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Illumina DNA Prep with Exome 2.5 Enrichment

Product Documentation

ILLUMINA PROPRIETARY
Document #1000000157112 v07
May 2026

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Overview

This documentation explains how to prepare up to 96 uniquely dual-indexed, paired-end, whole exome, and mitochondrial genome libraries from DNA using the Illumina DNA Prep with Exome 2.5 Enrichment workflow.

The Illumina DNA Prep with Exome 2.5 Enrichment kit provides the necessary consumables to create high-quality whole exome libraries for human samples. These libraries include hybridization-based enrichment of the exome portion of the library. Each hybridization reaction contains 12 libraries (12-plex enrichment) for a total output of 96 exome-enriched, human libraries.

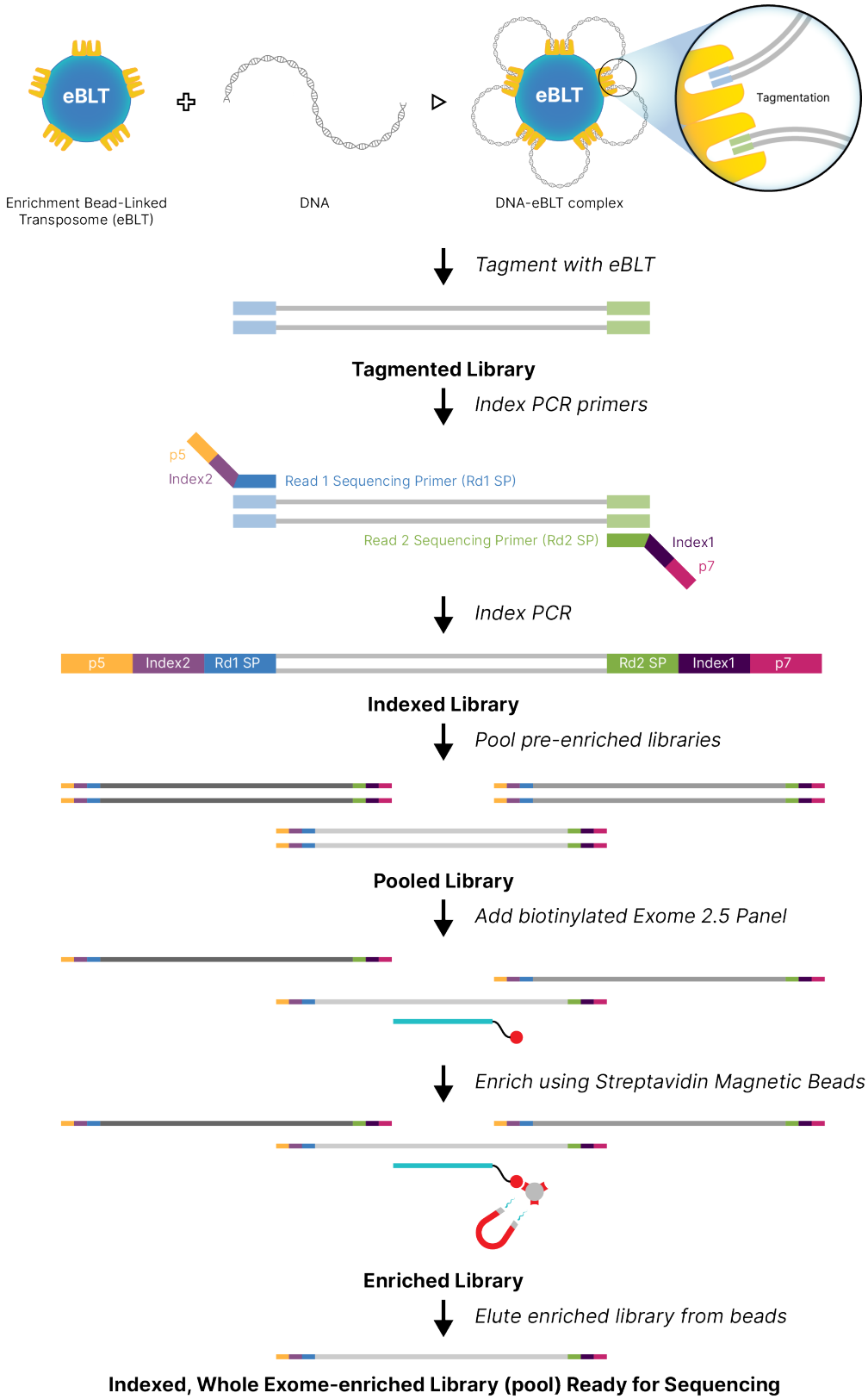
Reducing the number of libraries in each enrichment reaction requires optimization and reduces the total number of samples enriched per kit.

How the IDPE Exome 2.5 Assay Works

The IDPE Exome 2.5 workflow uses a bead-based transposome complex to tagment genomic DNA. Tagmentation is a process that fragments DNA and then tags the DNA with adapter sequences in one step. After it is saturated with input DNA, the bead-based transposome complex fragments a set number of DNA molecules. This fragmentation provides flexibility to use a wide DNA input range to generate normalized pre-enrichment libraries of consistent tight fragment size distribution.

Following tagmentation, a limited-cycle PCR adds adapter sequences to the ends of a DNA fragment. This step enables compatibility across all Illumina sequencing systems. A subsequent target enrichment workflow is then applied. Following pooling, the double-stranded DNA libraries are denatured and biotinylated oligonucleotide probes are hybridized to the denatured library fragments.

After hybridization, Streptavidin Magnetic Beads 4 (SMB4) then capture the complexes containing the biotinylated exome panel probes hybridized to the complementary exome. The exome-enriched and indexed libraries are eluted from beads and further amplified before sequencing. A subsequent Illumina Purification Beads (IPB) cleanup step then purifies libraries for use on an Illumina sequencing system.



Panel Details

The Twist Bioscience for Illumina Exome 2.5 Panel is a 120 bp, double-stranded oligo panel. The Exome 2.5 Panel can be combined with the Twist Bioscience for Illumina Mitochondrial Panel, and/or an Illumina Custom Enrichment Panel v2 (120 bp) to add or boost target coverage.

Table 1 Twist Bioscience for Illumina Exome 2.5 Panel Details

Targets	Coverage
Overall Total Target Regions*	287,879
ACMG73_RefSeqCuratedCDS_hg38	99.91%
ACMG73_RefSeqCuratedCDS_PathVar_hg38	99.90%
CCDS_01212021_cds_hg38	99.91%
ClinVar_03212022_SNV-PathLikelyPath_CDS	98.55%
Cosmic_CGC_11142021_RefSeqCuratedCDS_hg38	99.91%
CosmicMutCensus_11142021_PathSomatic_hg38	88.69%
Gencode_v39_hg38_CDS	98.96%
OMIM_03242022_Gencodev39CDS_hg38	99.08%
RefSeq_11142021_Curated_1stExonCDS_hg38	99.40%
RefSeq_11142021_Curated_CDS_hg38	99.08%

* Comparative bioinformatic analysis of target region coverage between this exome panel and the targets in the various public databases. Visit the Illumina support site to download the complete BED file for this design.

Both the Mitochondrial Panel and the recommended Illumina Custom Enrichment Panel v2 are 120 bp, double-stranded oligo panels. Spike-in panels in formats other than these 120 bp, double-stranded oligo products have not been tested.

The Twist Bioscience for Illumina Mitochondrial Panel is designed to cover the 37 genes (16,659 base pairs) of the human mitochondrial genome. The panel is purchased separately and can be used as a spike-in with the Illumina DNA Prep with Exome 2.5 Enrichment kits. Refer to *Product Contents* on page 6 for descriptions and part numbers.

The Illumina Custom Enrichment Panel v2 is a user-defined custom enrichment panel designed on the DesignStudio platform. Refer to *Product Contents* on page 6 for descriptions and part numbers.

DNA Input Recommendations

The Illumina DNA Prep with Exome 2.5 Enrichment protocol, including optional mitochondrial DNA Enrichment is compatible with high-quality, double-stranded human genomic DNA (gDNA) inputs of 10–1000 ng. The recommended minimum gDNA input is 50 ng for optimal performance given the

complexity and size of the human genome.

Assess gDNA purity to make sure that the initial gDNA sample does not contain any organic contaminants, such as phenol and ethanol. The input DNA must also contain less than 1 mM EDTA. These substances can interfere with the tagmentation reaction and result in assay failure or poor results.

gDNA Input ≥ 50 ng

For gDNA inputs between 50–1000 ng, quantifying and normalizing the initial gDNA sample is not required. The pre-enrichment library yield (before pooling and enrichment) is automatically normalized during library prep.

gDNA Input < 50 ng

This protocol does not normalize final pre-enrichment library yields when input gDNA ranges from 10–49 ng. Therefore, quantification and normalization of libraries before and after enrichment is required.

If using 10–49 ng gDNA input, quantifying the initial gDNA sample to determine the number of PCR cycles required for the pre-enrichment PCR is recommended. 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.

Assess gDNA Purity

UV absorbance is a common method used for assessing the purity of a gDNA sample. The ratio of absorbance at 260 nm to 280 nm provides an indication of sample purity. This protocol is optimized for gDNA with A260/280 ratios of 1.8–2.0, which indicates a gDNA sample with high purity.

For a secondary indication of sample purity, use an A260/230 ratio. Target an A260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of contaminants that may impact the tagmentation reaction. Incomplete tagmentation caused by contaminants can lead to library preparation failure, poor clustering, or low quality sequencing results.

These contaminants include general inhibitors of enzymatic reactions such as:

- Proteins that coat/bind DNA, preventing library prep enzymes from binding to the DNA substrate.
- Chelators such as EDTA, salts, and polysaccharides that bind-required cofactors of the library prep enzymes.
- Other enzymes such as proteases and reagents such as detergents and phenol that degrade or unfold the library prep enzymes.

If this testing produces ratios outside of the acceptable limits, one option is to repurify the gDNA sample using methods with Illumina Purification Beads outlined in *Single IPB Methodology (Optional)* on page 23.

Blood and Saliva Input Recommendations

The IDPE Exome 2.5 protocol is compatible with fresh whole blood (requires the Flex Lysis Reagent Kit), dried blood (requires eBLT), and saliva sample inputs. For information about protocols specific to blood and saliva, refer to *Blood Lysis (Optional)* on page 19 or *Saliva Lysis (Optional)* on page 21.

When starting with 10 µl liquid whole blood in EDTA tubes or 30 µl saliva in Oragene tubes, expect normalization of pre-enriched libraries equal to results observed when using 50–1000 ng gDNA input. Blood and saliva are heterogeneous sample types. Therefore the ability of IDPE Exome 2.5 to generate normalized libraries depends on the total amount of DNA obtained from the lysed sample. The following factors can adversely affect normalization of library independent of kit performance:

- Viscosity of the saliva samples
- Blood sample age
- Sample storage conditions
- Underlying medical conditions affecting white blood cell counts

Sample Input Recommendations

The IDPE Exome 2.5 workflow is compatible with purified gDNA from various samples. The kit is also compatible with samples when using the following protocols and reagent kits:

- Illumina Blood Lysis Protocol (blood) with the Flex Lysis Reagent Kit
- Illumina Saliva Lysis Protocol (saliva)

The recommended number of PCR cycles for the eBLT PCR program are adjusted based on sample input concentration and quality. For more information, refer to *Amplify Tagmented DNA* on page 29.

Sample Input Type	Quantification of Input DNA Required	Required DNA Input Quality	Normalized Pre-Enrichment Library Yield
10–49 ng genomic DNA	Yes	A260/280 ratio of 1.8–2.0 and A260/230 ratio of 2.0–2.2	No
50–1000 ng genomic DNA	No	A260/280 ratio of 1.8–2.0 and A260/230 ratio of 2.0–2.2	Yes
Saliva	No	Not applicable	Yes
Blood	No	Not applicable	Yes

Consumables & Equipment

The IDPE Exome 2.5 protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

The protocols have been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

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

Product Contents

Completing the Illumina DNA Prep with Exome 2.5 Enrichment protocol requires library prep and enrichment reagents, the exome panel, clean up/size selection beads, and index adapters. All are included in the full kits listed. The number of index adapters required depends on the number of samples to be uniquely indexed for your experiment. Depending on the sample input type and sequencing requirements, the protocol might require additional, optional consumables.

Component	Kit Options	Illumina Catalog #
Library prep and enrichment reagents	Illumina DNA Prep with Exome 2.5 Enrichment Kit, Tagmentation Set B (96 Samples, 12-plex)	20077595
	Illumina DNA Prep with Exome 2.5 Enrichment Kit, Tagmentation Set D (96 Samples, 12-plex)	20077596
[Optional] Supplemental Probe Panels	Twist Bioscience for Illumina Mitochondrial Panel (96 Samples, 12-plex)	20093180
	Illumina Custom Enrichment Panel v2 via DesignStudio (32 µl, 120 bp)	20073953
	Illumina Custom Enrichment Panel v2 via DesignStudio (384 µl, 120 bp)	20073952
	Illumina Custom Enrichment Panel v2 via DesignStudio (1536 µl, 120 bp)	20111339

Component	Kit Options	Illumina Catalog #
[Optional] Index adapters	Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20091654
	Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	20091656
	Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20091658
	Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20091660
[Optional] Blood lysis*	Flex Lysis Reagent Kit (96 samples)	20018706

* Required when starting the protocol from fresh whole blood samples and not purified blood gDNA.

Library Prep and Enrichment Kit Contents

Table 2 Illumina DNA/RNA Prep - IPB Tagmentation Buffers, Store at 15°C to 30°C*

Tube Quantity (96 Samples)	Acronym	Reagent Name
4	ST2	Stop Tagment Buffer 2
1	TWB	Tagmentation Wash Buffer
2	IPB	Illumina Purification Beads

* The reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Table 3 Illumina DNA Prep - Tagmentation (S) Beads, Store at 2°C to 8°C

Tube Quantity (96 Samples)	Acronym	Reagent Name
4	eBLT*	Enrichment Bead-Linked Transposomes
2	RSB	Resuspension Buffer

* Store the eBLT stock tube upright so that the beads are always submerged in the buffer.

Table 4 Illumina DNA/RNA Prep - Tagmentation PCR Reagents, Store at -25°C to -15°C*

Tube Quantity (96 Samples)	Acronym	Reagent Name
4	TB1	Tagmentation Buffer 1
4	EPM	Enhanced PCR Mix

* The reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Table 5 Twist BioScience for Illumina Exome 2.5 Panel, Store at -25°C to -15°C

Tube Quantity (96 Samples)	Acronym	Reagent Name
1	Not Applicable	Twist BioScience for Illumina Exome 2.5 Panel

Table 6 Illumina DNA Fast Hyb - Enrichment Beads + Buffers, Store at 2°C to 8°C

Tube Quantity (96 Samples)	Acronym	Reagent Name
2	SMB4	Streptavidin Magnetic Beads 4
1	RSB	Resuspension Buffer
1	EHB2	Enrich Hyb Buffer 2
1	ET2	Elute Target Buffer 2

Table 7 Illumina DNA Fast Hyb - Enrichment PCR + Buffers, Store at -25°C to -15°C*

Tube Quantity (96 Samples)	Acronym	Reagent Name
1	EE1	Enrichment Elution Buffer 1
4	EEW	Enhanced Enrichment Wash
1	PPC	PCR Primer Cocktail
1	HP3	2 N NaOH
1	NHB2	Hyb Buffer 2 + IDT NXT Blockers
1	EPM	Enhanced PCR Mix

* The reagents are shipped at 2°C to 8°C. Promptly store reagents at the indicated temperature to ensure proper performance.

Index Adapter Kit Contents

For index adapter sequences, refer to *Illumina Adapter Sequences*.

The index set received depends on the kit ordered with one containing Set B and the other Set D.

Table 8 Illumina DNA/RNA UD Indexes, Store at -25°C to -15°C

Description
Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)
Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)
[Optional] Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)
[Optional] Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)

[Optional] Twist Bioscience for Illumina Mitochondrial Panel

Table 9 Store at -25°C to -15°C

Description
Twist Bioscience for Illumina Mitochondrial Panel (96 samples, 12-plex)

[Optional] Illumina Custom Enrichment Panel v2

Table 10 Store at -25°C to -15°C

Description
Illumina Custom Enrichment Panel v2 (32 µl, 120 bp)
Illumina Custom Enrichment Panel v2 (384 µl, 120 bp)
Illumina Custom Enrichment Panel v2 (1536 µl, 120 bp)

[Optional] Flex Lysis Reagent Kit

The following reagents are shipped at -25°C to -15°C. Promptly store reagents at the indicated storage temperature to ensure proper performance.

Quantity	Acronym	Reagent Name	Storage Temperature
4	BLB	Blood Lysis Buffer	15°C to 30°C
4	PK1	Proteinase K	-25°C to -15°C

User-Supplied Consumables & Equipment

Make sure that you have the required consumables and equipment 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 consumables and equipment.

Consumables

Consumable	Supplier
Microcentrifuge tubes, 1.7 ml	General lab supplier
Pipette tips, 10 µl	General lab supplier
Pipette tips, 20 µl	General lab supplier
Pipette tips, 200 µl	General lab supplier
Pipette tips, 1000 µl	General lab supplier
96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)	Thermo Fisher Scientific, part # AB-0859
Nuclease-free water	General lab supplier
Distilled water	General lab supplier
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Eppendorf twin.tec 96-well LoBind PCR plate, skirted (or similar)	Eppendorf, catalog # 0030129512
Hard-Shell 96-well PCR plates	Bio-Rad, catalog # HSP-9601
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
Microseal 'F' foil seals	Bio-Rad, catalog # MSF-1001
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR **[Optional]** catalog # 89094-658
Ethanol 200 proof (absolute) for molecular biology (500 ml)	General lab supplier
One of the following kits, depending on quantification method: • **[Bioanalyzer]** Agilent High Sensitivity DNA Kit (2) • **[Fragment Analyzer]** High Sensitivity NGS Fragment Analysis Kit • **[Bioanalyzer]** Agilent DNA 1000 Kit (2)	One of the following suppliers, depending on instrument: • Agilent, catalog # 5067–4626* • Advanced Analytical, catalog DNF-474 • Agilent, catalog # 5067-1504
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856

Consumable	Supplier
One of the following kits for quantification: • Qubit dsDNA BR Assay Kit	• Thermo Fisher Scientific, catalog # Q32850 or Q32853
Tris-HCl 10 mM, pH 8.5	General lab supplier

* End of life announced. Refer to vendor site for more information.

Consumables for Plate Workflow

Consumable	Supplier
96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)	Thermo Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier
Hard-Shell 96-well PCR plates	Bio-Rad, part # HSP-9601
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
Microseal 'F' foil seals	Bio-Rad, part # MSF-1001

Consumables for Tube Workflow

Consumable	Supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
1.7 ml microcentrifuge tubes	General lab supplier

Consumables for Blood and Saliva Input

Consumable	Supplier
Illumina Purification Beads	Illumina, 1 x 100 ml, catalog # 20060057 Illumina, 4 x 100 ml, catalog # 20060058

Equipment

Equipment	Supplier
Pipettes, multichannel, 10 µl	General lab supplier

Equipment	Supplier
Pipettes, multichannel, 20 µl	General lab supplier
Pipettes, multichannel, 200 µl	General lab supplier
Pipettes, single channel, 10 µl	General lab supplier
Pipettes, single channel, 20 µl	General lab supplier
Pipettes, single channel, 200 µl	General lab supplier
Pipettes, single channel, 1000 µl	General lab supplier
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
Heat block for 96 well plate The use of thermal cyclers or heat blocks with active cooling (eg, Peltier, thermoelectric cooled) is not recommended for the hybridization step. The passive cooling step is critical for proper hybridization. The heat block must meet the following performance specifications: • Heated lid • Temperature range: Ambient +5°C to 99°C • Temperature regulation: ±0.1°C at 37°C; ±0.4°C at 60°C	General lab supplier
Illumina MIDI heat block insert One incubator (hybridization oven) is required. The incubator must meet the following performance specifications: • Temperature range: 10°C to 100°C • Temperature regulation: ±0.2°C	Illumina, catalog # BD-60-601
Qubit Fluorometer 3.0	Thermo Fisher Scientific, catalog # Q33216 or Q33217
Vortexer	General lab supplier

Equipment	Supplier
One of the following analyzers: • Fragment Analyzer • 2100 Bioanalyzer Desktop System	Agilent Technologies catalog #: • Refer to web product pages for catalog # • G2939BA* or G2940CA
[Saliva] Water bath or air incubator capable of reaching 50°C	As recommended by DNA Genotek, refer to Genotek's product pages.
[Optional] Vacuum concentrator Note: Use when concentrating a pooled library.	General lab supplier

* No longer available for purchase.

Equipment for Tube Workflow

Equipment	Supplier
MagneSphere Technology Magnetic Separation Stands (12 position, 1.5 ml)	Promega, catalog # Z5342

Equipment for Plate Workflow

Equipment	Supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
High-Speed Microplate Shaker	BioShake iQ High-Speed Thermal Mixer • Q Instruments, model # 1808-0506 BioShake XP High-Speed Thermal Mixer • Q Instruments, model # 1808-0505
Microplate centrifuge	General lab supplier

Thermal Cyclers

The following table lists recommended thermal cyclers or specifications. PCR thermal cyclers must be capable of supporting the sample volumes and temperature profiles used in this workflow, with appropriate thermal accuracy and block uniformity to ensure consistent incubation and amplification performance. Validate the thermal cycler before performing the protocol.

Performance may vary depending on the specific thermal cycler and consumables used. Minor workflow optimization may be required to account for instrument and consumable specific differences.

Thermal Cycler	Supplier

Thermal cycler with the following specifications:
• Heated lid
• Block ramp rate: ≥ 2.5°C/sec
• Temperature control range:
  • Min ≤ 4°C
  • Max ≥ 99°C
• Temperature accuracy: ±0.25°C
• Temperature uniformity: ±0.5°C
• Capable of supporting reaction volumes of 100 µl
• Compatible with 96-well PCR plates (full or semi-skirted), or suitable for the applicable workflow.
	General lab supplier

Protocol

This section describes the Illumina DNA Prep with Exome 2.5 Enrichment protocol, with optional Mitochondrial DNA and/or custom target enrichment.

- 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 consumables. This protocol includes the library prep and enrichment reagents, Twist BioScience for Illumina Exome 2.5 Panel, IPB, and index adapter plates. Refer to *Consumables & Equipment* on page 6.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

Illumina DNA Prep with Exome 2.5 Enrichment Workflow

The following diagram illustrates the Illumina DNA Prep with Exome 2.5 Enrichment workflow. Safe stopping points are marked between steps.

Time estimates are based on processing 12 samples with a single, 12-plex enrichment reaction.

Tips and Techniques

Safe Stopping Point

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding 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 tubes or plates from the working area.

Sealing the Plate

- Always seal the 96-well plate with the adhesive seal using a rubber roller to cover the plate before the following steps in the protocol:
  - Shaking steps
  - Thermal cycling steps
  - Centrifuge steps
- Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' seals for thermal cycling or short-term storage.
- Microseal 'F' foil seals are effective at temperatures down to -70°C and are suitable for storing the 96-well plates containing the final libraries long term.

Handling Enrichment Bead-Linked Transposomes (eBLT)

- Store the eBLT stock tube upright in the refrigerator so that the beads are always submerged in the buffer.
- Vortex the eBLT stock tube thoroughly until the beads are resuspended before use. 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 beads:
  - Use the appropriate magnetic stand for the plate.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand.
  - Do not disturb the bead pellet.

- 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).
- Dispense Tagmentation Wash Buffer (TWB) directly onto the beads.
- If liquid becomes adhered to the side or top of the tube or well, centrifuge at 280 × g for 3 seconds to pull volume into liquid.

Preparing Illumina DNA/RNA Unique Dual (UD) Indexes Plate

- If using a NextSeq 500 system, the read lengths must be modified to accommodate 10 base pair indexes. Visit the compatible products page on the Illumina support site.
- Illumina DNA Prep with Exome 2.5 Enrichment is compatible with Illumina DNA/RNA Unique Dual (UD) Index sets.

Prepare for Pooling

Record the index information for each sample before starting the library prep. The preplated index adapter plates provided with these kits contain a unique combination of 10 bp dual i5 and i7 indexes per well. There are a total of 96 unique dual index combinations per plate. Each plate contains a different set of unique dual indexes. For information on the tools compatible with your sequencing system, visit the product compatibility page on the Illumina support site.

- For low-plexity pooling strategies (2-plex to 9-plex), refer to the *Index Adapters Pooling Guide*.
- For index adapter sequences and information about recording the sequences, refer to *Illumina Adapter Sequences*.

Supported Enrichment Plexities

Illumina DNA Prep with Exome 2.5 Enrichment reagents are configured and tested at 12-plex enrichment plexity. Although lower enrichment plexities are possible, some plexities require additional pre-enrichment library prep and enrichment probe panel reagents.

Obtaining suitable enrichment yields for nonstandard enrichment plexities might require additional optimization.

- **Enrichment plexity**—The number of pre-enrichment libraries (12 recommended, 1-11 optional) pooled together in one enrichment reaction for hybridization with the Twist BioScience for Illumina Exome 2.5 Panel and the optional Twist Bioscience for Illumina Mitochondrial Panel. For example, combining 12 pre-enrichment libraries together creates a 12-plex enrichment pool.
- **Enrichment reaction**—The number of unique enrichment reaction preparations, regardless of the number of pre-enrichment libraries pooled per reaction (8 reactions provided in these kits).

To calculate the total number of post-enrichment 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-enrichment libraries.

When pooling pre-enrichment libraries, Illumina DNA Prep with Exome 2.5 Enrichment reagents support the number of enrichment reactions and plexity indicated.

Illumina DNA Prep with Exome 2.5 Enrichment Reagents	Enrichment Reactions	Enrichment Plexity
96-sample kit	8 reactions	12-plex
Twist Bioscience for Illumina Mitochondrial Panel (32 µl oligo panel)[2]	8 reactions[1]	12-plex
Illumina Custom Enrichment Panel v2 (32 µl, 120 bp)[2]	8 reactions	12-plex

[1] Mitochondrial Panel may be diluted before use. Read the protocol carefully to determine the volume and dilution of oligo panel needed for your experiment.
[2] Sold separately. Refer to *Product Contents* on page 6 for descriptions and catalog numbers.

Blood Lysis (Optional)

Use this protocol when performing the Illumina DNA Prep with Exome 2.5 Enrichment workflow using blood sample inputs with the Flex Lysis Reagent Kit. This protocol has been validated using fresh whole blood collected in EDTA collection tubes. Store the blood at 4°C and process it within 3 days. The use of frozen blood has not been validated and cannot be recommended.

This protocol is expected to generate > 100 ng of DNA output at the end of the blood lysis step.

Blood is a potential source of infectious diseases. Follow site-specific procedures to ensure the safe handling of blood samples. During the lysis protocol, make sure that the entire blood sample is fully lysed (brown in color following the heat incubation step) before proceeding to subsequent steps. A fully lysed sample makes sure that any blood borne pathogens are eliminated and the sample is no longer biohazardous.

Consumables

- BLB (Blood Lysis Buffer)
- IPB (Illumina Purification Beads)
- PK1 (Proteinase K)
- EtOH (Freshly prepared 80% ethanol)
- EDTA collection tubes (for blood sample collection)
- Nuclease-free water
- 96-well PCR plate

About Reagents

- IPB
  - Must be at room temperature before use.
  - Resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
BLB	Room temperature	If frozen, thaw at room temperature. If precipitates are observed, heat at 37°C for 10 minutes and vortex until resuspended.
IPB	Room temperature	Use at room temperature.
PK1	-25°C to -15°C	Place on ice until needed.

2. Prepare fresh 80% EtOH from absolute ethanol.
3. Save the following BLP program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 56°C for 10 minutes

Procedure

1. Combine the following volumes to prepare the Lysis Master Mix. Multiply each volume by the number of samples being processed.
   - BLB (8.4 µl)
   - PK1 (2.4 µl)
   - Nuclease-free water (37.2 µl)

   Reagent overage is included in the volume to ensure accurate pipetting.
2. Vortex and centrifuge the Lysis Master Mix.
3. Invert the EDTA tube 10 times to mix.
4. Transfer 10 µl blood from the tube to one well of a 96-well PCR plate.
5. Add 40 µl Lysis Master Mix to each sample.
6. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.

b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.

7. Add 20 µl IPB to the well.
8. Using a pipette set to 50 µl, slowly pipette 10 times to mix, and then seal.
9. Place on the preprogrammed thermal cycler and run the BLP program.
10. Place on a magnetic stand and wait 5 minutes.
    The dark brown color of the blood from the lysis reaction keeps the liquid from becoming clear. The beads migrate after 5 minutes.
11. Without disturbing the beads, remove and discard supernatant.
12. If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and then wait until the liquid is clear (~2 minutes).
13. Add 150 µl fresh 80% EtOH to the well.
14. Incubate on the magnetic stand for 30 seconds.
15. Pipette to remove and discard the EtOH.
16. Use a 20 µl pipette to remove and discard all residual EtOH.
17. Remove the plate from the magnetic stand.
18. Add 32 µl nuclease-free water and pipette to resuspend.
19. Seal the plate.
20. Centrifuge plate at 280 × g for 30 seconds.
21. Remove seal, place on magnetic stand and wait until the liquid is clear (~2 minutes).
22. Transfer 30 µl supernatant to a new 96-well PCR plate.
23. If you are not stopping, proceed immediately to step 3 of *Tagment Genomic DNA* on page 26.

SAFE STOPPING POINT

Seal the plate with a Microseal 'B' adhesive seal and store the plate at 2°C to 8°C for up to 3 days.

Saliva Lysis (Optional)

Use this protocol when performing the Illumina DNA Prep with Exome 2.5 Enrichment workflow using saliva sample inputs. This protocol is validated for saliva collected only in Oragene DNA saliva collection tubes. The saliva is mixed with the Oragene Dx solution contained in the collection tube, making it stable at room temperature.

This protocol is expected to generate > 100 ng of DNA output at the end of the saliva lysis step.

Saliva is a potential source of infectious diseases. Follow site-specific procedures to ensure the safe handling of saliva samples.

Consumables

- IPB (Illumina Purification Beads)
- 96-well PCR plate
- EtOH (Freshly prepared 80% ethanol)
- Nuclease-free water
- Oragene DNA collection tubes (for saliva sample collection)

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
IPB	Room temperature	Vortex and invert to mix.
Saliva samples in Oragene DNA collection tubes	Room temperature	For information on sample preparation and storage, refer to the DNA Genotek website.

2. For each sample, prepare 150 µl fresh 80% EtOH from absolute ethanol.

Procedure

1. For each sample, add 20 µl nuclease-free water to one well of a 96-well PCR plate.
2. Vortex the heat-treated Oragene DNA collection tube.
3. Transfer 30 µl saliva sample from the tube to the well containing nuclease-free water.
4. Slowly pipette to mix.
   For viscous samples, use a wide-bore pipette tip for more accurate pipetting.
5. Vortex and invert IPB multiple times to resuspend.
6. Add 20 µl IPB to the well.
7. Using a pipette set to 50 µl, slowly pipette 10 times to mix.
8. Incubate at room temperature for 5 minutes.

9. Place on a magnetic stand and wait 5 minutes.
10. Without disturbing the beads, remove and discard supernatant.
11. If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
12. Add 150 µl fresh 80% EtOH to the well.
13. Incubate on the magnetic stand for 30 seconds.
14. Use a 20 µl pipette to remove and discard all residual EtOH.
15. Remove the plate from the magnetic stand.
16. Add 32 µl nuclease-free water and pipette to resuspend.
17. Seal the plate with Microseal 'B', and then centrifuge at 280 × g for 30 seconds.
18. Remove seal, place on a magnetic stand and wait until the liquid is clear (~2 minutes).
19. Transfer 30 µl supernatant to a new 96-well PCR plate.
20. If you are not stopping, proceed immediately to step 3 of *Tagment Genomic DNA* on page 26.

SAFE STOPPING POINT

Seal the plate with a Microseal 'B' adhesive seal and store at 2°C to 8°C for up to 3 days.

Single IPB Methodology (Optional)

The Single IPB Methodology uses the bead purification procedure to purify the amplified pre-enrichment libraries. It is an alternative to the standard double-sided IPB *Clean Up Libraries* on page 32.

- Single IPB cleanup removes the need of pre-enrichment library concentration before the hybridization step, which could save about 20 minutes.
- This methodology gives better diversity and lowers the number of duplicate fragments. However, it may associate with a larger deviation of percent on-target reads due to a wider range of fragment sizes.
- Double-sided IPB cleanup and size selection provides more uniform fragment size and on target metrics.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (80% EtOH)
- Nuclease-free water
- 96-well 0.8 ml Polypropylene deep-well storage plate (MIDI plate) (2)
- 96-well PCR plate

- 1.7 ml microcentrifuge tubes
- Microseal 'B' adhesive seal
- Microseal 'F' foil seal

About Reagents

- IPB
  - Must be at room temperature before use.
  - 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
IPB	15°C to 30°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB	2°C to 8°C	Thaw and bring to room temperature. Vortex to mix.

2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.
3. If purifying your gDNA sample before the library preparation, start at step 4 and then add 1.8x IPB to your gDNA sample. For example, if your gDNA sample volume is 50 µl, add 90 µl IPB. Then proceed to step 6 (mix IPB and samples) and continue with the rest of the procedure.

Procedure

1. Shake the 96-well PCR plate at 1800 rpm for 1 minute.
2. Place the plate on the magnetic stand and wait until the liquid is clear (~1 minute).
3. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate.
4. Vortex and invert IPB multiple times to resuspend.
5. Add 81 µl IPB to each MIDI plate well containing supernatant.
6. Pipette each well 10 times to mix. Alternatively, seal the plate and shake at 1800 rpm for 1 minute.
7. Incubate the sealed MIDI plate at room temperature for 5 minutes.
8. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
9. Without disturbing the beads, remove and discard all supernatant.

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. Use a 20 µl pipette to remove and discard residual EtOH.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand.
14. Add 17 µl RSB to the beads.
15. Seal the plate, and then use a plate shaker to shake at 1800 rpm for 2 minutes.
16. Incubate at room temperature for 2 minutes.
17. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
18. Transfer 15 µl supernatant to a new 96-well PCR plate.
19. Seal the plate.

SAFE STOPPING POINT

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

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 11 15°C to 30°C Storage

Reagent	Box Name	Instructions
ST2	Illumina DNA/RNA Prep -Tagmentation Buffers	Use at room temperature.
TWB	Illumina DNA/RNA Prep -Tagmentation Buffers	Use at room temperature.

Table 12 2°C to 8°C Storage

Reagent	Box Name	Instructions
eBLT	Illumina DNA Prep - Tagmentation (S) Beads	Bring to room temperature by incubating at room temperature for 10 minutes.

Table 13 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Illumina DNA/RNA Prep - Tagmentation PCR Reagents	Thaw on ice.
Index adapter plate	Illumina DNA/RNA UD Indexes	Thaw at room temperature, then keep on ice.
TB1	Illumina DNA/RNA Prep - Tagmentation PCR Reagents	Bring to room temperature.

Tagment Genomic DNA

This step uses the Enrichment Bead-Linked Transposomes (eBLT) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences.

Consumables

- eBLT (Enrichment Bead-Linked Transposomes)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 8-tube strip
- 96-well PCR plate
- Microcentrifuge tubes, 1.7 ml
- Microseal 'B' adhesive seal

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 SDS at support.illumina.com/sds.html.

About Reagents

- eBLT
  - Must be stored upright so that the beads are always submerged in the buffer.
  - Do not use eBLT that has been stored below 2°C.

Preparation

1. Prepare the following consumables:
   - eBLT—Vortex to mix. Do not centrifuge before pipetting.
   - TB1—Vortex to mix.
2. 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.
2. If DNA volume is < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
3. Vortex eBLT for 10 seconds to resuspend. Repeat as necessary.
4. For each sample, combine the following volumes to prepare the Tagmentation Master Mix. Multiply each volume by the number of samples being processed.
   - eBLT (11.5 µl)
   - TB1 (11.5 µl)

   These volumes produce 23 µl Tagmentation Master Mix per sample, which includes extra volume to ensure accurate pipetting.
5. Vortex the Tagmentation Master Mix for 10 seconds to resuspend.
6. Divide the Tagmentation Master Mix volume equally into an 8-tube strip.
7. Using a multichannel pipette, transfer 20 µl Tagmentation Master Mix from the 8-tube strip to each well of the plate containing a sample.
   Use fresh tips for each sample column.
8. Discard the 8-tube strip after the Tagmentation Master Mix has been dispensed.
9. Using a multichannel pipette set to 40 µl, pipette each sample 10 times to resuspend, and then seal the plate. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
10. Place on the preprogrammed thermal cycler and run the TAG program.
11. Wait until the TAG program has reached the 10°C hold temperature before removing the plate and proceeding.

Post Tagmentation Clean Up

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

Consumables

- ST2 (Stop Tagment Buffer 2)
- TWB (Tagmentation Wash Buffer)
- Microseal 'B' adhesive seal

About Reagents

- TWB
  - Pipette slowly to minimize foaming.
  - A deliberately slow pipetting technique minimizes the potential of foaming to avoid incorrect volume aspiration and incomplete mixing.

Preparation

1. Prepare the following consumables:
   - ST2—If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved.
   - TWB—Use at room temperature. Vortex to mix.

Procedure

1. Let the 96-well PCR plate stand at room temperature for 2 minutes.
2. Add 10 µl ST2 to each well of the plate. If you are using a multichannel pipette, pipette ST2 into an 8-tube strip, and then transfer the 10 µl volumes.
3. Using a 200 µl pipette set to 50 µl, slowly pipette each well 10 times to resuspend the beads, and then seal. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute. Repeat as needed to resuspend the beads.
4. Make sure that the plate is sealed and centrifuge the samples for approximately 2 seconds.
5. Incubate the samples at room temperature for 5 minutes.
6. Place the samples on a magnetic stand, and then wait until the liquid is clear (~3 minutes).
7. **[≤ 48 samples]** Wash as follows.
   a. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
   b. Remove from the magnetic stand.
   c. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
   d. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
   e. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
   f. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.

g. Repeat steps b–e for a **second** wash.

h. Repeat step b and step c for a **third** wash.

8. **[> 48 samples]** Wash as follows.

a. To avoid excessive drying of the beads, perform steps b–d in one or two column increments until all columns have been processed.

b. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.

c. Remove from the magnetic stand.

d. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.

e. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

f. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).

g. To avoid excessive drying of the beads, perform steps h–k in one or two column increments until all columns have been processed.

h. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.

i. Remove from the magnetic stand.

j. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.

k. Repeat steps e–j for a **third** wash.

9. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

10. Place the plate on the magnetic stand and wait until the liquid is clear.

11. Keep on the magnetic stand and proceed to *Amplify Tagmented DNA* on page 29.
The TWB remains in the wells to prevent drying 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. To confirm the indexes of libraries have the appropriate color balance, refer to the *Index Adapters Pooling Guide*.

For a list of compatible index adapters for use with this protocol, refer to *Product Contents* on page 6.

Consumables

- EPM (Enhanced PCR Mix)
- Microcentrifuge tubes, 1.7 ml
- Eppendorf Lo Bind PCR Plate
- Microseal 'B' adhesive seal

- Nuclease-free water
- Index adapter plate (A, B, C, or D plates)

About Reagents

- Index adapter plates
  - A well can contain > 10 µl index adapters.
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.
  - You can use plates for a maximum of four freeze-thaw cycles if not using all 96 indexes in a single experiment.
  - Each index plate well contains a unique, prepared mix of Index 1 (i7) and Index 2 (i5).

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, then centrifuge briefly.
   - Index adapter plates
2. Save the following eBLT PCR program on a thermal cycler using the appropriate number of PCR cycles indicated in the table:
   - 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 genomic DNA	12
50–1000 ng genomic DNA	9

Sample Input Type	Number of PCR Cycles (X)
Saliva	9
Blood	9

Procedure

1. For each sample, combine the following volumes to prepare the PCR Master Mix. Multiply each volume by the number of samples being processed. Reagent overage is included in the volume to ensure accurate pipetting.
   - EPM (23 µl)
   - Nuclease-free water (23 µl)
2. Vortex, and then centrifuge the PCR Master Mix at 280 × g for 10 seconds.
3. With the plate on the magnetic stand, use a 200 µl multichannel pipette set to 100 µl to remove and discard supernatant.
   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 sample well.
6. Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker 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 Eppendorf PCR plate above the index adapter plate and press down to puncture the foil seal. Discard the Eppendorf PCR plate used to puncture the foil seal.
10. Using a new pipette tip, add 10 µl pre-paired Index 1 (i7) and Index 2 (i5) index adapters to each well.
11. Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
12. Seal the plate with Microseal 'B', and then centrifuge at 280 × g for 30 seconds.
13. Place on the preprogrammed thermal cycler and run the eBLT PCR program.

SAFE STOPPING POINT

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

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 14 15°C to 30°C Storage

Reagent	Box Name	Instructions
IPB	Illumina DNA/RNA Prep - IPB Tagmentation Buffers	Use at room temperature.

Table 15 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA Prep - Tagmentation (S) Beads	Bring to room temperature.

Clean Up Libraries

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

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (Freshly prepared 80% ethanol)
- Nuclease-free water
- 96-well 0.8 ml Polypropylene Deepwell Storage Plate (MIDI plate) (2)
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' foil seal

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. RSB—Vortex to mix.
2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Use a plate shaker to shake the 96-well PCR plate at 1800 rpm for 1 minute.
2. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
3. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate.
4. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
5. For gDNA, blood, or saliva, perform the following steps.

   For faster turnaround, use the *Single IPB Methodology (Optional)* on page 23. The two-step, double-sided cleanup process here produces optimal results. If speed is more important, a one-step cleanup can be performed.

   a. Add 77 µl nuclease-free water to each well-containing supernatant.
   b. Add 88 µl IPB to each well-containing supernatant.
   c. Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
   d. Seal the plate and incubate at room temperature for 5 minutes.
   e. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
   f. During incubation, thoroughly vortex and resuspend the IPB, and then add 20 µl to each well of a *new* MIDI plate.
   g. Remove seal and transfer 200 µl supernatant from each well of the first plate to the corresponding well of the new MIDI plate containing 20 µl IPB.
   h. Pipette each well in the MIDI plate 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
   i. Discard the first plate.
6. Incubate the sealed MIDI plate at room temperature for 5 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
8. Without disturbing the beads, remove and discard supernatant.

9. 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.
10. Wash beads a **second** time.
11. Use a 20 µl pipette to remove and discard residual EtOH.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand.
14. Add 17 µl RSB to the beads.
15. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
16. Incubate at room temperature for 2 minutes.
17. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
18. Transfer 15 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

Qualify Pre-Enriched Libraries

It is recommended to check the quality or qualify pre-enriched libraries before proceeding to enrichment.

If you elect not to check pre-enriched libraries, perform the following procedure instead to reserve samples for potential troubleshooting later.

Store for Potential Troubleshooting.

1. Transfer 1 µl of each pre-enriched library to a new 96-well PCR plate.
2. Add 4 µl RSB to each pre-enriched library.
3. Seal the plate with Microseal 'F' foil seal.
4. Store at -25°C to -15°C for up to 30 days for future troubleshooting if necessary.

Qualify Pre-Enriched Libraries.

Pre-enriched libraries can be qualified individually (one library at a time) or as a pool before enrichment.

Assess quality of 1 µl library or pooled libraries using one of the following methods.

- Add 1 µl RSB to the 1 µl library or pooled libraries, and then analyze the 2 µl volume using the Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit.
- Analyze 1 µl library or pooled libraries using the Agilent Technology 2100 Bioanalyzer using a DNA 1000 kit.

Expect the mean fragment size to be between 300 bp and 400 bp and a distribution of DNA fragment size range of 150–1500 bp as shown in the figures below.

Figure 1 Fragment Analyzer Trace: Example

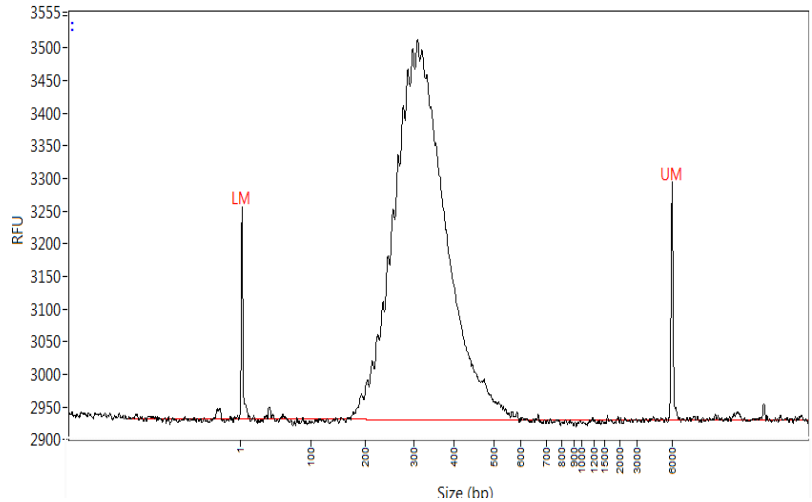
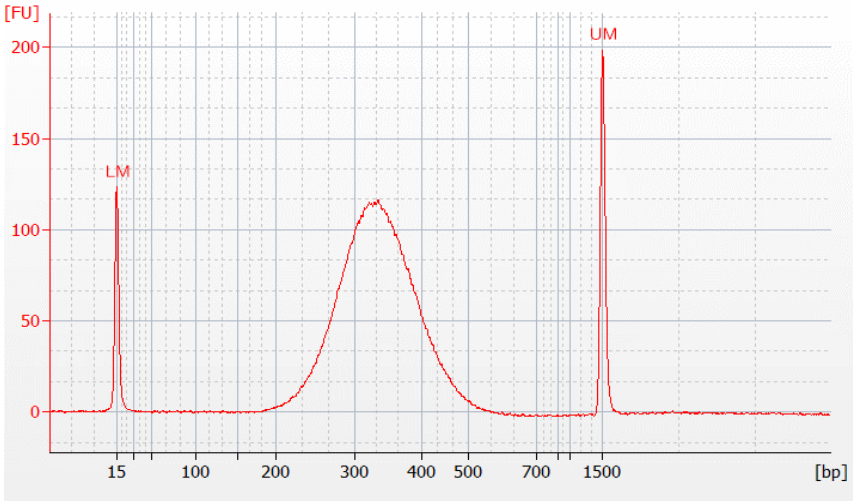


Figure 2 Bioanalyzer Trace: Example



Pool Pre-Enrichment Libraries

This step combines DNA libraries with unique indexes into one pool of up to 12 libraries. Fewer pre-enrichment libraries may be pooled, but you may need to perform additional optimization. If using fewer pre-enrichment libraries, you cannot process the full 96 samples through enrichment, as only eight enrichment reactions are supported with this kit.

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

Table 16 Recommended Pooling Methods

Sample Input	Pooling Method
10–49 ng gDNA	Mass only
50–1000 ng gDNA	Mass or volume*
Saliva	Volume
Blood	Volume

* If starting with ≥ 50 ng DNA input, the pre-enrichment library yields were normalized during tagmentation, which uses eBLT. This normalization enables you to pool equal volumes of each pre-enrichment library in a final pool volume ≤ 30 µl (target 250–500 ng per sample).

After pre-enrichment library quantification, all sample input types can be pooled by mass to achieve optimal library balance and a similar number of sequencing reads per library.

The final yield of pre-enrichment libraries generated in separate experimental preparations can vary. Therefore, pooling by mass is recommended to achieve optimal library balance when pooling samples from multiple experimental preparations.

If pre-enrichment libraries are not quantified, then proceed to pooling by volume. Pooling by volume can only be performed when starting with ≥ 50 ng gDNA input.

Quantify Pre-Enrichment Libraries

Determine library concentration (ng/µl) by proceeding as follows:

1. Quantify 1 µl of each pre-enrichment library using the Qubit dsDNA BR Assay Kit to determine library concentration (ng/µl).
   Expect the following pre-enrichment library yield based on sample type and input.

Sample Input Type (ng)	Pre-enrichment Library Yield (ng)
10–49 gDNA	≥ 100
50–1000 gDNA, blood, saliva	≥ 500

Concentration results may differ depending on the quantification method used. The Qubit dsDNA BR Assay is recommended, but validation will be needed when using an alternative method.

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-enrichment library samples prepared by the same user, reagent lot, index adapter plate, and experimental batch.

If starting from 50–1000 ng gDNA input, saliva input, or blood input, you can pool the pre-enrichment libraries using the following standard protocol.

1. Using the sample tracking method you chose in *Prepare for Pooling* on page 18, record the indexes for the libraries you plan to pool in this step.
2. Pool pre-enrichment libraries based on the sample volumes in the following table.
   - **[12-plex]** Combine 2.5 µl of each pre-enrichment library in a 1.7 ml microcentrifuge tube to generate a 12-plex pool at a total final pool volume of 30 µl.
   - **[< 12-plex]** Combine 2.5 µl of each pre-enrichment library in a 1.7 ml microcentrifuge tube, then add RSB to bring the total final pool volume up to 30 µl.

Pool by Mass

1. Using the sample tracking method you chose in *Prepare for Pooling* on page 18, record the indexes for the libraries you plan to pool in this step.
2. Combine each library in a 1.7 ml microcentrifuge tube to generate a 12-plex pool shown in the following table. Repeat as needed for additional pools.

When pooling by mass, always pool equivalent masses of each pre-enrichment library to obtain similar sequencing read output from each final, enriched library.

If the volume of your 12 pooled libraries is > 30 µl, concentrate the pooled libraries to 30 µl. Refer to *Concentrate Pooled Libraries (Optional)* on page 38.

If the final pool volume is less than 30 µl, add RSB to bring the total final pool volume up to 30 µl.

Table 17 Pooling by Mass Guidelines

	Inputs per Pre-enrichment Library (ng)			Total Mass per Final 12-Plex Pool (ng)		
Sample Input	**Minimum**	**Maximum**	**Recommended**	**Minimum**	**Maximum**	**Recommended**
10-49 ng gDNA	100	500	250–500	1200	6000	3000–6000
[Optional] 50–1000 ng gDNA*	250	500	250–500	3000	6000	3000–6000
[Optional] Quantified saliva and blood gDNA*	250	500	250–500	3000	6000	3000–6000

*If starting from 50–1000 ng input, saliva input, or blood input, you can pool pre-enrichment libraries by volume instead of by mass. Pooling by mass will produce more consistent sequencing reads from each enriched library within the pool.

SAFE STOPPING POINT

If you are stopping, cap the 1.5 ml microcentrifuge tube and store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 18 15°C to 30°C Storage

Reagent	Box Name	Instructions
[Optional] IPB	Illumina DNA/RNA Prep - IPB Tagmentation Buffers	Use at room temperature.

Table 19 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature if using one of the following: • **[Optional]** Illumina Custom Enrichment Panel v2 • **[Optional]** Twist Bioscience for Illumina Mitochondrial Panel.

Concentrate Pooled Libraries (Optional)

If the total volume of the pooled pre-enriched libraries is > 32 µl, the pool of pre-enriched libraries must be concentrated to a final volume of 32 µl. From the final volume, 30 µl is transferred for hybridization. Use this bead-based method to achieve a final volume of 32 µl. For more information, refer to *Pool Pre-Enrichment Libraries* on page 35.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (Freshly prepared 80% ethanol)
- **[Plate]** Microseal 'B' adhesive seals
- One of the following containers:

  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - [**Tube**] 1.7 ml microcentrifuge tubes
- One of the following magnets:
  - **[Plate]** Magnetic Stand-96
  - **[Tube]** MagneSphere Technology Magnetic Separation Stands (12 position, 1.7 ml). Used in place of MIDI plates when pool volumes ≥ 178 µl.

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following reagents.

Item	Storage	Instructions
IPB	15°C to 30°C	Use at room temperature.
RSB	2°C to 8°C	Bring to room temperature for > 30 minutes. Vortex to mix.

2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Centrifuge the sample tube at 280 × g for 1 minute.
2. Transfer samples to the corresponding well of a new MIDI plate or a new 1.7 ml microcentrifuge tube.

   If the pool volume is ≥ 178 µl, use a 1.7 ml microcentrifuge tube to prevent MIDI plate wells from overflowing.

3. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.

4. Add 2.5x pool volume of IPB to each well or to the microcentrifuge tube, and then mix thoroughly as follows.
   - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or the tube at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 1 minute.
7. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
8. Remove and discard all supernatant from each well or from the tube.
9. Wash as follows.
   a. Keep on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well or to the tube.
   b. Wait 30 seconds.
   c. Using a pipette set to 200 µl, remove and discard all supernatant from each well or from the tube.
10. Wash a **second** time.
11. Use a 20 µl pipette to remove and discard residual 80% EtOH.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand and add 32 µl RSB to each well or to the tube.
14. Mix thoroughly as follows.
    - **[Plate]** Seal plate and shake at 1800 rpm for 1 minute.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
15. Incubate the sample plate or the tube at room temperature for 5 minutes.
16. Centrifuge at 280 × g for 1 minute.
17. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
18. Transfer 30 µl supernatant to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
19. Resume the protocol at *Hybridize Probes* on page 42.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, or cap the tube, and store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 20 2°C to 8°C Storage

Reagent	Box Name	Instructions
EHB2	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature.
ET2	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends.
[Optional] RSB	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature if using one of the following: • **[Optional]** Illumina Custom Enrichment Panel v2 • **[Optional]** Twist Bioscience for Illumina Mitochondrial Panel.
SMB4	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.

Table 21 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EE1	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	Thaw at room temperature.
EEW	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	Bring to room temperature for 2 hours.
EPM	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	Thaw on ice.
HP3	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	Thaw at room temperature.
[Optional] Illumina Custom Enrichment Panel v2	Not applicable	Thaw at room temperature.
NHB2	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	Thaw at room temperature.

Reagent	Box Name	Instructions
PPC	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	Thaw on ice.
Twist BioScience for Illumina Exome 2.5 Panel	Not applicable	Bring to room temperature.
[Optional] Twist Bioscience for Illumina Mitochondrial Panel	Not applicable	Bring to room temperature.

Hybridize Probes

This step binds target regions of DNA within the pre-enrichment library with the exome capture probes, and optional mitochondrial genome or custom capture probes.

This protocol allows up to 10 µl probe panels to be added to each hybridization reaction.

Consumables

- EHB2 (Enrich Hyb Buffer 2)
- NHB2 (Hyb Buffer 2 + IDT NXT Blockers)
- Twist BioScience for Illumina Exome 2.5 Panel
- **[Optional]** RSB (Resuspension Buffer)
- **[Optional]** Twist Bioscience for Illumina Mitochondrial Panel (sold separately)
- **[Optional]** Illumina Custom Enrichment Panel v2 (sold separately)
- Nuclease-free water
- One of the following containers:
  - **[Plate]** 96-well PCR plate
  - **[Tube]** 8-tube strip
- One of the following seals:
  - **[Plate]** Microseal 'B' adhesive seal
  - **[Tube]** 8-tube strip caps

About Reagents

- NHB2 precipitates and separates during storage. Follow the NHB2 preparation instructions before first use.
- If precipitate or the bead pellet is present, make sure to reach room temperature, pipette up and down to release the pellet, and then vortex to resuspend.

Preparation

1. Prepare the following consumables:
   - EHB2:
     a. Vortex to mix.
     b. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
   - NHB2:
     a. When at room temperature, preheat to 50°C on a microheating system for 5 minutes.
     b. Vortex at maximum speed 3 times for 10 seconds each to resuspend.
     c. Centrifuge briefly.
     d. 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 well until the solution is clear.
     Use while warm to prevent precipitate from reforming.
   - Twist BioScience for Illumina Exome 2.5 Panel—Vortex to mix.
   - SMB4—If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, bring to room temperature. If you are extending the hold time, bring to room temperature at least 30 minutes before the NF-HYB program ends.
   - **[Optional]** Twist Bioscience for Illumina Mitochondrial Panel—Vortex to mix.
   - **[Optional]** Illumina Custom Enrichment Panel v2—Vortex to mix.
   - **[Optional]** RSB (Resuspension Buffer)—Vortex to mix.
2. Save the following IEE (Illumina Exome Enrichment)-HYB program.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 100 µl.
   - 98°C for 5 minutes
   - 18 cycles of 1 minute each, starting at 97°C for the first cycle, then decreasing 2°C per cycle (the 18th cycle is at 63°C)
   - Hold for 1.5 hours at 62°C.

- **[Optional]** For slight performance improvements or convenience, the hybridization hold at 62°C can be increased from 1.5 to 16 hours.

Total running time is ~2 hours.

Procedure

1. **[Optional]** Dilute Twist Bioscience for Illumina Mitochondrial Panel.
   Mitochondrial DNA (ChrM) is present in greater abundance relative to nuclear DNA in the cell, therefore the Mitochondrial Panel may be diluted before use. A dilution series of the Mitochondrial Panel is recommended before a spike-in to the Exome 2.5 Panel to achieve the desired coverage ratio. Determine the appropriate dilution empirically based on the application and sample type.
   - The Mitochondrial Panel can either be used as a single spike-in with the Exome 2.5 Panel, or as a double spike-in with both the Exome 2.5 Panel and a custom panel. Mitochondrial Panel dilution recommendations are provided for each scenario. After the appropriate dilution is determined, the diluted Mitochondrial Panel can be added to the hybridization reaction as described in step 2.

   The following tables provide examples of dilution series and the resultant sequencing coverage. Water or RSB may be used as the diluent.

Table 22 Serial Dilutions of Mitochondrial Panel for Single Spike-In

Dilution	Description	Mito Panel Starting Concentration	Mito Panel Final Concentration	Dilution Factor	Volume (µl) of Mito Panel	Volume (µl) of Diluent
1	1:10 Dilution	Stock	1:10	10	5	45
2	1:100 Dilution	1:10	1:100	10	5	45
3	1:500 Dilution	1:100	1:500	5	10	40
4	1:1000 Dilution	1:500	1:1000	2	25	25

Table 23 Mitochondrial Genome: Exome Coverage Ratios for Single Spike-In*

Pool	Mito: Exome Panel Ratio	Exome Mean Target Coverage	chrM Mean Target Coverage	chrM / Exome Coverage
1	1:1	61	4574	75
2	1:10	62	921	15

Pool	Mito: Exome Panel Ratio	Exome Mean Target Coverage	chrM Mean Target Coverage	chrM / Exome Coverage
3	1:100	63	239	4
4	1:500	64	167	3
5	1:1000	63	155	2

* Example of empirical data showing the coverage ratio for various Mitochondrial Panel dilutions. Data in Mitochondrial Genome: Exome Coverage Ratios was generated using human cell line DNA from the Coriell Institute (NA24143, NA24149, and NA24385). Enrichment for each pool was performed in 12-plex. Sequencing was performed on a NovaSeq 6000 using the S4 Reagent Kit v1.5. Analysis was performed in BaseSpace Sequence Hub. All FASTQs were downsampled to 50M total reads using FASTQ Toolkit Version 2.2.5 and enrichment analysis was performed using DRAGEN Enrichment Version 4.0.3.

Table 24 Serial Dilutions of Mitochondrial Panel for Double Spike-In

Dilution	Description	Mito Panel Starting Concentration	Mito Panel Final Concentration	Dilution Factor	Volume (µl) of Mito Panel	Volume (µl) of Diluent
1	1:5 Dilution	Stock	1:5	5	5	20
2	1:50 Dilution	1:5	1:50	10	5	45
3	1:250 Dilution	1:50	1:250	5	10	40
4	1:500 Dilution	1:250	1:500	2	25	25

2. Add the following volumes to each well of a new PCR plate or 8-tube strip *in the order listed*. For X µl volumes, refer to the following table.

> Creating a master mix of NHB2 and EHB2 negatively impacts enrichment performance. Reagents must be added in the correct order and amounts listed.

- Pre-enrichment library pool (30 µl)
- Twist BioScience for Illumina Exome 2.5 Panel (4 µl)
- **[Optional]** Diluted Twist Bioscience for Illumina Mitochondrial Panel (X µl)
- **[Optional]** Illumina Custom Enrichment Panel v2 (X µl)
- Nuclease-free water (X µl)
- NHB2 (50 µl)
- EHB2 (10 µl)

Spike-In Options	Reagent Volume (X µl)			
	Exome 2.5 Panel	Diluted Mitochondrial Panel	Custom Enrichment Panel v2	Nuclease-free water
Exome 2.5 Panel only	4	N/A	N/A	6
Single spike-in with Mitochondrial Panel	4	4	N/A	2
Single spike-in with Custom Enrichment Panel v2	4	N/A	4	2
Double spike-in with Mitochondrial Panel and Custom Enrichment Panel v2	4	2*	4	N/A

* The dilution of the Mitochondrial Panel probes should be adjusted for a final volume of 2 µl. Refer to the table *Serial Dilutions of Mitochondrial Panel for Double Spike-In* on page 45.

3. Using a pipette set to 90 µl, pipette each well 10 times to mix.
4. Centrifuge as follows.
   - **[Plate]** Seal the plate with Microseal 'B' and centrifuge at 280 × g for 30 seconds.
   - **[Tube]** Cap the tubes and centrifuge at 280 × g for 30 seconds.
5. Place the sample plate or tubes on the preprogrammed thermal cycler and run the IEE-HYB program.
6. Proceed immediately to the next procedure when the IEE-HYB program hold temperature time ends.

> Do not allow the hybridization reactions to cool. Precipitation occurs if the temperature of the hybridization reaction falls to room temperature.

Capture Hybridized Probes

This step uses magnetic beads to capture probes hybridized to the target regions of interest within the libraries. Heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- EEW (Enhanced Enrichment Wash)
- ET2 (Elute Target Buffer 2)

- HP3 (2N NaOH)
- SMB4 (Streptavidin Magnetic Beads 4)
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - **[Tube]** 1.7 ml microcentrifuge tubes and 8-tube strip
- One of the following seals:
  - **[Plate]** Microseal 'B' adhesive seal
  - **[Tube]** 8-tube strip caps
- One of the following magnetic stands:
  - **[Plate]** Magnetic Stand-96
  - **[Tube]** MagneSphere Technology Magnetic Separation Stands (12 position, 1.7 ml)

About Reagents

- EEW
  - Can be cloudy after reaching room temperature.
  - Can appear yellow.
  - Heat before use as instructed.
  - Do not centrifuge.
- SMB4
  - Make sure to use SMB4 and not Illumina Purification Beads for this procedure.
  - Must be at room temperature before use.

Preparation

1. Prepare the following consumables:
   - EE1—Pipette to mix. Centrifuge briefly before use.
   - EEW—Vortex three times for 30 seconds each. The reagent is heated during the procedure.
   - ET2—Vortex to mix. Centrifuge briefly before use.
   - HP3—Vortex to mix. Centrifuge briefly before use.
   - SMB4—Vortex to resuspend. If precipitate or the bead pellet is present, make sure to reach room temperature, pipette up and down to release the pellet, and then vortex to resuspend.
2. Preheat a minimum of one microheating system with a MIDI heat block insert to incubate the sample plate to 62°C. An optional second microheating system can be used to preheat EEW.

Procedure

Capture

1. Centrifuge the sample plate or tube at 280 × g for 30 seconds.
2. Using a pipette set to 100 µl, transfer each sample from the 96-well PCR plate or from the 8-strip tube, to the corresponding well of a new MIDI plate or to a new 1.7 ml microcentrifuge tube.
3. Vortex SMB4 to resuspend, and then add 250 µl to each well or tube, and then mix thoroughly as follows:
   - **[Plate]** Seal the plate and shake at 1200 rpm for 4 minutes.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
4. Place the sample plate or tube on the MIDI heat block insert on the microheating system, close the lid, and incubate for 15 minutes at 62°C.
   Proceed to step 5 while the sample incubates.
5. Preheat EEW (amber tube) by laying the tube on its side on the MIDI heat block insert on the second microheating system to the following temperature. If a second microheating system is not available, lay EEW on top of the MIDI plate or next to the 1.7 ml microcentrifuge tube on the MIDI heat block insert during the incubation in step 4. Keep EEW heated until step 2 of the *Wash on page 48*.
6. Centrifuge the sample plate or tube at 280 × g for 30 seconds.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Using a pipette set to 350 µl, remove and discard all supernatant from each well or tube.

Wash

1. Remove from the magnetic stand.
2. Add 200 µl preheated EEW (amber tube) to each well or microcentrifuge tube, and then mix thoroughly as follows.
   - **[Plate]** Seal and shake at 1800 rpm for 4 minutes. If splashing occurs, reduce the speed to 1600 rpm.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
3. Return unused EEW to the microheating system and keep heated.
4. Place the sample plate or tube on the MIDI heat block insert on the microheating system, close the lid, and incubate for 5 minutes at 62°C.
5. **[Tube]** Centrifuge at 280 × g for 3 seconds.
6. Immediately place the plate or microcentrifuge tube on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Using a pipette set to 200 µl, remove and discard all supernatant from each well or tube.
8. Repeat steps 1–7 two additional times for a total of three washes.

Transfer Wash

1. Remove the plate or tube from the magnetic stand.
2. Add 200 µl preheated EEW (amber tube) to each well or microcentrifuge tube, and then mix thoroughly as follows.
   - **[Plate]** Seal and shake at 1800 rpm for 4 minutes. If splashing occurs, reduce the speed to 1600 rpm.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
3. Transfer 200 µl to a new MIDI plate or a new tube.

   Transferring the reagent minimizes carryover of residual reagents that can inhibit downstream PCR.
4. Place the sample plate or tube on the MIDI heat block insert on the microheating system, close the lid, and incubate for 5 minutes at 62°C.
5. Centrifuge at 280 × g for 3 seconds.
6. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Using a pipette set to 200 µl, remove and discard all supernatant from each well or tube.
8. Centrifuge the plate or the tube at 280 × g for 30 seconds.
9. Place on a magnetic stand for 10 seconds.
10. Use a 20 µl pipette to remove and discard residual liquid from each well or from the tube.
11. Immediately proceed to *Elute* on page 49 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 samples being processed.
   Additional reagent is included in the volume to ensure accurate pipetting due to the potential of reagent foaming.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)
2. Vortex, and then centrifuge the master mix at 280 × g for 10 seconds.
3. Remove the sample plate or tube from the magnetic stand.
4. Add 23 µl Elution Master Mix to each well or tube, and then mix thoroughly as follows.
   - **[Plate]** Seal plate and shake at 1800 rpm for 2 minutes.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or tube at room temperature for 2 minutes.
6. Centrifuge at 280 × g for 30 seconds.

7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Transfer 21 µl supernatant from the MIDI plate or 1.5 ml microcentrifuge tube to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
9. Add 4 µl ET2 to each well or to the tube containing 21 µl supernatant.
10. Set pipette to 20 µl and slowly pipette each well or the tube 10 times to mix.
11. Centrifuge the sample plate or the tube at 280 × g for 30 seconds.

Amplify Exome-Enriched Library

This step uses PCR to amplify the targeted IDPE Exome 2.5 library.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- **[Plate]** Microseal 'B' adhesive seal
- **[Tube]** 8-tube strip caps

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, then centrifuge briefly.
   - PPC—Invert to mix, then centrifuge briefly.
2. Save the following AMP program on the thermal cycler.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 45 seconds
   - 12 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.

Procedure

1. Add 5 µl PPC to each well or tube.

2. Add 20 µl EPM to each well or tube and mix thoroughly as follows.
   - **[Plate]** Seal plate and shake at 1200 rpm for 1 minute.
   - **[Tube]** Pipette 10 times to mix, and then cap the 8-tube strip.
3. Centrifuge the plate or 8-tube strip at 280 × g for 30 seconds.
4. 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.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 25 15°C to 30°C Storage

Reagent	Box Name	Instructions
IPB	Illumina DNA/RNA Prep - IPB Tagmentation Buffers	Bring to room temperature for 30 minutes.

Table 26 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes.

Clean Up Amplified Exome-Enriched Library

This step uses IPB to purify the enriched library pools and remove unwanted products.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- **[Plate]** Microseal 'B' adhesive seals
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate

  - **[Tube]** 1.7ml tube
- One of the following magnetic stands:
  - **[Plate]** Magnetic Stand-96
  - **[Tube]** MagneSphere Technology Magnetic Separation Stands (12 position, 1.7 ml)

About Reagents

- IPB
  - Must be at room temperature before use.
  - Resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. RSB—Vortex to mix.
2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Centrifuge the PCR samples at 280 × g for 30 seconds.
2. Transfer 45 µl supernatant from each well of the PCR plate or from the 8-tube strip, to the corresponding well of a new MIDI plate or a new 1.7 ml microcentrifuge tube.
3. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
4. Add 40.5 µl IPB to each well or tube, and then mix thoroughly as follows.
   - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or tube at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 1 minute.
7. Place on a magnetic stand and wait until liquid is clear (~5 minutes).
8. Using a pipette set to 85 µl, remove and discard all supernatant from each well or tube.
9. Wash beads as follows.
   a. Keep on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.

b. Wait for 30 seconds.
c. Without disturbing the beads, remove and discard supernatant.

10. Wash beads a **second** time.
11. Use a 20 µl pipette to remove and discard residual EtOH from each well or tube.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand and add 32 µl RSB to each well or tube.
14. Mix thoroughly as follows.
    - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
15. Incubate the plate or tube at room temperature for 5 minutes.
16. Centrifuge at 280 × g for 30 seconds.
17. Place on a magnetic stand and wait until liquid is clear (~2 minutes).
18. Transfer 30 µl supernatant from the 96-well PCR plate or from the 8-tube strip, to the corresponding well of a new 96-well PCR plate or a new 1.7 ml microcentrifuge tube.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, or cap the tube, and store at -25°C to -15°C for up to 7 days.

Check Exome-Enriched Libraries

Perform the following to check the concentration and quality of the exome-enriched libraries.

1. Quantify 1 µl enriched libraries using the Qubit dsDNA BR Assay Kit to determine library concentration. Follow the manufacturer's recommendations.
2. Run 1 µl pooled library or the individual libraries on a Bioanalyzer using a high sensitivity DNA kit. Expect a mean fragment size ~350 bp and distribution of DNA fragments with a size range from ~200 bp to ~1000 bp.

Figure 3 Bioanalyzer Trace: Example 1

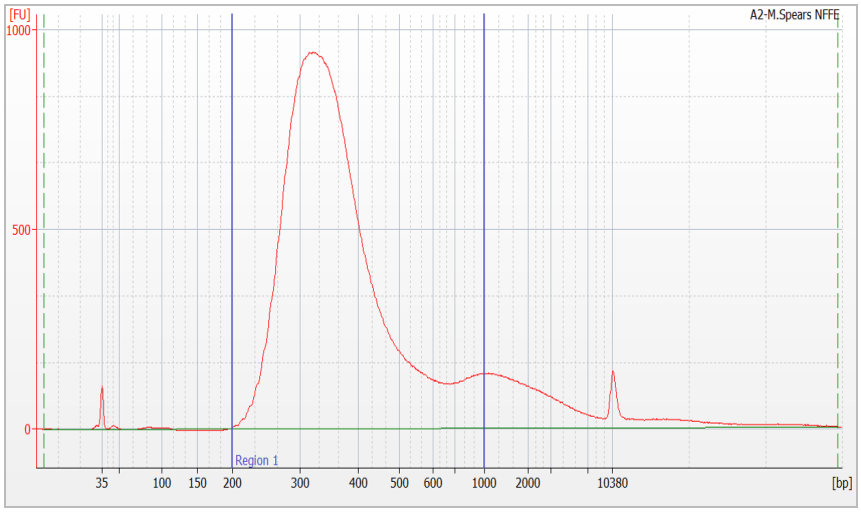
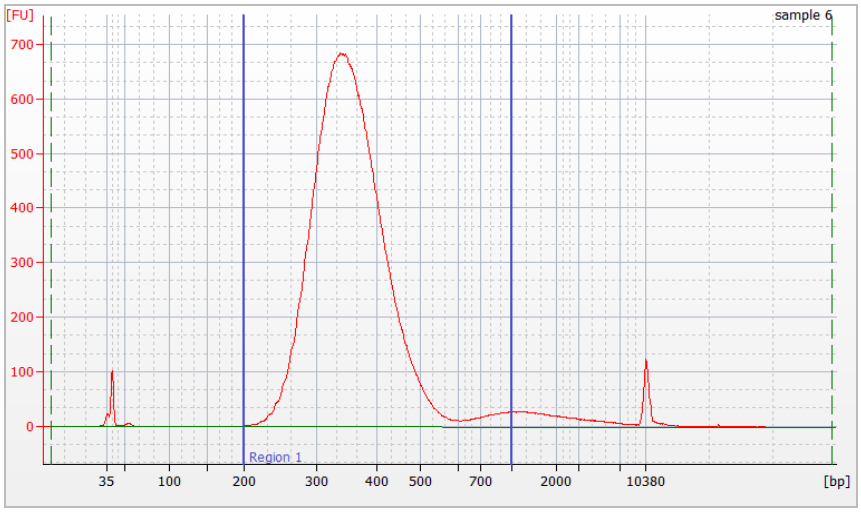


Figure 4 Bioanalyzer Trace: Example 2



Dilute Libraries to the Starting Concentration

This step dilutes exome-enriched 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 101 cycles per read (2 × 101) and 10 cycles per Index Read. If you want optional and additional overlapped reads/raw coverage, you can sequence up to 2 × 126 or 2 × 151.

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

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

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	Starting Concentration (nM)	Final Loading Concentration (pM)
NextSeq 550 and NextSeq 500	2	1.4–1.5
NextSeq 1000 and NextSeq 2000*	2	1000
NovaSeq 6000	0.875–0.925	175–185
NovaSeq X	2	150

* For the NextSeq 1000 and NextSeq 2000 systems, use the RSB with Tween 20 supplied with the system to dilute below 10 nM.

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 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.
   - Refer to the Illumina Denature and Dilute protocol generator and the Illumina support site for pool and denature instructions.
   - 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.

Resources & References

The support pages on the Illumina website provide software, training resources, product compatibility information, and the following documentation. Always check support pages for the latest versions.

Additional Resources

Resource	Description
DesignStudio support page	Provides information about DesignStudio and guidelines to create and order an Illumina custom enrichment panel.
Index Adapters Pooling Guide	Provides pooling guidelines and dual-index strategies for using the 10-base pair Illumina DNA/RNA UD Indexes.
Illumina Adapter Sequences	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
Illumina DNA/RNA UD Indexes support page	Provides information about Illumina DNA/RNA Unique Dual (UD) indexes.

Revision History

Document	Date	Description of Change
Document #1000000157112 v07	May 2026	Correction to Amplify Exome-Enriched Library preparation section, starting concentration for NovaSeq 6000, and Post Tagmentation Clean Up procedure. Standardized thermal cycler specifications.
Document #1000000157112 v06	October 2025	Updated SMB3 to SMB4. Updated SMB4 thaw and preparation instructions. Updated thermal cyclers. Updated Protocol procedural format. Added NovaSeq X.
Document #1000000157112 v05	March 2024	Added option to use an Illumina Custom Enrichment Panel v2.

Document	Date	Description of Change
Document #1000000157112 v04	January 2024	Changed product name from Illumina DNA Prep with Exome 2.0 Plus Enrichment to Illumina DNA Prep with Exome 2.5 Enrichment. Added Illumina DNA/RNA UD Indexes to kit contents, and list of optional index adapters. Removed reference to IDT for Illumina DNA/RNA UD Indexes throughout the document. Removed reference of obsolesced HiSeq 2500 and HiSeq 2000 Systems. Added HTML format.
Document #1000000157112 v03	May 2023	Updated protocol to include optional mitochondrial DNA enrichment. Updated verbiage from guide to documentation throughout. Changed verbiage from pre-enriched to pre-enrichment. Removed the Nextera brand index since it is discontinued. Removed document resources no longer in use in additional recommended resources.
Document #1000000157112 v02	August 2022	Removed the following: • Nextera XT reference from index adapters pooling reference in addition resources. • 1-plex pool plexity. Updated • the link number to DNA Prep Checklist in additional resources. • reference link for index adapter sequences. • reference for NextSeq 1000/2000. • Hybridize Probes total time to 2 hour minimum. • 10-49 ng recommended input to 250–500. • reagents to vortex instead of invert and thaw times to 2 hours for SMB3, EE1, and EEW. • shipping temperatures for Twist Exome kit and Fast Hyb kit.

Document	Date	Description of Change
Document #1000000157112 v01	June 2022	Updated document title. Added overview of Exome 2.0 plus kit information and panel coverage details table. Updated required DNA input quality in the sample input recommendations table. Updated gDNA Input ≥ 50 ng is automatically normalized during library prep. Added contamination list to assessing gDNA purity. Removed custom protocol selector from resource list. Updated guide workflow image to reflect using exome panel. Removed protocol introduction section. Added protocols and reagent kit list to sample input recommendations. Updated instructions for a secondary assessment of the gDNA sample. Added instructions for preparing IDT for Unique Dual index plates using the NextSeq 500 System. Updated prepare for UD indexes instructions to give expanded explanation for preparing fewer than 96 samples. Updated eBLT storage instructions to include incubation and specify to store vertically. Added more detailed steps for Tagment Genomic DNA. Updated preparation steps for Post Tagmentation Cleanup to include a multichannel pipette and processing large numbers of samples. Updated prepare for pooling instructions to record the index before starting library prep. Updated the Single Methodology to IPB. Removed free Adapter Blocking Reagent references due to end of life. Removed Nextera XT Troubleshooting technical note reference. Removed appendix labels.
Document #1000000157112 v00	April 2021	Initial release.

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