FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Intended Use

The Illumina® DNA Prep with Enrichment Dx Kit is a set of reagents and consumables used to prepare sample libraries from genomic DNA derived from human cells and tissue to develop *in vitro* diagnostic assays. User-supplied probe panels are required for the preparation of libraries targeting specific genomic regions of interest. The generated sample libraries are intended for use on Illumina sequencing systems. The Illumina® DNA Prep with Enrichment Dx includes software for sequencing run setup, monitoring, and analysis.

Principles of Procedure

Illumina DNA Prep with Enrichment Dx Kit is intended for manual preparation of DNA sequencing libraries enriched for targeted regions from genomic DNA derived from human cells and tissue.

User-supplied biotinylated oligonucleotide panels are required for target enrichment. Illumina DNA Prep with Enrichment Dx Kit is compatible with a range of panel sizes, including small panels (< 20.000 probes) to large panels (> 200.000 probes). The generated enriched libraries are intended for sequencing on the Illumina sequencing systems.

The Illumina DNA Prep with Enrichment Dx Kit procedure consists of the following steps:

- **Tagment Genomic DNA**—Uses Enrichment BLT Small (eBLTS) to tagment the DNA input. During tagmentation, gDNA is fragmented and tagged with adapters in a single step. A minimum DNA input of 50 ng is required to saturate the eBLTS in the tagmentation reaction. When saturated, the eBLTS fragments a set number of DNA molecules to generate normalized libraries of consistent fragment size distribution.
- **Post Tagmentation Cleanup**—Cleans up the adapter-tagged DNA on the eBLTS to use in amplification.
- **Amplify Tagmented DNA**—Amplifies the tagmented DNA using a limited-cycle PCR program. Unique dual (UD) indexes are added at the ends of the DNA fragments, which enable dual unique barcoding of the DNA libraries and cluster generation during sequencing.
- **Clean Up Libraries**—Uses a bead-purification procedure to purify and size select the amplified DNA libraries.
- **Pool Libraries**—Combines DNA libraries with unique indexes into one pool of up to 12 libraries. You can pool libraries by volume or by mass.
- **Hybridize Probes**—Consists of a hybridization reaction during which the double-stranded DNA libraries are denatured and a panel of biotinylated DNA probes is hybridized to targeted genomic regions.
  - Illumina DNA Prep with Enrichment Dx Kit is compatible with multiple panels. Illumina DNA Prep with Enrichment Dx Kit does not include an enrichment panel. Probe panels are supplied by the user and must meet the required specifications. Illumina DNA Prep with Enrichment Dx Kit reagents are

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compatible with both Illumina and third-party enrichment DNA oligonucleotide panels that meet the required specifications. For information on the required specifications for third-party panels, refer to *Enrichment Probe Panel Requirements* on page 10

- **Capture Hybridized Probes**—Uses Streptavidin Magnetic Beads (SMB3) to capture the biotinylated probes hybridized to the targeted regions of interest.
- **Amplify Enriched Libraries**—Uses PCR to amplify the enriched libraries.
- **Clean Up Amplified Enriched Libraries**—Uses a bead purification procedure to purify the enriched libraries ready for sequencing.
- **Sequencing**—Sequencing of the enriched libraries is performed on MiSeqDx, NextSeq 550Dx, or NovaSeq 6000Dx sequencing systems. For MiSeqDx and NextSeq 550Dx, the integrated DNA GenerateFASTQ Dx Local Run Manager Module is used for sequencing run setup, run monitoring, and FASTQ generation from base calls. For NextSeq 550Dx with DRAGEN Server and NovaSeq 6000Dx, the DRAGEN for Illumina DNA Prep with Enrichment Dx Application is used for run setup and secondary analysis with several available workflows.

Limitations of Procedure

- For *in vitro* diagnostic use.
- Illumina DNA Prep with Enrichment Dx Kit is compatible with genomic DNA derived from human cells and tissue.
- Illumina DNA Prep with Enrichment Dx Kit is compatible with double-stranded gDNA inputs of 50–1000 ng. Performance is not guaranteed with inputs outside these thresholds.
- Illumina DNA Prep with Enrichment Dx Kit does not include reagents for DNA extraction. The analytical testing results, including interference testing, provided in *Performance Characteristics* on page 56 have been obtained with whole blood and FFPE as representative sample types with representative DNA extraction kits. All diagnostic tests developed for use with Illumina DNA Prep with Enrichment Dx Kit reagents require full validation for all aspects of performance with the DNA extraction kit of choice.
- Illumina DNA Prep with Enrichment Dx Kit is not recommended for poor quality FFPE samples with ΔCq > 5. Using samples with ΔCq > 5 might increase the chances of library preparation failure and decrease assay performance.
- Illumina DNA Prep with Enrichment Dx Kit reagents have been configured and tested for the sample input, enrichment reactions, and plexity indicated in the following table.

Illumina DNA Prep with Enrichment Dx Kit	Sample Input	Enrichment Reactions	Enrichment Plexity
16-sample kit	Low quality (FFPE)	16 reactions	1-plex
96-sample kit	High quality (eg, whole blood)	8 reactions	12-plex

- FFPE input processing has been tested and is recommended exclusively for 1-plex enrichment reactions with use of the 16-sample kit.
- For the 96-sample kit, nonstandard plexities (2-plex to 11-plex) are possible, but have the following limitations:
  - Processing of samples in 2-plex to 11-plex enrichment reactions reduces the throughput of the kit.
  - Optimal results are not guaranteed. Obtaining suitable enrichment yield for nonstandard plexities might require additional optimization.
  - For low plexity pooling strategies (2-plex to 8-plex), selecting index adapters with diverse sequences is required to optimize color balance for successful sequencing and data analysis. The DNA GenerateFASTQ Dx module on MiSeqDx and NextSeq 550Dx provides options for color-balanced index combinations during run set up. For more information on pooling strategies, refer to *Pooling Methods* on page 33.
- Illumina DNA Prep with Enrichment Dx Kit is limited to delivering enriched libraries that are sequenced on the MiSeqDx, NextSeq 550Dx, and NovaSeq 6000Dx only. Use of other sequencing systems requires full validation for all aspects of performance.
- Enrichment panels are not included as part of this product. The analytical testing results provided in *Performance Characteristics* on page 56 have been obtained with representative enrichment panels and are provided for information purposes only. The analytical performance characteristics serve to exemplify the general capabilities of the assay and do not establish the capabilities or suitability concerning any specific assay claims. All diagnostic tests developed for use with these reagents require full validation for all aspects of performance.
- Illumina DNA Prep with Enrichment Dx Kit is compatible with both Illumina and third-party enrichment panels. However, performance with third-party enrichment panels that do not meet the panel requirements is not guaranteed. For information on panel requirements, refer to *Enrichment Probe Panel Requirements* on page 10.
- Illumina DNA Prep with Enrichment Dx Kit uses a 2 hour hybridization time. Using a longer hybridization time can impact performance metrics.
- The DNA GenerateFASTQ Dx Local Run Manager modules for MiSeqDx and NextSeq 550Dx only deliver FASTQ files. If you are using these modules, you are required to perform secondary analysis validation.

- The DRAGEN for Illumina DNA Prep with Enrichment Dx Application is available on NextSeq 550Dx with DRAGEN Server and NovaSeq 6000Dx. The application supports multiple secondary analysis workflows, including FASTQ generation, FASTQ and VCF generation for germline variant detection, and FASTQ and VCF generation for somatic variant detection. If you are using the application for VCF generation, you do not need to perform secondary analysis validation. Limitations of the application include the following:
  - Insertions of length > 18 bp and deletions of length > 21 bp have not been validated.
  - Large variants, including multinucleotide variants (MNVs) and large indels, might be reported as separate smaller variants in the output VCF file.
  - Small MNVs are reported as separate variants in the output VCF file.
  - Deletions are reported in the VCF file at the coordinate of the preceding base per VCF format. Therefore, consider adjacent variants before reporting that an individual base call is a homozygous reference.
  - Germline-specific limitations:
    - The Germline FASTQ and VCF generation analysis workflow of the DRAGEN for Illumina DNA Prep with Enrichment Dx Application is designed to deliver qualitative results for germline variant calling (eg, homozygous, heterozygous, wild type).
    - Copy number variation can affect whether a variant is identified as homozygous or heterozygous.
    - The system will not report more than two variants at a single locus, even in the presence of copy number variation.
  - Somatic-specific limitations:
    - The Somatic FASTQ and VCF generation analysis workflow of the DRAGEN for Illumina DNA Prep with Enrichment Dx Application is designed to deliver qualitative results for somatic variant calling (ie, presence of a somatic variant).
    - The Somatic FASTQ and VCF generation analysis workflow cannot differentiate between germline and somatic variants. The workflow is designed to detect variants across a range of variant frequencies, but variant frequency cannot be used to differentiate somatic variants from germline variants.
    - Normal tissue in the specimen impacts the detection of variants. The reported limit of detection is based on a variant frequency relative to the total DNA extracted from both tumor and normal tissue.
    - If more than one variant allele is called at the same locus, none of the alleles will be reported as passing variants. Instead, the full set of alleles will be reported but filtered via the multiallelic tag.

Product Components

The Illumina DNA Prep with Enrichment Dx Kit consists of the following components.

- Illumina DNA Prep with Enrichment Dx with UD Indexes Set A, catalog # 20051354 (16 samples), or # 20051352 (96 samples)

- Illumina DNA Prep with Enrichment Dx with UD Indexes Set B, catalog # 20051355 (16 samples), or # 20051353 (96 samples)
- Local Run Manager DNA GenerateFASTQ Dx Module for NextSeq 550Dx, catalog # 20063024
- Local Run Manager DNA GenerateFASTQ Dx Module for MiSeqDx, catalog # 20063022
- DRAGEN for Illumina DNA Prep with Enrichment Dx Application for NovaSeq 6000Dx, catalog # 20074609
- DRAGEN for Illumina DNA Prep with Enrichment Dx Application for NextSeq 550Dx, catalog # 20074730

Reagents Provided

Completing the Illumina DNA Prep with Enrichment Dx requires Illumina DNA Prep with Enrichment Dx with UD Indexes Set A or Illumina DNA Prep with Enrichment Dx with UD Indexes Set B. You can perform the following number of library prep and enrichment reactions using a 16 sample or 96 sample kit.

Illumina DNA Prep with Enrichment Dx Kit	**Sample Input**	**Enrichment Reactions**	**Enrichment Plexity**
16-sample kit	Low quality (FFPE)	16 reactions	1-plex
96-sample kit	High quality (eg, whole blood)	8 reactions	12-plex

Illumina DNA Prep with Enrichment Dx with UD Indexes Set A/B

Illumina Prep Dx Tagmentation Reagents 1, Store at 15°C to 30°C

The following reagents are shipped at room temperature. Promptly store reagents at the indicated storage temperature to ensure proper performance.

Reagent Name	Tube Quantity		Cap Color	Fill Volume	Active Ingredients
	16 Samples (# 20050020)	96 Samples (# 20050025)			
Stop Tagment Buffer 2 (ST2)	1	4	Red	350 µl	Detergent solution in water.
Tagment Wash Buffer 2 (TWB2)	1	1	Green	41 ml	Buffered aqueous solution containing detergent and salt.
Cleanup Beads (CB)	1	N/A*	Red	10 ml	Solid-phase paramagnetic beads in buffered aqueous solution.

* Cleanup Beads for 96 samples are included in Illumina Prep Dx Cleanup Beads 96 Samples (# 20050030).

Illumina Prep Dx Cleanup Beads (96 Samples), Store at 15°C to 30°C

For 96 sample kits, Cleanup Beads are included in the Illumina Prep Dx Cleanup Beads (catalog # 20050030). The following reagent is shipped at room temperature. Promptly store reagents at the indicated storage temperature to ensure proper performance. For 16 sample kits, Cleanup Beads are included in Illumina Prep Dx Tagmentation Reagents 1 (catalog # 20050020).

Reagent Name	Quantity	Cap Color	Fill Volume	Active Ingredients
Cleanup Beads (CB)	4	Red	10 ml	Solid-phase paramagnetic beads in buffered aqueous solution.

Illumina DNA Prep Dx Tagmentation Reagents 2, Store at 2°C to 8°C

The following reagents are shipped refrigerated. Promptly store reagents at the indicated storage temperature to ensure proper performance. Store the eBLTS stock tube upright so that the beads are always submerged in the buffer.

Reagent Name	Tube Quantity		Cap Color	Fill Volume		Active Ingredients
	16 Samples (# 20050021)	96 Samples (# 20050026)		16 Samples	96 Samples	
Enrichment BLT Small (eBLTS)	1	4	Yellow	200 µl	290 µl	Streptavidin Magnetic Beads linked with transposomes in buffered aqueous solution containing glycerol, EDTA, dithiothreitol, salt, and detergent.
Resuspension Buffer (RSB)	1	4	Clear	1.8 ml	1.8 ml	Buffered aqueous solution.

Illumina Prep Dx Tagmentation Reagents 3, Store at -25°C to -15°C

The following reagents are shipped frozen. Promptly store reagents at the indicated storage temperature to ensure proper performance.

Reagent Name	Tube Quantity		Cap Color	Fill Volume		Active Ingredients
	16 Samples (# 20050022)	96 Samples (# 20050027)		16 Samples	96 Samples	
Tagmentation Buffer 1 (TB1)	1	4	Clear	290 µl	290 µl	Buffered aqueous solution containing magnesium salt and dimethylformamide.
Enhanced PCR Mix (EPM)	2	4	Clear	200 µl	610 µl	DNA polymerase and dNTPs in buffered aqueous solution.

Illumina DNA Prep Dx Enrichment Reagents 1 (16 samples), Store at 2°C to 8°C

For 16 sample kits, the following reagents are included in Illumina DNA Prep Dx Enrichment Reagents 1 (catalog # 20050023). For 96 sample kits, the reagents are included in Illumina Prep Dx Enrichment Reagents 1 (catalog # 20050028).

The following reagents are shipped refrigerated. Promptly store reagents at the indicated storage temperature to ensure proper performance.

Reagent Name	Tube Quantity	Cap Color	Fill Volume	Active Ingredients
Streptavidin Magnetic Beads (SMB3)	4	Clear	1.2 ml	Streptavidin Magnetic Beads in buffered aqueous solution containing formamide, detergent, and salt.
Resuspension Buffer (RSB)	1	Clear	1.8 ml	Buffered aqueous solution.
Enrichment Hyb Buffer 2 (EHB2)	1	Clear	200 µl	Buffered aqueous solution containing detergent and salt.
Elute Target Buffer 2 (ET2)	1	Clear	200 µl	Buffered aqueous solution.

Illumina Prep Dx Enrichment Reagents 1 (96 samples), Store at 2°C to 8°C

For 96 sample kits, the following reagents are included in Illumina Prep Dx Enrichment Reagents 1 (catalog # 20050028). For 16 sample kits, the reagents are included in IlluminaDNA Prep Dx Enrichment Reagents 1 (catalog # 20050023).

The following reagents are shipped refrigerated. Promptly store reagents at the indicated storage temperature to ensure proper performance.

Reagent Name	Tube Quantity	Cap Color	Fill Volume	Active Ingredients
Streptavidin Magnetic Beads (SMB3)	2	Clear	1.2 ml	Streptavidin Magnetic Beads in buffered aqueous solution containing formamide, detergent, and salt.
Resuspension Buffer (RSB)	4	Clear	1.8 ml	Buffered aqueous solution.
Enrichment Hyb Buffer 2 (EHB2)	1	Clear	200 µl	Buffered aqueous solution containing detergent and salt.
Elute Target Buffer 2 (ET2)	1	Clear	200 µl	Buffered aqueous solution.

Illumina DNA Prep Dx Enrichment Reagents 2, Store at -25°C to -15°C

The following reagents are shipped frozen. Promptly store reagents at the indicated storage temperature to ensure proper performance.

Reagent Name	Tube Quantity		Cap Color	Fill Volume	Active Ingredients
	16 Samples (# 20050024)	96 Samples (# 20050029)			
Enrichment Elution Buffer 1 (EE1)	1	1	Clear	580 µl	Detergent solution in water.
Enhanced Enrichment Wash Buffer (EEW)	4	4	Amber	4.1 ml	Buffered aqueous solution containing salts and detergent.
PCR Primer Cocktail (PPC)	1	1	Clear	320 µl	PCR primers (oligonucleotides) mix.
2N NaOH (HP3)	1	1	Clear	200 µl	2N sodium hydroxide (NaOH) solution.
HYB Buffer 2 + IDT NXT Blockers (NHB2)	2	1	Blue	480 µl	Buffered aqueous solution with Cot-1 DNA, crowding agent, and formamide
Enhanced PCR Mix (EPM)	2	1	Clear	200 µl	DNA polymerase and dNTPs in buffered aqueous solution.

Illumina Unique Dual Index Dx Set A/B, Store at -25°C to -15°C

The following reagents are shipped frozen. Promptly store reagents at the indicated storage temperature to ensure proper performance. For index adapter sequences, refer to *Appendix: Illumina UD Indexes Adapter Sequences* on page 61.

Component	Quantity
Illumina Unique Dual Index Dx Set A (96 Indexes), # 20050038	1
Illumina Unique Dual Index Dx Set B (96 Indexes), # 20050039	1

Reagents Not Provided

Reagents Required, Not Provided

- DNA extraction and purification reagents
- DNA quantification reagents
- Ethanol (200 proof for molecular biology)
- Nuclease-free water
- 10 mM Tris-HCl, pH 8.5
- 1N NaOH solution, molecular biology grade
- If using the NextSeq 550Dx sequencing system:
  - 200 mM Tris, pH 7.0 (can be diluted from 1 M Tris-HCL, pH 7.0)
  - NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) (catalog # 20028871)
- If using the MiSeqDx sequencing system:
  - MiSeqDx Reagent Kit v3 (catalog # 20037124)
- If using the NovaSeq 6000Dx sequencing system:
  - 400 mM Tris, pH 8.0 (can be diluted from 1 M Tris-HCL, pH 8.0)
  - NovaSeq 6000Dx S2 Reagent Kit (300 cycles) (catalog # 20046931)
  - NovaSeq 6000Dx S4 Reagent Kit (300 cycles) (catalog # 20046933)
  - NovaSeq 6000Dx S2 Buffer Cartridge (catalog # 20062292)
  - NovaSeq 6000Dx S4 Buffer Cartridge (catalog # 20062293)
  - NovaSeq 6000Dx Library Tube (catalog # 20062290)
  - NovaSeq 6000Dx Library Tube, 24 Pack (catalog # 20062291)

Enrichment Probe Panel Requirements

Illumina DNA Prep with Enrichment Dx Kit reagents are compatible with both Illumina and third-party enrichment DNA oligonucleotide panels. If using third-party biotinylated DNA probes (fixed or custom panels), make sure that they meet the required specifications.

Illumina DNA Prep with Enrichment Dx Kit has been optimized and validated using the following third-party panel specifications. Comparable performance is not guaranteed when using third-party panels that do not meet the specifications.

- 80 bp or 120 bp probe length
- Between 500 to 675,000 probes
- Single- or double-stranded DNA
- Total probe input of ≥ 3 pmols for enrichment at plexities from 1-plex to 12-plex

Storage and Handling

- Room temperature is defined as 15°C to 30°C.
- Reagents are stable when stored as indicated until the specified expiration date on the kit labels. For storage temperatures, refer to *Reagents Provided* on page 5.
- The frozen reagents are stable for a maximum of four freeze-thaw cycles that occur before the specified expiration date.
- The Illumina DNA Prep with Enrichment Dx Kit procedure contains the following safe stopping points:
  - After *Amplify Tagmented DNA* on page 28, the amplified libraries are stable for up to 30 days when stored at -25°C to -15°C.
  - After *Clean Up Libraries* on page 30, the cleaned-up amplified libraries are stable for up to 30 days when stored at -25°C to -15°C.
  - After *Pool Pre-Enriched Libraries* on page 32, the pooled libraries are stable for up to 30 days when stored at -25°C to -15°C.
  - After *Amplify Enriched Library* on page 43, the enriched, amplified libraries plate can remain on the thermal cycler for up to 24 hours. Alternatively, the plate can be stored at 2°C to 8°C for up to 48 hours.
  - The final cleaned up enriched libraries are stable for up to 7 days when stored at -25°C to -15°C.
- If any of the packaging or contents of the Illumina DNA Prep with Enrichment Dx Kit are damaged or compromised, contact Illumina Customer Service.
- Stop Tagment Buffer 2 (ST2) can form visible precipitates or crystals. If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates dissolve.
- Hybridization Oligos (HYB) and Enhanced Enrichment Wash Buffer (EEW) must be preheated to the same temperature as the hybridization hold temperature applicable per sample type and probe panel. For more information on handling NHB2 and EEW, refer to *Procedural Notes* on page 16.
- Enrichment Hyb Buffer 2 (EHB2) and HYB Buffer+IDT NXT Blockers (NHB2) can develop crystals and cloudiness. If crystals and cloudiness are observed, vortex or pipette up and down to mix until the solution is clear. Make sure to preheat NHB2 before pipetting.
- When handling Cleanup Beads (CB), use the following best practices:
  - Never freeze the beads.
  - Immediately before use, vortex the beads until resuspended and the color appears homogeneous.
- When handling Enrichment BLT Small (eBLTS), use the following best practices:

  - Store the eBLTS tube upright so that the beads are always submerged in the buffer.
  - Vortex the eBLTS thoroughly until the beads are resuspended. To avoid resettling the beads, centrifuging before pipetting is not recommended.
  - If beads are adhered to the side or top of a 96-well plate, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
- When handling index adapter plates, use the following best practices:
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.

Equipment and Materials Required, Not Provided

In addition to the Illumina DNA Prep with Enrichment Dx Kit, make sure that you have the required equipment and materials before starting the protocol.

Equipment

Make sure that you have the required equipment before starting the protocol.

The protocol has been optimized and validated using items with the listed specifications. Comparable performance is not guaranteed when using equipment outside the specifications.

Some items are required only for specific workflows. These items are specified in separate tables.

- Thermal cycler with the following specifications:
  - Heated lid
  - Minimum temperature control range of 10°C to 98°C
  - Minimum temperature accuracy of ±0.25°C
  - Maximum reaction volume of 100 µl
  - Compatible with full-skirted 96-well PCR plates
- Microsample incubator with the following specifications:
  - Temperature range of ambient +5.0°C to 99.0°C
  - Compatible with 96-well MIDI plates
- Microsample incubator inserts compatible with 96-well MIDI plates
- High-speed microplate shaker with a mixing speed range of 200–3000 rpm
- Magnetic stand compatible with 96-well PCR plates
- Magnetic stand compatible with 96-well MIDI plates
- Fluorometer compatible with your quantification method
- DNA fragment analyzer
- Precision pipettes:

  - 10 µl single-channel and multichannel pipettes
  - 20 µl single-channel and multichannel pipettes
  - 200 µl single-channel and multichannel pipettes
  - 1000 µl single-channel pipettes
  - Precision pipettes ensure accurate reagent and sample delivery. Single-channel or multichannel pipettes can be used if they are calibrated regularly and are accurate within 5% of the stated volume.
- Microplate centrifuge
- Microcentrifuge
- One of the following Illumina sequencing systems:
  - MiSeqDx Instrument, catalog # DX-410-1001
  - NextSeq 550Dx Instrument, catalog # 20005715 with optional Illumina DRAGEN Server for NextSeq 550Dx, catalog # 20086130
  - NovaSeq 6000Dx Instrument, catalog # 20068232
- **[Optional]** Vacuum concentrator
- **[FFPE]** Real-time PCR detection system

Materials

Make sure that you have the required materials before starting the protocol.

Some items are required only for specific workflows. These items are specified in separate tables.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate materials.

- Filtered pipette tips
- Conical centrifuge tubes, 15 ml or 50 ml
- 1.5 ml microcentrifuge tubes
- RNase/DNase-free multichannel reagent reservoirs, disposable
- RNase/DNase-free 8-tube strips and caps
- Serological Pipettes
- 96-well polypropylene deep-well storage plate, 0.8 ml (MIDI plate)
- Hard-Shell 96-well full-skirted PCR plates
- **[FFPE]** qPCR plates compatible with qPCR instrument
- Adhesive seals for 96-well plates with the following specifications:
  - Peelable, optically clear polyester
  - Suitable for skirted PCR plates
  - Strong adhesive that withstands multiple temperature changes of -40°C to 110°C

  - DNase/RNase-free
- Plastic consumables compatible with quantification method of choice
- Fluorometric dsDNA quantification kit compatible with chosen quantification system:
  - For quantifying pre-enriched amplified libraries, a broad range quantification kit can be used.
  - For quantifying enriched libraries, the range of the quantification kit depends on probe panel used.
- Fragment analysis kit for library qualification with chosen qualification system:
  - For qualifying pre-enriched amplified libraries, a broad range kit can be used.
  - For qualifying enriched libraries, the range of the qualification kit depends on probe panel used.
- **[Optional]** Kit for DNA extraction from human cells and tissue. You can use any validated extraction method.

Specimen Collection, Transport, and Storage

CAUTION

Handle all specimens as if they are potentially infectious agents.

- This assay is compatible with genomic DNA derived from human cells and tissue.
- For commercially available purified gDNA, make sure that the samples have been transported under the correct conditions and stored according to instructions from the manufacturer. Follow best practices for storage and freeze-thaw cycles of the gDNA.
- For whole blood input, follow the blood collection, transport, and storage requirements applicable to the DNA extraction method of choice. Any validated extraction method can be used. Transportation of whole blood must comply with country, federal, state, and local regulations for the transport of etiologic agents.
- For extraction of DNA from FFPE tissue, any validated extraction method can be used. Follow the instructions and recommendations applicable to the extraction method of choice for determining the following practices:
  - Formalin-fixation and paraffin-embedding method for tissues, to ensure best quality of extracted DNA.
  - Storage of FFPE specimens.
  - The starting material requirements, such as the number and thickness of the FFPE sections. Most purification methods recommend using freshly cut sections.

Warnings and Precautions

- Illumina DNA Prep with Enrichment Dx Kit reagents contain potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents

as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the Safety Data Sheets (SDS) at support.illumina.com/sds.html.

- Immediately report any serious incidents related to this product to Illumina and the Competent Authorities of the member states in which the user and the patient are established.
- Handle all blood specimens as if they are known to be infectious for Human Immunodeficiency Virus (HIV), Human hepatitis B virus (HBV), and other blood-borne pathogens (universal precautions).
- Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and kit reagents.
- To prevent sample or reagent degradation, make sure that all sodium hypochlorite vapors from cleaning have fully dissipated prior to starting the protocol.
- Contamination of the samples with other PCR products/amplicons can cause inaccurate and unreliable results. To avoid contamination, use the following best practices:
  - Use proper laboratory practices and laboratory hygiene.
  - Execute the workflow steps in the designated pre-amplification or post-amplification areas.
  - Store used reagents before cleaning up libraries in a pre-amplification area.
  - Separate pre-amplification reagents from post-amplification reagents.
  - Make sure that pre-amplification and post-amplification areas have dedicated equipment, such as pipettes, pipette tips, vortexer, and centrifuge.
- Avoid cross-contamination. Use fresh pipette tips between samples and between dispensing reagents. Using filtered tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.
  - When adding or transferring samples or reagent master mixes, change tips between each sample.
  - When adding index adapters with a multichannel pipette, change tips between each row or each column. If using a single-channel pipette, change tips between each sample.
  - Remove unused index adapter plates from the working area.
- Use the following best practices for ethanol wash steps:
  - Always prepare fresh 80% ethanol. Ethanol can absorb water from the air, which can impact results.
  - Make sure that all ethanol is removed from the bottom of the wells during wash steps. Residual ethanol can impact results.
  - Adhere to the specified drying time for magnetic stand steps to ensure complete evaporation. Residual ethanol can impact the performance of subsequent reactions.
- Always prepare master mixes before use and never store the combined working solutions.
- The performance of Illumina DNA Prep with Enrichment Dx Kit is not guaranteed when procedures are not followed as outlined in the package insert.
- Do not use any kit components beyond the stated expiration date on the kit label.

- Do not interchange kit components from different Illumina DNA Prep with Enrichment Dx kits. Kits are identified on the kit label.

Procedural Notes

DNA Input Recommendations

The Illumina DNA Prep with Enrichment Dx Kit protocol is compatible with high quality, double-stranded genomic DNA (gDNA) inputs of 50–1000 ng.

Make sure that the initial gDNA sample does not contain > 1 mM EDTA and is free of organic contaminants, such as phenol and ethanol. These substances can interfere with the tagmentation reaction and result in assay failure.

gDNA Input ≥ 50 ng

For gDNA inputs between 50–1000 ng, quantifying and normalizing the initial gDNA sample is not required.

gDNA Input < 50 ng

DNA inputs of 10-50ng can be used, with the following adjustments:

- If using 10–49 ng gDNA input, quantifying the initial gDNA sample is recommended to determine the number of PCR cycles required after tagmentation. Use a fluorometric-based method to quantify double-stranded gDNA input. Avoid methods that measure total nucleic acid, such as NanoDrop or other UV absorbance methods.
- This protocol does not normalize final pre-enriched library yields from 10–49 ng gDNA and therefore, quantification and normalization of libraries before and after enrichment is required.
- Illumina DNA Prep with Enrichment Dx Kit has been characterized and verified for DNA inputs of 50–1000 ng. Equivalent product performance cannot be guaranteed for gDNA inputs < 50 ng.

Blood Input Recommendations

Illumina DNA Prep with Enrichment Dx Kit is compatible with gDNA extracted from peripheral whole blood. Any validated extraction method can be used. When extracting gDNA from whole blood, initial quantification of the input DNA is not required and the Illumina DNA Prep with Enrichment Dx Kit produces normalized pre-enriched library yields.

The following factors can adversely affect the amount of DNA obtained from whole blood samples and therefore the library normalization:

- Blood sample age
- Storage conditions
- Underlying medical conditions affecting white blood cell counts

FFPE Tissue Sample Input Recommendations

Use the following FFPE DNA quality criteria to determine the appropriate input for successful library preparation:

- For FFPE samples with ΔCq value of ≤ 5, the recommended DNA input is 50–1000 ng.
- Illumina DNA Prep with Enrichment Dx is not recommended for poor quality FFPE samples with ΔCq > 5. Using samples with ΔCq > 5 is possible, but might increase chances of library preparation failure or decrease assay performance.

FFPE Extraction

Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. You can use any validated method for DNA extraction from FFPE samples. For gDNA extracted from FFPE tissue, initial quantification of input DNA is required and Illumina DNA Prep with Enrichment Dx Kit does not produce normalized pre-enriched library yields.

FFPE DNA Qualification

The gDNA extracted from FFPE tissue shall be qualified prior to use. For optimal performance, assess DNA sample quality using a validated extraction method for qualification of DNA extracted from FFPE samples. The Illumina DNA Prep with Enrichment Dx Kit protocol is compatible with FFPE DNA samples with ΔCq value of ≤ 5. Illumina DNA Prep with Enrichment Dx Kit is not recommended for poor quality FFPE samples with ΔCq > 5. Using samples with ΔCq > 5 is possible, but might increase chances of library preparation failure or decrease assay performance.

[Optional] FFPE Reference Samples

Use characterized reference materials such as Horizon HD799 (DNA) as a positive control when performing the protocol. Qualified FFPE materials from cell line derived xenografts can also be used as reference samples. Use a fluorometric-based method to quantify reference materials before use.

NOTE Running a positive control reference sample or no template control consumes reagents and reduces the total number of unknown samples that can be processed.

Sample Input Recommendations

The sample input recommendations for the Illumina DNA Prep with Enrichment Dx Kit are summarized in the following table.

Table 1 Sample Input Recommendations

Sample Input Type	Sample Input Amount	Quantification of Input DNA Required	Required DNA Input Quality	Normalized Pre-Enriched Library Yield
gDNA	10–49 ng	Yes	260/280 ratio of 1.8–2.0	No
gDNA	50–1000 ng	No	260/280 ratio of 1.8–2.0	Yes
gDNA from blood	50–1000 ng	No	260/280 ratio of 1.8–2.0	Yes
gDNA from FFPE	50–1000 ng	Yes	ΔCq value of ≤ 5	No

The recommended PCR cycles for the eBLTS PCR program are adjusted based on sample input concentration and quality. For more information, refer to *Amplify Tagmented DNA* on page 28.

Tips and Techniques

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding index adapters with a multichannel pipette, change tips between ***each row*** or ***each column***. If using a single-channel pipette, change tips between each sample.

Sealing the Plate

- Always seal the 96-well plate with a new adhesive seal using a rubber roller to cover the plate before the following steps in the protocol:
  - Shaking steps
  - Incubation steps. Failure to seal the plate properly can lead to evaporation during incubation.
  - Centrifuge steps
  - Hybridization steps
- Make sure the edges and wells are completely sealed to reduce risk of cross-contamination and evaporation.
  - If any fluid or condensation is observed on the seal or sides of the plate wells, centrifuge as needed before unsealing.
- Place the plate on a flat surface before slowly removing the seal.

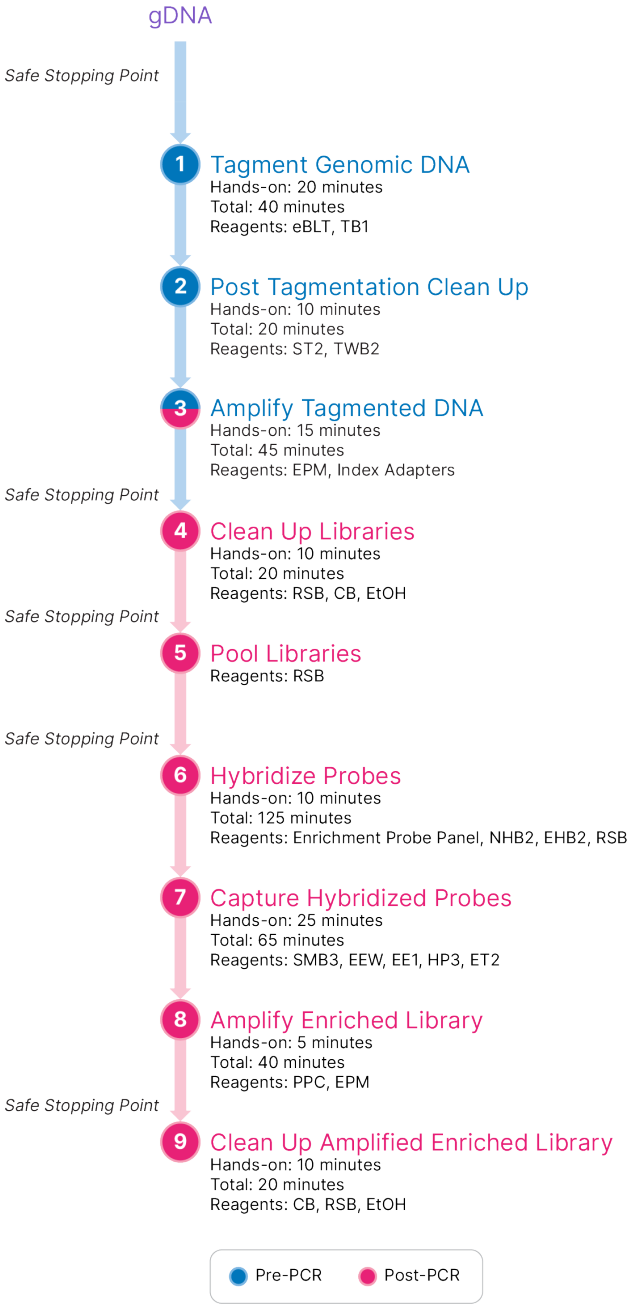
Handling Enrichment BLT Small (eBLTS)

- Store the eBLTS stock tube upright in the refrigerator so that the beads are always submerged in the buffer.

- Immediately before use, vortex the eBLTS stock tube thoroughly until the beads are resuspended. To avoid resettling the beads, centrifugation before pipetting is not recommended.
- If beads are adhered to the side or top of a 96-well plate, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
- When washing eBLTS:
  - Use the appropriate magnetic stand for the plate.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - If beads are aspirated into pipette tips, dispense back into the plate on the magnetic stand and wait until the liquid is clear (2 minutes).

Illumina DNA Prep with Enrichment Dx Kit Workflow

The following diagram illustrates the Illumina DNA Prep with Enrichment Dx Kit workflow. Safe stopping points are marked between steps. Time estimates are based on processing 12 samples at 12-plex enrichment.



Instructions For Use

This chapter describes the Illumina DNA Prep with Enrichment Dx Kit protocol.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required components, equipment, and materials.
  - Third-party biotinylated probes must meet specific requirements. Refer to *Enrichment Probe Panel Requirements* on page 10 to make sure that your third-party probes meet the requirements.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.
- When creating a master mix, overage is included in the provided volumes.
- Make sure to use the appropriate magnetic stand for your plate type.

Prepare for Pooling

This step is required to ensure successful sequencing of enriched libraries. Pooling of libraries can happen prior to enrichment and prior to sequencing.

Prior to enrichment—Individual indexed amplified libraries are pooled together for enrichment with the selected probe panel. This creates a multiplexed pool of enriched libraries. For FFPE sample input, processing has been tested and is recommended exclusively for 1-plex enrichment reactions. For high quality gDNA, 12-plex has been tested, but 2-plex through 11-plex are possible.

Prior to sequencing—1-plex enriched libraries and/or multiplex enriched libraries are pooled together prior to sequencing. The number of enriched libraries that can be sequenced depends on the target read depth for each sample on your sequencing system.

Unique Dual Indexing

Illumina DNA Prep with Enrichment Dx Kit uses unique dual indexes.

- Dual-indexed libraries add Index 1 (i7) and Index 2 (i5) sequences to generate uniquely tagged libraries.
- UD indexes have distinct, unrelated index sequences for the i7 and i5 Index Read. Indexes are 10 bases long.

Selecting index adapters with diverse sequences for pooled libraries optimizes color balance for successful sequencing and data analysis. Plexity pools that are ≥ 10-plex are inherently color-balanced, so you can use any index adapter combination. During your sequencing run, the DNA GenerateFASTQ Dx Local Run Manager Module provides options for color-balanced index combinations and notifies you if there is not sufficient diversity in the selected index combinations.

For information on Illumina UD index adapter sequences and plate layouts, refer to *Appendix: Illumina UD Indexes Adapter Sequences* on page 61

Supported Enrichment Plexities

Illumina DNA Prep with Enrichment Dx Kit reagents are configured and tested at 1-plex and 12-plex enrichment plexity. Although other enrichment plexities are possible, some plexities require additional pre-enrichment library prep and enrichment probe panel reagents.

Obtaining suitable enrichment yield for nonstandard enrichment plexity might require additional optimization. Optimal results are not guaranteed.

- **Enrichment plexity**– The number of pre-enriched libraries (1–12) pooled together in one enrichment reaction for hybridization with the enrichment probe panels. For example, combining 12 pre-enriched libraries together creates a 12-plex enrichment pool.
- **Enrichment reaction**– The number of unique enrichment reaction preparations, regardless of the number of pre-enriched libraries pooled per reaction. For example, a single enrichment reaction can prepare a 1-plex or 12-plex enrichment pool.

To calculate the total number of post-enriched libraries, multiply the enrichment plexity per reaction by the number of enrichment reactions. For example, a single enrichment reaction of a 12-plex enrichment pool produces a pool of 12 post-enriched libraries.

When pooling pre-enriched libraries, Illumina DNA Prep with Enrichment Dx Kit reagents support the following enrichment reactions and plexity.

Illumina DNA Prep with Enrichment Dx Kit Reagents	Enrichment Reactions	Enrichment Plexity
16-sample kit	16 reactions	1-plex
96-sample kit	8 reactions	12-plex

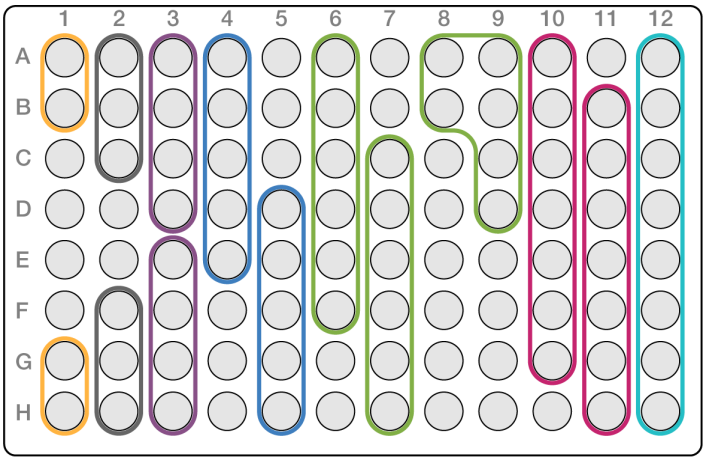
Two-Plex Through Eight-Plex Pooling Strategies

The following table shows index adapters (wells) that can be combined in a 2–8-plex pool, while the color-coded figure illustrates each combination.

Pool any plexity ≥ 2 from the top or bottom of a column. Do not pool across a row.

Plexity	Combinations	Color in Figure
2	The first two or last two wells in a column: • A and B • G and H Rows C–F are not used.	Orange

Plexity	Combinations	Color in Figure
3	The first three or last three wells in a column: • A–C • F–H Rows D and E are not used.	Gray
4	The first four or last four wells in a column: • A–D • E–H	Purple
5	The first five or last five wells in a column: • A–E • D–H	Blue
6	[Option 1] The first six or last six wells in a column: • A–F • C–H [Option 2] The first two wells (A and B) or last two wells (G and H) in one column and any four wells in an adjacent column.	Green
7	The first seven or last seven wells in a column: • A–G • B–H	Pink
8	The entire column.	Teal

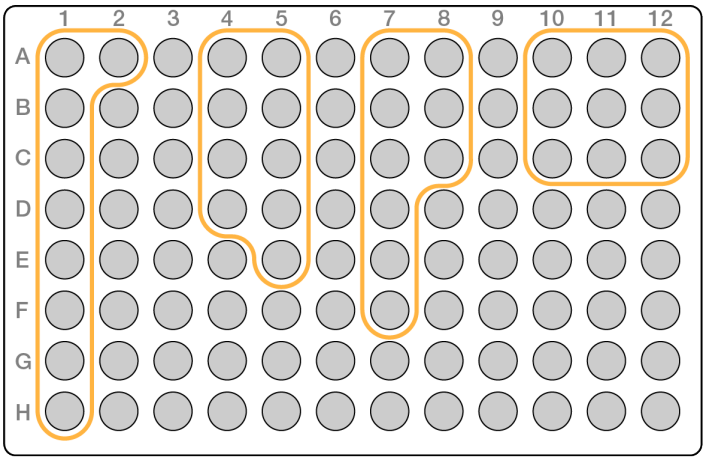


Nine-Plex Pooling Strategies

Use index adapters from any wells that optimize color balance in a sequencing run, for example:

- A1–H1 and A2
- A4–D4 and A5–E5
- A7–F7 and A8–C8
- A10–C10, A11–C11, and A12–C12

The following figure depicts all four examples.



Tagment Genomic DNA

This step uses the Enrichment BLT Small (eBLTS) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences.

Consumables

- eBLTS (Enrichment BLT Small) (yellow cap)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 96-well PCR plate
- Adhesive seal
- 1.7 ml microcentrifuge tubes
- 8-tube strip
- Pipette tips
  - 200 µl multichannel pipettes

⚠ CAUTION

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the Safety Data Sheets (SDS) at support.illumina.com/sds.html.

About Reagents

- eBLTS must be stored at temperatures at 2°C to 8°C. Do not use eBLTS that has been stored below 2°C.
- Do not centrifuge eBLTS.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
eBLTS (yellow cap)	2°C to 8° C	Bring to room temperature. Vortex immediately before use to mix. Do not centrifuge before pipetting.
TB1	-25°C to -15°C	Bring to room temperature. Vortex to mix.

2. Vortex or pipette DNA, and then centrifuge briefly.
3. Save the following TAG program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 55°C for 5 minutes
   - Hold at 10°C

Procedure

1. Add 2–30 µl DNA to each well of a 96-well PCR plate so that the total input amount is 50–1000 ng.
   If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
2. Vortex eBLTS thoroughly until the beads are fully resuspended.
3. Combine the following volumes in a tube to prepare the Tagmentation Master Mix. Multiply each volume by the number of samples being processed.
   - eBLTS (11.5 µl)
   - TB1 (11.5 µl)

   Reagent overage is included in the volume.

4. Pipette the Tagmentation Master Mix thoroughly to mix.
5. Divide the Tagmentation Master Mix volume equally into an 8-tube strip.
6. Using a 200 µl multichannel pipette, transfer 20 µl Tagmentation Master Mix to each well of the PCR plate containing a sample. Use fresh tips for each sample column or row.
7. Discard the 8-tube strip after the Tagmentation Master Mix has been dispensed.
8. Using a 200 µl multichannel pipette set to 40 µl, pipette each sample 10 times to mix. Use fresh tips for each sample column.
   Alternatively, seal the PCR plate and use a plate shaker at 1600 rpm for 1 minute.
9. Seal the plate, and then place on the preprogrammed thermal cycler, and run the TAG program.
10. Wait until the TAG program has reached the 10°C hold temperature, and then remove the plate immediately.
11. Let the 96-well PCR plate stand at room temperature for 2 minutes, and then proceed to the next step.

Post Tagmentation Cleanup

This step washes the adapter-tagged DNA on the eBLTS before PCR amplification.

Consumables

- ST2 (Stop Tagment Buffer 2)
- TWB2 (Tagment Wash Buffer 2)
- 96-well PCR plate magnetic stand
- Adhesive seal
- 8-tube strip
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes
- Prepare for later procedure:
  - EPM (Enhanced PCR Mix)
  - Index adapter plate

About Reagents

- Make sure to use the appropriate magnetic stand for your plate. Using a MIDI plate magnetic stand for a PCR plate could prevent TWB2 from adhering to beads.
- Pipette TWB2 slowly to minimize foaming to avoid incorrect volume aspiration and incomplete mixing.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice for 1 hour. Invert to mix, then briefly centrifuge.
ST2	15°C to 30°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Use at room temperature.
TWB2	15°C to 30°C	Use at room temperature.
Index adapter plate	-25°C to -15°C	Thaw at room temperature for 30 minutes.

Procedure

1. Add 10 µl ST2 to each tagmentation reaction. If you are using a multichannel pipette, pipette ST2 into an 8-tube strip, and then transfer the appropriate volumes to the PCR plate. Use fresh tips for each sample column or row.
2. Using a 200 µl pipette set to 50 µl, slowly pipette each well 10 times to resuspend the beads. Alternatively, seal the plate and shake at 1600 rpm for 1 minute. Repeat as needed.
3. Seal the plate, and then centrifuge at 280 × g for 10 seconds.
4. Incubate at room temperature for 5 minutes.
5. Place on the PCR plate magnetic stand and wait until liquid is clear (3 minutes).
6. [≤ 48 samples] Wash three times as follows.
   a. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant without disturbing the bead pellet.
   b. Remove from the magnetic stand.
   c. Immediately after, slowly add 100 µl TWB2 directly onto the beads.
   d. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
   e. If splashing occurs, spin down at 280 × g for 10 seconds.
   f. Place on the PCR plate magnetic stand and wait until the liquid is clear (3 minutes).
      Leave the plate on the magnetic stand and TWB2 in wells to prevent overdrying when performing the third wash. Remove and discard supernatant after you have prepared the PCR Master Mix.
   g. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
   h. Repeat steps c–f two times for a total of three washes.
7. [> 48 samples] Wash three times as follows.
   a. Perform steps b and c in 1-column to 2-column increments until all columns have been processed to prevent overdrying.
   b. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
   c. Remove from the magnetic stand.

d. Immediately after, slowly dispense 100 µl TWB2 directly onto the beads.
e. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
f. If splashing occurs, spin down at 280 × g for 10 seconds.
g. Place on the PCR plate magnetic stand and wait until the liquid is clear (3 minutes).
Leave the plate on the magnetic stand and TWB2 in wells to prevent overdrying when performing the third wash. Remove and discard supernatant after you have prepared the PCR Master Mix.
h. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
i. Remove from the magnetic stand and slowly add 100 µl TWB2 directly onto the beads.
j. Repeat steps h and i in 1- or 2-column increments until all columns have been processed.
k. Repeat steps e–h two times for a total of three washes.

8. Keep on the magnetic stand until step 4 of the *Procedure* section in *Amplify Tagmented DNA*.
The TWB2 remains in the wells to prevent overdrying of the beads.

Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation.

Consumables

- EPM (Enhanced PCR Mix)
- Index adapter plate
- 96-well PCR plate
- Nuclease-free water
- Adhesive seal
- 1.5 ml microcentrifuge tubes
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

About Reagents

- Index adapter plates
  - A well may contain > 10 µl of index adapters.
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw at 4°C or on ice for 1 hour. Invert to mix, then briefly centrifuge.
Index adapter plate	-25°C to -15°C	Thaw at room temperature for 30 minutes.

2. Save the following eBLTS PCR program on a thermal cycler using the appropriate number of PCR cycles indicated in the table below.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 72°C for 3 minutes
   - 98°C for 3 minutes
   - X cycles of:
     - 98°C for 20 seconds
     - 60°C for 30 seconds
     - 72°C for 1 minute
   - 72°C for 3 minutes
   - Hold at 10°C

   Total running time is ~38 minutes for 9 cycles and ~46 minutes for 12 cycles.

Sample Input Type	Number of PCR Cycles (X)
10–49 ng gDNA	12
50–1000 ng gDNA	9
50–1000 ng gDNA extracted from FFPE	12
gDNA extracted from blood	9

Procedure

1. Combine the following to prepare the PCR Master Mix. Multiply each volume by the number of samples being processed.
   - EPM (23 µl)
   - Nuclease-free water (23 µl)

   Reagent overage is included in the volume.
2. Pipette the PCR Master Mix 10 times to mix, and then centrifuge briefly.

3. With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard TWB2. Foam that remains on the well walls does not adversely affect the library.
4. Remove from the magnetic stand.
5. Immediately add 40 µl PCR Master Mix directly onto the beads in each well.
6. Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
7. Seal the sample plate and centrifuge at 280 × g for 10 seconds.
8. Centrifuge the index adapter plate at 1000 × g for 1 minute.
9. Prepare the index adapter plate.
   - [< 96 samples] Pierce the foil seal on the index adapter plate with a new pipette tip for each well for only the number of samples being processed.
   - [96 samples] Align a new semiskirted PCR plate above the index adapter plate and press down to puncture the foil seal. Discard the PCR plate used to puncture the foil seal.
10. Using a new pipette tip, add 10 µl pre-paired index adapters to each well.
11. Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
12. Seal the plate, and then centrifuge at 280 × g for 10 seconds.
13. Place on the thermal cycler and run the eBLTS PCR program.

SAFE STOPPING POINT

If you are stopping, store at -25°C to -15°C for up to 30 days.

Clean Up Libraries

This step uses double-sided bead purification procedure to purify the amplified libraries.

Consumables

- CB (Cleanup Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well 0.8 ml Polypropylene Deepwell Storage Plate (MIDI plate)
- 96-well PCR plate
- MIDI plate magnetic stand
- PCR plate magnetic stand
- 1.5 ml microcentrifuge tubes
- Nuclease-free water

About Reagents

- Cleanup Beads
  - Vortex before each use.
  - Vortex frequently to make sure that beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
CB	Room temperature	Vortex and invert to mix until the color of the liquid is homogeneous.
RSB	2°C to 8°C	Thaw for 30 minutes at room temperature. Vortex to mix.

Procedure

1. Shake the 96-well PCR plate at 1800 rpm for 1 minute, and then centrifuge briefly.
2. Place on the PCR plate magnetic stand and wait until the liquid is clear (1 minute).
3. Vortex CB 3 times for 10 seconds, and then invert multiple times to resuspend.
4. For high quality gDNA, do as follows.
   a. Add 77 µl nuclease-free water to each well of a new MIDI plate.
   b. Add 88 µl CB to each well of the MIDI plate.
   c. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of the MIDI plate.
   d. Discard the PCR plate.
   e. Pipette each well 10 times to mix. Alternatively, seal the plate and shake at 1800 rpm for 1 minute.
   f. Seal the plate and incubate at room temperature for 5 minutes.
   g. Check for air bubbles. If observed, spin down.
   h. Place on the MIDI plate magnetic stand and wait until the liquid is clear (5 minutes).
   i. During incubation, thoroughly vortex the CB, and then add 20 µl to each well of a *new* MIDI plate.
   j. Transfer 200 µl supernatant from each well of the first MIDI plate into the corresponding well of the new MIDI plate (containing 20 µl CB).
   k. Discard the first MIDI plate.
   l. Pipette each well of the new MIDI plate 10 times to mix. Alternatively, seal the plate and shake at 1800 rpm for 1 minute.
5. For extracted FFPE, do as follows.
   a. Add 81 µl CB to each well of a new MIDI plate.
   b. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of the MIDI plate.

c. Discard the PCR plate.
d. Pipette each well 10 times to mix. Alternatively, seal the plate and shake at 1800 rpm for 1 minute.

6. Incubate at room temperature for 5 minutes.
7. Check for air bubbles. If observed, spin down.
8. Place on the MIDI plate magnetic stand and wait until the liquid is clear (5 minutes).
9. Without disturbing the beads, remove and discard supernatant.
10. Wash beads as follows.
    a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
    b. Incubate for 30 seconds.
    c. Without disturbing the beads, remove and discard supernatant.
11. Wash beads a **second** time.
12. Air-dry on the magnetic stand for 5 minutes.
13. While air-drying, use a 20 µl pipette to remove and discard residual EtOH.
14. Remove from the magnetic stand.
15. Add 17 µl RSB to the beads.
16. Seal the plate and shake at 1800 rpm for 2 minutes.
17. Incubate at room temperature for 2 minutes.
18. Check for air bubbles. If observed, spin down.
19. Place the plate on the MIDI plate magnetic stand and wait until the liquid is clear (2 minutes).
20. Transfer 15 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Pool Pre-Enriched Libraries

This step combines DNA libraries with unique indexes into one pool of up to 12 libraries.

Pooling Methods

You can pool by volume or mass. Use the following table to determine the appropriate method for your input.

Table 2 Recommended Pooling Methods

Sample Input	Pooling Method
10–49 ng gDNA	Mass
50–1000 ng gDNA	Volume
gDNA extracted from FFPE	Mass
gDNA extracted from blood	Volume

- One-plex enrichment does not require pooling pre-enriched libraries. However, adding RSB might be necessary.
- After pre-enriched library quantification, all sample input types can be pooled by mass to achieve optimal index balance.
- The final yield of pre-enriched libraries generated in separate experimental preparations can vary. Therefore, pooling by mass is recommended to achieve optimal index balance.
- Use 1-plex enrichment for the following situations.
  - 10–49 ng gDNA
  - 50–1000 ng gDNA extracted from FFPE
  - Low minor allele frequency detection for somatic variant calling.

Pool by Mass

For the following situations, quantify your libraries to use a DNA mass per library for enrichment specified in *Pool Pre-Enriched Libraries at Equal Concentration* on page 34.

- 10–49 ng gDNA sample input
- 50–1000 ng gDNA extracted from FFPE sample input
- Low minor allele frequency detection for somatic variant calling
- gDNA extracted from blood for optimal index balance

Quantify Pre-Enriched Libraries

1. Run 1 µl of the pre-enriched libraries using your preferred fluorescence-based quantification method that uses dsDNA intercalating dye.
   - For 50–1000 ng high quality gDNA, expect a ≥ 500 ng pre-enriched library yield.
   - For 50–1000 ng gDNA extracted from FFPE, expect a 500–6000 ng pre-enriched library yield, depending on the quality of the initial sample.

NOTE For quantification methods with different biases, qualify the quantification method for this workflow. Concentration results might differ depending on the method used.

Pool Pre-Enriched Libraries at Equal Concentration

Use the following table to determine the DNA mass per library required for enrichment, according to sample type and enrichment plexity. Optimal enrichment yields and assay performance are not guaranteed when using lower pre-enriched library yields than recommended.

The total DNA mass in the enrichment reaction should not exceed 6000 ng.

Sample Input	Enrichment Plexity	DNA Mass per Library (ng)	Total DNA Library Mass (ng)
High quality gDNA	12	250–500	3000–6000
gDNA extracted from FFPE	1	200	200

1. Record the indexes for the libraries that you plan to pool in this step.
2. Based on the concentration of each library, calculate the volume that must be added to the enrichment reaction to achieve the required DNA mass.
   - High quality gDNA: Calculate the volume of library needed for 250–500 ng input.
   - gDNA extracted from FFPE: Calculate the volume of library needed for 200 ng input.
3. Add the calculated volume for each library into the same well of the PCR plate.
4. If using high quality gDNA, perform one of the following based on the total volume of pooled pre-enriched libraries:
   - If pre-enriched library volume = 30 µl, proceed to *Hybridize Probes* on page 36.
   - If pre-enriched library volume < 30 µl, add RSB to reach 30 µl total volume.
   - If pre-enriched library volume > 30 µl, use a bead-based method or a vacuum concentrator to concentrate the pooled sample. Add RSB to the concentrated pooled sample to reach 30 µl total volume.
5. If using gDNA extracted from FFPE, perform one of the following based on the total volume of pooled pre-enriched libraries.
   - If pre-enriched library volume = 7.5 µl, proceed to *Hybridize Probes* on page 36.

- If pre-enriched library volume < 7.5 µl, add RSB to reach 7.5 µl total volume.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Pool by Volume

When the input is 50–1000 ng gDNA, quantifying and normalizing individual libraries generated in the same experiment is not required.

To achieve optimal performance, only pool pre-enriched library samples prepared by the same user, reagent lot, and index adapter plate.

1. Record the indexes for the libraries that you plan to pool in this step.
2. Combine the following pre-enriched library and RSB volumes for your enrichment plexity into the same well of a new PCR plate.
   The resulting volume is 30 µl.

Enrichment Plexity *	Each Pre-Enriched Library Volume (µl)	RSB Volume (µl)
1-plex	14	16
2-plex	14	2
3-plex	10	0
4-plex	7.5	0
5-plex	6	0
6-plex	5	0
7-plex	4.2	0.6
8-plex	3.7	0.4
9-plex	3.3	0.3
10-plex	3	0
11-plex	2.7	0.3
12-plex	2.5	0

*For information on nonstandard plexities (2-plex through 11-plex), see *Limitations of Procedure* on page 2.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

[Optional] Qualify Pre-Enriched Libraries

If pooling by volume, to quantify the pre-enriched libraries use a fluorometric-based method that uses dsDNA intercalating dye. To qualify the pre-enriched libraries, use a DNA fragment analyzer with the appropriate fragment analysis kit.

Use 1 µl in total for library qualification. Pre-enriched libraries are concentrated enough to allow for small dilutions for quantification or fragment analysis.

Hybridize Probes

This step binds targeted regions of the DNA. with capture probes.

Illumina DNA Prep with Enrichment Dx Kit reagents are compatible with both Illumina and third-party enrichment DNA oligonucleotide panels. For information on the required specifications for third-party panels, refer to *Enrichment Probe Panel Requirements* on page 10.

Consumables

- EHB2 (Enrichment Hyb Buffer 2)
- NHB2 (HYB Buffer 2 + IDT NXT Blockers) (blue cap)
- Enrichment probe panel
- 96-well PCR plate
- Adhesive seal
- Prepare for later procedure:
  - SMB3 (Streptavidin Magnetic Beads)
  - EEW (Enhanced Enrichment Wash Buffer) (amber cap)

About Reagents

- NHB2 precipitates and separates during storage.
- Enrichment probe panel refers to the chosen enrichment oligonucleotide panel from Illumina vendor.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
EHB2	2°C to 8°C	Bring to room temperature. Vortex to mix. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix until the solution is clear.

Item	Storage	Instructions
Enrichment Probe Panel	-25°C to -15°C (Illumina)	For both Illumina and third-party panels, bring to room temperature. Vortex to mix.
NHB2 (blue cap)	-25°C to -15°C	Thaw at room temperature. When at room temperature, preheat on a microsample incubator to the same temperature as the probe you are using for 5 minutes. Vortex at maximum speed 3 times for 10 seconds each to resuspend. Centrifuge briefly. Pipette up and down from the bottom of the tube. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix until the solution is clear. Use while warm to avoid precipitates from reforming.
SMB3*	2°C to 8°C	If you are proceeding to the next procedure immediately after the 90-minute hold in the HYB program, bring to room temperature at least 2 hours before starting the HYB program.
EEW* (amber tube)	-25°C to -15°C	If you are proceeding to the next procedure immediately after the 90-minute hold in the HYB program, bring to room temperature at least 2 hours before starting the HYB program. When at room temperature, preheat on a microsample incubator to the applicable hybridization and capture temperature for 30 minutes before the HYB program ends.

*If you are stopping before the next procedure, delay preparing this reagent until you reach that procedure.

2. Save the following HYB program on the thermal cycler using the appropriate number of cycles, which are listed in Table 3.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume
     - **[High quality gDNA]** 100 µl
     - **[gDNA extracted from FFPE]** 25 µl
   - 98°C for 5 minutes
   - X cycles of 1 minute each, starting at 98°C for the first cycle, then decreasing 2°C per cycle
   - Hold for 90 minutes at the applicable temperature:
     - **[gDNA extracted from FFPE]** 58°C
     - **[80 mer probe panels]** 58°C
     - **[Somatic variant calling]** 58°C
     - **[All others]** 62°C

   Total running time is ~115 minutes.

Table 3 Cycle Number per Sample or Panel

Sample and Panel Type	Number of Cycles (X)
gDNA extracted from FFPE (regardless of panel type)	20
80 mer probe panels (regardless of sample type)	20
Somatic variant calling	20
All other samples and panels	18

Procedure

1. **[High quality gDNA]** Add the following reagents ***in the order listed*** to each pooled library in the PCR plate. Do not create a master mix. Creating a master mix of NHB2 and EHB2 negatively impacts enrichment performance.
   - NHB2 (blue cap) (50 µl)
   - Enrichment probe panel (10 µl)
   - EHB2 (10 µl)
2. **[High quality gDNA]** Using a pipette set to 90 µl, pipette each well 10 times to mix.
3. **[gDNA extracted from FFPE]** Add the following reagents ***in the order listed*** to each pooled library in the PCR plate.
   Do not create a master mix. Creating a master mix of NHB2 and EHB2 negatively impacts enrichment performance.
   - NHB2 (blue cap) (12.5 µl)

- Enrichment probe panel (2.5 µl)
- EHB2 (2.5 µl)

4. **[gDNA extracted from FFPE]** Using a pipette set to 20 µl, pipette each well 10 times to mix.
5. Seal the plate and centrifuge at 280 × g for 10 seconds.
6. Place the sample plate on the preprogrammed thermal cycler and run the HYB program.
7. Proceed immediately to the next procedure when the HYB program hold temperature time ends.

CAUTION

Precipitation occurs if the temperature of the hybridization reaction falls below room temperature.

Capture Hybridized Probes

This step uses Streptavidin Magnetic Beads (SMB3) to capture probes hybridized to the targeted regions of interest.

Consumables

- EEW (Enhanced Enrichment Wash Buffer) (amber cap)
- EE1 (Enrichment Elution Buffer 1)
- ET2 (Elute Target Buffer 2)
- HP3 (2N NaOH)
- SMB3 (Streptavidin Magnetic Beads)
- 1.5 ml microcentrifuge tube
- 96-well MIDI plate
- 96-well PCR plate
- Adhesive seal
- MIDI plate magnetic stand
- Prepare for later procedure:
  - Enhanced PCR Mix (EPM)
  - PCR Primer Cocktail (PPC)

About Reagents

- EEW
  - Make sure EEW has been thawed at room temperature for at least 2 hours before preheating on a microsample incubator.
  - Make sure EEW has been heated in a microsample incubator for 30 minutes before HYB program ends.

  - Leave EEW in microsample incubator when not in use. EEW should remain heated throughout the protocol.
  - Can be cloudy after reaching room temperature.
  - Can appear yellow.
- SMB3
  - SMB3 must be at room temperature before use.

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
SMB3	2°C to 8°C	Let stand for 2 hours to bring to room temperature. Invert, and then vortex until fully resuspended.
EEW (amber tube)	-25°C to -15°C	After 2 hour room temperature incubation, preheat on a microsample incubator to the applicable hybridization and capture temperature for 30 minutes before the HYB program ends.
EE1	-25°C to -15°C	Thaw at room temperature, and then vortex.
HP3	-25°C to -15°C	Thaw at room temperature, and then vortex.
ET2	2°C to 8°C	Bring to room temperature. Vortex to mix.
EPM	-25°C to -15°C	Thaw on ice for one hour. Invert to mix, then centrifuge briefly. Set aside on ice.
PPC	-25°C to -15°C	Thaw on ice for one hour. Vortex to mix, then centrifuge briefly. Set aside on ice.

2. Preheat one microsample incubator with a MIDI heat block insert to incubate the sample plate to one of the following temperatures. An optional second microsample incubator can be used to preheat EEW. Rest EEW on top of the MIDI heat block insert.
   - [FFPE] 58°C
   - [80 mer per probe panels] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C

Procedure

Capture

1. Add SMB3 to the corresponding well of a new MIDI plate as follows.
   - **[High quality gDNA]** Add 250 µl SMB3.

- **[gDNA extracted from FFPE]** Add 62.5 µl SMB3.

2. Using a pipette set to 100 µl for high quality gDNA or 25 µl for FFPE, transfer each pooled library from the 96-well PCR plate to the corresponding well of the new MIDI plate.
3. Seal the plate and shake at 1200 rpm for 4 minutes.
4. If splashing occurs, centrifuge the plate briefly.
5. Place the pooled libraries plate on the MIDI heat block insert on the microsample incubator, under the EEW tube, close the lid, and then incubate for 15 minutes at the applicable temperature:
   - [FFPE] 58°C
   - [80 mer probe panel] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C
6. Remove the pooled libraries plate and centrifuge at 280 × g for 30 seconds.
7. Immediately place on a MIDI plate magnetic stand and wait until the liquid is clear (2 minutes).
8. **[High quality gDNA]** Using a pipette set to 200 µl, remove and discard all supernatant from each well without disturbing the bead pellet.
9. **[gDNA extracted from FFPE]** Using a pipette set to 90 µl, remove and discard all supernatant from each well without disturbing the bead pellet.
10. Remove and discard all residual supernatant.

Wash

1. Remove from the magnetic stand.
2. **[High quality gDNA]** Quickly remove EEW from the microsample incubator and add 200 µl to each well.
3. **[gDNA extracted from FFPE]** Quickly remove EEW from the microsample incubator and add 50 µl to each well.
4. Return unused EEW to microsample incubator and keep heated.
5. Seal and shake at 1800 rpm for 4 minutes.
6. Place the sample plate on the MIDI heat block insert in the microsample incubator, under the EEW tube, close the lid, and then incubate for 5 minutes at the applicable temperature:
   - [FFPE] 58°C
   - [80 mer probe panels] 58°C
   - [Somatic variant calling] 58°C
   - [All other panels] 62°C
7. Immediately place on a MIDI plate magnetic stand and wait until the liquid is clear (2 minutes).
8. Using a pipette set to 200 µl for high quality gDNA or 50 µl for FFPE, remove and discard all supernatant from each well.

9. Repeat steps 1–8 two times for a total of three washes.

Transfer Wash

1. Remove from the magnetic stand.
2. **[High quality gDNA]** Quickly remove EEW from the microsample incubator and add 200 µl to each well.
3. **[gDNA extracted from FFPE]** Quickly remove EEW from the microsample incubator and add 50 µl to each well.
4. Seal and shake at 1800 rpm for 4 minutes. If splashing occurs, reduce the speed to 1600 rpm.
5. Transfer resuspended bead solution to a new MIDI plate.
   Some sample might remain in wells.

   CAUTION

   Transferring the reagent minimizes carryover of residual reagents that can inhibit downstream PCR.
6. Place the sample plate on the MIDI heat block insert on the microsample incubator, close the lid, and then incubate for 5 minutes at the applicable temperature:
   - [FFPE] 58°C
   - [80 mer probe panels] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C
7. Immediately place on a MIDI plate magnetic stand and wait until the liquid is clear (2 minutes).
8. Using a pipette set to 200 µl for high quality gDNA or 50 µl for FFPE, remove and discard all supernatant from each well.
9. Centrifuge the plate at 280 × g for 30 seconds.
10. Place on a MIDI plate magnetic stand for 10 seconds.
11. Use a 20 µl pipette to remove and discard residual liquid from each well.
12. Immediately proceed to *Elute* on page 42 to prevent excessive drying of the beads and library yield loss.

Elute

1. Combine the following volumes to prepare an Elution Master Mix. Multiply each volume by the number of pooled libraries being processed.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)

   Additional reagent overage is included in the volume.
2. Vortex, and then centrifuge briefly.
3. Remove the MIDI plate from the magnetic stand.

4. Add 23 µl Elution Master Mix to each well.
5. Seal plate and shake at 1800 rpm for 2 minutes.
6. Incubate the plate at room temperature for 2 minutes.
7. Centrifuge at 280 × g for 30 seconds.
8. Place on a MIDI plate magnetic stand and wait until the liquid is clear (2 minutes).
9. Transfer 21 µl supernatant from the MIDI plate to the corresponding well of a new 96-well PCR plate.
10. Discard the MIDI plate.
11. Add 4 µl ET2 to each well containing 21 µl supernatant.
12. Set pipette to 20 µl and slowly pipette each well 10 times to mix.
13. Seal the plate, and then centrifuge at 280 × g for 10 seconds.
14. Incubate the plate for 1 minute at room temperature.

Amplify Enriched Library

This step uses PCR to amplify the enriched library.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- Adhesive seal

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw at 4°C or on ice for one hour. Invert to mix, then centrifuge briefly. Set aside on ice.
PPC	-25°C to -15°C	Thaw at 4°C on ice for one hour. Vortex to mix, then centrifuge briefly. Set aside on ice.

2. Save the following AMP program on the thermal cycler using the appropriate number of PCR cycles, which are listed in the following table.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 45 seconds
   - (X) cycles of:
     - 98°C for 30 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 10°C

   Total running time is ~35 minutes.

Sample and Panel Type	(X) Cycles
FPPE	14
Illumina Exome Panel (CEX) for high quality gDNA	10
Illumina Exome Panel (CEX) for FFPE	12
All other samples and panels	12[1234]

[1] Can be adjusted up to 15 cycles for small third-party panels through subsequent optimization. If using FFPE, the number of cycles can be adjusted up to 17.
[2] Can be adjusted up to 17 cycles for third-party panels that only have 500 probes. If using FFPE, the number of cycles can be adjusted up to 19.
[3] Can be adjusted up to 14 cycles for FFPE samples.
[4] Increasing the number of PCR cycles might result in a higher duplicate rate and smaller fragment sizes for FFPE samples.

Procedure

1. Add 5 µl PPC to each well.
2. Add 20 µl EPM to each well.
3. Seal plate and shake at 1200 rpm for 1 minute.
4. Centrifuge the plate at 280 × g for 10 seconds.
5. Place on the preprogrammed thermal cycler and run the AMP program.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to two days. Alternatively, leave on the thermal cycler for up to 24 hours.

Clean Up Amplified Enriched Library

This step uses Cleanup Beads to purify the enriched library and remove unwanted products.

Consumables

- CB (Cleanup Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- Adhesive seals
- 96-well MIDI plate
- 96-well PCR plate
- MIDI plate magnetic stand

About Reagents

- Cleanup Beads
  - Vortex before each use.
  - Vortex frequently to make sure that beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
CB	Room temperature	Vortex and invert to mix until the color of the liquid is homogeneous.
RSB	2°C to 8°C	Bring to room temperature. Vortex to mix.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Centrifuge the PCR plate at 280 × g for 10 seconds.
2. Vortex CB 3 times for 10 seconds, and then invert.
3. Add 40.5 µl CB to each well of a new **MIDI** plate.
4. Transfer 45 µl from each well of the PCR plate to the corresponding well of the MIDI plate.
5. Seal the plate and shake at 1800 rpm for 1 minute.
6. Incubate the MIDI plate at room temperature for 5 minutes.

7. Centrifuge at 280 × g for 10 seconds.
8. Place on a MIDI plate magnetic stand and wait until liquid is clear (5 minutes).
9. Using a pipette set to 95 µl, remove and discard all supernatant from each well.
10. Wash two times as follows.
    a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
    b. Incubate for 30 seconds.
    c. Without disturbing the beads, remove and discard supernatant.
11. Air-dry on the magnetic stand for 5 minutes.
12. While air-drying, use a 20 µl pipette to remove and discard residual EtOH from each well.
13. Remove from the magnetic stand and add 32 µl RSB to each well.
14. Seal the plate and shake at 1800 rpm for 1 minute.
15. Incubate the plate at room temperature for 5 minutes.
16. Centrifuge at 280 × g for 10 seconds.
17. Place on a MIDI plate magnetic stand and wait until liquid is clear (2 minutes).
18. Transfer 30 µl supernatant from the 96-well MIDI plate to the corresponding well of a new PCR plate.
19. Discard the MIDI plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Check Enriched Libraries

To quantify double-stranded gDNA input, use a fluorescence-based method that uses intercalating dye. Avoid methods that measure total nucleic acid, such as NanoDrop or other UV absorbance methods.

1. Run 1 µl of the enriched libraries using your quantification method.

NOTE Total probe molarity proportionally impacts the post-enrichment library yield.

Expect a mean insert size of 125–235 bp and distribution of library fragments with a size range from ~200 bp to ~1000 bp.

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, regardless of the enrichment probe panel you are using, Illumina recommends setting up a paired-end run with 151 cycles per read (2 × 151) and 10 cycles per Index Read. If you would like fewer overlapped reads or less raw coverage, you can sequence down to 2 × 126 or 2 x 101.

1. Calculate the molarity value of the library or pooled libraries using the following formula.
   - For libraries qualified on a DNA fragment analyzer, use the average size obtained for the library.
   - For all other qualification methods, use 350 bp as the average library size.

   $$\frac{ng/\mu l \times 10^6}{660\frac{g}{mol} \times average\ library\ size\ (bp)} = Molarity\ (nM)$$

   For example, if your library concentration is 20 ng/µl and average size is 350 bp, the resulting molarity value is 86.58 nM.

   $$\frac{20\ ng/\mu l \times 10^6}{660\frac{g}{mol} \times 350(bp)} = 86.58(nM)$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Minimum Required Library Volume (µl)	Starting Concentration (nM)	Final Loading Concentration (pM)
NextSeq 550Dx	10	2	1.2
MiSeqDx	5	4	11
NovaSeq 6000Dx	150 (S2) or 310 (S4)	1.75	350

[NovaSeq 6000Dx] 1.75 nM is the starting concentration for a final loading concentration of 350 pM. If necessary, adjust the final loading concentration using the following table.

Final Loading Concentration (pM)	Pooled Library Concentration (nM)
100	0.50
150	0.75
200	1
250	1.25
300	1.50
350	1.75

Final Loading Concentration (pM)	Pooled Library Concentration (nM)
400	2
450	2.25
500	2.50

3. Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
   - **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl of each diluted library to a tube to create a multiplexed library pool.
4. Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
   - For the NextSeq 550Dx System, refer to *NextSeq 550Dx Sequencing Preparation* on page 48.
   - For the MiSeqDx System, refer to *MiSeqDx Sequencing Preparation* on page 50.
   - For the NovaSeq 6000Dx System, refer to *NovaSeq 6000Dx Sequencing Preparation* on page 51.

   The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

NextSeq 550Dx Sequencing Preparation

Use the following instructions for denaturing and diluting libraries for sequencing on the NextSeq 550Dx sequencing system.

Consumables

- HT1 (Hybridization Buffer)
- 1N NaOH
- 200 mM Tris-HCl, pH 7.0

Preparation

Prepare a ***fresh*** dilution of 0.2N NaOH to denature libraries for sequencing. To prevent small pipetting errors from affecting the final NaOH concentration, extra volume is prepared.

CAUTION

Freshly diluted 0.2N NaOH is essential to the denaturation process. Improper denaturation can reduce yield.

1. Prepare the following consumables.

Item	Storage	Instructions
HT1	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C until you are ready to dilute denatured libraries.

2. Combine the following volumes in a microcentrifuge tube to prepare a fresh dilution of NaOH:
   - Laboratory-grade water (800 µl)
   - 1N NaOH (200 µl)

   The result is 1 ml 0.2N NaOH.
3. Invert the tube several times to mix.
4. Combine the following volumes in a microcentrifuge tube to prepare 200 mM Tris-HCl, pH 7.0.
   - Laboratory-grade water (800 µl)
   - 1M Tris-HCl, pH 7.0 (200 µl)

   The result is 1 ml 200 mM Tris-HCl, pH 7.0

NOTE Keep the tube capped. Use the fresh dilution within **12 hours**.

Denature Libraries

1. Combine the following volumes of library and freshly diluted 0.2N NaOH in a microcentrifuge tube.
   - 10 µl library
   - 10 µl 0.2N NaOH
2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Incubate at room temperature for 5 minutes.
4. Add 10 µl 200 mM Tris-HCl, pH 7.

Dilute Denatured Libraries to 20 pM

1. Add 970 µl prechilled HT1 to the tube of denatured libraries.
   The result is a 20 pM denatured library.
2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Place the 20 pM libraries on ice until you are ready to proceed to final dilution.

Dilute Libraries to Loading Concentration

1. Add the following volumes to dilute the denatured 20 pM library solution to 1.2 pM.
   - Denatured library solution (78 µl)
   - Prechilled HT1 (1222 µl)

   The total volume is 1.3 ml at 1.2 pM.

2. Invert to mix, and then pulse centrifuge.
3. Proceed to sequencing. For instructions, refer to the *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*, and *Local Run Manager DNA Generate FASTQ Dx Workflow Guide for NextSeq 550Dx (document # 200015671)*, or *DRAGEN for Illumina DNA Prep with Enrichment Dx on NextSeq 550Dx Application User Guide (document # 200025238)*.

MiSeqDx Sequencing Preparation

Use the following instructions for denaturing and diluting libraries for sequencing on the MiSeqDx sequencing system.

Consumables

- HT1 (Hybridization Buffer)
- 1N NaOH

Preparation

Prepare a ***fresh*** dilution of 0.2N NaOH to denature libraries for sequencing. To prevent small pipetting errors from affecting the final NaOH concentration, extra volume is prepared.

⚠ CAUTION

Freshly diluted 0.2N NaOH is essential to the denaturation process. Improper denaturation can reduce yield.

1. Prepare the following consumables.

Item	Storage	Instructions
HT1	-25°C to -15°C	Thaw at room temperature. Store at 2°C to 8°C until you are ready to dilute denatured libraries.

2. Combine the following volumes in a microcentrifuge tube to prepare a fresh dilution of NaOH:
   - Laboratory-grade water (800 µl)
   - 1N NaOH (200 µl)

   The result is 1 ml 0.2N NaOH.

NOTE Keep the tube capped. Use the fresh dilution within **12 hours**.

Denature a 4 nM Library

1. Combine the following volumes in a microcentrifuge tube.
   - 4 nM library (5 µl)

- 0.2N NaOH (5 µl)

2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Incubate at room temperature for 5 minutes.
4. Add 990 µl prechilled HT1 to the tube containing denatured library.
   The result is 1 ml 20 pM denatured library.

Dilute Denatured 20 pM Library

1. Dilute to the desired concentration using the following volumes.

Concentration	6 pM	8 pM	10 pM	11 pM	12 pM	15 pM	20 pM
20 pM library	180 µl	240 µl	300 µl	330 µl	360 µl	450 µl	600 µl
Prechilled HT1	420 µl	360 µl	300 µl	270 µl	240 µl	150 µl	0 µl

2. Invert to mix, and then pulse centrifuge.
3. Proceed to sequencing. For instructions, refer to *MiSeqDx Instrument Reference Guide for MOS v4 (document # 1000000157953)* and the *Local Run Manager DNA Generate FASTQ Dx Workflow Guide for MiSeqDx (document # 200015661)*.

NovaSeq 6000Dx Sequencing Preparation

Use the following instructions for denaturing and diluting libraries for sequencing on the NovaSeq 6000Dx sequencing system.

Consumables

- HP3 (2N NaOH)
- RSB (Resuspension Buffer)
- 1N NaOH
- 10 mM Tris-HCl, pH 8.5
- 400 mM Tris-HCl, pH 8.0
- NovaSeq 6000Dx library tube

Preparation

Prepare a ***fresh*** dilution of 0.2N NaOH to denature libraries for sequencing. To prevent small pipetting errors from affecting the final NaOH concentration, extra volume is prepared.

CAUTION

Freshly diluted 0.2N NaOH is essential to the denaturation process. Improper denaturation can reduce yield.

1. Combine the following volumes in a microcentrifuge tube to dilute 1N NaOH to 0.2N NaOH:

   Table 4 S2 Mode

Reagent	Volume for One Flow Cell (µl)	Volume for Two Flow Cells (µl)
Laboratory-grade water	40	80
Stock 1N NaOH	10	20

   These volumes result in 50 µl 0.2N NaOH for one flow cell or 100 µl 0.2N NaOH for two flow cells.

   Table 5 S4 Mode

Reagent	Volume for One Flow Cell (µl)	Volume for Two Flow Cells (µl)
Laboratory-grade water	80	160
Stock 1N NaOH	20	40

   These volumes result in 100 µl 0.2N NaOH for one flow cell or 200 µl 0.2N NaOH for two flow cells.
2. Invert several times to mix, or vortex thoroughly.
3. Combine the following volumes in a microcentrifuge tube to prepare 400 mM Tris-HCl, pH 8.0.
   - Laboratory-grade water (600 µl)
   - 1M Tris-HCl, pH 8.0 (400 µl)

   The result is 1 ml 400 mM Tris-HCl, pH 8.0

NOTE Keep the tube capped. Use the fresh dilution within **12 hours**.

Create a Normalized Library Pool

Loading concentration can vary depending on library preparation, quantification, and normalization methods.

Use the following instructions to normalize libraries to the appropriate concentration and then pool. Libraries sequenced on the same flow cell must be combined into a single normalized pool.

NOTE The maximum number of samples that can be run per lane with the Illumina DNA Prep with Enrichment Dx Kit is 192. This limit is due to the total number of UD Indexes in Set A and B.

Normalize Libraries for Pooling

1. Determine the required pooled library concentration based on the desired final loading concentration.
   - For a final loading concentration of 350 pM, the required pooled library concentration is 1.75 nM.

- To determine the pooled library concentration for a different final loading concentration, refer to *Dilute Libraries to the Starting Concentration* on page 47.

2. Normalize libraries to the desired pooled library concentration using 10 mM Tris-HCl, pH 8.5.
   For assistance diluting libraries to the appropriate concentration, refer to the Pooling Calculator on the Illumina website.

Recommended Loading Concentrations

The optimal DNA loading concentration depends on the library type and insert size. For libraries > 450 bp, higher loading concentrations might be necessary.

Pool Normalized Libraries and Add Optional PhiX Control

1. Combine the appropriate volume of each normalized library in a new microcentrifuge tube to result in one of the following final volumes:

Mode	Final Volume (µl)
S2	150
S4	310

2. **[Optional]** Spike-in 1% nondenatured PhiX> as follows.
   a. Dilute 10 nM PhiX to 2.5 nM using 10 mM Tris-HCl, pH 8.5.
   b. Add the appropriate volume of nondenatured 2.5 nM PhiX to the tube of nondenatured library pool.

Mode	Nondenatured 2.5 nM PhiX (µl)	Nondenatured Library Pool (µl)
S2	0.9	150
S4	1.9	310

When spiking in PhiX, 1% is the recommended amount for well balanced libraries. Low-diversity libraries can require more. To use a PhiX control with low diversity libraries, contact Illumina Technical Support for guidance.

Denature Library Pool and Optional PhiX Control

1. Add 0.2N NaOH to the tube of nondenatured library pool and optional PhiX as follows.

Flow Cell	0.2N NaOH	Nondenatured Library Pool (µl)	Resulting Volume
S2	37	150	187 µl, or 187.9 µl with PhiX
S4	77	310	387 µl, or 388.9 µl with PhiX

2. Cap and then vortex briefly.
3. Centrifuge at 280 × g for up to 1 minute.
4. Incubate at room temperature for 8 minutes to denature.

5. Add 400 mM Tris-HCl, pH 8.0 as follows to neutralize.

Mode	400 mM Tris-HCl, pH 8.0 (µl)	Resulting Volume
S2	38	225 µl, or 225.9 µl with PhiX
S4	78	465 µl, or 466.9 µl with PhiX

6. Cap and then vortex briefly.
7. Centrifuge at 280 × g for up to 1 minute.
8. Transfer the entire volume of denatured library or denatured library and PhiX to the NovaSeq 6000Dx library tube.
9. Proceed to sequencing. For instructions, refer to *NovaSeq 6000Dx Instrument Product Documentation* (*document # 200010105*) and *DRAGEN for Illumina DNA Prep with Enrichment Dx for NovaSeq 6000Dx (document # 200014776)*.

Troubleshooting

Use the following table to troubleshoot issues in the workflow. If a sequencing run or library preparation for a sample fails two times, additional troubleshooting may be necessary. Contact Illumina Technical Support.

Observation	Possible Cause	Recommended Action
Sequencing run does not pass run Quality Control Specifications	User or lab equipment error in the assay workflow	Qualify enriched libraries to ensure appropriate library yield and fragment size distribution. Repeat library preparation from one of the following steps depending on where suspected use or equipment error occurred. If unknown, or other errors occurred, contact Illumina Technical Support to troubleshoot your run. • Resequence libraries. Refer to *NextSeq 550Dx Sequencing Preparation* on page 48, *MiSeqDx Sequencing Preparation* on page 50 , or *NovaSeq 6000Dx Sequencing Preparation* on page 51. • Reenrich libraries. Refer to *Hybridize Probes* on page 36. • Start library preparation from the beginning of the workflow. Refer to *Instructions For Use* on page 21.
	Instrument issue	Contact Illumina Technical Support.
Error with FASTQ generation or general sequencing system error (eg, network error, errors loading/unloading reagents, etc)	Software or instrument issue	Refer to the module or application guide for help with analysis, or refer to *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*, *MiSeqDx Instrument Reference Guide for MOS v4 (document # 1000000157953)*, or *NovaSeq 6000Dx Instrument Product Documentation (document # 200010105)*. Contact Illumina Technical Support for additional help.

Observation	Possible Cause	Recommended Action
DNA library does not generate sufficient yield for sequencing loading	Requirements for sample input were not met	Ensure appropriate sample input and repeat library preparation. Refer to *Sample Input Recommendations* on page 17.
	Use or equipment error in the assay workflow	Repeat library preparation from one of the following steps depending on where suspected use or equipment error occurred. If unknown, or other errors occurred, contact Illumina Technical Support to troubleshoot your run. • Resequence libraries. Refer to *NextSeq 550Dx Sequencing Preparation* on page 48, *MiSeqDx Sequencing Preparation* on page 50 , or *NovaSeq 6000Dx Sequencing Preparation* on page 51. • Reenrich libraries. Refer to *Hybridize Probes* on page 36. • Start library preparation from the beginning of the workflow. Refer to *Instructions For Use* on page 21.
	Requirements for enrichment probe panel were not met	Ensure appropriate enrichment probe panel and repeat library preparation. Refer to *Enrichment Probe Panel Requirements* on page 10.

Performance Characteristics

Performance With Whole Exome Panels

Exome panel performance was tested by using the lowest (50 ng) and the highest (1000 ng) recommended input of the Coriell Cell Line gDNA NA12878, with a known truth set for germline variant detection (Coriell platinum genome). Exome panel 1 (45 Mb) and exome panel 2 (36.8 Mb) were used as representative panels. 24 technical replicates were tested through the Illumina DNA Prep with Enrichment Dx assay using exome panel 1 (45 Mb) in two 12-plex enrichment reactions. 12 technical replicates were tested through the Illumina DNA Prep with Enrichment Dx assay using exome panel 2 (36.8 Mb) in a single 12-plex enrichment reaction. The enriched libraries were sequenced on the NextSeq 550Dx sequencing system with the DNA GenerateFASTQ Dx Local Run Manager module.

The following table displays the mean values of secondary sequencing and variant calling performance metrics for the technical replicates tested with each panel.

Table 6 Assay Performance With Two Whole Exome Panels

Panel	Padded Unique Read Enrichment	Uniformity of Coverage	Fragment Length Median	SNV Recall[1]	SNV Precision[2]	Indel Recall[1]	Indel Precision[2]
Exome panel 1 (45 Mb)	80%	96%	186 bp	96%	99%	90%	89%
Exome panel 2 (36.8 Mb)	93%	98%	188 bp	96%	99%	92%	93%

[1]Recall=Positives/(true positives + false negatives)
[2]Precision=True positives/(true positives + false positives)

Limit of Detection

The Horizon HD799 DNA reference standard was used to test the limit of detection. HD799 consists of moderately compromised formalin-treated DNA with known SNVs in allelic frequencies ranging from 1–24.5%. The lowest-recommended DNA input (50 ng) was used and the detection rate of SNVs with ≥ 5.0% variant allele frequency (VAF) was evaluated. 16 technical replicates were tested through the Illumina DNA Prep with Enrichment Dx assay using the FFPE workflow, enriched with a pan-cancer enrichment panel (1.94 Mb) in 16 (1-plex) enrichments, and then sequenced on a NextSeq 550Dx instrument with the DNA GenerateFASTQ Dx module.

All samples passed the panel-specific sample performance requirements as shown in the following table.

Table 7 Sample Performance for Limit of Detection

Panel	Variant Detection Rate of SNVs of ≥ 5.0% VAF	Average Uniformity of Coverage
Pan-cancer enrichment panel (1.94 Mb, 523 genes)	100%	99%

Interfering Substances

The impact of potential interferents was assessed in Illumina DNA Prep with Enrichment Dx by evaluating the performance of the assay in the presence of interfering substances.

Interference in Whole Blood

Acetaminophen (exogenous compound, drug), creatinine, and triglycerides (endogenous metabolites) were tested by spiking them into whole human blood specimens before DNA extraction. To assess interference resulting from blood collection (short draw), EDTA was also spiked into whole blood specimens. Additionally, to assess interference resulting from sample preparation, molecular grade ethanol was spiked into DNA extracted from whole blood.

The following table shows the test concentrations per interferent.

Table 8 Potentially Interfering Substances and Concentrations Tested in Whole Blood

Test Substance	Test Concentration
Acetaminophen	15.6 mg/dL* Three times the highest concentration expected following a drug therapeutic dose.
Creatinine	15 mg/dL* Highest-observed concentration in population.
Triglycerides	1.5 g/dL* Highest-observed concentration in population.
EDTA	6 mg/mL Three times the concentration expected in blood, collected in EDTA tubes.
Molecular grade ethanol	15% v/v In the eluate post DNA extraction.

*As per CLSI EP37-ED1:2018

Per interfering substance, 12 technical replicates were tested through the Illumina DNA Prep with Enrichment Dx assay, enriched with exome panel 1 (45 Mb) in a single (12-plex) enrichment, and then sequenced on a NextSeq 550Dx instrument with the DNA GenerateFASTQ Dx module.

For the substances tested, all 12 specimens met the sample performance requirements and no interference with the performance of the assay was observed.

Interference in FFPE Tissue

Two colorectal FFPE samples were tested in the presence and absence of hemoglobin at 0.1 mg per 10 µm FFPE section to represent a worst-case scenario of 50% FFPE tissue sample contamination with high hemoglobin level blood. The specimens were tested through the Illumina DNA Prep with Enrichment Dx assay using pan-cancer enrichment panel 1 (1.94 Mb) as a representative panel in single-plex enrichments. The enriched libraries were then sequenced on a NextSeq 550Dx instrument with the DNA GenerateFASTQ Dx module. All specimens met the sample performance requirements, and it was demonstrated that hemoglobin does not interfere with the performance of the assay.

To assess interference resulting from sample preparation, two exogenous compounds were spiked into DNA extracted from a bladder cancer FFPE tissue sample. The exogenous substances tested are extraction solutions commonly used during the DNA extraction process and are listed with tested quantities in the following table.

The test substance solutions are commercially available in column-based DNA isolation kits.

Table 9 Potentially Interfering Exogenous Substances and Concentrations Tested in FFPE

Test Substance	Test Concentration (µl / 30 µl Eluate)
Deparaffinization Solution	113×10^{-6}
Wash Buffer AW2	0.417

Per interfering substance, eight technical replicates were tested through the Illumina DNA Prep with Enrichment Dx assay, enriched with a pan-cancer enrichment panel (1.94 Mb) in single-plex enrichments, and then sequenced on a NextSeq 550Dx instrument with the DNA GenerateFASTQ Dx module.

For both substances tested, all eight specimens met the sample performance requirements and no interference in the performance of the assay was observed.

Cross-Contamination

Coriell Cell Line gDNA NA12878 (female, 10 samples), Coriell Cell Line gDNA NA12877 (male, 12 samples), and no template controls (NTC, 2 samples) were tested through the Illumina DNA Prep with Enrichment Dx assay in a checkerboard plate layout. All samples used the highest (1000 ng) gDNA input recommendation as the most stringent condition for evaluating sample cross-contamination. Testing was performed twice by two separate operators. Exome panel 1 (45 Mb) was used in 12-plex enrichment reactions. The enriched libraries were sequenced on NextSeq 550Dx with the DNA GenerateFASTQ Dx. Evaluation was done by assessing the coverage of the male specific Y-chromosome in the female samples by comparing to background levels of a full plate of female samples as well as index representation of the NTC samples.

Table 10 Cross-Contamination Results

Female Samples With Male Y-Chromosome Coverage in < 3x Baseline Noise	Index Representation in NTC
100%	< 0.0005%

DRAGEN for Illumina DNA Prep with Enrichment Dx Application Performance

Performance characteristics of the DRAGEN for Illumina DNA Prep with Enrichment Dx Application for NovaSeq 6000Dx are provided in the *NovaSeq 6000Dx Instrument Package Insert (document # 200025276*).

The DRAGEN for Illumina DNA Prep with Enrichment Dx on NextSeq 550Dx provides the same secondary analysis workflows as the application on the NovaSeq 6000Dx, including the following three workflows: FASTQ generation, FASTQ and VCF generation for germline variant detection, and FASTQ and VCF generation for somatic variant detection.

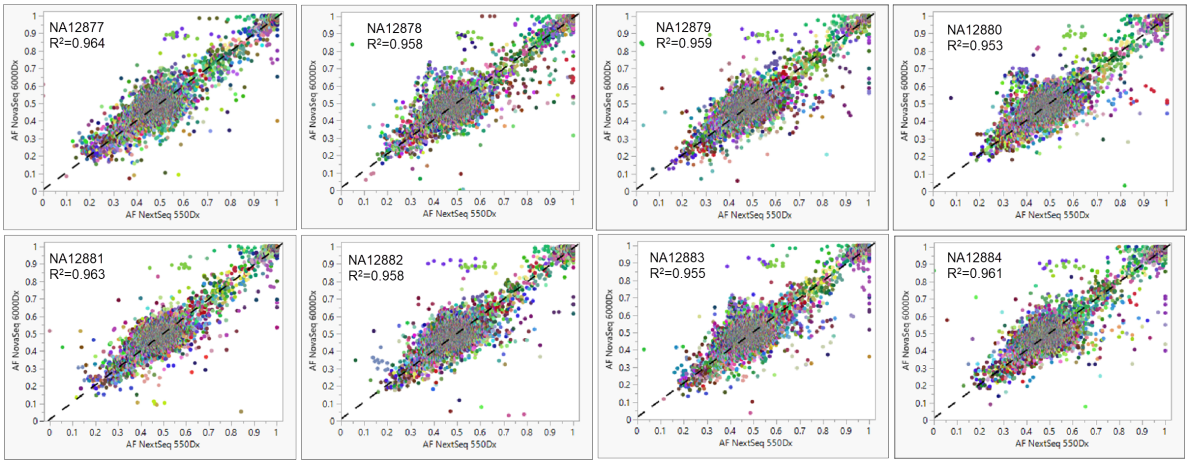
Comparable secondary analysis performance was obtained from same library preparation sequenced on both platforms. Variant detection rate (Table 11) and frequency agreement (Figure 1) for Coriell Cell Line gDNA samples were evaluated using a representative assay designed to query a variety of genes covering 1,970,505 bases (9,232 targets) across all 23 human chromosomes. Eight Platinum Genome DNA samples were tested, seven in replicates of six (NA12877, NA12878, NA12879, NA12880, NA12882, NA12883, NA12884) and one (NA12881) in replicates of five (See Figure 1). Libraries were sequenced with three runs each on NovaSeq 6000Dx and NextSeq 550Dx Instruments and variant calling was performed using the FASTQ and VCF generation for germline variant detection analysis workflow of the DRAGEN for Illumina DNA Prep with Enrichment Dx Application.

Based on the strong correlation between application performance on NovaSeq 6000Dx and NextSeq 550Dx instruments, performance characteristics related to secondary analysis provided in the *NovaSeq 6000Dx Instrument Package Insert (document # 200025276*) are also determined to be applicable to DRAGEN for Illumina DNA Prep with Enrichment Dx on the NextSeq 550Dx application.

Table 11 Application Performance – Variant Detection Rate for SNVs, Insertions and Deletions

Panel	Variant Detection Rate on NovaSeq 6000Dx	Variant Detection Rate on NextSeq 550Dx
Pan-genome panel (1.97 Mb, 9,232 targets, 23 Chr.)	99.9%	99.9%

Figure 1 Variant Frequency comparison for NovaSeq 6000Dx and NextSeq 550Dx runs with DRAGEN for IDPE Dx Application analysis



Appendix: Illumina UD Indexes Adapter Sequences

These unique dual (UD) index adapters are arranged in the plate to enforce the recommended pairing strategy. The index adapters are 10 bases long, instead of the typical eight bases.

Index 1 (i7) Adapters

`CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG`

Index 2 (i5) Adapters

`AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC`

The following sequence is used for Read 1 and Read 2 adapter trimming.

`CTGTCTCTTATACACATCT`

Plate A/Set 1 Index Adapters

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0001	CGCTCAGTTC	TCGTGGAGCG
UDP0002	TATCTGACCT	CTACAAGATA
UDP0003	ATATGAGACG	TATAGTAGCT
UDP0004	CTTATGGAAT	TGCCTGGTGG
UDP0005	TAATCTCGTC	ACATTATCCT
UDP0006	GCGCGATGTT	GTCCACTTGT
UDP0007	AGAGCACTAG	TGGAACAGTA

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0008	TGCCTTGATC	CCTTGTTAAT
UDP0009	CTACTCAGTC	GTTGATAGTG
UDP0010	TCGTCTGACT	ACCAGCGACA
UDP0011	GAACATACGG	CATACACTGT
UDP0012	CCTATGACTC	GTGTGGCGCT
UDP0013	TAATGGCAAG	ATCACGAAGG
UDP0014	GTGCCGCTTC	CGGCTCTACT
UDP0015	CGGCAATGGA	GAATGCACGA
UDP0016	GCCGTAACCG	AAGACTATAG
UDP0017	AACCATTCTC	TCGGCAGCAA
UDP0018	GGTTGCCTCT	CTAATGATGG
UDP0019	CTAATGATGG	GGTTGCCTCT
UDP0020	TCGGCCTATC	CGCACATGGC
UDP0021	AGTCAACCAT	GGCCTGTCCT
UDP0022	GAGCGCAATA	CTGTGTTAGG
UDP0023	AACAAGGCGT	TAAGGAACGT
UDP0024	GTATGTAGAA	CTAACTGTAA
UDP0025	TTCTATGGTT	GGCGAGATGG
UDP0026	CCTCGCAACC	AATAGAGCAA
UDP0027	TGGATGCTTA	TCAATCCATT
UDP0028	ATGTCGTGGT	TCGTATGCGG
UDP0029	AGAGTGCGGC	TCCGACCTCG
UDP0030	TGCCTGGTGG	CTTATGGAAT
UDP0031	TGCGTGTCAC	GCTTACGGAC
UDP0032	CATACACTGT	GAACATACGG
UDP0033	CGTATAATCA	GTCGATTACA
UDP0034	TACGCGGCTG	ACTAGCCGTG
UDP0035	GCGAGTTACC	AAGTTGGTGA
UDP0036	TACGGCCGGT	TGGCAATATT
UDP0037	GTCGATTACA	GATCACCGCG

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0038	CTGTCTGCAC	TACCATCCGT
UDP0039	CAGCCGATTG	GCTGTAGGAA
UDP0040	TGACTACATA	CGCACTAATG
UDP0041	ATTGCCGAGT	GACAACTGAA
UDP0042	GCCATTAGAC	AGTGGTCAGG
UDP0043	GGCGAGATGG	TTCTATGGTT
UDP0044	TGGCTCGCAG	AATCCGGCCA
UDP0045	TAGAATAACG	CCATAAGGTT
UDP0046	TAATGGATCT	ATCTCTACCA
UDP0047	TATCCAGGAC	CGGTGGCGAA
UDP0048	AGTGCCACTG	TAACAATAGG
UDP0049	GTGCAACACT	CTGGTACACG
UDP0050	ACATGGTGTC	TCAACGTGTA
UDP0051	GACAGACAGG	ACTGTTGTGA
UDP0052	TCTTACATCA	GTGCGTCCTT
UDP0053	TTACAATTCC	AGCACATCCT
UDP0054	AAGCTTATGC	TTCCGTCGCA
UDP0055	TATTCCTCAG	CTTAACCACT
UDP0056	CTCGTGCGTT	GCCTCGGATA
UDP0057	TTAGGATAGA	CGTCGACTGG
UDP0058	CCGAAGCGAG	TACTAGTCAA
UDP0059	GGACCAACAG	ATAGACCGTT
UDP0060	TTCCAGGTAA	ACAGTTCCAG
UDP0061	TGATTAGCCA	AGGCATGTAG
UDP0062	TAACAGTGTT	GCAAGTCTCA
UDP0063	ACCGCGCAAT	TTGGCTCCGC
UDP0064	GTTCGCGCCA	AACTGATACT
UDP0065	AGACACATTA	GTAAGGCATA
UDP0066	GCGTTGGTAT	AATTGCTGCG
UDP0067	AGCACATCCT	TTACAATTCC

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0068	TTGTTCCGTG	AACCTAGCAC
UDP0069	AAGTACTCCA	TCTGTGTGGA
UDP0070	ACGTCAATAC	GGAATTCCAA
UDP0071	GGTGTACAAG	AAGCGCGCTT
UDP0072	CCACCTGTGT	TGAGCGTTGT
UDP0073	GTTCCGCAGG	ATCATAGGCT
UDP0074	ACCTTATGAA	TGTTAGAAGG
UDP0075	CGCTGCAGAG	GATGGATGTA
UDP0076	GTAGAGTCAG	ACGGCCGTCA
UDP0077	GGATACCAGA	CGTTGCTTAC
UDP0078	CGCACTAATG	TGACTACATA
UDP0079	TCCTGACCGT	CGGCCTCGTT
UDP0080	CTGGCTTGCC	CAAGCATCCG
UDP0081	ACCAGCGACA	TCGTCTGACT
UDP0082	TTGTAACGGT	CTCATAGCGA
UDP0083	GTAAGGCATA	AGACACATTA
UDP0084	GTCCACTTGT	GCGCGATGTT
UDP0085	TTAGGTACCA	CATGAGTACT
UDP0086	GGAATTCCAA	ACGTCAATAC
UDP0087	CATGTAGAGG	GATACCTCCT
UDP0088	TACACGCTCC	ATCCGTAAGT
UDP0089	GCTTACGGAC	CGTGTATCTT
UDP0090	CGCTTGAAGT	GAACCATGAA
UDP0091	CGCCTTCTGA	GGCCATCATA
UDP0092	ATACCAACGC	ACATACTTCC
UDP0093	CTGGATATGT	TATGTGCAAT
UDP0094	CAATCTATGA	GATTAAGGTG
UDP0095	GGTGGAATAC	ATGTAGACAA
UDP0096	TGGACGGAGG	CACATCGGTG

Plate B/Set 2 Index Adapters

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0097	CTGACCGGCA	CCTGATACAA
UDP0098	GAATTGAGTG	TTAAGTTGTG
UDP0099	GCGTGTGAGA	CGGACAGTGA
UDP0100	TCTCCATTGA	GCACTACAAC
UDP0101	ACATGCATAT	TGGTGCCTGG
UDP0102	CAGGCGCCAT	TCCACGGCCT
UDP0103	ACATAACGGA	TTGTAGTGTA
UDP0104	TTAATAGACC	CCACGACACG
UDP0105	ACGATTGCTG	TGTGATGTAT
UDP0106	TTCTACAGAA	GAGCGCAATA
UDP0107	TATTGCGTTC	ATCTTACTGT
UDP0108	CATGAGTACT	ATGTCGTGGT
UDP0109	TAATTCTACC	GTAGCCATCA
UDP0110	ACGCTAATTA	TGGTTAAGAA
UDP0111	CCTTGTTAAT	TGTTGTTCGT
UDP0112	GTAGCCATCA	CCAACAACAT
UDP0113	CTTGTAATTC	ACCGGCTCAG
UDP0114	TCCAATTCTA	GTTAATCTGA
UDP0115	AGAGCTGCCT	CGGCTAACGT
UDP0116	CTTCGCCGAT	TCCAAGAATT
UDP0117	TCGGTCACGG	CCGAACGTTG
UDP0118	GAACAAGTAT	TAACCGCCGA
UDP0119	AATTGGCGGA	CTCCGTGCTG
UDP0120	GGCCTGTCCT	CATTCCAGCT
UDP0121	TAGGTTCTCT	GGTTATGCTA
UDP0122	ACACAATATC	ACCACACGGT
UDP0123	TTCCTGTACG	TAGGTTCTCT
UDP0124	GGTAACGCAG	TATGGCTCGA

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0125	TCCACGGCCT	CTCGTGCGTT
UDP0126	GATACCTCCT	CCAGTTGGCA
UDP0127	CAACGTCAGC	TGTTCGCATT
UDP0128	CGGTTATTAG	AACCGCATCG
UDP0129	CGCGCCTAGA	CGAAGGTTAA
UDP0130	TCTTGGCTAT	AGTGCCACTG
UDP0131	TCACACCGAA	GAACAAGTAT
UDP0132	AACGTTACAT	ACGATTGCTG
UDP0133	CGGCCTCGTT	ATACCTGGAT
UDP0134	CATAACACCA	TCCAATTCTA
UDP0135	ACAGAGGCCA	TGAGACAGCG
UDP0136	TGGTGCCTGG	ACGCTAATTA
UDP0137	TAGGAACCGG	TATATTCGAG
UDP0138	AATATTGGCC	CGGTCCGATA
UDP0139	ATAGGTATTC	ACAATAGAGT
UDP0140	CCTTCACGTA	CGGTTATTAG
UDP0141	GGCCAATAAG	GATAACAAGT
UDP0142	CAGTAGTTGT	AGTTATCACA
UDP0143	TTCATCCAAC	TTCCAGGTAA
UDP0144	CAATTGGATT	CATGTAGAGG
UDP0145	GGCCATCATA	GATTGTCATA
UDP0146	AATTGCTGCG	ATTCCGCTAT
UDP0147	TAAGGAACGT	GACCGCTGTG
UDP0148	CTATACGCGG	TAGGAACCGG
UDP0149	ATTCAGAATC	AGCGGTGGAC
UDP0150	GTATTCTCTA	TATAGATTCG
UDP0151	CCTGATACAA	ACAGAGGCCA
UDP0152	GACCGCTGTG	ATTCCTATTG
UDP0153	TTCAGCGTGG	TATTCCTCAG
UDP0154	AACTCCGAAC	CGCCTTCTGA

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0155	ATTCCGCTAT	GCGCAGAGTA
UDP0156	TGAATATTGC	GGCGCCAATT
UDP0157	CGCAATCTAG	AGATATGGCG
UDP0158	AACCGCATCG	CCTGCTTGGT
UDP0159	CTAGTCCGGA	GACGAACAAT
UDP0160	GCTCCGTCAC	TGGCGGTCCA
UDP0161	AGATGGAATT	CTTCAGTTAC
UDP0162	ACACCGTTAA	TCCTGACCGT
UDP0163	GATAACAAGT	CGCGCCTAGA
UDP0164	CTGGTACACG	AGGATAAGTT
UDP0165	CGAAGGTTAA	AGGCCAGACA
UDP0166	ATCGCATATG	CCTTGAACGG
UDP0167	ATCATAGGCT	CACCACCTAC
UDP0168	GATTGTCATA	TTGCTTGTAT
UDP0169	CCAACAACAT	CAATCTATGA
UDP0170	TTGGTGGTGC	TGGTACTGAT
UDP0171	GCGAACGCCT	TTCATCCAAC
UDP0172	CAACCGGAGG	CATAACACCA
UDP0173	AGCGGTGGAC	TCCTATTAGC
UDP0174	GACGAACAAT	TCTCTAGATT
UDP0175	CCACTGGTCC	CGCGAGCCTA
UDP0176	TGTTAGAAGG	GATAAGCTCT
UDP0177	TATATTCGAG	GAGATGTCGA
UDP0178	CGCGACGATC	CTGGATATGT
UDP0179	GCCTCGGATA	GGCCAATAAG
UDP0180	TGAGACAGCG	ATTACTCACC
UDP0181	TGTTCGCATT	AATTGGCGGA
UDP0182	TCCAAGAATT	TTGTCAACTT
UDP0183	GCTGTAGGAA	GGCGAATTCT
UDP0184	ATACCTGGAT	CAACGTCAGC

Index Name	i7 Bases in Adapter	i5 Bases in Adapter
UDP0185	GTTGGACCGT	TCTTACATCA
UDP0186	ACCAAGTTAC	CGCCATACCT
UDP0187	GTGTGGCGCT	CTAATGTCTT
UDP0188	GGCAGTAGCA	CAACCGGAGG
UDP0189	TGCGGTGTTG	GGCAGTAGCA
UDP0190	GATTAAGGTG	TTAGGATAGA
UDP0191	CAACATTCAA	CGCAATCTAG
UDP0192	GTGTTACCGG	GAGTTGTACT

Revision History

Document	Date	Description of Change
Document # 200038118 v00	July 2023	Initial Release. Previous document 200019584 replaced by this one. Changes from document 200019584 v2 to this new document: • Added content to support sequencing on NextSeq 550Dx Instrument using DRAGEN for Illumina DNA Prep with Enrichment Dx Application for NextSeq 550Dx. • Clarified Reagents Not Provided list. • Added incidence reporting information to Warnings and Precautions. • Clarified Enrichment Libraries expectation. • Added instruction to prepare 400 mM Tris-HCl, pH 8.0. • Removed Sequencing Preparation step typographical error. Changes previously made to document 200019584: • Added content to support sequencing on NovaSeq 6000Dx Instrument. • Added sequencing system names and catalog numbers. • Removed unique dual indexing information for single-indexed libraries.

Patents and Trademarks

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Product Labeling

For a complete reference of symbols that appear on product packaging and labeling, refer to the symbol key at support.illumina.com on the *Documentation* tab for your kit.