Illumina Cell-Free DNA Prep with Enrichment

Fast, flexible solution for detecting low-abundance mutations in cfDNA

- Detect rare variants with allele frequencies as low as 0.2% from only 20 ng cfDNA extracted from plasma

- Prepare sequencing-ready libraries from user-supplied panels in ~8.5–9.5 hours with 2.5–3 hours hands-on time

- Analyze data and call variants with high analytical sensitivity using DRAGEN™ secondary analysis

- Enable user-defined interpretation and research report generation with Illumina Connected Insights

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Introduction

Circulating cell-free DNA (cfDNA) in plasma has emerged as an important noninvasive disease biomarker in cancer, cardiovascular disease, and organ transplantation. In the field of cancer research, sequencing cfDNA from liquid biopsies provides valuable insight into tumor heterogeneity, enables biomarker profiling, and serves as a complement or an alternative to tissue biopsy samples when tissue is not readily available. Because plasma samples typically contain low amounts of cfDNA from cells of interest, a robust and sensitive assay is necessary to detect rare somatic variants. Fixed gene panels enable variant identification, but are of limited utility for studying novel targets and accommodating changes in genes of interest.

Illumina Cell-Free DNA Prep with Enrichment is a versatile library preparation solution (Table 1) that leverages the power of next-generation sequencing (NGS) technology

Table 1: Overview of Illumina Cell-Free DNA Prep with Enrichment

Parameter	Specification
DNA type	cfDNA from plasma or whole blood
DNA input[a]	10–30 ng
Sample multiplexing	192 unique dual indexes
Duplicate marking	Nonrandom unique molecular identifiers (UMIs)
Enrichment plexity	1-plex or 4-plex
Supported sequencing systems	NextSeq 550 System NextSeq 2000 System, P3 or P4 flow cells NovaSeq 6000 or NovaSeq 6000Dx (in research mode) Systems, P3 or P4 flow cells NovaSeq X Series, 1.5B or 10B flow cells
Total workflow time[b]	~8.5–9.5 hours[c]
Total hands-on time	~2.5–3 hours

a. Recommended 20 ng of cfDNA input.
b. Includes library preparation, enrichment, and normalization steps.
c. Workflow times for single-stranded and double-stranded probes, respectively.

to achieve highly sensitive detection of low-abundance variants in cfDNA samples. This single-vendor solution comprises the library preparation kit, custom panels, and Illumina mid- to high-throughput sequencing systems, including the NovaSeq™ X Series. Data analysis is performed using the DRAGEN for ILMN cfDNA Prep with Enrichment App. Illumina Connected Insights can be used to enable user-defined analysis and interpretation.

Streamlined workflow

Illumina Cell-Free DNA Prep with Enrichment is part of an integrated cfDNA sequencing workflow, delivering excellent performance and data quality. The scalable workflow starts with cfDNA extracted from plasma or whole blood, followed by sequencing on Illumina mid- and high-throughput systems, and highly accurate variant calling using the DRAGEN for ILMN cfDNA Prep with Enrichment App (Figure 1). This user-friendly solution delivers high performance across a wide range of content sizes, is compatible with liquid-handling automation, and accommodates sample multiplexing for efficient scaling.

Fast, flexible library preparation

Illumina Cell-Free DNA Prep with Enrichment is a ligation-based assay that uses a single hybridization step for rapid library preparation (Figure 2). Illumina Cell-Free DNA Prep with Enrichment is compatible with user-supplied enrichment oligonucleotides from Illumina. Source custom enrichments panels based on your specified target gene list using the free online DesignStudio™ tool from Illumina. The DesignStudio tool is compatible with single-stranded DNA (ssDNA) enrichment probes and double-stranded DNA (dsDNA) enrichment v2 probes. For enhanced content portability, Illumina Cell-Free DNA Prep with Enrichment can be used with ssDNA probes from Integrated DNA Technologies and dsDNA probes from Twist Bioscience. The kit accommodates 55–2000 kb ssDNA and 70–2000 kb dsDNA panel content, enabling flexible study design. Sequencing-ready libraries are prepared in ~8.5–9.5 hours, with only ~2.5–3 hours of hands-on time, enabling researchers to go from extracted cfDNA to sequencing in a single day. For maximum efficiency and flexibility, the kit is compatible with cfDNA extracted directly from peripheral blood or plasma using commercially available column- or bead-based purification methods.
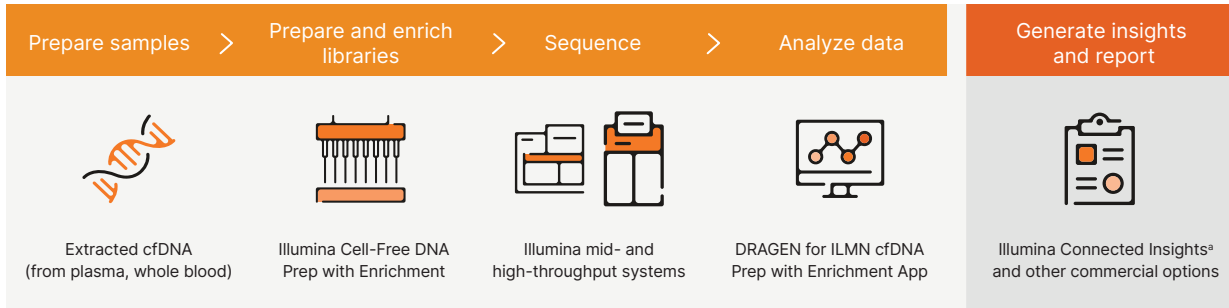
Figure 1: cfDNA-to-results from a single partner—Illumina supports a streamlined workflow for cfDNA sequencing, spanning library preparation, sequencing, and data analysis.

a. The Illumina Connected Insights product line supports user-defined analysis through application programming interface (API) calls to third-party knowledge sources.
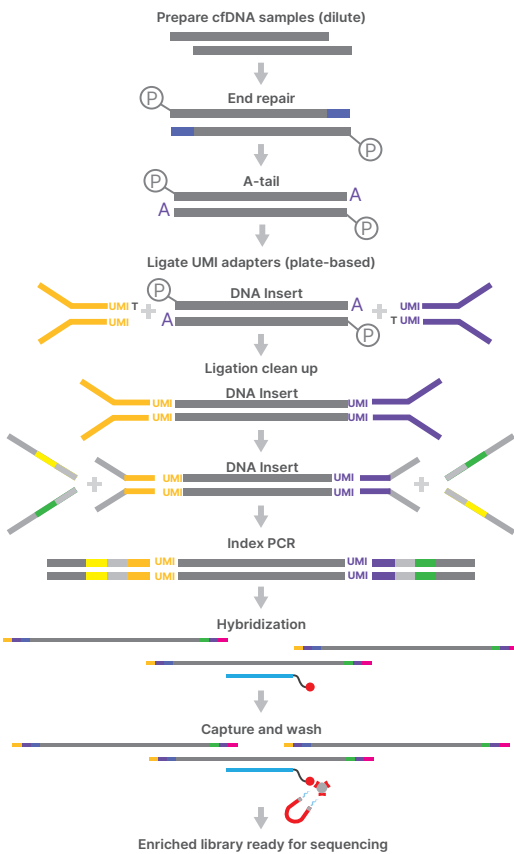

Figure 2: Illumina Cell-Free DNA Prep with Enrichment chemistry—First, cfDNA fragments are repaired and ligated to nonrandom unique molecular identifiers (UMIs). Unique dual indexes are incorporated for multiplexing during PCR amplification. Next, libraries are enriched for targeted regions of interest with biotinylated probes using a single hybridization step. Enriched libraries are amplified and normalized for sequencing on Illumina mid- or high-throughput sequencing systems.

To demonstrate the compatibility of Illumina Cell-Free DNA Prep with Enrichment with a range of enrichment panel sizes and formats, libraries were prepared from 20 ng cfDNA with small, medium, or large enrichment panels (Table 2). Prepared libraries were sequenced on the NextSeq™ 550 System, NovaSeq 6000 System, or NovaSeq X System. Data were analyzed with DRAGEN for ILMN cfDNA Prep with Enrichment app in BaseSpace™ Sequence Hub. The results demonstrate that Illumina Cell-Free DNA Prep with Enrichment delivers > 1500× depth of UMI-collapsed coverage and high coverage uniformity, evaluated by the percentage of targets with > 1000× coverage, across enrichment panels with varying sizes and formats (Figure 3).

Table 2: Parameters used for enrichment panel design

Panel	Size	Probe format	Variant types
Small-A[a]	55 kb	80 bp ssDNA	SNVs, indels
Small-B[a]	180 kb	80 bp ssDNA	SNVs, indels
Medium-A[b]	250 kb	120 bp dsDNA	SNVs, indels, fusions
Medium-B[c]	300 kb	80 bp ssDNA	SNVs, indels, fusions, CNVs
Large[d]	2000 kb	80 bp ssDNA	SNVs, indels, fusions, CNVs

a. Probes were tiled with 20 bp overlap across coding regions for genes of interest.
b. Probes were tiled end-to-end across coding regions for genes of interest. Fusion breakpoints were targeted with overlapping probes at 2× tiling.
c. Probes were tiled with 20 bp overlap across coding regions for genes of interest, and across fusion breakpoints. For CNV detection of genes with small CDS regions (eg, *MYC*), probes were supplemented at low density across introns.
d. Custom design with wet lab optimization.
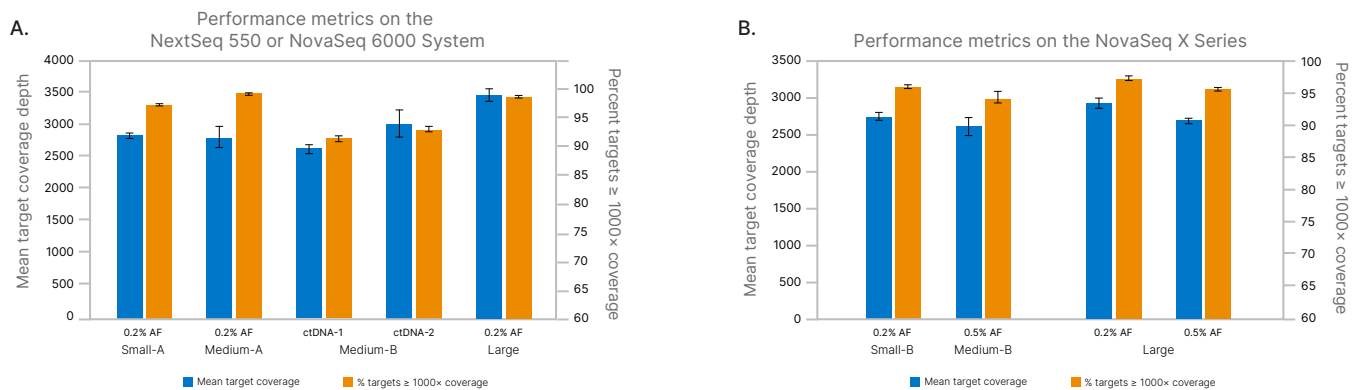SNV, single nucleotide variant; indel, insertion-deletion; CNV, copy number variant

Figure 3: Compatibility with a range of panel sizes—Multiple library replicates (4–8) were prepared from 20 ng cfDNA from plasma, 20 ng cfDNA from whole blood samples spiked with SNVs at a VAF of 0.2%, or 20 ng Seraseq ctDNA Complete Mutation Mix AF0.5% (SeraCare,* Catalog no. 0710-0531) using Illumina Cell-Free DNA Prep with Enrichment. Data were analyzed with the DRAGEN for ILMN cfDNA Prep with Enrichment app in BaseSpace Sequence Hub. (A) Libraries were sequenced on the NextSeq 550 System (small-A panel) or the NovaSeq 6000 System (medium-A, medium-B, and large panels) at an average read depth of 10M, 46M, 54M, or 450M single reads for small, medium, and large panels, respectively. Small-A and medium-A panels were sequenced at ~30,000× and the large panel was sequenced at ~35,000× on-target coverage. (B) Small-B and medium-B panel libraries were sequenced on individual lanes of a 10B flow cell and the large panel libraries on a 1.5B flow cell of the NovaSeq X System. The average read depth was 32M, 54M, and 340M single reads for small-B, medium-B, and large panels, respectively. All panels were sequenced at ~30,000× on-target coverage. * Seracare is part of LGC Diagnostics.

Sensitive low-frequency variant detection

Illumina Cell-Free DNA Prep with Enrichment features enhancements to library preparation chemistry to improve library conversion efficiency and detect low-abundance variants with variant allele frequencies (VAF) as low as 0.2%. To demonstrate the high-quality results achieved using Illumina Cell-Free DNA Prep with Enrichment, Illumina scientists performed studies evaluating the ability to call single nucleotide variants (SNVs), copy number variations (CNVs), and gene fusions. Libraries prepared using Illumina Cell-Free DNA Prep with Enrichment were sequenced on the NextSeq 550 System, NovaSeq 6000 System, or NovaSeq X System. Variant calling was performed using DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub. The results demonstrate the ability to detect mutations at 0.2% VAF from as little as 20 ng cfDNA for small variants, with more than 90% analytical sensitivity (Table 3, Figure 4–5) and 99.98% analytical specificity.

Illumina Cell-Free DNA Prep with Enrichment supports sample multiplexing and has been verified to provide accurate SNV, insertion-deletion (indel), CNV, and gene fusion recall for 1-plex and 4-plex enriched libraries (Figures 5–7).

Table 3: Detection of low-abundance variants with high accuracy

Variant type	Analytical sensitivity[a]
Small variants (0.2% VAF)	≥ 90%
Indels (0.5% VAF)	≥ 90%
Gene amplifications (1.3-fold change)	≥ 95%
Gene deletions (0.6-fold change)	≥ 95%
Gene rearrangements (0.5% VAF)	≥ 95%

a. Analytical sensitivity is defined as percent detection at the stated variant level.
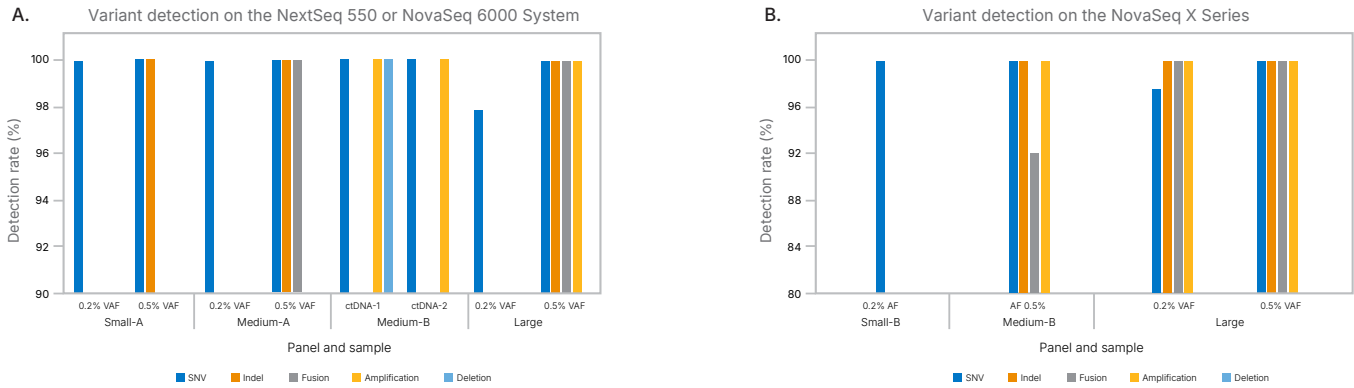
Figure 4: Variant detection at low VAF—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared using 20 ng from plasma, 20 ng cfDNA from whole blood samples spiked with SNVs at VAF of 0.2%, or 20 ng cfDNA from Seraseq ctDNA Complete Mutation Mix AF0.5% (SeraCare, Catalog no. 0710-0531). Variant calling was performed using DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub. (A) Prepared libraries were sequenced on the NextSeq 550 System (55-kb ssDNA small-A panel) or NovaSeq 6000 System (250-kb medium-A, 300-kb medium-B, and 2000-kb large panels) at an average read depth of 10M, 46M, 54M, or 450M single reads for small, medium, and large panels, respectively. Small-A, medium-A, and medium-B panels were sequenced at ~30,000× and the large panel at ~35,000× on-target coverage. (B) Small-B and medium-B panel libraries were sequenced on individual lanes of a 10B flow cell and the large panel libraries on a 1.5B flow cell of the NovaSeq X System. The average read depth was 32M, 54M, and 340M single reads for small-B, medium-B, and large panels, respectively. All panels were sequenced at ~30,000× on-target coverage.
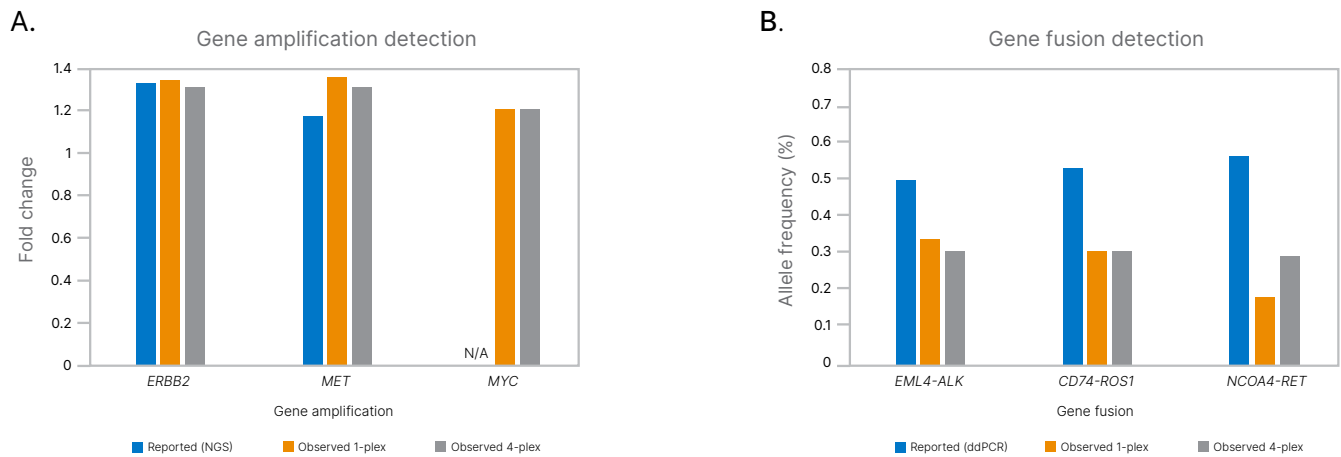

Figure 5: Detection of low-abundance gene amplifications and gene fusions—Illumina Cell-Free DNA Prep with Enrichment demonstrates excellent performance for detecting (A) gene amplifications and (B) gene fusions using both 1-plex and 4-plex enriched libraries with custom content. Libraries were prepared from 20 ng cfDNA from Seraseq ctDNA Complete Mutation Mix AF-0.5% (SeraCare, Catalog no.0710-0531). Four libraries were individually enriched with a 80-bp ssDNA 2000-kb size panel (1-plex) and the same four libraries were re-enriched with the same panel following the multiplex (4-plex) format. Libraries were sequenced on the NovaSeq 6000 System at an average read depth of 400M single reads (≥ 35,000× on-target coverage). Data were analyzed with DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub. The three gene amplifications and fusions in the reference sample were detected in all replicates of 1-plex and 4-plex enriched libraries at the indicated fold change and allele frequency. Discrepancies in VAF for fusions are attributed to differences between testing methods. Note: SeraCare does not verify *MYC* gene amplification by NGS methods.

A. Single nucleotide variant detection

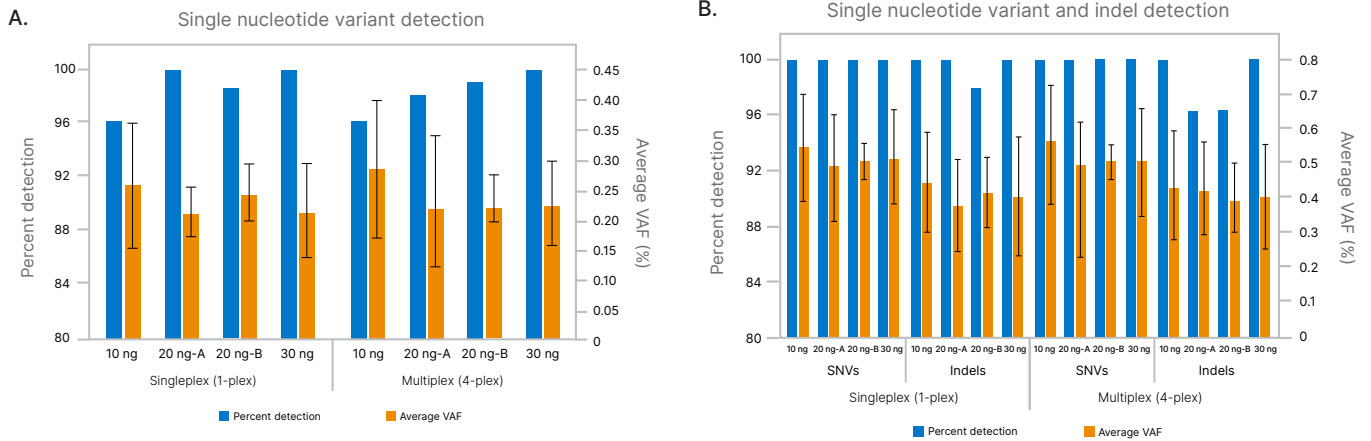B. Single nucleotide variant and indel detection

Figure 6: Sensitive variant detection with 1-plex and 4-plex enriched libraries—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared from cfDNA samples (10 ng, 20 ng, or 30 ng) spiked with SNVs at (A) 0.2% VAF or (B) 0.5% VAF using cfDNA from Seraseq ctDNA Complete Mutation Mix AF-0.5% (SeraCare, Catalog no. 0710-0531). Four libraries were individually enriched with an 80-bp ssDNA 180-kb panel (10 ng, 20 ng-A, and 30 ng) or 80-bp dsDNA 180-kb panel (20 ng-B) for the singleplex (1-plex) format. The same four libraries were re-enriched with the same panel for the multiplex (4-plex) format. Libraries were sequenced on the NextSeq 550 System at an average read depth of 33M single reads (≥ 30,000× on-target coverage). The DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub was used to analyze data and call variants.

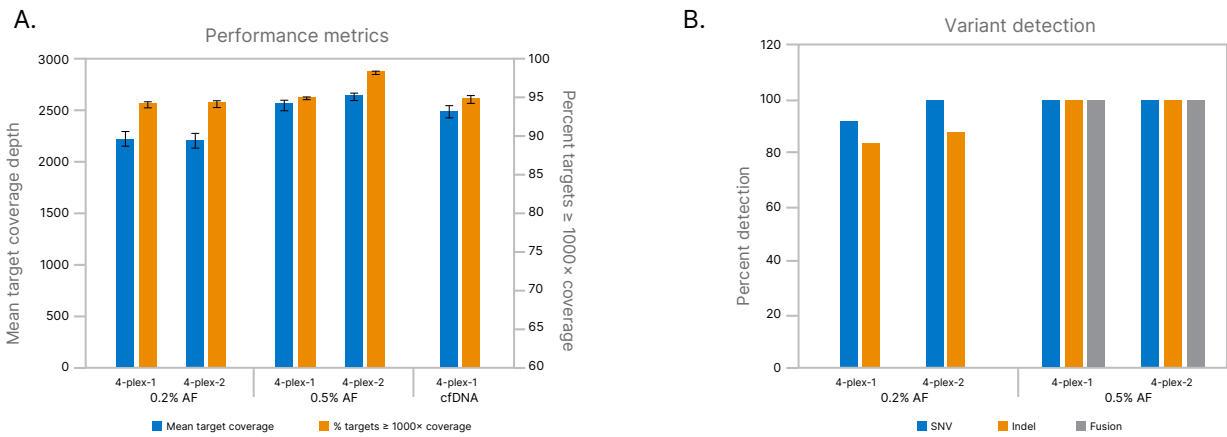
A. Performance metrics

B. Variant detection

Figure 7: Compatibility of 4-plex enriched libraries with the NovaSeq X Series—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared from 20 ng of cfDNA from healthy donors (cfDNA), cfDNA spiked with SNVs at VAF of 0.2% (0.2% AF), or Seraseq ctDNA Complete Mutation Mix AF-0.5% (0.5% AF). Libraries were indexed with IDT for Illumina UMI DNA/RNA UD Indexes (0.2% AF and cfDNA) or Illumina UMI DNA/RNA UD v3 indexes (0.5% AF) and enriched with a 120-bp dsDNA 250-kb panel following the 4-plex enrichment format. Libraries were sequenced on a 1.5B flow cell of the NovaSeq X System at an average read depth of 46M single reads (~30,000× on-target coverage). Library performance metrics (A) and variant call analysis for expected variants (B) were performed using DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub.

Optimized performance across Illumina sequencing systems

To demonstrate the excellent performance of Illumina Cell-Free DNA Prep with Enrichment on Illumina mid- and high-throughput systems, prepared libraries were enriched and sequenced on multiple Illumina systems. The robust and straightforward Illumina Cell-Free DNA Prep with Enrichment solution yields reliable results across all Illumina sequencing systems, providing > 1500× depth of UMI-collapsed coverage and high coverage uniformity, as evaluated by the percentage of targets with > 1000× coverage (Figure 8A). A high variant detection rate was achieved for all variant types across the different sequencing systems (Figure 8B).

Integrated data analysis

The DRAGEN for ILMN cfDNA Prep with Enrichment App uses accelerated, fully integrated bioinformatics algorithms to ensure optimal assay performance. The software performs UMI-based error correction, sequence alignment, and somatic variant calling of small variants, CNVs, and gene fusions. The DRAGEN for ILMN cfDNA Prep with Enrichment App runs locally on a phase 4 Illumina DRAGEN Server v4.0.3 or onboard the NovaSeq 6000Dx System (in research mode). The analysis pipeline can also be run as a cloud application on BaseSpace Sequence Hub or accessed via Illumina Connected Analytics (ICA), a secure, cloud-based genomics platform to scale up secondary analysis without the need to acquire and maintain more local infrastructure.

The integrated analysis pipeline gives users the flexibility to analyze their data based on the panels used for target enrichment, with options to align their sequencing data to hg19 or hg38, and perform specific analyses and customize workflows to suit their research objectives. User-provided noise files can be used to filter out site-specific noise and enhance small variant detection. The software also allows users to mark clonal hematopoiesis variants, exclude specified regions from small variant calling, perform accurate CNV calling, and detect somatic hotspots with high analytical sensitivity using a custom somatic hotspot file or, alternatively, using the built-in DRAGEN somatic hotspots regions. Users accessing the cloud-based DRAGEN for ILMN cfDNA Prep with Enrichment App can explore even more options to optimize their analysis by modifying thresholds for UMI collapsing and small variant calling.
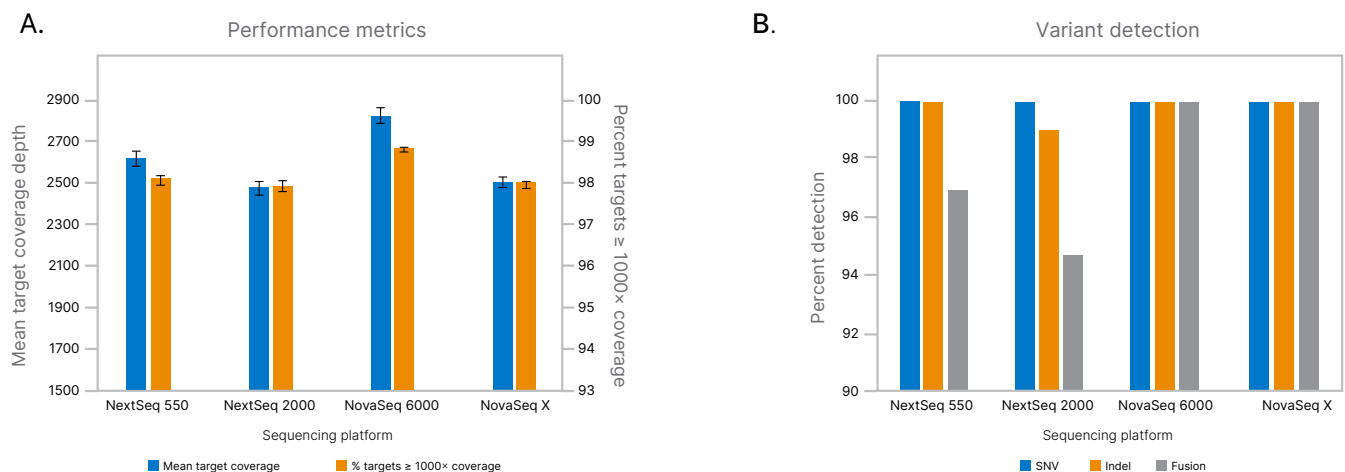

Figure 8: Compatibility with Illumina mid- and high-throughput systems—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared from 20 ng of cfDNA from Seraseq ctDNA Complete Mutation Mix AF-0.5% (SeraCare, Catalog no.0710-0531) and enriched with a 120-bp dsDNA 250-kb panel. Libraries were sequenced on the NextSeq 550, NextSeq 2000, NovaSeq 6000, or NovaSeq X Systems at an average read depth of 46M single reads and ≥ 30,000× on-target coverage. Eight libraries were pooled for the NextSeq 550 System run, 25 libraries for the NextSeq 2000 System run, 51 libraries on one lane of the S4 flow cell for the NovaSeq 6000 System run, and 27 libraries on a single lane of a 10B flow cells of the NovaSeq X System run. The DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub was used to analyze data and call variants.

Labs can leverage Illumina Connected Insights to support user-defined interpretation and analysis. Variant calling files, produced either locally or via the cloud with DRAGEN on Illumina Connected Analytics, can be automatically ingested into Illumina Connected Insights. When combined with sequencing system integration and autolaunch capabilities of Illumina Connected Analytics, the analysis workflow can be fully automated, eliminating the need for manual touchpoints, streamlining the workflow from sequencing to insights and draft report generation.

Automation-enabled workflow

Illumina Cell-Free DNA Prep with Enrichment supports liquid-handling systems for automating library preparation, enabling labs to adjust for variable throughput needs. With an automated workflow, labs can achieve highly reproducible sample handling, maintain consistent results, and drive efficiency. Automation also allows for the rapid scaling of throughput without the need for additional hands-on time. Additional efficiency gains can be achieved by adopting Illumina Qualified Methods,* available from our automation partners and reviewed by Illumina to ensure method performance and data quality.

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* Illumina Qualified Methods available in H1 2025.

Enhanced product attributes

Illumina offers high levels of service and support to ensure operational success for laboratories. To enable greater efficiency, Illumina Cell-Free DNA Prep with Enrichment features:

- **Extended shelf life**: The minimum guaranteed shelf life for Illumina Cell-Free DNA Prep with Enrichment reagents is extended to six months, reducing the risk of product expiration and enabling labs to use reagents according to current testing needs

- **Advanced change notification**: Illumina notifies laboratories six months before any significant changes are made to a product in the Illumina Cell-Free DNA Prep with Enrichment kit

Summary

Illumina Cell-Free DNA Prep with Enrichment is a single-vendor, versatile library preparation solution optimized for use with low-input cfDNA extracted from plasma samples. The user-friendly solution supports a range of panel sizes and is compatible with Illumina or third-party enrichment panels, enabling content flexibility. With the Illumina Cell-Free DNA Prep with Enrichment solution, researchers can detect low-frequency somatic variation with exceptional analytical sensitivity. The high-performance Illumina Cell-Free DNA Prep with Enrichment solution combined with sequencing on powerful Illumina sequencing systems and accelerated data analysis delivers a high-quality cfDNA sequencing workflow, spanning sample processing to data analysis, from a single trusted partner.

Learn more

Illumina Cell-Free DNA Prep with Enrichment

Illumina Connected Insights

Ordering information

Product	Catalog no.
Illumina Cell-Free DNA Prep, Ligation (16 samples)	20104105
Illumina Cell-Free DNA Prep, Ligation (96 samples)	20104106
Illumina Cell-Free DNA Prep, Enrichment (16 reactions)	20104107
Illumina Cell-Free DNA Prep with Enrichment, Ligation (192 samples, 4-plex)	20104103
Illumina Cell-Free DNA Prep with Enrichment, Ligation (192 samples, 4-plex) On-premises	20104104
Illumina Custom Enrichment Panel v2 (32 µl, 120-bp)	20073953
Illumina Custom Enrichment Panel v2 (384 µl, 120-bp)	20073952

Product	Catalog no.
Illumina Custom Enrichment Panel v2 (1536 µl, 120-bp)	20111339
IDT for Illumina UMI DNA/RNA UD Indexes Set A, Ligation (96 Indexes, 96 Samples)	20034701
IDT for Illumina UMI DNA/RNA UD Indexes Set B, Ligation (96 Indexes, 96 Samples)	20034702
IDT for Illumina UMI DNA/DNA Index Anchors Set A for Automation	20066404
IDT for Illumina UMI DNA/DNA Index Anchors Set B for Automation	20063213
Illumina UMI DNA/RNA UD v3 indexes Set A, Ligation (96 indexes, 96 samples)	20126235
Illumina UMI DNA/RNA UD v3 indexes Set B, Ligation (96 indexes, 96 samples)	20126237
Illumina Connected Insights—Genome Equivalent Sample—VCF	20090138
Illumina Connected Insights Starter Implementation Package	20071787
Illumina Connected Insights Expanded Implementation Package	20071787 (as scoped)

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