APPLICATION NOTE

Sensitive variant profiling in tissue and liquid biopsy samples

Using a custom 79-gene panel with Illumina Cell-Free DNA Prep with Enrichment enables low-frequency variant detection in tumor samples

- Custom predesigned panel targets somatic variants across 79 genes relevant to solid tumors
- Unique molecular identifiers for error correction enhance accuracy during sequencing
- Single workflow for both tissue and liquid biopsy samples improves laboratory efficiency

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Introduction

Next-generation sequencing (NGS) has powered crucial breakthroughs in cancer research by uncovering associations between genomic variants and tumorigenesis. Instead of running iterative single-gene assays, NGS allows researchers to examine multiple cancer-associated alterations in parallel from both solid tumors and circulating tumor DNA (ctDNA), maximizing discovery power while reducing turnaround times significantly. Additionally, 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 includes a single, fast hybridization workflow for targeted sequencing applications in cancer research. This ligation-based assay incorporates unique molecular identifiers (UMIs) to enable high-sensitivity detection of rare variants in both formalin-fixed paraffin-embedded (FFPE) tissue and cell-free DNA (cfDNA) from liquid biopsy samples. The kit is part of an integrated workflow that includes DNA extracted from plasma, whole blood, or FFPE tissue; library preparation and enrichment; sequencing on Illumina mid- and high-throughput systems; and highly accurate variant calling using DRAGEN™ secondary analysis pipelines (Figure 1).

Illumina Cell-Free DNA Prep with Enrichment supports flexible experimental design and is compatible with user-supplied custom enrichments panels designed based on a specified target gene list using the free online DesignStudio™ assay design tool from Illumina. This technical note demonstrates the excellent performance of Illumina Cell-Free DNA Prep with Enrichment using a predesigned custom probe panel covering 79 commonly altered genes in solid tumors.

Methods

Samples

Several control samples were used to test the performance of the 79-gene panel with Illumina Cell-Free DNA Prep with Enrichment (Table 1). For FFPE curls (SeraCare Life Sciences,* Catalog no. 0710-0137), DNA was extracted using AllPrep DNA/RNA FFPE kits (Qiagen, Catalog no. 80234). A 0.3% ctDNA control sample was prepared by mixing equal volumes of the 0.5% and 0.1% Seraseq ctDNA Complete Reference Material (SeraCare Life Sciences, Catalog nos. 0710-0672 and 0710-0673, respectively).

* SeraCare Life Sciences is now part of LCG Diagnostics

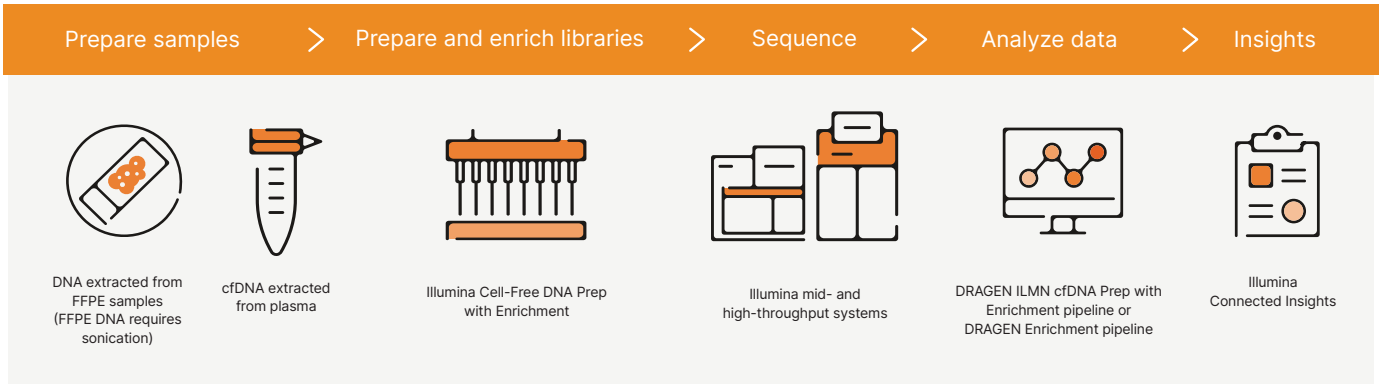

Figure 1: Illumina Cell-Free DNA Prep with Enrichment workflow for processing cfDNA and FFPE samples—This fast, streamlined workflow is optimized for integration into current lab workflows, from samples to variant report, and is compatible with liquid-handling systems for automated library preparation. Automation methods coming soon.

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Table 1: Samples analyzed using Illumina Cell-Free DNA Prep with Enrichment with the 79-gene custom panel

Sample	Sample type	Description	Source	Catalog no.
Seraseq Tri-Level Tumor Mutation DNA Mix v2	DNA	Contains 25 SNVs, 13 indels and, 2 SVs at different allele frequencies (4%, 7%, 10%)	SeraCare Life Sciences[a]	0710-0097
Seraseq FFPE WT (DNA/RNA) Reference Material	FFPE curl	Wild-type/normal reference control based on GM24385	SeraCare Life Sciences[a]	0710-0137
Seraseq ctDNA Complete Reference Material AF5%	ctDNA	Contains 12 SNVs, 7 indels, 3 CNVs, and 3 SVs at 5% VAF	SeraCare Life Sciences[a]	0710-0669
Seraseq ctDNA Complete Reference Material AF2.5%	ctDNA	Contains 12 SNVs, 7 indels, 3 CNVs, and 3 SVs at 2.5% VAF	SeraCare Life Sciences[a]	0710-0670
Seraseq ctDNA Complete Reference Material AF1%	ctDNA	Contains 12 SNVs, 7 indels, 3 CNVs, and 3 SVs at 1% VAF	SeraCare Life Sciences[a]	0710-0671
Seraseq ctDNA Complete Reference Material AF0.5%	ctDNA	Contains 12 SNVs, 7 indels, 3 CNVs, and 3 SVs at 0.5% VAF	SeraCare Life Sciences[a]	0710-0672
Seraseq ctDNA Complete Reference Material AF0.1%	ctDNA	Contains 12 SNVs, 7 indels, 3 CNVs, and 3 SVs at 0.1% VAF	SeraCare Life Sciences[a]	0710-0673
Seraseq ctDNA Complete Reference Material AF0%	ctDNA	Wild-type/normal reference control based on GM24385	SeraCare Life Sciences[a]	0710-0674
Wild-type/normal control reference DNA	DNA	Normal control DNA	Coriell Institute	NA12878
Wild-type/normal control reference DNA	DNA	Normal control DNA	Coriell Institute	NA24385

a. SeraCare Life Sciences is now part of LCG Diagnostics.
CNV, copy number variant; indel, insertion-deletion; SNV, single nucleotide variant; SV, structural variant; VAF, variable allele frequency; WT, wild type.

DNA fragmentation

Mechanical shearing was used for DNA fragmentation for FFPE samples. DNA extracted from FFPE samples (FFPE DNA) was diluted in Tris-EDTA (TE) buffer to 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 ME220 Focused-ultrasonicator with the settings recommended in the Detect rare somatic variants in FFPE tumor samples using Illumina Cell-Free DNA Prep with Enrichment application note. DNA fragmentation was not performed on cfDNA samples.

Custom enrichment panel

The custom enrichment panel used in this study covers all coding exons and targets hundreds of mutations across 79 key genes associated with solid tumors (Table 2). Gene content was selected based on published literature, current guidelines, and insights from key opinion leaders. This panel can be used to investigate single nucleotide variants (SNVs), multinucleotide variants (MNVs), insertion/deletions (indels), and copy number variants (CNVs) in DNA samples. By harnessing the expertise of recognized authorities in the oncology community, this solid tumor panel provides researchers with comprehensive coverage of the variants that are most likely to play a role in tumorigenesis.

Table 2: Custom 79-gene solid tumor panel content

AKT1	*CD274*	*DDR2*	*FGFR4*	*IDH1*	*MYC*	*PDGFRA*	*RNF43*
ALK	*CDH1*	*EGFR*	*FOXL2*	*IDH2*	*MYCN*	*PDGFRB*	*ROS1*
APC	*CDK4*	*EPCAM*	*GNA11*	*KEAP1*	*NF1*	*PIK3CA*	*SF3B1*
AR	*CDK6*	*ERBB2*	*GNAQ*	*KIT*	*NF2*	*PMS2*	*SMAD4*
ARID1A	*CDKN2A*	*ERBB3*	*GNAS*	*KRAS*	*NRAS*	*POLE*	*STK11*
ATM	*CDKN2B*	*ESR1*	*H3F3A*	*MAP2K1*	*NRG1*	*POLD1*	*TERT*
BAP1	*CHEK1*	*FBXW7*	*H3F3B*	*MET*	*NTRK1*	*PPP2R1A*	*TP53*
BRAF	*CHEK2*	*FGFR1*	*HIST1H3B*	*MLH1*	*NTRK2*	*PTEN*	*TSC1*
BRCA1	*CTNNB1*	*FGFR2*	*HIST1H3C*	*MSH2*	*NTRK3*	*RB1*	*VHL*
BRCA2	*CYSLTR2*	*FGFR3*	*HRAS*	*MSH6*	*PALB2*	*RET*	

Library preparation and enrichment

Illumina Cell-Free DNA Prep with Enrichment libraries were prepared from either fragmented FFPE DNA or cfDNA according to the instructions detailed in the Illumina Cell-Free DNA Prep with Enrichment user guide. For FFPE DNA, after sonication, 45 µl fragmented DNA (~40 ng) was transferred to a 96-well PCR plate to perform the end repair reaction. For ctDNA samples, 20 ng DNA was input into the library prep. Changes to the 'Concentrate indexed libraries' step were made to pool by mass instead of volume to accommodate 1-plex, 4-plex, and 12-plex pooling of libraries from a single library preparation that were tested during this study. Libraries were quantified using the Qubit dsDNA BR assay (Thermo Fisher Scientific, Catalog no. Q32853). To adjust for higher volumes, 250 ng of each library were pooled with some modifications to the protocol. Enrichment was performed using a custom 79-gene probe panel as described in the Illumina Cell-Free DNA Prep with Enrichment user guide.

Pre-enrichment library pooling

For 1-plex libraries, 250 ng prepared libraries were diluted with resuspension buffer (RSB) to a final volume of 7.5 µl and transferred to the corresponding well of the HYB plate to be used during the 'Hybridize double-stranded probes' step.

For 4-plex and 12-plex libraries, pools were prepared by mass rather than by volume during the 'Bind' step of 'Concentrate indexed libraries' part of the protocol. Pooling was performed in a 1.5-ml Eppendorf tube. For 4-plex and 12-plex pools, 1500 ng per sample was combined for a total multiplex pool of 6000 ng and 18,000 ng, respectively. These pools were then subjected to a 1.2× Illumina Purification Bead clean up and eluted in 22.5 µl of RSB, transferring 20 µl of eluate to a new 1.5 ml Eppendorf tube. Multiplex pool concentrations were calculated using the Qubit dsDNA BR assay. For 4-plex pools, 1000 ng of pool was diluted with RSB to a final volume of 7.5 µl and transferred to the HYB plate to be used during the 'Hybridize double-stranded probes' step. For 12-plex pools, 3000 ng of pool was diluted with RSB to a final volume of 7.5 µl and transferred to the HYB plate to be used during the 'Hybridize double-stranded probes' step.

Library normalization

Libraries were manually normalized with onboard denature and dilution of prepared libraries as described in the Denature and Dilute protocol generator.

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Sequencing

Prepared libraries were sequenced on the Illumina NextSeq™ 2000 System. Libraries prepared with Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene panel may also be sequenced on the NextSeq 550 or NextSeq 1000 Systems (Table 3) depending on sample throughput needs. Recommendations for FFPE DNA libraries are 10–20 million paired-end reads per sample and 60 million paired-end reads per sample for cfDNA libraries. The performance metrics for the custom 79-gene panel are detailed in Table 4.

Data analysis

Data analysis was performed using the DRAGEN Enrichment pipeline for FFPE DNA samples or the DRAGEN ILMN cfDNA Prep with Enrichment pipeline for cfDNA samples. Both DRAGEN pipelines enable identification of read families, UMI read collapsing, and error correction. For cloud-based data analysis, raw sequencing data (BCL files) were demultiplexed and converted to FASTQ files in BaseSpace™ Sequence Hub. Illumina Connected Insights or third-party reporting solutions are available for tertiary analysis.

For the FFPE experiment downstream secondary analysis was performed with the DRAGEN™ Enrichment app v4.2.7 enabling 'Somatic Small Variant Caller' with default settings. UCSC hg 38 Alt-Masked v3 was used as the reference human genome. UMI settings were enabled, with UMI- aware variant calling set to 'Low depth'.

Table 4: Expected sequencing performance for the custom 79-gene solid tumor panel

Parameter	Expected performance
Percent covered > 50× (FFPE DNA)	> 99%
Percent covered > 500× (cfDNA)	> 98%
Mean target coverage[a] (FFPE DNA)	800–1000×
Mean target coverage[a] (cfDNA)	2000–2500×
Percent on-target aligned reads	> 70%

a. Coverage after UMI collapsing of reads based on DRAGEN secondary analysis.

The minimum number of supporting reads for UMI was set to one. Variant Annotation by Illumina Connected Annotation (formerly known as Nirvana) for germline tagging was enabled. Under 'Advanced settings' duplicate marking was enabled and 'Combine phased variants' was enabled with the default 'Combine distance' of 15.

For the ctDNA experiment, secondary analysis was performed with the DRAGEN ILMN cfDNA Prep with Enrichment app v4.0.3. The built-in reference genome hg38 Alt-Aware was used for alignment. 'Map align' and 'UMI default' settings were used with two UMI minimum supporting reads. 'Small variant calling' and 'Structural variant calling' with 'DNA fusion filter calling' for hg38 were enabled.

Table 3: Sample throughput for the custom 79-gene solid tumor panel with FFPE DNA or cfDNA

System	Reagent kit or flow cell	Max reads	2 × 150 bp output	No. of FFPE DNA samples	No. of cfDNA samples
NextSeq 550 System	Mid-output	260M	39 Gb	24	4
	High-output	800M	120 Gb	64	12
NextSeq 1000 System	P1	200M	30 Gb	16	3
	P2	800M	120 Gb	64	12
NextSeq 2000 System	P3	2.4B	360 Gb	192[a]	40
	P4	3.6B	540 Gb	192[a]	60

a. Limited by the number of available sample barcodes (number of unique dual sample indexes = 192).

Results

Deep sequencing using NGS provided the high sensitivity needed to reveal low-frequency somatic variation in tumor subpopulations. Starting with as little as 10 ng cfDNA (recommend 20 ng for cfDNA and 40 ng for FFPE DNA), Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene solid tumor panel provided uniform coverage of target regions when paired with high-quality sequencing on Illumina sequencing systems, identifying somatic mutations as low as 2.5% variant allele frequency (VAF) for FFPE DNA and 0.1% VAF for cfDNA (Table 5).

Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene solid tumor panel demonstrated excellent target coverage, uniformity of coverage, and on-target percentage of aligned reads for FFPE DNA (Figure 2) and cfDNA (Figure 3) samples.

The analytical sensitivity of Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene solid tumor panel for detecting single nucleotide variants (SNVs) and insertion and deletion (indel) mutations was determined using FFPE DNA and cfDNA samples. Illumina Cell-Free DNA Prep with Enrichment demonstrated high analytical sensitivity, with the ability to detect all SNVs and indels down to 5% VAF in FFPE DNA samples (Table 6) and down to 0.3% VAF in the cfDNA samples (Table 7).

In addition to covering the coding regions of 78 genes, the custom enrichment panel covers the *TERT* promoter, which contains relatively high GC content and as a result, is often not fully covered by targeted panels. The custom 79-gene panel has been optimized to obtain full coverage for the *TERT* promoter region (Figure 4).

Table 5: Variant calling metrics for Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene panel

Sample	Input DNA	Plexity[a]	Paired-end reads per sample	Mean target coverage[b]	Mean reads per UMI family	Sensitivity 2.5% VAF[c]	Sensitivity 5% VAF[c]
FFPE DNA	40 ng	1-plex	12M	879	3	98.0%	99.4%
	40 ng	4-plex	12M	862	3	96.3%	98.2%
	40 ng	12-plex	12M	822	3	90.6%	94.7%
Sample	Input DNA	Plexity[a]	Paired-end reads per sample	Mean target coverage[b]	Mean reads per UMI family	Sensitivity 0.1% VAF[c]	Sensitivity 0.3% VAF[c]
cfDNA	20 ng	1-plex	60M	2404	6	93.3%	100%
	20 ng	4-plex	60M	2501	6	94.6%	98.9%
	20 ng	12-plex	60M	2430	6	95.5%	100%

a. 12-plex is not extensively tested and may result in lower performance with various samples.
b. Coverage after UMI collapsing of reads based on DRAGEN secondary analysis pipelines.
c. Based on Seraseq ctDNA Complete Reference Material (5%, 2.5%, 1.0%, 0.5%, and 0.1% VAF). A 1:1 mix of 0.5% and 0.1% was used to generate 0.3% VAF samples.

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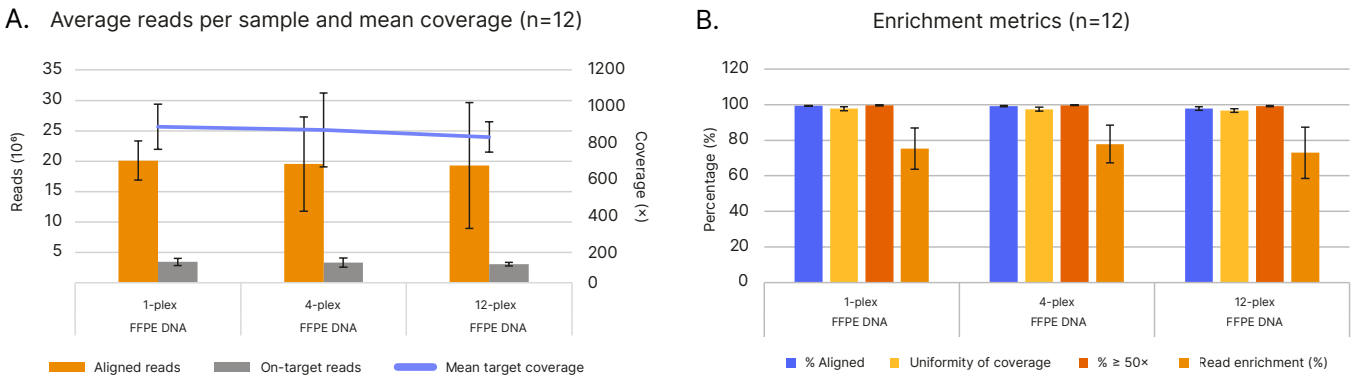

Figure 2: Excellent coverage, uniformity, and on-target alignment for FFPE DNA samples—DNA was fragmented by mechanical shearing. Libraries from a total of 12 FFPE DNA samples were prepared with Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene solid tumor panel. Prepared and enriched libraries were sequenced on the NextSeq 2000 System using a P1 flow cell with 2 × 150 paired-end reads yielding average reads ~20M and mean target coverage of ~900×. On-target reads and mean target coverage were calculated after UMI collapsing of reads (2–4 reads per UMI family). All metrics were generated using the DRAGEN Enrichment App.

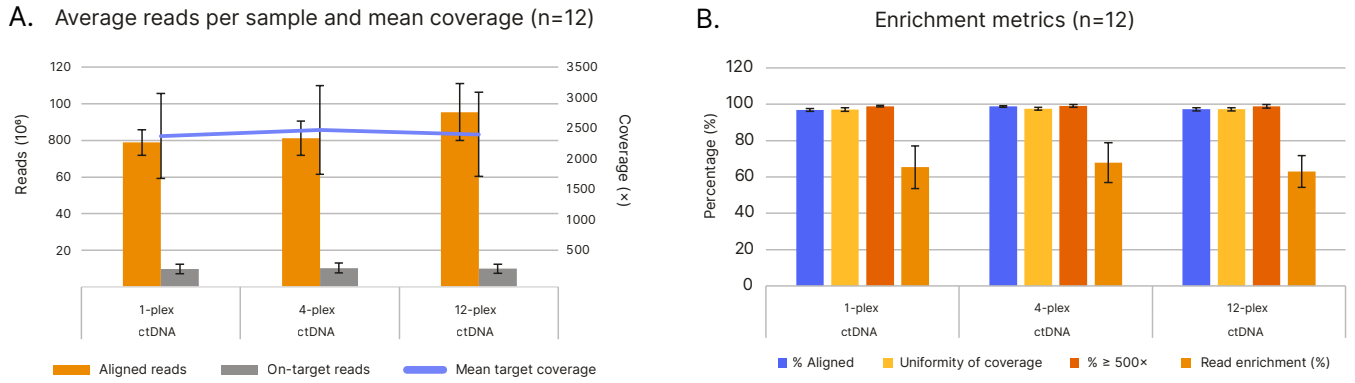

Figure 3: Excellent coverage, uniformity, and on-target alignment for cfDNA samples—Libraries were prepared with Illumina Cell-Free DNA Prep with Enrichment using the custom 79-gene solid tumor panel from a total of 12 cfDNA samples and sequenced on the NextSeq 2000 System using a P1 flow cell with 2 × 150 paired-end reads yielding average reads ~80M and mean target coverage of ~2500×. On-target reads and mean target coverage were calculated after UMI collapsing of reads (4–8 reads per UMI family). All metrics were generated using the DRAGEN for ILMN cfDNA Prep with Enrichment App, except percentage read enrichment (dark blue bars) which is calculated by taking the number of on target reads divided by the number of mapped reads times 100.

Table 6: Summary of individual somatic variants detected in FFPE DNA samples

Gene	COSM ID	Mutation type	HGVS nomenclature	Amino acid change	Variant depth	Total depth	Assay VAF
AKT1	COSM33765	Substitution	c.49G>A	p.E17K	18	397	4.53%
ALK	COSM144250	Insertion	c.3604G>A	p.G1202R	52	1321	3.94%
BRAF	COSM476	SNV	c.1799T>A	p.V600E	24	706	3.40%
BRCA1	COSM1383519	Substitution	c.1961delA	p.K654fs*47	167	3036	5.50%
BRCA2	COSM1738242	Substitution	c.7934delG	p.R2645fs*3	33	929	3.55%
EGFR	COSM6240	Substitution	c.2369C>T	p.T790M	50	1150	4.35%
EGFR	COSM6224	Insertion	c.2573T>G	p.L858R	50	1507	3.32%
EGFR	COSM12370	SNV	c.2240_2257del18	p.L747_P753>S	11	545	2.02%
EGFR	COSM6256	Substitution	c.2254_2277del24	p.S752_I759 del SPKANKEI	11	527	2.09%
EGFR	COSM6223	Substitution	c.2235_2249del15	p.E746_A750 del ELREA	11	546	2.01%
ERBB2	COSM20959	Substitution	c.2324_2325ins12	p.A775_G776 ins YVMA	117	3864	3.03%
KIT	COSM1314	Insertion	c.2447A>T	p.D816V	25	568	4.40%
KRAS	COSM516	Deletion	c.34G>T	p.G12C	26	888	2.93%
KRAS	COSM554	Substitution	c.183A>C	p.Q61H	19	1088	1.75%
NRAS	COSM584	Substitution	c.182A>G	p.Q61R	38	957	3.97%
PIK3CA	COSM775	Deletion	c.3140A>G	p.H1047R	82	2122	3.86%

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Table 7: Summary of individual somatic variants detected in cfDNA samples

Gene	COSM ID	Mutation type	HGVS nomenclature	Amino acid change	Variant depth	Total depth	Assay VAF
AKT1	COSM33765	Substitution	c.49G>A	p.E17K	3	1436	0.21%
ALK	COSM144250	Insertion	c.3604G>A	p.G1202R	11	3223	0.34%
BRAF	COSM476	SNV	c.1799T>A	p.V600E	14	2508	0.56%
BRCA1	COSM1383519	Substitution	c.1961delA	p.K654fs*47	6	3713	0.16%
BRCA2	COSM1738242	Substitution	c.7934delG	p.R2645fs*3	9	2992	0.30%
EGFR	COSM6240	Substitution	c.2369C>T	p.T790M	19	3331	0.57%
EGFR	COSM6224	Insertion	c.2573T>G	p.L858R	5	2986	0.17%
EGFR	COSM12370	SNV	c.2240_2257del18	p.L747_P753>S	7	2505	0.28%
EGFR	COSM6256	Substitution	c.2254_2277del24	p.S752_I759 del SPKANKEI	5	2398	0.21%
EGFR	COSM6223	Substitution	c.2235_2249del15	p.E746_A750 del ELREA	3	2546	0.12%
ERBB2	COSM20959	Substitution	c.2324_2325ins12	p.A775_G776 ins YVMA	7	3374	0.21%
KIT	COSM1314	Insertion	c.2447A>T	p.D816V	5	2337	0.21%
KRAS	COSM516	Deletion	c.34G>T	p.G12C	13	2723	0.48%
KRAS	COSM554	Substitution	c.183A>C	p.Q61H	6	3216	0.19%
NRAS	COSM584	Substitution	c.182A>G	p.Q61R	6	2532	0.24%
PIK3CA	COSM775	Deletion	c.3140A>G	p.H1047R	14	3524	0.40%

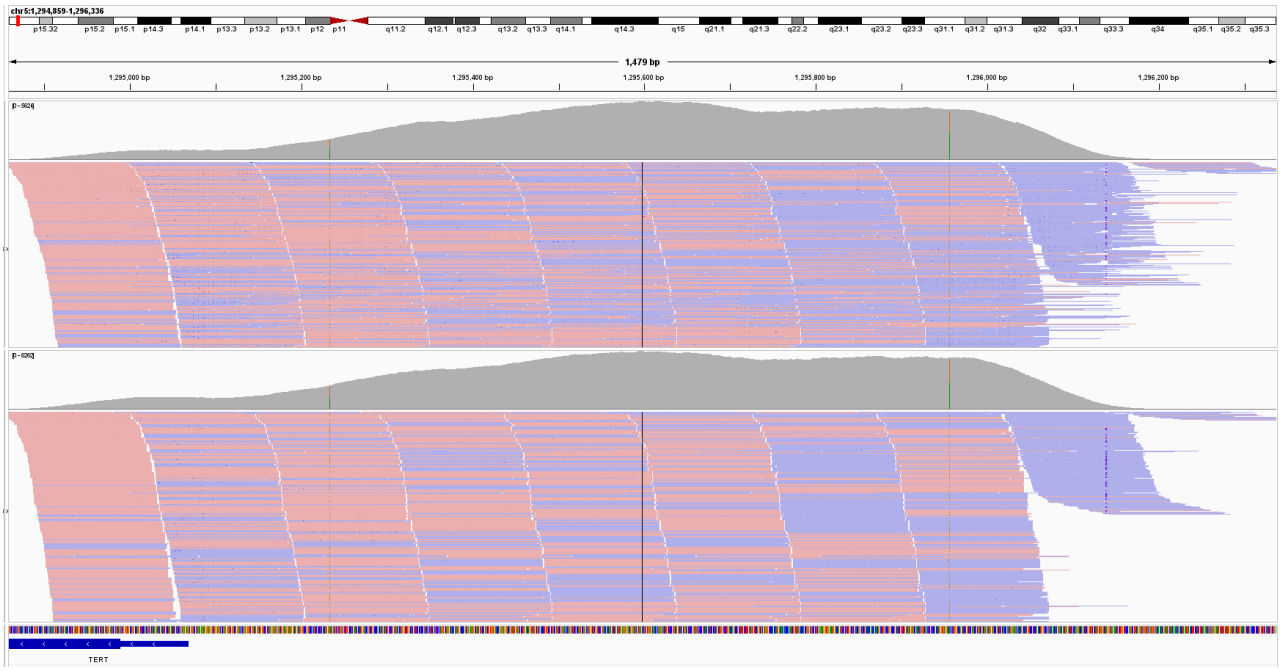

Figure 4: Coverage of the *TERT* promoter region using Illumina Cell-Free DNA Prep with Enrichment and the custom 79-gene solid tumor panel—Integrated Genomics Viewer (IGV) screenshot of two replicate BAM files showing excellent coverage of the *TERT* promoter (hg38). The blue track on the bottom left indicates the start of the *TERT* gene. The pink and purple lines indicate first and second sequencing reads, respectively, and the gray tracks indicate coverage.

The custom 79-gene panel covers select introns in *ALK*, *ROS*, and *RET* genes. This intronic coverage allows for detection of DNA-based gene rearrangements in liquid biopsy samples. *ALK*, *ROS1*, and *RET* DNA-based gene rearrangements are readily detected down to 0.5% VAF in Seraseq controls (Table 8) using the DRAGEN Structural Variant caller that is part of both the DRAGEN Enrichment and DRAGEN ILMN cfDNA Prep with Enrichment pipelines.

Table 8: Recall rate for fusions in *ALK*, *ROS1*, and *RET*

Plexity	0.3% Expected VAF[a]	0.5% Expected VAF[a]
1-plex	83%	100%
4-plex	83%	100%
12-plex	50%	100%

a. Expected VAF level based on sample dilution of the Seraseq ctDNA Complete Reference Material, not the level measured by NGS. The sample with variants at 0.3% VAF was made by mixing equal volumes of the 0.5% and 0.1% VAF control samples.

Summary

Illumina Cell-Free DNA Prep with Enrichment is a versatile library preparation solution optimized for use with low-input cfDNA and FFPE DNA samples. The kit supports a range of panel sizes and is compatible with Illumina or third-party enrichment panels, allowing flexible experimental design. This application note demonstrates the excellent library preparation performance of Illumina Cell-Free DNA Prep with Enrichment using a custom 79-gene panel for both solid tumors and cfDNA. High-quality libraries prepared using this approach paired with sequencing on Illumina sequencing systems accurately identify somatic mutations as low as 2.5% VAF for FFPE DNA and 0.1% VAF for cfDNA, enabling labs to adopt a single solution for assessing both tissue and liquid biopsy samples.

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Ordering information

Contact your Illumina sales representative to order the custom 79-gene panel. The panel design can be accessed via the online DesignStudio tool.

Learn more

Illumina Cell-Free DNA Prep with Enrichment

Illumina Custom Enrichment Panel v2

DRAGEN ILMN cfDNA Prep with Enrichment app

DRAGEN Enrichment app

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