DATA SHEET

# 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

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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 to achieve highly sensitive detection of low-abundance variants in cfDNA samples. This high-performance kit is part of an integrated cfDNA-to-results workflow that includes library preparation with user-supplied panels followed by sequencing on Illumina mid- to high-throughput systems. Data analysis is performed using the DRAGEN for ILMN cfDNA Prep with Enrichment App.

# 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 whole blood or plasma, 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.

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

| Parameter | Specification |
|---|---|
| DNA type | cfDNA from whole blood or plasma |
| 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 and NovaSeq Systems |
| 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.

# 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 or third-party vendors, including single-stranded DNA (ssDNA) from Integrated DNA Technologies (IDT) and double-stranded DNA (dsDNA) from Twist Bioscience, for enhanced content portability. 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.

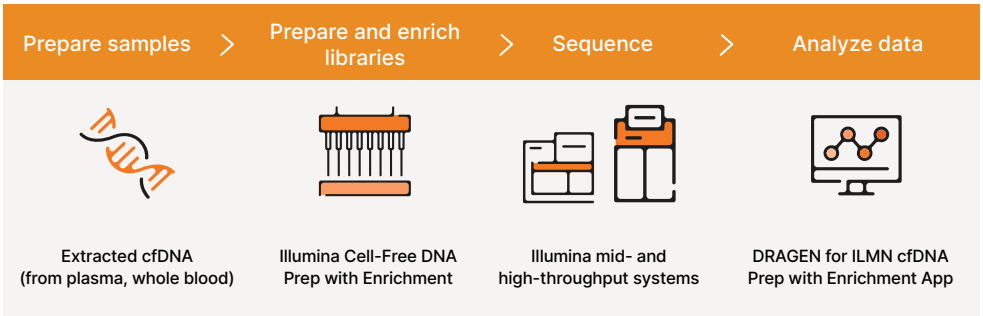


Figure 1: cfDNA-to-results from a single partner—Illumina supports a streamlined workflow for cfDNA sequencing, from library preparation to data analysis. Extracted cfDNA is input to library prep using Illumina Cell-Free DNA Prep with Enrichment. Libraries are sequenced according to scale and throughput needs on an Illumina sequencing system. Accurate, rapid secondary analysis and variant calling are performed with the DRAGEN for ILMN cfDNA Prep with Enrichment App.

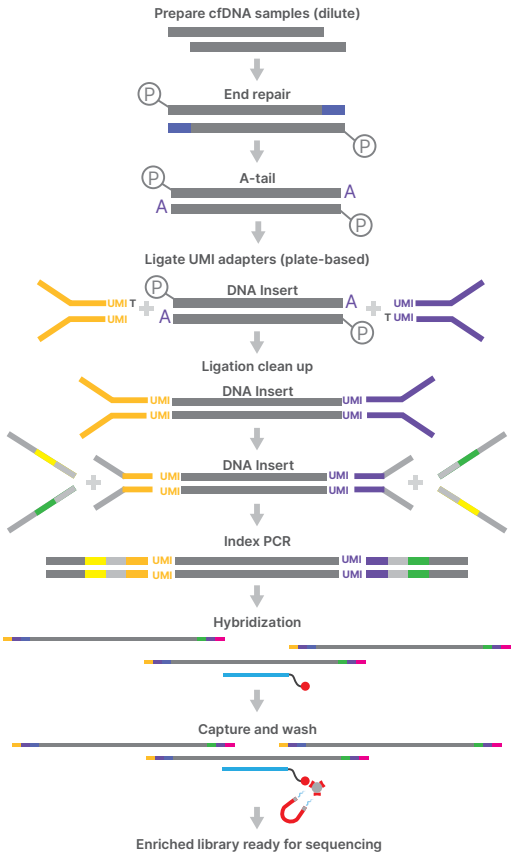


Figure 2: Illumina Cell-Free DNA Prep with Enrichment chemistry—First, cfDNA fragments are repaired and ligated to non-random Unique Molecular Identifiers (UMIs). Unique dual indexes are incorporated for multiplexing during PCR amplification. Next, libraries are enriched for targeted regions of interest 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 (55 kb, ssDNA), medium (250 kb) or large (2000 kb) enrichment panels (Table 2). Prepared libraries were sequenced either on the NextSeq™ 550 System (small panel at 10M paired-end reads per sample) or the NovaSeq™ 6000 System (medium and large panels at 46M and 450M paired-end reads per sample, respectively). 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] | 55 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.

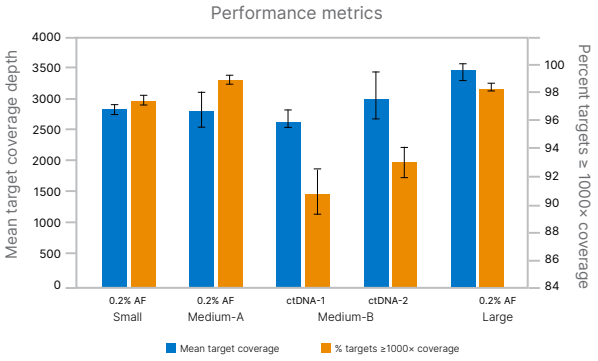


Figure 3: Compatibility with a range of panel sizes—Four library replicates were prepared from 20 ng cfDNA using Illumina Cell-Free DNA Prep with Enrichment and sequenced on the NextSeq 550 System (for small panel) or the NovaSeq 6000 System (for medium and large panels) at an average read depth of 10M, 46M, or 450M paired-end reads for small, medium, and large panels, respectively. Data were analyzed with DRAGEN for ILMN cfDNA Prep with Enrichment app in BaseSpace Sequence Hub. Small and medium panels were sequenced at ~30,000× and the large panel was sequenced at ~35,000× on-target coverage.

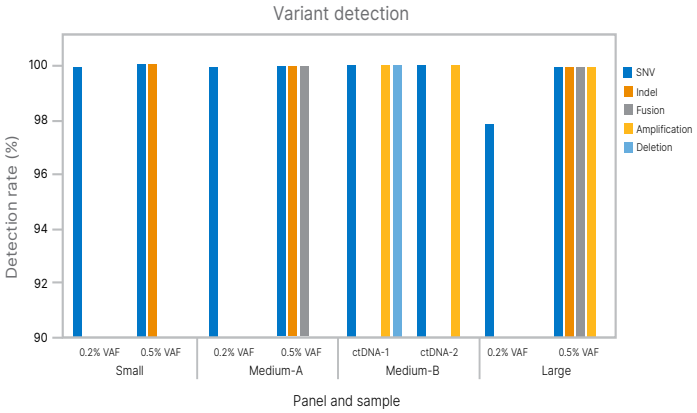


Figure 4: Variant detection at low variant allele frequency (VAF)—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared using 20 ng cfDNA from whole blood samples spiked with SNVs at variant allele frequency (VAF) of 0.2% or using 20 ng cfDNA from Seraseq ctDNA Complete Mutation Mix AF-0.5% (SeraCare, Catalog no. 0710-0531). Prepared libraries were sequenced on the NextSeq 550 System (55 kb ssDNA small panel) or NovaSeq 6000 (250 kb medium and 2000 kb large panels) platform at an average read depth of 10M, 46M, or 450M paired-end reads for small, medium, and large panels, respectively. Variant calling was performed using DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub. Small and medium panels were sequenced at ~30,000× and the large panel at ~35,000× on-target coverage.

# 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 (Figure 4, Figure 5). Libraries prepared using Illumina Cell-Free DNA Prep with Enrichment were sequenced on the NextSeq 550 System (10M paired-end reads per sample) or the NovaSeq 6000 System at high depth (46M and 450M paired-end reads per sample for medium and large panels, respectively). 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 low as 20 ng cfDNA for small variants, with more than 90% analytical sensitivity (Table 3) and 99.98% analytical specificity.

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.

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 (Figure 5, Figure 6).

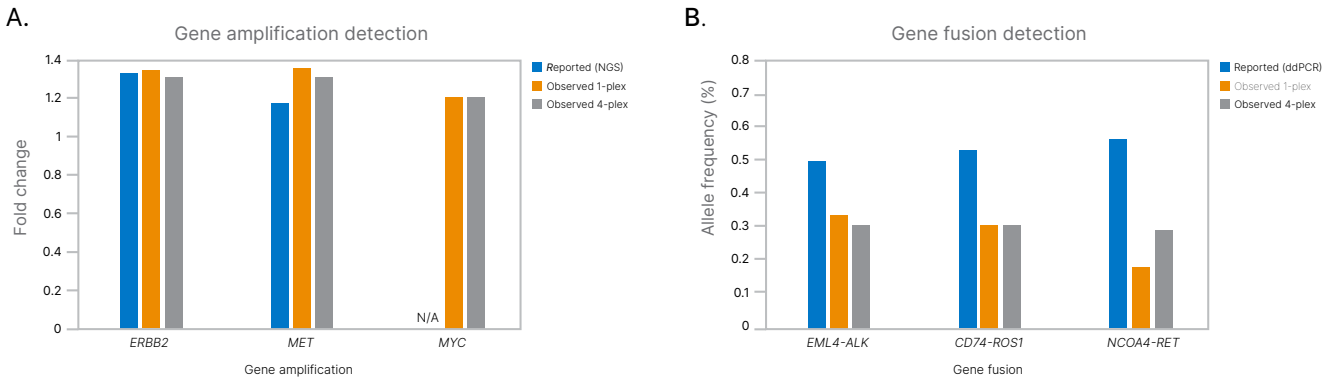


Figure 5: Detection of low-abundance gene amplifications and gene fusions—Illumina Cell-Free DNA Prep with Enrichment demonstrates excellent performance for detecting (A) gene amplifications and (B) gene fusions using both 1-plex and 4-plex enriched libraries with custom content. Libraries were prepared 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 80bp 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 paired-end reads (≥ 35,000X 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.

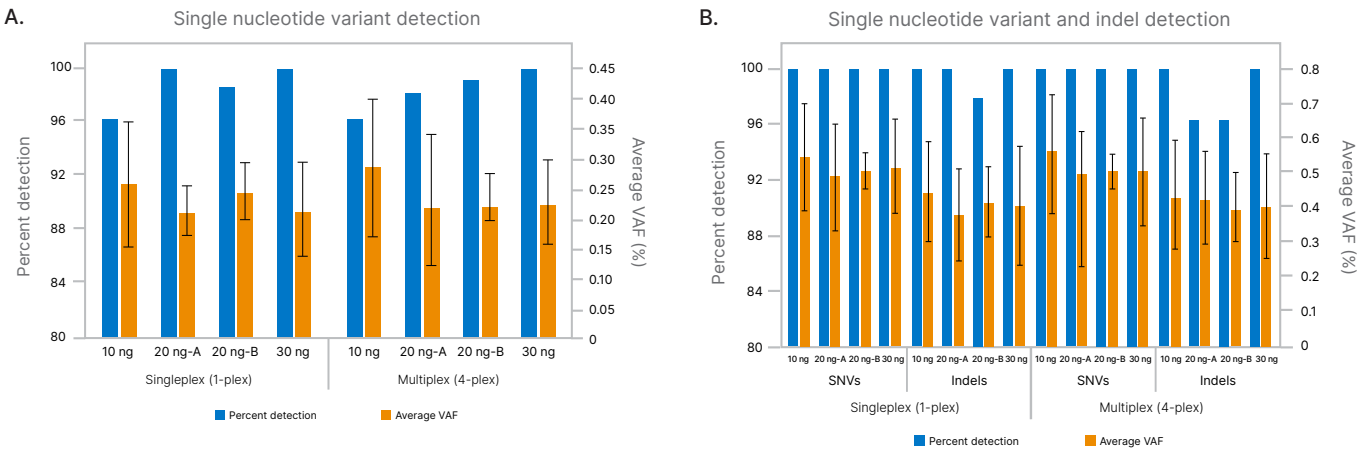


Figure 6: Sensitive variant detection with 1-plex and 4-plex enriched libraries—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared from cfDNA samples (10 ng, 20 ng, or 30 ng) spiked with SNVs at (A) 0.2% VAF or (B) 0.5% VAF using cfDNA from Seraseq ctDNA Complete Mutation Mix AF-0.5% (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 paired-end 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.

## 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, libraries were prepared from 20 ng of input cfDNA at 0.5% VAF from Seraseq ctDNA Complete Mutation Mix AF-0.5% (SeraCare, Catalog no. 0710-0531) enriched with a 120-bp dsDNA 250-kb panel and sequenced on the NextSeq 550 System, NextSeq 2000 System, or NovaSeq 6000 System at an average read depth of 92M reads per sample at ~30,000× on-target coverage. 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 7).

## 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 use 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.

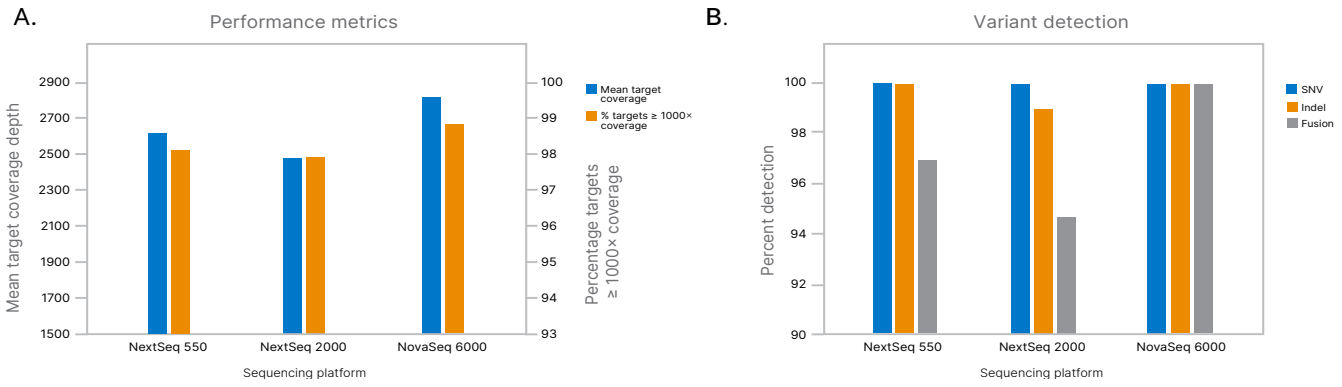


Figure 7: Compatibility with Illumina mid- and high-throughput systems—Illumina Cell-Free DNA Prep with Enrichment libraries were prepared from 20 ng of cfDNA with known VAF 0.5% and enriched with a 120-bp dsDNA 250-kb panel. Libraries were sequenced on the NextSeq 550, NextSeq 2000, or NovaSeq 6000 Systems at an average read depth of 46M paired-end reads and ≥ 30,000× on-target coverage. Eight libraries pooled for the NextSeq 550 System run, 25 libraries for the NextSeq 2000 System run, and 51 libraries on one lane of the S4 flow cell for the NovaSeq 6000 System run. The DRAGEN for ILMN cfDNA Prep with Enrichment App in BaseSpace Sequence Hub was used to analyze data and call variants.

## 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.

## Summary

Illumina Cell-Free DNA Prep with Enrichment is a 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

## 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 |
| 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 Qualified Methods available in late 2024.

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