determined using a custom FFPE cell line carrying cancer-associated variants (FFPE TST Custom DNAv2, SeraCare). Illumina Cell-Free DNA Prep with Enrichment demonstrated high analytical sensitivity, with the ability to detect ~90% of SNVs between 1% and 2% variant allele frequency (VAF) (Figure 3, Table 3). The sensitivity for detection of indels above 2% VAF was > 80% (Figure 3, Table 3).

Compatibility with 1-plex and 4-plex enrichment formats

Comparable performance metrics, including mean target coverage depth, percent of targets with ≥ 50× coverage, and percent read enrichment were achieved for both 1-plex and 4-plex enrichment formats across samples ranging in quality from ΔCq 1.5–4.2 (Figure 4).

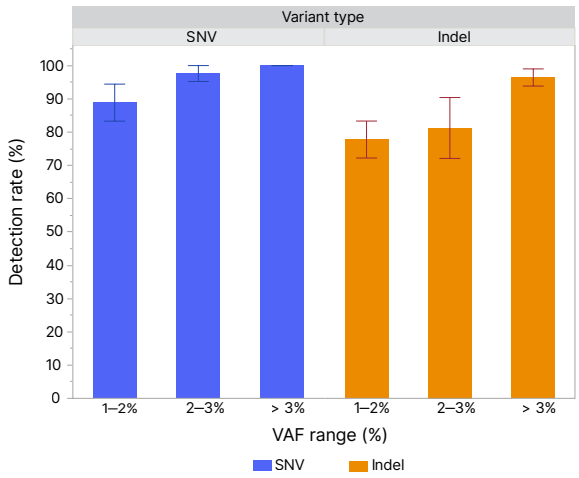


Figure 3: Variant detection at low allele frequencies—Variants were diluted to at least three targeted allele frequency levels with FFPE DNA from a wild-type cell line (GM24385) sample. Six library replicates were prepared with 10 ng input DNA per allele frequency level and enriched with an 80-bp ssDNA 2000-kb panel following the 1-plex enrichment workflow. Libraries were sequenced on the NextSeq 550 System at 2 × 149 bp. Sequencing reads were subsampled to 20M clusters. The detection rate (n/6) for each variant type at different VAF ranges was evaluated.

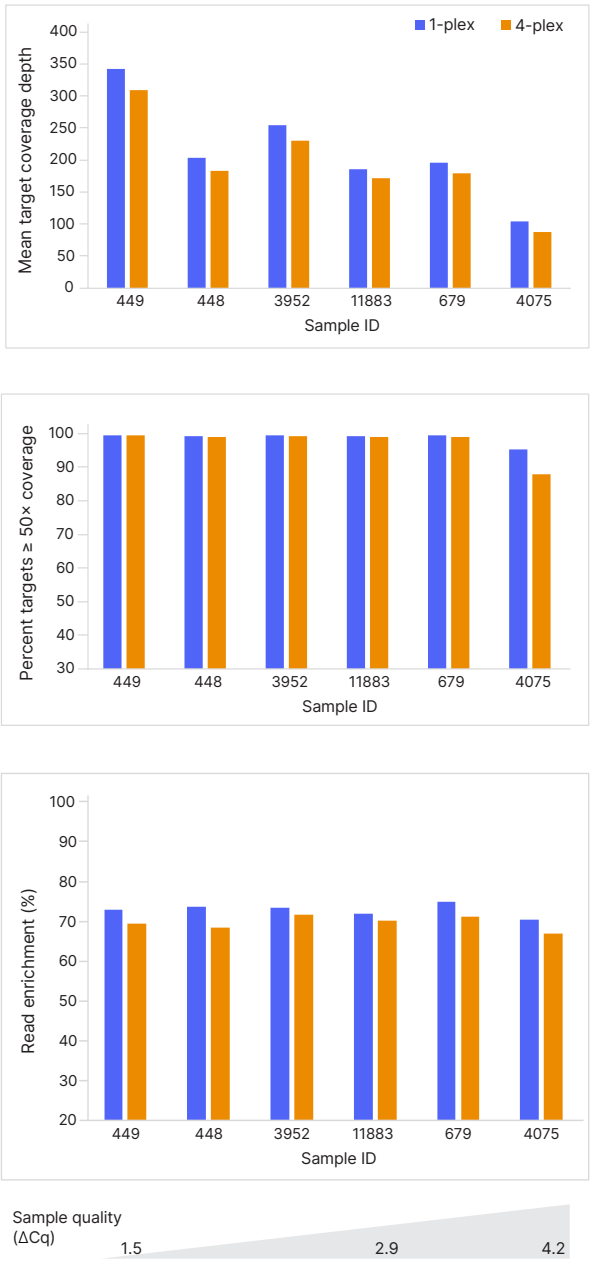


Figure 4: Performance metrics across 1-plex and 4-plex enrichment formats—Libraries from selected FFPE DNA tissue samples (20 ng input DNA) of different ΔCq were individually enriched with an 80-bp ssDNA 2000-kb panel (1-plex, blue bars). The same libraries were re-enriched using the same panel following the multiplex (4-plex, orange bars) enrichment format. Libraries were sequenced on the NextSeq 550 System at 2 × 149 bp, and sequencing reads were subsampled to 20M clusters.

Table 3: Summary of individual somatic variants detected using Illumina Cell-Free DNA Prep with Enrichment

COSMIC ID	Gene	Variant type	Amino acid change	Detected VAF (%)[a]	No. of replicates detected[b]
COSV51614178	*MLH1*	SNV	p.V384D	2.0	6
COSV57169334	*MYD88*	SNV	p.L273P	2.3	6
COSV58963463	*CSF3R*	SNV	p.T618I	2.6	6
COSV55545304	*KRAS*	SNV	p.K117N	2.9	6
COSV50630049	*CBL*	SNV	p.L380P	2.9	5
COSV61615239	*IDH1*	SNV	p.R132H	3.0	5
COSV52274101	*MSH6*	SNV	p.E1322*	3.5	6
COSV59205440	*SF3B1*	SNV	p.G742D	3.9	6
COSV56057713	*BRAF*	MNV	p.V600K	2.1	6
COSV64288359	*PTEN*	Del	p.C250Wfs*2	2.3	6
COSV56542602	*VHL*	Del	p.P99Qfs*60	2.5	6
COSV52740986	*TP53*	Del	p.K291Tfs*48	2.7	6
COSV55388067	*KIT*	Del	p.W557_E561del	3.2	5
COSV61376874	*ARID1A*	Del	p.A339Lfs*24	3.2	6
COSV62688630	*CTNNB1*	Del	p.S45del	4.7	6
COSV67575778	*JAK2*	Del	p.N542_E543del	5.3	5
COSV56060749	*BRAF*	Ins	p.T599dup	1.9	6
COSV55386625	*KIT*	Ins	p.A502_Y503dup	2.8	6
COSV64290304	*PTEN*	Ins	E242Lfs*15	3.1	6
COSV51766549	*EGFR*	Ins	p.A767_V769dup	3.5	6
COSV51772596	*EGFR*	Ins	p.N771_H773dup	3.6	6
COSV57195669	*CEBPA*	Ins	p.H24Afs*84	5.0	6

a. Average of detected replicates.
b. Out of 6 total replicates.

Excellent library performance with Illumina Exome 2.5 Enrichment Panel

The Illumina Cell-Free DNA Prep with Enrichment kit with the Illumina Exome 2.5 Enrichment Panel consistently generated libraries of > 150-bp length from FFPE cell lines (Figure 5). High performing metrics were achieved for all libraries prepared with ≥ 20 ng input, including read enrichment, percentage of aligned reads, and coverage uniformity. Performance metrics were comparable between 1-plex and 4-plex–enriched libraries.

Sensitive detection of variants with Illumina Exome 2.5 Enrichment panel

The impact of sequencing depth on mean target coverage and variant detection was assessed for libraries enriched using the Illumina Exome 2.5 Enrichment panel. Results demonstrate that up to 500× mean target coverage was achieved when eight exome libraries were sequenced in one NovaSeq 6000 S4 flow cell lane (Figure 6). When sequencing reads were subsampled to 200M and 400M total passing filter reads, libraries achieved a mean target coverage of ~150× and ~300×, respectively, independent of input amounts and enrichment plexity.

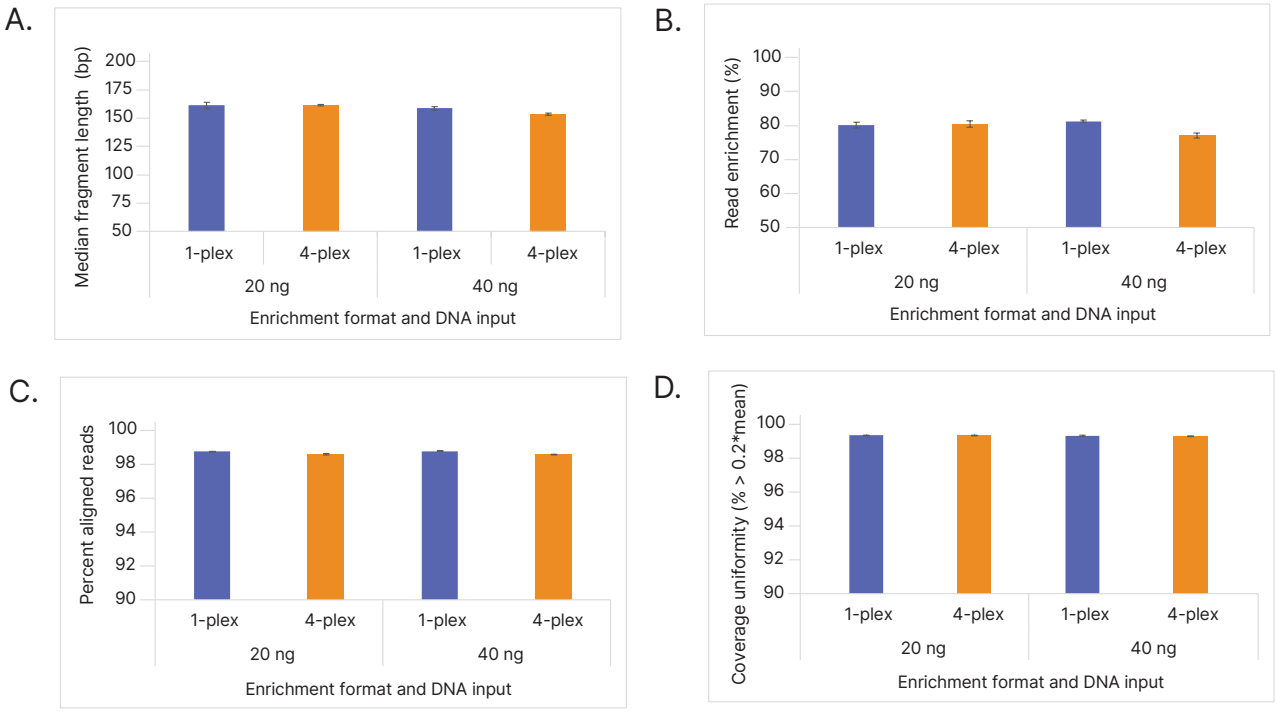


Figure 5: Library performance metrics with Illumina Exome 2.5 Enrichment panel—Four library replicates were prepared using Illumina Cell-Free DNA Prep with Enrichment from a custom FFPE cell line carrying cancer associated variants (FFPE TST Custom DNAv2, SeraCare). Variants were diluted to a targeted allele frequency of ~2%, and libraries were prepared with either 20 ng or 40 ng input DNA. Enrichment was performed with the Illumina Exome 2.5 Enrichment Panel following the 1-plex or 4-plex enrichment workflow for dsDNA probes in the Illumina Cell-Free DNA Prep with Enrichment user guide. Eight Illumina Exome 2.5 Enrichment panel-enriched libraries were sequenced per S4 lane on the NovaSeq 6000 System at 2 × 151 bp. Sequencing data were subsampled to 200M clusters (or 400M total passing filter reads), and library performance metrics were compared between the two inputs.

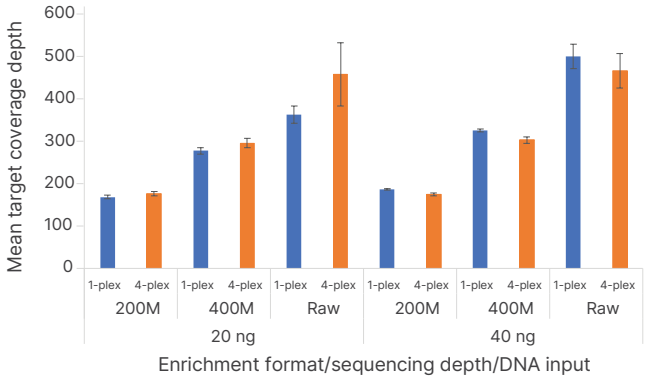
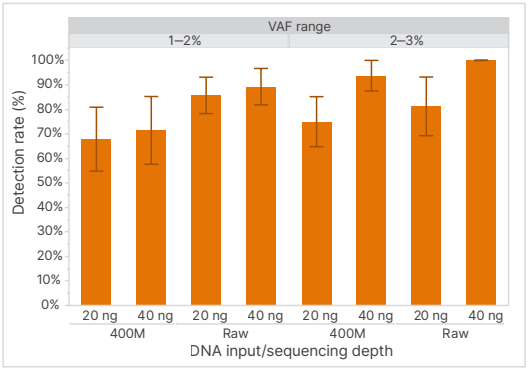


Figure 6: Mean target coverage achieved with the Illumina Exome 2.5 Enrichment panel—Four library replicates were prepared using Illumina Cell-Free DNA Prep with Enrichment from 20 ng or 40 ng DNA derived from a custom FFPE cell line carrying cancer-associated variants (FFPE TST Custom DNAv2, SeraCare). Variants were diluted to a targeted allele frequency of ~2%. Enrichment was performed with the Illumina Exome 2.5 Enrichment Panel following the 1-plex (blue bars) or 4-plex (orange bars) enrichment workflow for dsDNA probes. Eight libraries were sequenced per S4 lane on the NovaSeq 6000 System at 2 × 151 bp. Sequencing data were subsampled to 100M or 200M clusters (200M or 400M total passing filter reads), and library performance metrics were evaluated. Raw reads ranged from 296M–417M clusters (592M–834M total passing filter reads) for 1-plex or 319M–544M clusters (638M–1.08B total passing filter reads) for 4-plex enriched libraries.

Multiplexed (4-plex) libraries prepared using the Illumina Exome 2.5 Enrichment panel achieved 80% sensitivity for SNVs between 2%–3% VAF when the reads for 20 ng libraries were subsampled to 200M clusters. The detection rate of SNVs improved with 40 ng input or with deeper sequencing, particularly for variants within the 1%–2% VAF range (Figure 7A). Multiplexed libraries prepared using the Illumina Exome 2.5 Enrichment panel achieved 80%–90% sensitivity for indels between 2%–3% VAF, depending on the input amount and sequencing depth. Above 3% VAF, 100% of indels were detected at 20 ng and 40 ng input amounts (Figure 7B).

A. SNV detection



B. Indel detection

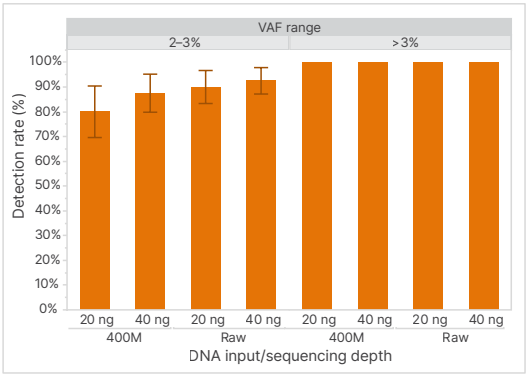


Figure 7: Somatic variant detection with the Illumina Exome 2.5 Enrichment panel—Four library replicates were prepared using Illumina Cell-Free DNA Prep with Enrichment from 20 ng or 40 ng DNA derived from a custom FFPE cell line carrying cancer-associated variants (FFPE TST Custom DNAv2, SeraCare). Variants were diluted to a targeted allele frequency of ~2%. Enrichment was performed with the Illumina Exome 2.5 Enrichment Panel following the 4-plex enrichment workflow for dsDNA probes. Eight libraries were sequenced per S4 lane on the NovaSeq 6000 System at 2 × 151 bp. Sequencing data were subsampled to 200M clusters (400M total passing filter reads), and library performance metrics were evaluated. Raw reads ranged from 319M–544M clusters (638M–1.08B total passing filter reads) for enriched libraries.

Summary

Preparing high-quality libraries from FFPE DNA is a key factor in the performance of NGS-based tumor molecular characterization. This application note demonstrates the excellent performance of Illumina Cell-Free DNA Prep with Enrichment in preparing sequencing-ready libraries from DNA extracted from FFPE samples, producing accurate data for somatic variant calling and downstream analysis. This versatile library preparation kit is compatible with user-supplied enrichment panels up to exome size and provides content portability, allowing labs to tailor their experimental design based on their research needs.

Learn more

Illumina Cell-Free DNA Prep with Enrichment

DRAGEN Enrichment App

DRAGEN recipe for analyzing FFPE sample libraries prepared using Illumina Cell-Free DNA Prep with Enrichment

Reference

1. Do H, Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. *Clin Chem*. 2015;61(1):64-71. doi:10.1373/clinchem.2014.223040

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