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Illumina DNA Prep with Exome 2.0 Plus Enrichment

Reference Guide

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Revision History

Document	Date	Description of Change
Document #10000000157112 v02	August 2022	Removed • 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 #10000000157112 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.

Document	Date	Description of Change
		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 #10000000157112 v00	April 2021	Initial release.

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Overview

The Illumina®DNA Prep with Exome 2.0 Plus Enrichment kits provide all the reagents necessary including library prep and enrichment reagents, clean up/size selection beads, human exome panel, and indexes needed to make high quality, human, whole genome libraries followed by hybridization-based enrichment of the exome portion of those libraries.

The kit offers the following:

- Enables the construction of 96 libraries followed by enrichment of those libraries in eight hybridization reactions, each containing 12 libraries (12-plex enrichment) for total output of 96 exome-enriched, human libraries.
- The number of libraries in each enrichment reaction may be reduced but will require optimization and will reduce the total number of samples enriched with each kit.

Table 1 Twist Bioscience® for Illumina Exome 2.0 Plus 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 product support page to download the complete BED file for this design.

This guide explains how to prepare up to 96 uniquely dual-indexed paired-end whole genome libraries from DNA using the Illumina DNA Prep with Exome 2.0 Plus Enrichment workflow.

The workflow:

- Uses on-bead tagmentation, an enzymatic reaction, to both fragment the input DNA and add adapter sequences in only 15 minutes.
- Uses innovative sample normalization at inputs ≥ 50 ng so that library quantitation is not required prior to hybridization.
- Provides sufficient volume of each reagent to enable automation on most liquid handler platforms.
- Enables preparation of libraries directly from whole blood or saliva samples when using our recommended protocol (refer to *Supplemental Procedures* on page 38).

DNA Input Recommendations

The Illumina DNA Prep with Exome 2.0 Plus Enrichment protocol 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-enriched library yield (prior to 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 and 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 the above testing produces ratios outside of the acceptable limits, one potential solution is to dilute the starting material in 10 mM Tris-HCl, pH 7.5–8.5 to dilute the contaminant to levels that will have no or minimal impact on the library prep enzymes. Another option is to repurify the gDNA sample using methods with Illumina Purification Beads outlined in *Single IPB Methodology* on page 54.

Blood and Saliva Input Recommendations

The Illumina DNA Prep with Exome 2.0 Plus Enrichment protocol is compatible with fresh whole blood (requires the Flex Lysis Reagent Kit) and saliva sample inputs. For information about protocols specific to blood and saliva, refer to *[Optional] Blood Lysis* on page 38 or *[Optional] Saliva Lysis* on page 40.

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 that observed when using 50–1000 ng gDNA input. Blood and saliva are heterogeneous sample types, therefore the ability of Illumina DNA Prep with Exome 2.0 Plus Enrichment 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 Illumina DNA Prep with Exome 2.0 Plus Enrichment 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 14.

Table 2 Sample Input Recommendations

Sample Input Type	Quantification of Input DNA Required	Required DNA Input Quality	Normalized Pre-Enriched 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

Additional Resources

The following resources provide instructions and guidelines for using the prepared libraries. Refer to the Illumina DNA Prep with Exome 2.0 Plus Enrichment support page for additional information.

- Compatible products and requirements for recording sample information, sequencing libraries, and analyzing data.
- Questions and answers about using the kit.
- Training videos about the kit and courses for related products and subjects.
- The latest versions of the kit documentation.

Table 3 Additional Recommended Resources

Resource	Description
Illumina DNA Prep with Enrichment Checklist (document # 1000000048601)	Provides a checklist of steps for the experienced users.
Illumina DNA Prep with Enrichment Consumables & Equipment List (document # 1000000048602)	Provides an interactive checklist of user-supplied consumables and equipment.
Index Adapters Pooling Guide (document #1000000041074)	Provides pooling guidelines and dual-index strategies for using the 10-base pair IDT for Illumina DNA/RNA UD Indexes or 8-base pair Nextera XT and Nextera XT v2 Indexes with the Illumina DNA Prep with Exome 2.0 Plus Enrichment kit.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
IDT for Illumina DNA/RNA UD Indexes support page	Provides information about IDT for Illumina DNA/RNA Unique Dual (UD) indexes.

Protocol

This section describes the Illumina DNA Prep with Exome 2.0 Plus Enrichment 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 consumables. This protocol includes the library prep and enrichment reagents, Twist BioScience for Illumina Exome 2.0 Plus Panel, IPB, and index adapter plates. Refer to *Supporting Information* on page 43.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

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 support website.

- For low-plexity pooling strategies (2-plex to 9-plex), refer to the *Index Adapters Pooling Guide (document #1000000041074)*.
- For index adapter sequences and information about recording the sequences, refer to Illumina Adapter Sequences (document # 1000000002694).

Supported Enrichment Plexities

Illumina DNA Prep with Exome 2.0 Plus 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-enriched libraries (12 recommended, 1-11 optional) pooled together in one enrichment reaction for hybridization with the Twist BioScience for Illumina Exome 2.0 Plus Panel. 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 (8 reactions provided in these kits).

To calculate the total number of postenriched 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 postenriched libraries.

When pooling pre-enriched libraries, Illumina DNA Prep with Exome 2.0 Plus Enrichment reagents support the number of enrichment reactions and plexity indicated.

Table 4 Supported Enrichment Plexities

Illumina DNA Prep with Exome 2.0 Plus Enrichment Reagents	Enrichment Reactions	Enrichment Plexity
96-sample kit	8 reactions	12-plex

Tips and Techniques

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
  - 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 (Enrichment BLT, 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. To avoid resettling of 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.

Handling Tagmentation Wash Buffer (TWB)

- Pipette slowly to minimize foaming.

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

- IDT for Illumina® DNA/RNA UD Indexes use 10-base pair index codes that differ from other Illumina index adapters, which use eight base pair index codes. Confirm that the sequencing system is configured for 10-base pair index codes. If using NextSeq 500 system, the read lengths must be modified to accommodate 10 base pair indexes. Visit the Compatible Products web page.
- Illumina DNA Prep with Exome 2.0 Plus Enrichment is compatible with IDT® for Illumina® DNA/RNA Unique Dual (UD), or IDT for Illumina Nextera DNA Unique Dual (UD).
- Each index plate is for single use only.

Illumina DNA Prep with Exome 2.0 Plus Enrichment Workflow

The following diagram illustrates the Illumina DNA Prep with Exome 2.0 Plus Enrichment workflow. Safe stopping points are marked between steps.

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

Figure 1 Illumina DNA Prep with Exome 2.0 Plus Enrichment Workflow

Tagment Genomic DNA

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

Consumables

- eBLT (Enrichment Bead-Linked Transposomes) (yellow cap)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 96-well PCR plate
- 1.7 ml microcentrifuge tubes
- 8-tube strip
- Microseal 'B' adhesive seal
- Pipette tips
  - 200 µl multichannel pipettes

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 vertically at temperatures above 2°C.
  - Do not use eBLT that has been stored below 2°C.

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
eBLT (yellow cap)	2°C to 8° C	Bring to room temperature by incubating at room temperature for 10 minutes. Vortex to mix. Do not centrifuge before pipetting.
TB1	-25°C to -15°C	Bring to room temperature. 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.
   - If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
2. Vortex eBLT (yellow cap) vigorously for 10 seconds to resuspend. Repeat as necessary.
3. 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 for accurate pipetting.
4. Vortex the Tagmentation Master Mix thoroughly to resuspend.
5. Divide the Tagmentation Master Mix volume equally.
   [Optional] Use a 8-tube strip if using an 8-channel multi-channel pipette.
6. Transfer 20 µl of Tagmentation Master Mix to each well of the plate or tube.
   [Optional] Using a 200 µl 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.
7. Using a 200 µl multichannel pipette set to 40 µl, pipette 10 times to mix the Tagmentation Master Mix and the gDNA sample, then seal the plate. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
8. Place on the preprogrammed thermal cycler and run the TAG program.
9. 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) (red cap)
- TWB (Tagmentation Wash Buffer)

- 96-well plate magnet
- 8-tube strips
- Microseal 'B' adhesive seal
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

About Reagents

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
ST2 (red cap)	15° to 30° C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved.
TWB	15° to 30° C	Vortex to mix.

Procedure

1. Let the 96-well PCR plate stand at room temperature for 2 minutes.
2. Add 10 µl ST2 (red cap) to each well of the plate. If you are using a multichannel pipette, pipette ST2 into an 8-tube strip, and then transfer the appropriate 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.
4. Seal the plate and incubate at room temperature for 5 minutes.
5. Place the plate on the magnetic stand and wait until liquid is clear (~3 minutes).
6. **[≤ 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 and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads. A deliberately slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.
   c. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
   d. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
   e. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
   f. Repeat steps b–e two times for a total of three washes.

7. **[> 48 samples]** Wash as follows.
    a. Perform steps b and c in 1- or 2-column increments until all columns have been processed.
    b. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
    c. Immediately after, slowly add 100 µl TWB directly onto the beads using a deliberately slow pipetting technique.
    d. Remove from the magnetic stand.
    e. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
    f. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
    g. Perform steps h and i in 1- or 2-column increments until all columns have been processed.
    h. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
    i. Immediately add 100 µl TWB directly onto the beads.
    j. Repeat steps d–g two times for a total of three washes.
8. Pipette each well slowly to resuspend the beads. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
9. Seal the plate and place on the magnetic stand until the liquid is clear (~3 minutes). Keep on the magnetic stand until step 4 of the *Procedure* section in *Amplify Tagmented DNA*.
   The TWB 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 prepaired 10-base pair Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for library clustering on the flow cells. To confirm the indexes of libraries being pooled for enrichment have the appropriate color balance, refer to *Index Adapters Pooling Guide (document #1000000041074)*.

For a list of compatible index adapter plates for use with this protocol, refer to *Kit Contents* on page 43.

Consumables

- EPM (Enhanced PCR Mix)
- Index adapter plate
- Low DNA binding PCR Plate
- Nuclease-free water
- 1.7 ml microcentrifuge tubes
- Microseal 'B' adhesive seal
- 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. Refer to the *Tips and Techniques* on page 7 for best practices when working with the Index plates.
  - 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:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Invert to mix, then briefly centrifuge.
Index adapter plate	-25°C to -15°C	Thaw at room temperature then keep on ice.

2. Save the following eBLT PCR program on a thermal cycler using the appropriate number of PCR cycles indicated in the table in this step.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 72°C for 3 minutes

   Note the initial 72°C incubation before the 98°C denaturation step. This 72°C incubation is critical to successful library preparation and must be performed.

   - 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 Amount/Type	Number of PCR Cycles (X)
10–49 ng genomic DNA	12

Sample Input Amount/Type	Number of PCR Cycles (X)
50–1000 ng genomic DNA	9
Saliva	9
Blood	9

Procedure

1. Combine the following volumes 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 to ensure accurate pipetting.
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 well.
6. Immediately pipette 10 times 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 x 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 low DNA binding PCR Plate above the index adapter plate and press down to puncture the foil seal. Discard the low DNA binding PCR Plate used to puncture the foil seal.
10. Using a new pipette tip for each sample, 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. Ensure the beads are fully resuspended before proceeding to the next step.
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.

Clean Up Libraries

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

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (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 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	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 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. 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 (~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. For gDNA, blood, or saliva, perform the following steps:

   For faster turnaround, use the *Single IPB Methodology* on page 54. The two-step, double sided clean up process here will produce optimal results. If speed is more important, a 1-step clean up can be performed. If you would like to attempt enrichment of FFPE samples, single plex enrichment and 1-sided cleanup can be attempted, but performance is not guaranteed.

   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 of the MIDI plate 10 times to mix. Alternatively, seal the plate and shake 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 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 of the MIDI plate 10 times to mix. Alternatively, seal the plate and shake at 1800 rpm for 1 minute.
   i. Discard the first plate.
6. Incubate 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 all supernatant.
9. Wash two times as follows.
   a. With the plate 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. Use a 20 µl pipette to remove and discard residual EtOH.
11. Air-dry on the magnetic stand for 5 minutes.
12. Remove from the magnetic stand.
13. Add 17 µl RSB to the beads.
14. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
15. Incubate at room temperature for 2 minutes.
16. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

17. 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 the pre-enriched libraries 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.

Storage procedure 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.

Perform the following procedure to qualify pre-enriched libraries.

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

1. Assess quality of 1 µl library or pooled libraries using one of the following methods.
   - Add 1 µl RSB to the 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 Figure 2 and Figure 3.

Figure 2 Example Fragment Analyzer Trace

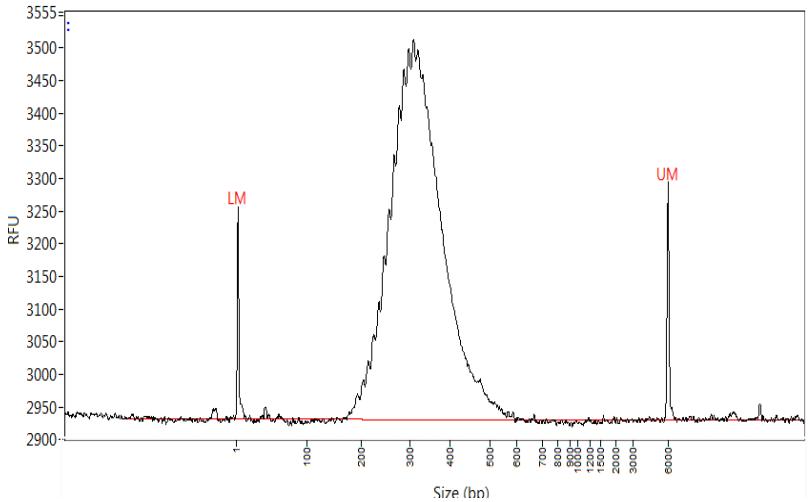
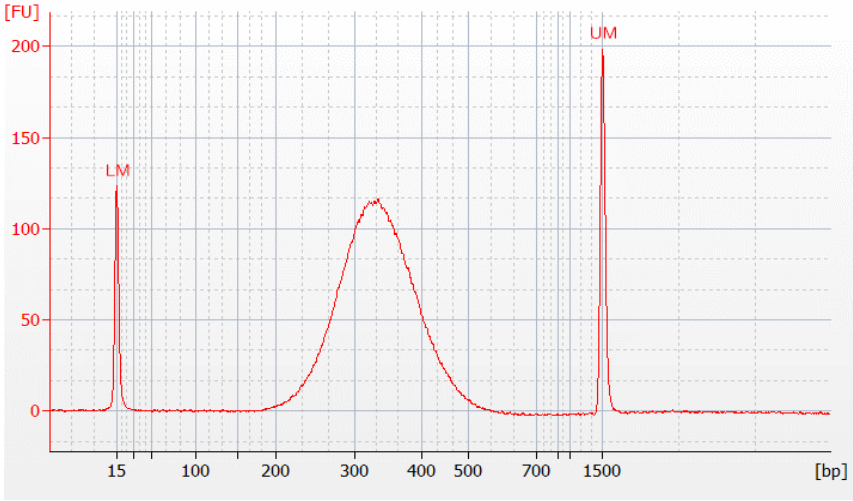


Figure 3 Example Bioanalyzer Trace



Pool Pre-Enriched Libraries

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

Pooling Methods

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

Table 5 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-enriched library yields were normalized during tagmentation, which uses eBLT. This normalization enables you to pool equal volumes of each pre-enriched library in a final pool volume ≤ 30 µl (target 250–500 ng per sample).

- After pre-enriched library quantification, all sample input types can be pooled by mass to achieve optimal library balance and similar number of sequencing reads per library.
- The final yield of pre-enriched 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-enriched libraries are not quantified, then proceed to pooling by volume. This can only be performed when starting with ≥ 50 ng gDNA input.

Quantify Pre-Enriched Libraries

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

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

   Table 6 Expected Pre-Enriched Library Yield

Sample Input Type (ng)	Pre-enriched 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-enriched library samples prepared by the same user, reagent lot, and index adapter plate.

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

For a standard 12-plex pool: Combine 2.5 µl of each pre-enriched library in a 1.7 ml microcentrifuge tube to generate a 12-plex pool at a total final pool volume of 30 µl.

When preparing a pool of lower plexity (<12 pre-enriched libraries per pool): Combine 2.5 µl of each pre-enriched library in a 1.7 ml microcentrifuge tube, then add RSB to bring the total final pool volume up to 30 µl.

1. Using the sample tracking method you chose in *Prepare for Pooling* on page 6, record the indexes for the libraries you plan to pool in this step.
2. Pool pre-enriched libraries based on the sample volumes in the following table.

Library Pool Plexity	Each Pre-Enriched Library Volume (µl)	Total Volume (µl)
1-plex	14	30 (with 16 RSB)
12-plex	2.5	30

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.

Pool Pre-enriched Libraries by Mass

1. Using the sample tracking method you chose in *Prepare for Pooling* on page 6, 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-enriched 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 *[Optional] Concentrate Pooled Libraries* on page 23.

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

Table 7 Pooling by Mass Guidelines

	Inputs per Pre-enriched 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	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-enriched libraries by volume instead of by mass. Pooling by mass will produce more consistent sequencing reads from each enriched library within the pool.

[Optional] Concentrate Pooled Libraries

If the total volume of the pooled pre-enriched libraries is > 30 µl, the pool must be concentrated to a final volume of 32 µl, from which 30 µl is transferred for hybridization. Use this bead-based method to achieve a final volume of 32 µl.

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**] 1.7 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 before each use
  - Vortex frequently to make sure that 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	Let stand at room temperature for 30 minutes. Vortex and invert to mix. Resuspend IPB beads.
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 instead of MIDI plate well to prevent overflowing of the reagents from the MIDI plate wells.

3. Vortex and invert IPB multiple times to resuspend.
4. Add 2.5x pool volume of IPB to each well or to the microcentrifuge tube, and then mix thoroughly as follows. For example, if your library pool volume is 100 µl, add 250 µl IPB to the well.
   - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed for 10 seconds. Repeat three times
5. Incubate the plate or the tube at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 10 seconds.
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 two times 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 the tube.

10. Use a 20 µl pipette to remove and discard residual 80% EtOH.
11. Air-dry on the magnetic stand for 5 minutes.
12. Remove from the magnetic stand and add 32 µl RSB to each well or to the tube.
13. 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 Repeat three times.
14. Incubate the sample plate or the tube at room temperature for 5 minutes.
15. Centrifuge at 280 × g for 10 seconds.
16. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
17. Transfer 30 µl supernatant to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
18. Resume the protocol at *Hybridize Probes* on page 25.

SAFE STOPPING POINT

If you are stopping:

Seal the plate with Microseal 'B' adhesive seal, Microseal 'F' foil seal, or cap the 1.7 ml microcentrifuge tube and store at -25°C to -15°C for up to 30 days.

Hybridize Probes

This step binds target regions of the DNA within the pre-enriched library with the exome capture probes.

This protocol requires only 4 µl exome probes per hybridization reaction, which is less than other Illumina enrichment protocols.

Consumables

- EHB2 (Enrich Hyb Buffer 2)
- Twist BioScience for Illumina Exome 2.0 Plus Panel (green cap)
- NHB2 (Hyb Buffer 2 + IDT NXT Blockers) (blue cap)
- RSB (Resuspension Buffer)
- 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.

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 well until the solution is clear.
Twist BioScience for Illumina Exome 2.0 Plus Panel	-25°C to -15°C	Bring to room temperature. Vortex to mix. Use a total probe volume of 4 µl per hybridization reaction.
NHB2 (blue cap)	-25°C to -15°C	Thaw at room temperature. When at room temperature, preheat to 50°C on a microheating system for 5 minutes. Vortex at maximum speed three 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 well 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 1.5-hour hold in the IEE (Illumina Exome Enrichment)-HYB program, bring to room temperature. If you are extending the hold time, bring to room temperature at least 2 hours before the IEE (Illumina Exome Enrichment)-HYB program ends.

Item	Storage	Instructions
EEW(amber tube)	-25°C to -15°C	If you are proceeding to the next procedure immediately after the 1.5-hour hold in the IEE (Illumina Exome Enrichment)-HYB program, bring to room temperature.

2. Save the following IEE (Illumina Exome Enrichment)-HYB program

   The IEE-HYB protocol has been optimized and validated using Bio-Rad C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module or Bio-Rad DNA Engine Tetrad 2 (refer to the equipment table in *Equipment* on page 50). Comparable performance is not guaranteed when using alternate equipment.

   - 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
   - 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. Add the following volumes to each well of a new PCR plate or 8-tube strip ***in the order listed***. Creating a master mix of NHB2 and EHB2 negatively impacts enrichment performance.
   - Pre-enriched library pool (30 µl)
   - NHB2 (blue cap) (50 µl)
   - Twist BioScience for Illumina Exome 2.0 Plus Panel (4 µl)
   - Nuclease-free water (6 µl)
   - EHB2 (10 µl)
2. Using a pipette set to 90 µl, pipette each well 10 times to mix.
3. 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.
4. Place the sample plate or tubes on the preprogrammed thermal cycler and run the IEE (Illumina Exome Enrichment)-HYB program.

5. Proceed immediately to the next procedure when the IEE (Illumina Exome Enrichment)-HYB program hold temperature time ends.

   Precipitation occurs if the temperature of the hybridization reaction falls below room temperature. Proceed directly from Hybridization to Capture.

Capture Hybridized Probes

This step uses Streptavidin Magnetic Beads (SMB3) to capture the Exome 2.0 Plus probes hybridized to the target regions of interest within the libraries.

Consumables

- SMB3 (Streptavidin Magnetic Beads)
- EEW (Enhanced Enrichment Wash) (amber cap)
- EE1 (Enrichment Elution Buffer 1)
- HP3 (2N NaOH)
- ET2 (Elute Target Buffer 2)
- 1.7 ml microcentrifuge tube
- 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 magnets:
  - [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
- SMB3
  - Make sure to use SMB3 and not Illumina Purification Beads for this procedure.

- 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. Vortex to mix before use.
EEW (amber tube)	-25°C to -15°C	Let stand for 2 hours to bring to room temperature. Vortex three times for 30 seconds each. The reagent is heated during the procedure.
EE1	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Centrifuge briefly before use.
HP3	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Centrifuge briefly before use.
ET2	2°C to 8°C	Bring to room temperature. Vortex to mix. Centrifuge briefly before use.

2. Preheat a 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 tubes of pooled enriched libraries at 280 × g for 30 seconds.
2. Using a pipette set to 100 µl, transfer each enriched pool from each well 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. Add 250 µl SMB3 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 vortex at high speed 3 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 pooled libraries incubates.
5. Preheat EEW (amber tube) by laying the tube on its side on the MIDI heat block insert on the microheating system to 62°C. Alternatively, 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 30.
6. Immediately centrifuge the plate or tube at 280 × g for 30 seconds.

7. Immediately 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 from the 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 3 times for 10 seconds each.
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 briefly 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 from the tube.
8. Repeat steps 1–7 two additional times for a total of 3 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 to the 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 3 times for 10 seconds each.
3. Transfer 200 µl resuspended bead solution to a new MIDI plate or to a new 1.7 ml microcentrifuge 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. **[Tube]** Centrifuge briefly for 3 seconds.
6. Immediately 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 from the 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 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.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)

   Reagent overage is included in the volume.
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 to the 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 3 times for 10 seconds each.
5. Incubate the plate or the 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 1.7 ml microcentrifuge tube to the corresponding well of a new 96-well PCR plate or to 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 Pools

This step uses PCR to amplify the enriched whole exome library pools.

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:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge briefly.
PPC	-25°C to -15°C	Thaw on ice. 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 strip tube 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 at 10°C.

Clean Up Amplified Exome-Enriched Library Pools

This step uses IPB to purify the amplified exome-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]** 1.7 ml microcentrifuge tubes
- One of the following magnets:
  - **[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
  - Vortex before each use
  - Vortex frequently to make sure that 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	15°C to 30°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
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 PCR samples at 280 × g for 30 seconds.
2. Transfer 45 µl from each well of the PCR plate or from the 8-tube strip to the corresponding well of a new MIDI plate or to a new 1.7 ml microcentrifuge tube.
3. 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 for 10 seconds. Repeat 2 times.
4. Incubate the plate or the tube at room temperature for 5 minutes.

5. Centrifuge at 280 × g for 1 minute.
6. Place on a magnetic stand and wait until liquid is clear (~5 minutes).
7. Using a pipette set to 85 µl, remove and discard all supernatant from each well or tube.
8. Wash two times as follows.
   a. With the plate 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.
9. Use a 20 µl pipette to remove and discard residual EtOH from each well or from the tube.
10. Air-dry on the magnetic stand for 5 minutes.
11. Remove from the magnetic stand and add 32 µl RSB to each well or to the tube.
12. 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 3 times for 10 seconds. Repeat 2 times.
13. Incubate the plate or the tube at room temperature for 5 minutes.
14. Centrifuge at 280 × g for 30 seconds.
15. Place on a magnetic stand and wait until liquid is clear (~2 minutes).
16. Transfer 30 µl supernatant from the 96-well PCR plate or from the strip tube 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 Library Pools

Perform the following to check the concentration and quality of each of the exome-enriched library pool.

1. Run 1 µl of the enriched libraries using the Qubit dsDNA BR Assay Kit to quantify library concentration.
2. Run 1 µl of the pooled library or the individual libraries on the Agilent Technology 2100 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 4 Bioanalyzer Trace: Example 1

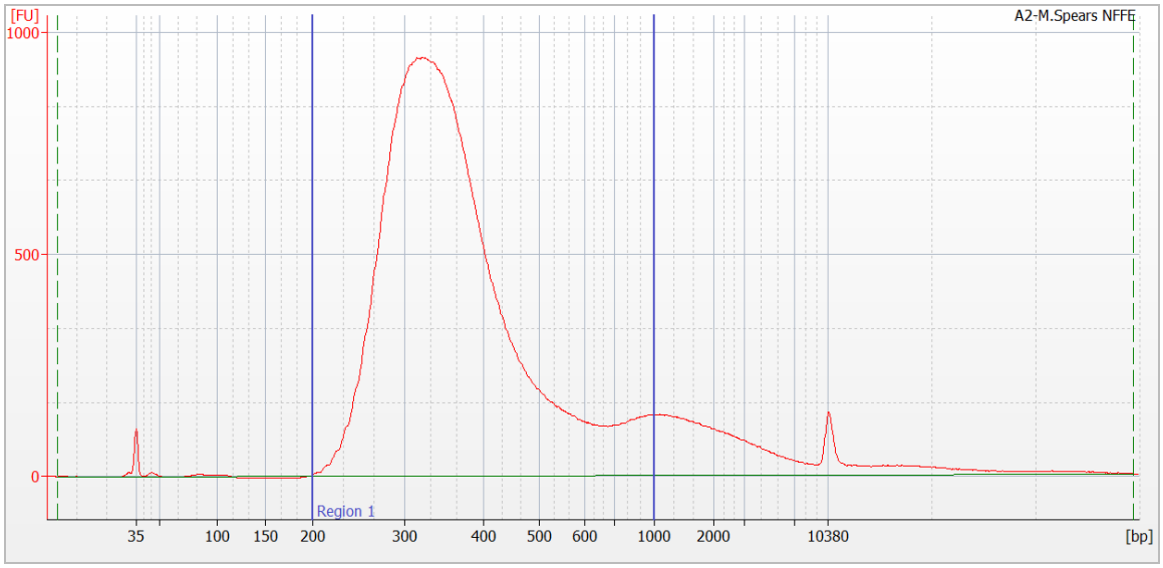
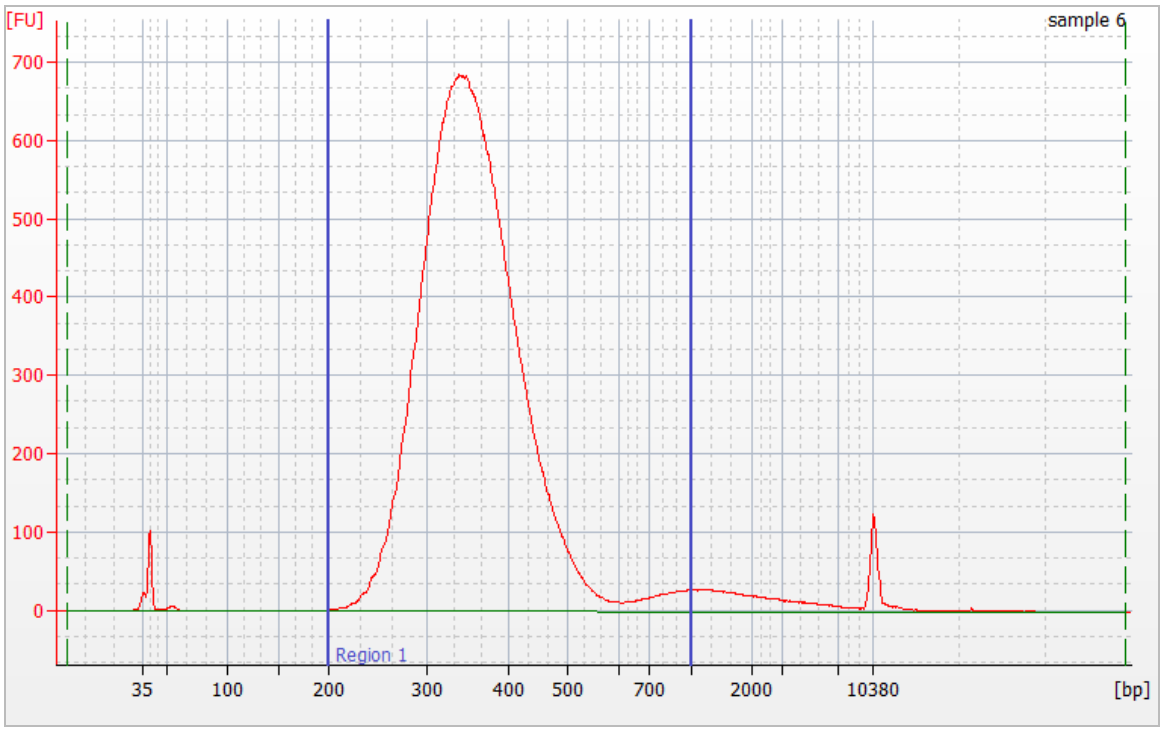


Figure 5 Bioanalyzer Trace: Example 2



Dilute Libraries to the Starting Concentration

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

Illumina recommends setting up a paired-end run with 101 cycles per read (2 × 101) and 10 cycles per Index Read. If you would like additional overlapped reads or additional raw coverage, you can sequence up to 2 × 126 or 2 × 151, but it is not required.

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 other qualification methods, use 350 bp as the average library size

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

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

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
HiSeq 4000 and HiSeq 3000 Systems	2–3	150–200
HiSeq 2500 and HiSeq 2000 Systems (high output modes)	2	16-18
NextSeq 550 and NextSeq 500 Systems	2	1.4–1.5
NextSeq 1000/2000 System	2	1000
NovaSeq 6000 System (standard workflow)	2	175–185

3. Dilute libraries using RSB as follows.
   - **Libraries quantified as a 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 NovaSeq 6000 System, refer to the Denature and Dilution Guide for denature instructions.
   - For the HiSeq 4000 and HiSeq 3000 Systems, see the cBot 2 or cBot system guide for reagent preparation instructions.
   - For the NextSeq 1000/2000 Systems loading at 1000 pM, refer to the System guide for denature instructions.

- For all other systems, see the respective denature and dilute libraries guide.

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.

Supplemental Procedures

This section provides instructions for optional procedures within the Illumina DNA Prep with Exome 2.0 Plus Enrichment workflow.

[Optional] Blood Lysis

Use this protocol when performing the Illumina DNA Prep with Exome 2.0 Plus 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. This will ensure that any bloodbourne pathogens are eliminated and the sample is no longer biohazardous.

Consumables

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

About Reagents

- IPB
  - Must be at room temperature before use
  - Vortex before each use
  - Vortex frequently to make sure that 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	15°C to 30°C	Vortex and invert to mix.
BLB	15°C to 30°C	If frozen, thaw at room temperature. If precipitates are observed, heat at 37°C for 10 minutes and vortex until resuspended.
PK1	-25°C to -15°C	Place on ice until needed.

2. Prepare 150 µl fresh 80% EtOH from absolute ethanol. Include a 20% overage.
3. Save the following BLP program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 70 µl
   - 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 (37.2µl)

   Reagent overage is included in the volume to ensure accurate pipetting.
2. Vortex and centrifuge the Lysis Master Mix.
3. Invert the EDTA collection 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. Vortex and invert IPB multiple times to resuspend.
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 will keep 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 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 80% 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 of 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 11.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 11, seal the plate with a Microseal 'B' adhesive seal and store the plate at 2°C to 8°C for up to 3 days.

[Optional] Saliva Lysis

Use this protocol when performing the Illumina DNA Prep with Exome 2.0 Plus 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.

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
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- Oragene DNA collection tubes (for saliva sample collection)

About Reagents

- IPB
  - Must be at room temperature before use
  - Vortex before each use
  - Vortex frequently to make sure that 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
Saliva samples in Oragene DNA collection tubes	Room temperature	Any time after sample collection, incubate for a minimum of 1 hour at 50°C in a water bath or an air incubator (as recommended by DNA Genotek) to lyse the cells. Following heat treatment, store at room temperature. For information on long-term storage of Oragene/saliva samples at room temperature and guarantees, see the DNA Genotek website.
IPB	15°C to 30°C	Vortex and invert to mix.

2. For each sample, prepare 150 µl fresh 80% EtOH from absolute ethanol. Include a 20% overage.

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 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 all 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 and centrifuge the plate 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 of 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 11

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.

Supporting Information

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed protocol contents, and obtained all required consumables and equipment.

Kit Contents

Completing the Illumina DNA Prep with Exome 2.0 Plus Enrichment protocol requires library prep and enrichment reagents, the exome panel, clean up/size selection beads, and index adapters, which are all 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 2.0 Plus Enrichment Kit, Tagmentation Set B (96 Samples, 12-plex)	20077595
	Illumina DNA Prep with Exome 2.0 Plus Enrichment Kit, Tagmentation Set D (96 Samples, 12-plex)	20077596
[Optional] Index adapters	IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20027213
	IDT for Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20042666
	IDT for Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20042667
	IDT for Illumina Nextera DNA UD Indexes Set C (96 Indexes, 96 Samples)	20027215
	IDT for Illumina Nextera DNA UD Indexes Set D (96 Indexes, 96 Samples)	20027216
[Optional] Blood lysis[1]	Flex Lysis Reagent Kit (96 samples)	20018706
[Optional] Additional reagents	Illumina Adapter Blocking Reagents (12 reactions)	20024144
	Illumina Adapter Blocking Reagents (48 reactions)	20024145

[1] Required when starting the protocol from fresh whole blood samples and not purified blood gDNA.

Illumina DNA Prep with Exome 2.0 Plus Enrichment - Kit Contents

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

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

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

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

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

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

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

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

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

Twist BioScience for Illumina Exome 2.0 Plus Panel, Store at -25°C to -15°C

The following reagents are shipped at frozen temperature.

Tube Quantity (96 Samples)	Acronym	Reagent Name	Tube Cap Color
1	Not Applicable	Twist BioScience for Illumina Exome 2.0 Plus Panel	Green

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

The following reagents are shipped at 4°C.

Tube Quantity (96 Samples)	Acronym	Reagent Name	Tube Cap Color
2	SMB	Streptavidin Magnetic Beads	Clear
1	RSB	Resuspension Buffer	Clear
1	EHB2	Enrich Hyb Buffer 2	Clear
1	ET2	Elute Target Buffer 2	Clear

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

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

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

IDT for Illumina DNA UD Indexes, Store at -25°C to -15°C

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

For index adapter sequences, refer to *Illumina Adapter Sequences (document # 1000000002694)*.

Description
IDT for Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)
IDT for Illumina Nextera DNA UD Indexes Set D (96 Indexes, 96 Samples)

[Optional] IDT for Illumina DNA/RNA UD Indexes, Store at -25°C to -15°C

For index adapter sequences, refer to *Illumina Adapter Sequences (document # 1000000002694)*

Description
IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)

[Optional] IDT for Illumina Nextera DNA UD Indexes, Store at -25°C to -15°C

For index adapter sequences, refer to *Illumina Adapter Sequences (document # 1000000002694)*.

Description
IDT for Illumina Nextera DNA UD Indexes Set C (96 Indexes, 96 Samples)

[Optional] Flex Lysis Reagent Kit

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

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

Symbol Descriptions

The following table describes the symbols on the shipment packaging, consumable, or consumable packaging.

Symbol	Description
	Indicates the direction to the top of the box.

Symbol	Description
	Indicates that the contents are fragile and must be handled with care.
	Storage temperature range in degrees Celsius. Store the consumable within the indicated range.[1]
	The date the consumable expires. For best results, use the consumable before this date.
	Indicates the manufacturer (Illumina).
	The intended use is Research Use Only (RUO).
	Indicates the part number so that the consumable can be identified.[2]
	Indicates the batch code to identify the manufacturing batch or lot of the consumable.[1]
	Indicates a health hazard.

[1] Storage temperature can differ from shipping temperature.
[2] Ref identifies the individual component, while LOT identifies the lot or batch the component belongs to.

Consumables and 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
1.7 ml microcentrifuge tubes	General lab supplier
10 µl pipette tips	General lab supplier
10 µl multichannel pipettes	General lab supplier
10 µl single channel pipettes	General lab supplier
20 µl pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl pipette tips	General lab supplier
200 µl single channel pipettes	General lab supplier
200 µl multichannel pipettes	General lab supplier
1000 µl pipette tips	General lab supplier
1000 µl single channel pipettes	General lab supplier
96-well 0.8 ml Polypropylene Deep well Storage Plate (MIDI plate)	Thermo Fisher Scientific, part # AB-0859
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Distilled water	General lab supplier
Eppendorf twin.tec™ 96 Well Low DNA Binding PCR Plates, 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, 0.2 ml	General lab supplier

Consumable	Supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, catalog # 89094-658
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, product # E7023
Nuclease-free water	General lab supplier
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, catalog # Q32850 or Q32853
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
One of the following kits, depending on quantification method: • [Fragment Analyzer] High Sensitivity NGS Fragment Analysis Kit • [Bioanalyzer] Agilent DNA 1000 Kit (2) • [Bioanalyzer] Agilent High Sensitivity DNA Kit (2)	One of the following suppliers, depending on instrument: • Advanced Analytical, catalog # DNF-474 • Agilent, catalog # 5067-1504 • Agilent, catalog # 5067-4626
Tris-HCl 10 mM, pH 8.5	General lab supplier

Consumables for Plate Workflow

Consumable	Supplier
96-well 0.8 ml Polypropylene Deep well Storage (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
[Blood] Flex Lysis Reagent Kit	Illumina, catalog # 20015884
[Blood] EDTA Blood Collection tubes	Various suppliers
[Salivia] Oragene DNA Collection Kit for Saliva	Genotek, catalog # OGR-500 or OGD-510

Equipment

Make sure that you have the required 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.

Equipment	Supplier
Microcentrifuge	General lab supplier
Microheating System-Hybex System for Illumina	SciGene, catalog # • 1057-30-0 (115 V) or • 1057-30-2 (230 V)
MIDI Heat Block Insert for SciGene Hybex System	Illumina, catalog # BD-60-601
Qubit Fluorometer 3.0	Thermo Fisher Scientific, catalog # Q33216 or Q33217
Vortexer	General lab supplier
One of the following analyzers: Advanced Analytical: • Fragment Analyzer™ Agilent Technologies: • 2100 Bioanalyzer Desktop System	Advanced Analytical, refer to web product pages for catalog numbers Agilent Technologies: • Part # G2940CA
[Saliva] Water or air incubator capable of reaching 50°C	DNA Genotek
[Optional] Vacuum concentrator Note: Use when concentrating a pooled library.	General lab supplier

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 the recommended settings for the thermal cyclers. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

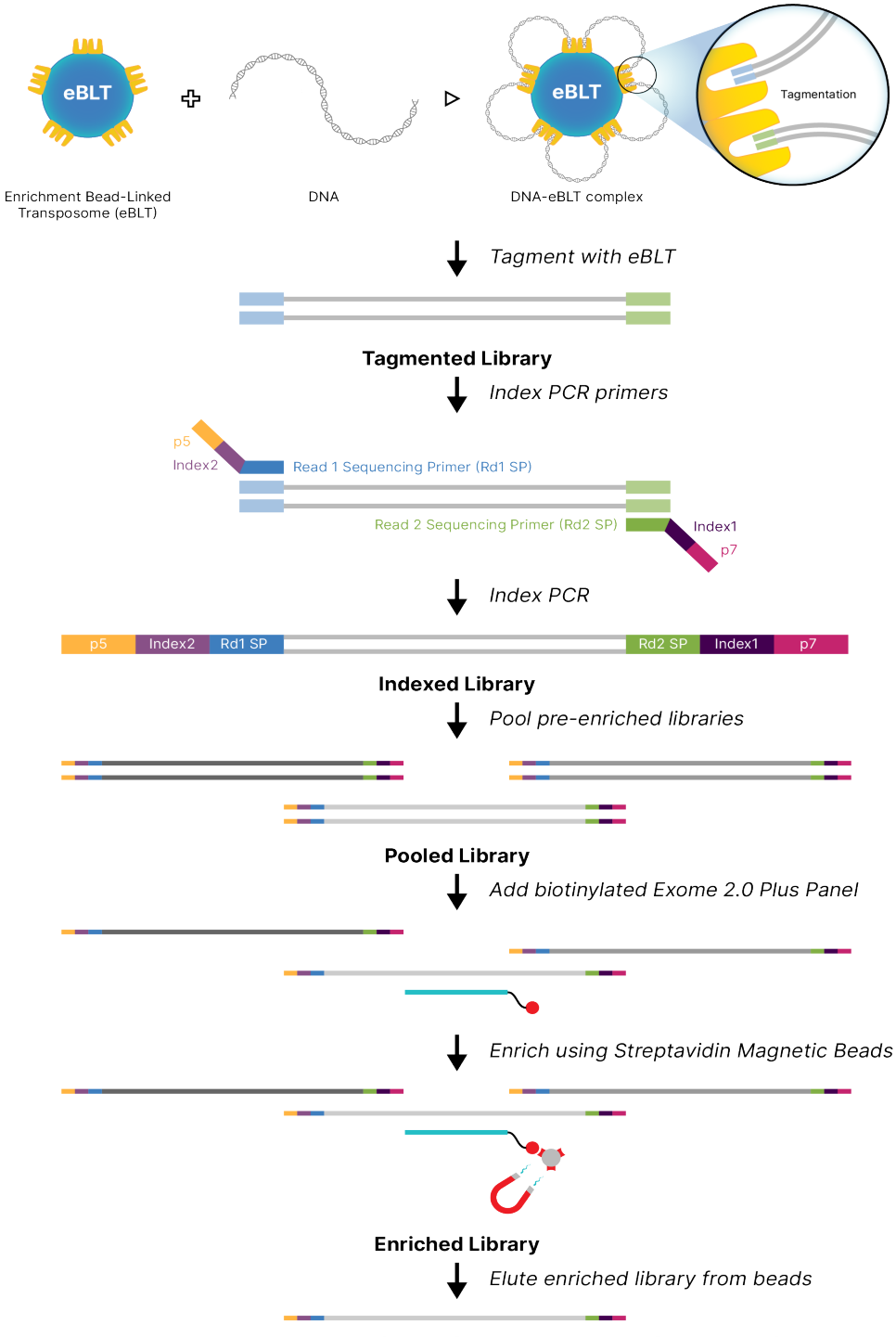
Thermal Cycler	Temp Mode	Lid Temp	Vessel Type
Bio-Rad C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module (part # 1851197)	Calculated	Heated	Plate
Bio-Rad DNA Engine Tetrad 2 (part # PTC-0240G)	Calculated	Heated, Constant at 100°C	Polypropylene plates and tubes

How the Illumina DNA Prep with Exome 2.0 Plus Enrichment Assay Works

The Illumina DNA Prep with Exome 2.0 Plus Enrichment workflow uses a bead-based transposome complex to tagment genomic DNA, which 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-enriched 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 (SMB3) 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.

Figure 6 Illumina DNA Prep with Exome 2.0 Plus Enrichment Workflow



Single IPB Methodology

The Single IPB Methodology uses the bead purification procedure to purify the amplified pre-enriched libraries. It is an alternative to the standard double-sided IPB *Clean Up Libraries* on page 17

- Single IPB cleanup removes the need of pre-enriched 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)
- 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
- Nuclease-free water

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

Acronyms

Acronym	Definition
BLB	Blood Lysis Buffer
BLT	Bead-Linked Transposome
eBLT	Enrichment Bead-Linked Transposome
EE1	Enrichment Elution Buffer 1
EEW	Enhanced Enrichment Wash
EHB2	Enrich Hyb Buffer 2
EPM	Enhanced PCR Mix
ET2	Elute Target Buffer 2
EtOH	Ethanol
HP3	2 N NaOH
IPB	Illumina Purification Beads
NHB2	Hyb Buffer 2 + IDT NXT Blockers
PK1	Proteinase K
PPC	PCR Primer Cocktail
RSB	Resuspension Buffer
SMB3	Streptavidin Magnetic Beads
ST2	Stop Tagment Buffer 2
TB1	Tagmentation Buffer 1
TWB	Tagment Wash Buffer
UD	Unique Dual

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Technical Support Telephone Numbers

Region	Toll Free	International
Australia	+61 1800 775 688	
Austria	+43 800 006249	+43 1 9286540
Belgium	+32 800 77 160	+32 3 400 29 73
Canada	+1 800 809 4566	
China		+86 400 066 5835
Denmark	+45 80 82 01 83	+45 89 87 11 56
Finland	+358 800 918 363	+358 9 7479 0110
France	+33 8 05 10 21 93	+33 1 70 77 04 46
Germany	+49 800 101 4940	+49 89 3803 5677
Hong Kong, China	+852 800 960 230	
India	+91 8006500375	
Indonesia		0078036510048
Ireland	+353 1800 936608	+353 1 695 0506
Italy	+39 800 985513	+39 236003759
Japan	+81 0800 111 5011	
Malaysia	+60 1800 80 6789	
Netherlands	+31 800 022 2493	+31 20 713 2960
New Zealand	+64 800 451 650	
Norway	+47 800 16 836	+47 21 93 96 93
Philippines	+63 180016510798	
Singapore	1 800 5792 745	
South Korea	+82 80 234 5300	
Spain	+34 800 300 143	+34 911 899 417

Region	Toll Free	International
Sweden	+46 2 00883979	+46 8 50619671
Switzerland	+41 800 200 442	+41 56 580 00 00
Taiwan, China	+886 8 06651752	
Thailand	+66 1800 011 304	
United Kingdom	+44 800 012 6019	+44 20 7305 7197
United States	+1 800 809 4566	+1 858 202 4566
Vietnam	+84 1206 5263	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download from support.illumina.com.

Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

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