Illumina DNA Prep with Enrichment

Reference Guide

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Table of Contents

Overview

This guide explains how to prepare up to 384 uniquely dual-indexed paired-end libraries from DNA using the Illumina DNA Prep with Enrichment workflow.

The Illumina DNA Prep with Enrichment workflow:

- Uses tagmentation, an enzymatic reaction, to fragment DNA and add adapter sequences in only 15 minutes.
- Uses innovative sample normalization at inputs ≥ 50 ng.
- Can prepare libraries directly from whole blood or saliva samples when using an extraction protocol.
- Is compatible with extracted formalin-fixed paraffin-embedded (FFPE) samples. ΔCq ≤ 5 is recommended for optimal performance.

DNA Input Recommendations

The Illumina DNA Prep with Enrichment protocol is compatible with high-quality, double-stranded genomic DNA (gDNA) inputs of 10–1000 ng. For human gDNA samples and other large complex genomes, the recommended minimum gDNA input is 50 ng.

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

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 absorbance at 280 nm provides an indication of sample purity. This protocol is optimized for gDNA with 260/280 absorbance ratio values of 1.8–2.0, which indicates a pure gDNA sample.

For a secondary indication of sample purity, use the ratio of absorbance at 260 nm to absorbance at 230 nm. Target a 260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of contaminants. For a complete list of contaminants, including sources, avoidance, and effects on the library preparation, see the *Nextera XT Troubleshooting Technical Note*.

Dilute the starting material in 10 mM Tris-HCl, pH 7.5–8.5. Incomplete tagmentation caused by contaminants can cause library preparation failure, poor clustering, or low quality sequencing results.

FFPE Tissue Sample Input Recommendations

To obtain reliable, reproducible results from formalin-fixed paraffin-embedded (FFPE) DNA samples, accurately assess DNA quality to determine the required number of PCR cycles in this protocol. For more information, see *Sample Input Recommendations* on page 3.

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

FFPE Extraction

Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. The QIAGEN AllPrep DNA/RNA FFPE Kit provides high yield of nucleic acids compared to other extraction methods tested for this assay.

FFPE DNA Qualification

For optimal performance, assess DNA sample quality using the Infinium FFPE QC Kit with KAPA qPCR MasterMix (Universal) and Primer Premix on the Bio-Rad CFX96 Touch Real-Time PCR Detection System or equivalent instrument. For more information on FFPE DNA qualification, see the *Infinium HD FFPE QC Assay Protocol (Part # 15020981)*.

FFPE Reference Samples (Optional)

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

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

Blood and Saliva Input Recommendations

The Illumina DNA Prep with 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, see *[Optional] Blood Lysis* on page 40 or *[Optional] Saliva Lysis* on page 42.

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 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
- Storage conditions
- Underlying medical conditions affecting white blood cell counts

Sample Input Recommendations

The Illumina DNA Prep with Enrichment workflow is compatible with blood, saliva, or FFPE 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)
- QIAGEN AllPrep DNA/RNA FFPE Kit for extraction of FFPE samples (FFPE)
- Infinium FFPE QC Kit for qualification (FFPE)

The recommended number of PCR cycles for the eBLT PCR program are adjusted based on sample input concentration and quality. For more information, see *Amplify Tagmented DNA* on page 13.

Table 1 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	260/280 ratio of 1.8–2.0	No
50–1000 ng genomic DNA	No	260/280 ratio of 1.8–2.0	Yes
50–1000 ng extracted FFPE	Yes	Δ Cq value ≤ 5	No
Saliva	No	Not applicable	Yes
Blood	No	Not applicable	Yes

Additional Resources

The Illumina DNA Prep with Enrichment 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.

- 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 2 Sample Input Recommendations

Resource	Description
Custom Protocol Selector	A tool for generating end-to-end instructions tailored to your library prep method, run parameters, and analysis method, with options to refine the level of detail.
Illumina DNA Prep with Enrichment Checklist (document # 1000000048601)	Provides a checklist of steps for the experienced users.
Illumina DNA Prep with Enrichment Consumables and Equipment List (document # 1000000048602)	Provides an interactive checklist of user-supplied consumables and equipment.
Illumina DNA Prep with Enrichment with RNA Probes (document #1000000070581)	Provides the protocol to use Illumina DNA Prep with Enrichment with third-party, RNA-based probes.
Index Adapters Pooling Online Help (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 Enrichment kit.
Illumina Adapter Sequences (document # 1000000002694)	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
Infinium HD FFPE QC Assay Protocol (document # 15020981)	Provides the protocol to assess DNA input quality for FFPE samples.
Illumina Free Adapter Blocking Reagent (document # 1000000047585)	Provides the protocol to block excess free adapter, minimize index hopping, and enhance data quality.
IDT for Illumina DNA/RNA UD Indexes support page	Provides information about IDT for Illumina DNA/RNA Unique Dual (UD) indexes.

Protocol

This chapter describes the Illumina DNA Prep with 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 requires library prep and enrichment reagents, an enrichment probe panel, and index adapters. Enrichment probe panels and index adapters are sold separately. See *Supporting Information* on page 45.
    - Third-party biotinylated probes must meet specific requirements. See *Third-Party Panel Requirements* on page 49 to make sure that your third-party probes meet the requirements.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

Supported Enrichment Plexities

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

Obtaining suitable enrichment yield for nonstandard enrichment plexity might require additional optimization. Optimal results are not guaranteed. For information on reagents required for other enrichment plexities, see *Non-Standard Enrichment Plexity Reagents* on page 49.

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

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

When pooling pre-enriched libraries, Illumina DNA Prep with Enrichment reagents support the following enrichment reactions and plexity indicated in the table below.

Illumina DNA Prep with Enrichment Reagents	Enrichment Reactions	Enrichment Plexity
16-sample kit	16 reactions	1-plex

Illumina DNA Prep with 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
  - 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 (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 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.

IPB 100 ml Bottle Resuspension

1. Mix the bead reagent by inverting manually, at a rate of at least a single inversion per second.
2. Rotate bottle 90 degrees every 30 seconds, and then continue inverting for a total of 2 minutes.
3. If beads are adhered to the walls of the container, invert manually for an additional 1 minute.

Handling Tagment Wash Buffer (TWB)

- Pipette slowly to minimize foaming.

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

- Illumina DNA Prep with Enrichment is compatible with IDT® for Illumina® DNA/RNA Unique Dual (UD), IDT for Illumina Nextera DNA Unique Dual (UD).
- Each index plate is for single use only.
- 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 the sequencing system is configured for 10 base pair index codes.

Prepare IDT for Illumina DNA/RNA UD Indexes as follows.

- Centrifuge at 1000 × g for 1 minute to settle liquid away from the seal.
- [< 96 samples] Pierce the foil seal on the index adapter plate using a new pipette tip for each well for only the number of samples being processed.
- [96 samples] Align a new Eppendorf 96-well PCR plate above the index adapter plate and press down to puncture the foil seal on all 96 wells. Press down slowly to avoid tipping the volume over.
- Discard the empty Eppendorf plate used to puncture the foil seal.

Illumina DNA Prep with Enrichment Workflow

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

gDNA

Extracted FFPE

Blood Lysis
Hands-on: 20 minutes
Total: 40 minutes
Reagents: BLB, PK1, EtOH, IPB

Safe Stopping Point

Saliva Lysis
Hands-on: 20 minutes
Total: 40 minutes
Reagents: IPB, EtOH

Safe Stopping Point

1 Tagment Genomic DNA
Hands-on: 20 minutes
Total: 40 minutes
Reagents: eBLT, TB1

2 Post Tagmentation Clean Up
Hands-on: 10 minutes
Total: 20 minutes
Reagents: ST2, TWB

3 Amplify Tagmented DNA
Hands-on: 15 minutes
Total: 45 minutes
Reagents: EPM, Index Adapters

Safe Stopping Point

4 Clean Up Libraries
Hands-on: 10 minutes
Total: 20 minutes
Reagents: RSB, IPB, EtOH

Safe Stopping Point

5 Pool Libraries
Reagents: RSB

Safe Stopping Point

6 Hybridize Probes
Hands-on: 10 minutes
Total: 125 minutes
Reagents: Enrichment Probe Panel, NHB1, EHB2, RSB

(Optional)
Overnight Hybridization

7 Capture Hybridized Probes
Hands-on: 25 minutes
Total: 65 minutes
Reagents: SMB, EEW, EE1, HP3, ET2

8 Amplify Enriched Library
Hands-on: 5 minutes
Total: 40 minutes
Reagents: PPC, EPM

Safe Stopping Point

9 Clean Up Amplified Enriched Library
Hands-on: 10 minutes
Total: 20 minutes
Reagents: IPB, RSB, EtOH

🔵 Pre-PCR 🔴 Post-PCR

Tagment Genomic DNA

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

Consumables

- (eBLT) (yellow cap)
- Tagmentation Buffer 1 (TB1)
- 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, see the SDS at support.illumina.com/sds.html.

About Reagents

- eBLT must be stored 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. 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.
2. If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
3. Vortex eBLT (yellow cap) vigorously for 10 seconds to resuspend. Repeat as necessary.
4. 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)

   Reagent overage is included in the volume to ensure accurate pipetting.
5. Vortex the Tagmentation Master Mix thoroughly to resuspend.
6. Divide the Tagmentation Master Mix volume equally into an 8-tube strip.
7. 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.
8. Discard the 8-tube strip after the Tagmentation Master Mix has been dispensed.
9. Using a 200 µl multichannel pipette set to 40 µl, pipette each sample 10 times, and then seal the plate. Use Alternatively, seal the plate and use a plate shaker 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 Cleanup

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

Consumables

- Stop Tagment Buffer 2 (ST2) (red cap)
- Tagmentation Wash Buffer (TWB)
- 96-well plate magnet
- 8-tube strip

- Microseal 'B' adhesive seal
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

About Reagents

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

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
STB ST2 (red cap)	Room temperature	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved.
TWB	Room temperature	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 (red cap) to the plate. If you are using a multichannel pipette, pipette ST2 into an 8-tube strip, and then transfer the appropriate 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. **[≤ 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.
   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 one time for a total of two washes.

6. [**> 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 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 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. Perform steps g and h 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 one time for a total of two washes.
7. Remove the plate 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.
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 pre-paired 10 base pair Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation. To confirm the indexes of libraries being pooled for enrichment have the appropriate color balance, see the Index Adapters Pooling Online Help (document # 1000000041074).

Index adapter plates are ordered separately from the library prep and enrichment components. For a list of compatible index adapter plates for use with this protocol, see *Kit Contents* on page 47

Consumables

- Enhanced PCR Mix (EPM)
- Index adapter plate
- Eppendorf Lo Bind 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.

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.

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

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

Sample Input Type	Number of PCR Cycles (X)
10–49 ng genomic DNA	12

Sample Input Type	Number of PCR Cycles (X)
50–1000 ng genomic DNA	9
50–1000 ng extracted FFPE	12
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. Add 40 µl PCR Master Mix directly onto the beads in each well.
6. Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
7. Seal the sample plate and centrifuge at 280 × g for 3 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.

Clean Up Libraries

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

Consumables

- Illumina Purification Beads (IPB)
- Resuspension Buffer (RSB)
- Freshly prepared 80% ethanol (EtOH)
- 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
- 1.7 ml microcentrifuge tubes

About Reagents

- Illumina Purification Beads
  - 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
IPB	15°C to 30°C	Resuspend IPB beads
RSB	2°C to 8°C	Thaw and bring to room temperature. Vortex to mix.

2. Prepare fresh 80% EtOH from absolute ethanol.

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. Resuspend Illumina Purification Beads.
5. For gDNA, blood, or saliva, perform the following steps.
    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 resuspend the IPB, and then add 20 µl to each well of a *new* MIDI plate.
    g. 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. For extracted FFPE, perform the following steps.
    a. Add 81 µl IPB to each well of the MIDI plate containing supernatant.
    b. Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
7. Incubate 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 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 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

If you are not checking library quality, perform the following procedure to reserve samples 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.

Perform the following procedure to qualify pre-enriched libraries.

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 when analyzed using a size range of 150–1500 bp as shown in Figure 1 and Figure 2.

   For FFPE samples, the mean fragment size can be as low as 250 bp.

Figure 1 Example Fragment Analyzer Trace

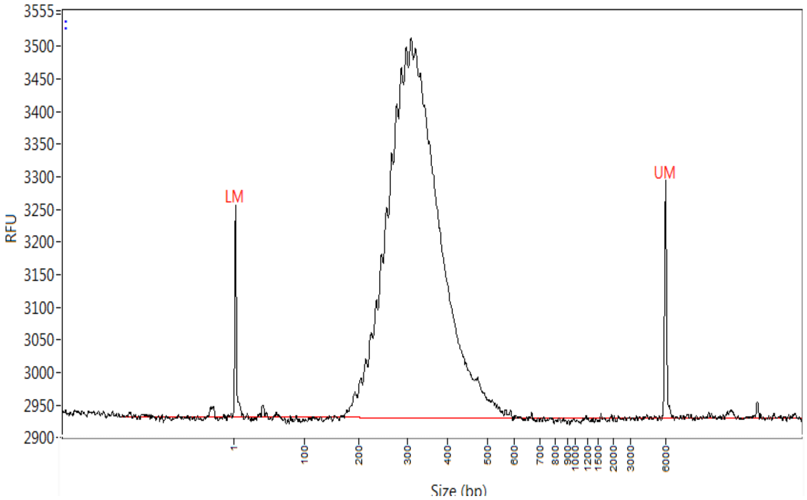
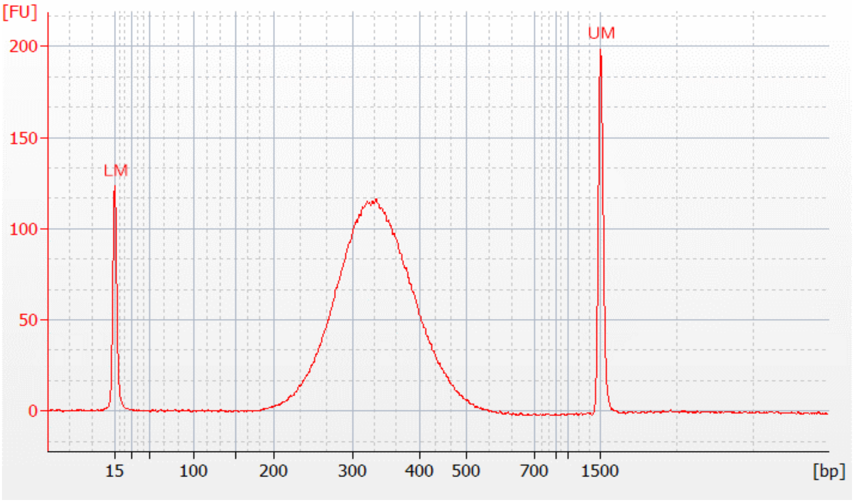


Figure 2 Example Bioanalyzer Trace



Pool Pre-Enriched Libraries

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

Pooling Methods

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

Table 3 Recommended Pooling Methods

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

* For volume, use 1-plex enrichment.

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

Pool by Volume

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

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

1. Using the sample tracking method you chose in *Prepare for Pooling* on page 1, 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 by Mass

To achieve optimal sample balancing when starting with input of 10-49 ng gDNA or 50–1000 ng extracted FFPE samples, pool your libraries at equal concentration after dsDNA quantification.

Quantify Pre-Enriched Libraries

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

   Table 4 Expected Pre-Enriched Library Yield

Sample Input Type (ng)	Pre-Enriched Library Yield (ng)
10–49 gDNA	≥ 100
50–1000 gDNA , blood , saliva	≥ 250

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

Pool Pre-Enriched Libraries at Equal Concentration

For the following sample input types and applications, a minimum of 500 ng per pre-enriched library for 1-plex enrichment is tested and recommended. Other enrichment plexities might be possible. Optimal results are not guaranteed.

- Degraded samples, such as FFPE
- Somatic variant calling
- Low minor allele frequency detection

When pooling by mass for samples derived from high-quality DNA, as little as 100 ng per pre-enriched library can be used for enrichment. Additional optimization might be required to obtain suitable enrichment yield when pooling pre-enriched libraries < 250 ng. Optimal results are not guaranteed.

If proceeding with < 500 ng per pre-enriched library, decrease the mass of each library to equal concentration based on your sample quantification results. Confirm the total DNA library mass (ng) remains 500–6000 ng. For example, if you have 250 ng per pre-enriched library, you can proceed with 12-plex enrichment with 3000 ng of total DNA library mass and proceed with steps 2 in *Pool by Mass* on page 21.

1. Prepare each pre-enriched library. Dilute with RSB as needed.
2. Using the sample tracking method you chose in *Prepare for Pooling* on page 1, record the indexes for the libraries you plan to pool in this step.
3. Combine each library in a 1.5 ml microcentrifuge tube into the plexities shown in the following table.

Library Pools	Each Pre-Enriched Library Sample (ng)	Total DNA Library Mass (ng)
1	500	500
12	500	6000

4. Perform one of the following based on the total volume of the pooled pre-enriched libraries:
   - If pre-enriched library volume = 30 µl, proceed to *Hybridize Probes* on page 22.
   - If pre-enriched library volume < 30 µl, add RSB to reach 30 µl total volume.
   - If pre-enriched library volume > 30 µl, use one of the following methods to concentrate the pooled sample:
     - **Bead-based method**—Follow the instructions in *[Optional] Concentrate Pooled Library* on page 38.
     - **Vacuum concentrator**— Use a no heat setting and a medium drying rate.

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.

Hybridize Probes

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

This protocol requires half the probe volume as previous Illumina enrichment protocols.

Illumina DNA Prep with Enrichment reagents are compatible with both Illumina and third-party enrichment DNA oligonucleotide panels. For information the required specifications for third-party panels, see *Third-Party Panel Requirements* on page 49.

Consumables

- Enrich Hyb Buffer 2 (EHB2)
- Enrichment probe panel
- Hyb Buffer 2 + IDT NXT Blockers (NHB2) (blue cap)
- Resuspension Buffer (RSB)
- 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.
- Enrichment probe panel refers to the chosen enrichment oligonucleotide panel from Illumina or a third-party vendor to run with this workflow.

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.
Enrichment Probe Panel	-25°C to -15°C (Illumina)	Bring to room temperature. Vortex to mix. Use a total probe volume of 10 µl.
Third-Party Probes	Varies for third-party probes	Bring to room temperature. Vortex to mix. Use the vendor-recommended probe volume per reaction for the third party or Illumina enrichment assay. Use the vendor-recommended volume of probes meeting the requirements listed in *Third-Party Panel Requirements* on page 49. Add RSB, if needed, for a total volume of 10 µl per enrichment reaction, regardless of the enrichment plexity. For example, if the vendor recommends using 4 µl of probes for their assay or the Illumina enrichment assay, add 6 µl RSB for a total probe volume of 10 µl per enrichment reaction to use with this protocol.

Item	Storage	Instructions
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.
RSB	2°C to 8°C	If using third-party probe panels, bring to room temperature. Vortex to mix.
SMB3	2°C to 8°C	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.
EEW (amber tube)	-25°C to -15°C	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, bring to room temperature.

2. Save the following NF-HYB program on the thermal cycler using the appropriate number of cycles, which are listed in Table 5.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume
     - 50 µl for the C1000 thermal cycler
     - 100 µl for the Tetrad 2 thermal cycler
   - 95°C for 5 minutes
   - X cycles of 1 minute each, starting at 94°C for the first cycle, then decreasing 2°C per cycle
   - Hold for 90 minutes at the applicable temperature:
     - [FFPE] 58°C
     - [CEX panel] 58°C
     - [Somatic variant calling] 58°C
     - [All others] 62°C

   If optimizing percent duplicates for third-party panels is needed, increasing the 90 minute hold time to 2.5–16 hours might improve performance. See example data sets with increased hybridization times in BaseSpace Sequence Hub Public Data.

   - **[Optional]** Extend the hold to a maximum of 24 hours

   Total running time is ~115 minutes.

Table 5 Cycle Number per Sample or Panel

Sample and Panel Type	Number of Cycles (X)
FFPE (regardless of panel type)	18
CEX panel (regardless of sample type)	18
Somatic variant calling	18
All other samples and panels	16

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 sample or pool (30 µl)
   - NHB2 (blue cap) (50 µl)
   - Enrichment probe panel (10 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 NF-HYB program.
5. Proceed immediately to the next procedure when the NF-HYB program hold temperature time ends.

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

Capture Hybridized Probes

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

Consumables

- SMB (Streptavidin Magnetic Beads) (SMB3)
- Enhanced Enrichment Wash (EEW) (amber cap)
- Enrichment Elution Buffer 1 (EEB1)
- 2N NaOH (HP3)
- Elute Target Buffer 2 (ETB2)
- 1.7 ml microcentrifuge tube
- One of the following containers:
    - [Plate] 96-well MIDI plate and 96-well PCR plate
    - [Tube] 1.5 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, 1.5 ml)

About Reagents

- EEW
    - Can be cloudy after reaching room temperature

  - Can appear yellow
  - Heated before use
- 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 30 minutes to bring to room temperature. Vortex to mix before use.
EEW (amber tube)	-25°C to -15°C	Thaw at room temperature. Vortex 3 times for 30 seconds each. The reagent is heated during the procedure.
EE1	-25°C to -15°C	Thaw at room temperature. Pipette 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 minimum of one microheating system with a MIDI heat block insert to incubate the sample plate to one of the following temperatures. An optional second microheating system can be used to preheat EEW.
   - [FFPE] 58°C
   - [CEX panel] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C

Procedure

Capture

1. Centrifuge the sample plate or tubes 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 1.5 ml microcentrifuge tube.
3. Add 250 µl SMB3 to each well or to the 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 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 :
   - [FFPE] 58°C
   - [CEX panel] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C

   Proceed to step 11 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 1.5 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 1.
   - [FFPE] 58°C
   - [CEX panel] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C
6. Immediately centrifuge the sample 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 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. 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 the following temperature:
   - [FFPE] 58°C
   - [CEX panel] 58°C
   - [Somatic variant calling] 58°C

  - [All others] 62°C
5. **[Tube]** Centrifuge briefly.
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. Repeat steps 1–7 two times for a total of 3 washes.

Transfer Wash

1. Remove 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. Do not centrifuge.
3. Transfer 200 µl resuspended bead solution to a new MIDI plate or to a new 1.5 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 the applicable temperature:
   - [FFPE] 58°C
   - [CEX panel] 58°C
   - [Somatic variant calling] 58°C
   - [All others] 62°C
5. **[Tube]** Centrifuge briefly.
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)

   Additional reagent overage is included in the volume to ensure accurate pipetting due to the potential of reagent foaming.
2. Vortex, and then centrifuge the master mix at 280 × g for 10 seconds.
3. Remove 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 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 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 Enriched Library

This step uses PCR to amplify the enriched library.

Consumables

- Enhanced PCR Mix (EPM)
- PCR Primer Cocktail (PPC)
- [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 using the appropriate number of PCR cycles, which are listed in the following table.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 30 seconds
   - (X) cycles of:
     - 98°C for 10 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.

Panel Type	(X) Cycles
Illumina Exome Panel (CEX)	10
All other panels	12[1][2][3]

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

Procedure

1. Add 5 µl PPC to each well or to the tube.
2. Add 20 µl EPM to each well or to the 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 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.

Clean Up Amplified Enriched Library

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

Consumables

- Illumina Purification Beads (IPB)
- Resuspension Buffer (RSB)
- 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] 1.5 ml microcentrifuge tubes
- One of the following magnets:
  - [Plate] Magnetic Stand-96
  - [Tube] MagneSphere® Technology Magnetic Separation Stands (12 position, 1.5 ml)

About Reagents

- Illumina Purification Beads
  - 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
IPB	15°C to 30°C	Resuspend IPB beads.
RSB	2°C to 8°C	Bring to room temperature. Vortex to mix.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Centrifuge the PCR samples at 280 × g for 30 seconds.
2. Transfer 50 µl supernatant from each well of the PCR plate or from the 8-strip tube to the corresponding well of a new MIDI plate or to a new 1.5 ml microcentrifuge tube.
3. Resuspend IPB.
4. Add 45 µl IPB to each well or to the 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 3 times for 10 seconds each.
5. Incubate the sample 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 liquid is clear (~5 minutes).
8. Using a pipette set to 95 µl, remove and discard all supernatant from each well or from the tube.
9. 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.
10. Use a 20 µl pipette to remove and discard residual EtOH from each well or from the tube.
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 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 each.
14. Incubate the plate or the tube at room temperature for 5 minutes.
15. Centrifuge at 280 × g for 30 seconds.
16. Place on a magnetic stand and wait until liquid is clear (~2 minutes).
17. Transfer 30 µl supernatant from the 96-well PCR plate or from the 8-strip tube to the corresponding well of a new 96-well PCR plate or a new 1.5 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 Enriched Libraries

Perform the following to check the concentration and quality of the enriched library.

1. Run 1 µl of the enriched libraries using the Qubit dsDNA BR Assay Kit to quantify library concentration.
   Expect post-enrichment library yields of ≥ 3 ng/µl. Library yields may be less for small third-party panels.

   Total probe molarity proportionally impacts the post-enrichment library yield. Probe panels from third-party vendors may produce proportionally lower enriched library yields. However, sequencing metrics are still expected to meet specification.

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 3 Example Bioanalyzer Trace

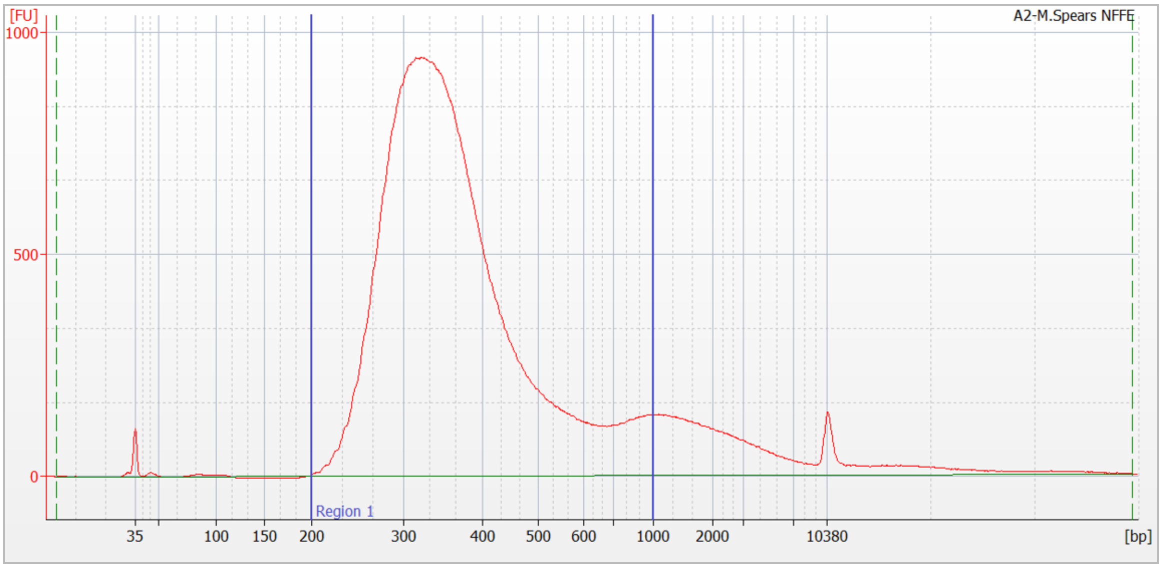
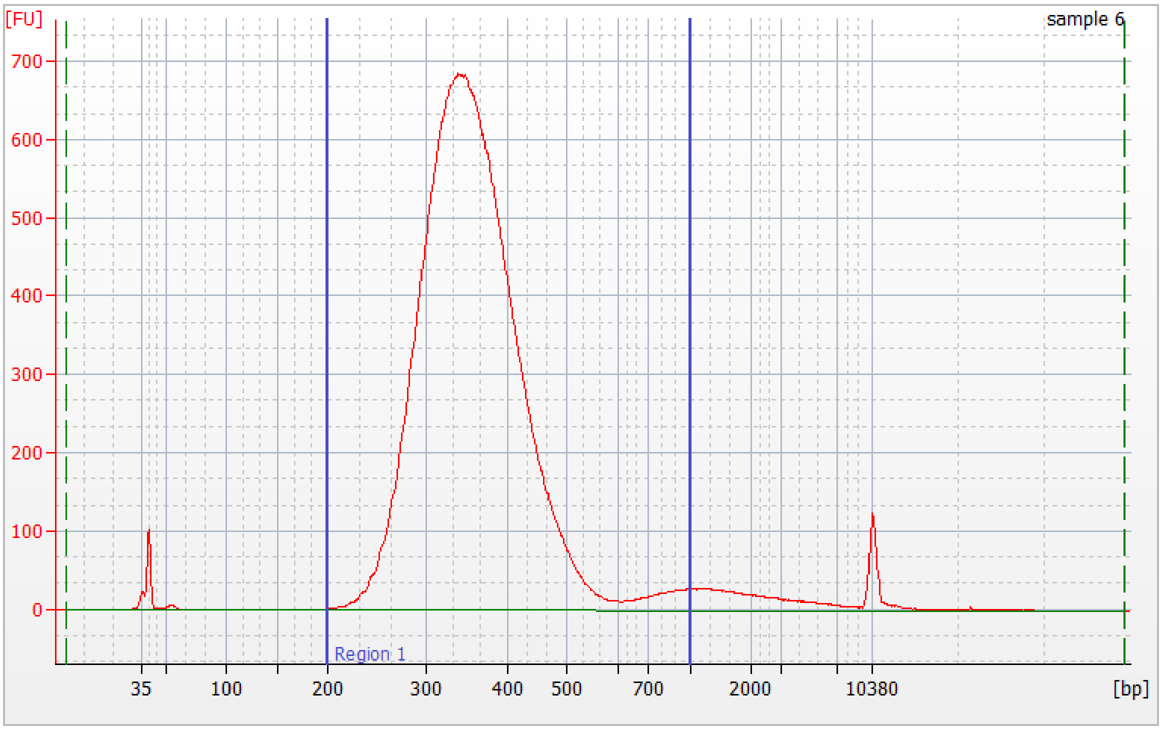


Figure 4 Example Bioanalyzer Trace



Dilute Libraries to the Starting Concentration

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

For sequencing, regardless of the enrichment probe panel you are using, Illumina recommends setting up a paired-end run with 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.
   - Use 350 bp as the average library size for qualification methods other than the bioanalyzer.

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

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

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
HiSeq 2500 and HiSeq 2000 Systems (high output modes)	2	16–18
HiSeq 2500 System (rapid run mode)	2	7–8
HiSeq 4000 and HiSeq 3000 Systems	2–3	150–200
iSeq 100 System	2	100
MiniSeq System	2	1.7–1.8
MiSeq System (v3 reagents)	4	10–12
NextSeq 550 and NextSeq 500 Systems	2	1.4–1.5
NovaSeq 6000 System (standard workflow)	2	175–185

3. Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
   - **Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl of each diluted library to a tube to create a multiplexed library pool.
4. Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
   - For the iSeq 100 System, see the system guide for dilution instructions (libraries are automatically denatured).
   - For the NovaSeq 6000 System, see the system guide for pool and denature instructions.
   - For the HiSeq 4000 and HiSeq 3000 Systems, see the cBot 2 or cBot system guide for reagent preparation 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 Enrichment workflow.

[Optional] Concentrate Pooled Library

If the total volume of the pooled pre-enriched libraries is > 30 µl, the sample must be concentrated to a final volume of 30 µl. Use this bead-based method to achieve a final volume of 30 µl. For more information, see *Pool by Mass* on page 21.

Consumables

- Illumina Purification Beads (IPB)
- Resuspension Buffer (RSB)
- 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] 1.5 ml microcentrifuge tubes
- One of the following magnets:
  - [Plate] Magnetic Stand-96
  - [Tube] MagneSphere Technology Magnetic Separation Stands (12 position, 1.5 ml)

About Reagents

- Illumina Purification Beads
  - 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
IPB	15°C to 30°C	Resuspend IPB beads
RSB	2°C to 8°C	Bring to room temperature. Vortex to mix.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Centrifuge the sample tube at 280 × g for 1 minute.
2. Transfer samples to the corresponding well of a new MIDI plate or a new 1.5 ml microcentrifuge tube.

> If the pool volume is ≥178 µl, use a 1.5 ml microcentrifuge tube to prevent MIDI plate wells from overflowing.

3. Resuspend IPB.
4. Add 1.8x pool volume of IPB to each well or to the 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 3 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 2 times as follows.
    a. With the plate on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well or to the tube.
    b. Incubate for 30 seconds.
    c. Using a pipette set to 200 µl, remove and discard all supernatant from each well or from the tube.
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 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 3 times for 10 seconds each.

14. Incubate the sample plate or the tube at room temperature for 5 minutes.
15. Centrifuge at 280 × g for 1 minute.
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 22.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal, Microseal 'F' foil seal, or cap the 8-tube strip and store at -25°C to -15°C for up to 30 days.

[Optional] Blood Lysis

Use this protocol when performing the Illumina DNA Prep with 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 any bloodbourne pathogens are eliminated and the sample is no longer biohazardous.

Consumables

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

About Reagents

- Illumina Purification Beads
  - 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
IPB	Room temperature	Resuspend IPB beads.
BLB	Room temperature	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 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 (7 µl)
   - PK1 (2 µl)
   - Nuclease-free water (31 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.

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 as clear as possible (~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 30 µl nuclease-free water and pipette to resuspend.
19. Proceed immediately to step 3 of *Tagment Genomic DNA* on page 10 or stop and store the sample bead mixture.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 10, seal the plate with a Microseal 'B' adhesive seal and store the sample bead mixture at 2°C to 8°C for up to 3 days.

[Optional] Saliva Lysis

Use this protocol when performing the Illumina DNA Prep with 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

- Illumina Purification Beads (IPB)
- Oragene DNA collection tubes (for saliva sample collection)

- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plate

About Reagents

- Illumina Purification Beads
    - 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
Saliva samples in Oragene DNA collection tubes	Room temperature	For information on sample preparation and storage, see the DNA Genotek website.
IPB	Room temperature	Resuspend IPB beads.

2. Prepare 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 water. Slowly pipette to mix. For viscous samples, use a wide-bore pipette tip for more accurate pipetting.
4. Resuspend IPB.
5. Add 20 µl IPB to the well.
6. Using a pipette set to 50 µl, slowly pipette 10 times to mix.
7. Incubate at room temperature for 5 minutes.
8. Place on a magnetic stand and wait 5 minutes.
9. Without disturbing the beads, remove and discard supernatant.
10. 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).

11. Add 150 µl fresh 80% EtOH to the well.
12. Incubate on the magnetic stand for 30 seconds.
13. Use a 20 µl pipette to remove and discard all residual EtOH.
14. Remove the plate from the magnetic stand.
15. Add 30 µl nuclease-free water and pipette to resuspend.
16. Proceed immediately to step 3 of *Tagment Genomic DNA* on page 10 or stop and store the sample bead mixture.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 10, seal the plate with a Microseal 'B' adhesive seal and store the sample bead mixture 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.

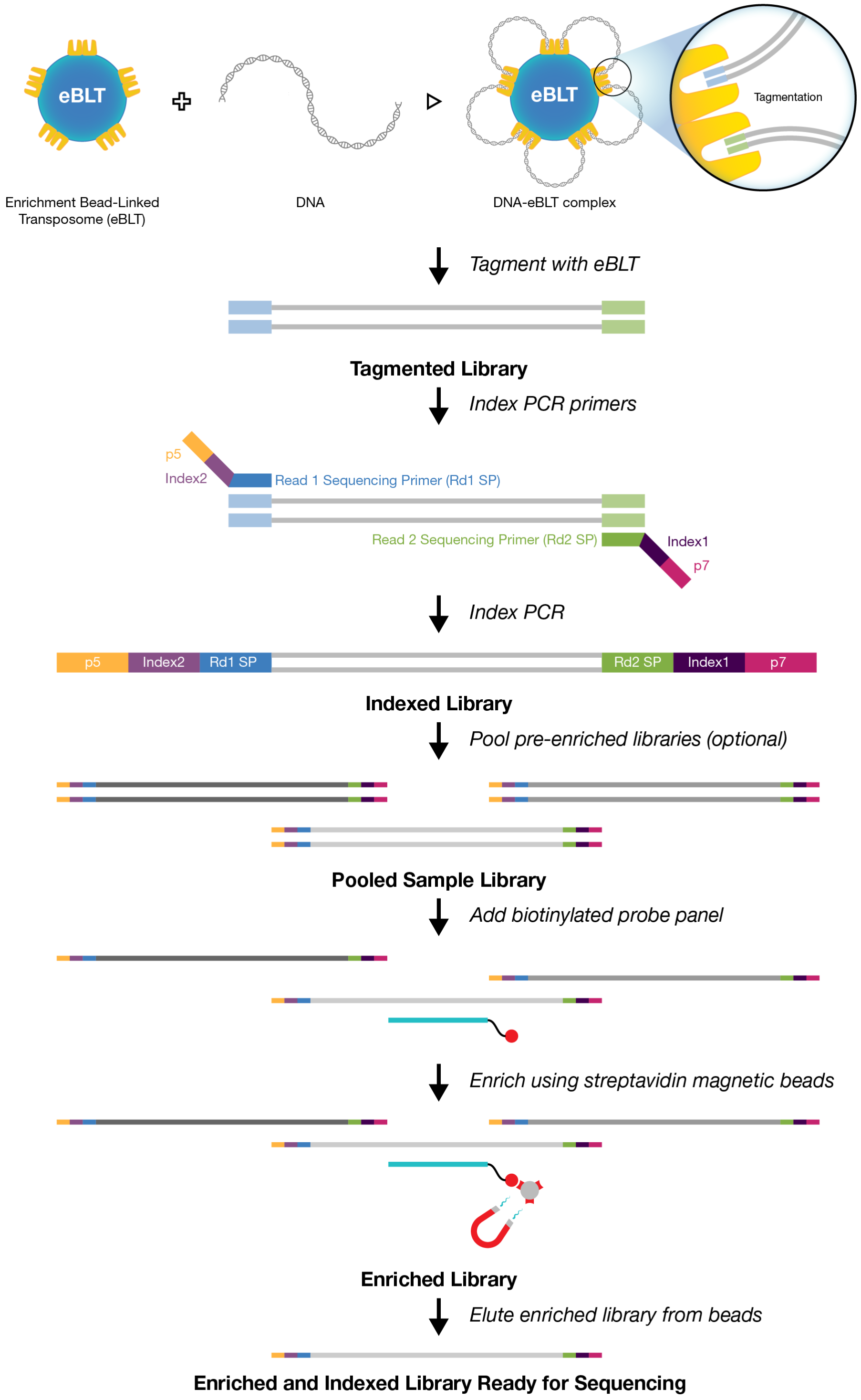
Introduction

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.

How the Illumina DNA Prep with Enrichment Assay Works

The Illumina DNA Prep with 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 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 capability across all Illumina sequencing systems. A subsequent target enrichment workflow is then applied. Enrichment can be performed with either individually prepared libraries (1-plex) or pooled libraries (up to 12-plex). 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 targeted library fragments within the regions of interest. The captured and indexed libraries are eluted from beads and further amplified before sequencing.

Figure 5 Illumina DNA Prep with Enrichment Workflow



Kit Contents

Completing the Illumina DNA Prep with Enrichment protocol requires library prep and enrichment reagents, an enrichment probe panel, and index adapters. 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[1]	Illumina DNA Prep with Enrichment - (S) Tagmentation, 16 Samples	20025523
	Illumina DNA Prep with Enrichment - (S) Tagmentation, 96 Samples	20025524
[Optional] Library prep only reagents Use for enrichment plexities between 1-plex and 12-plex. See *Non-Standard Enrichment Plexity Reagents* on page 49.	Illumina DNA Prep - (S) Tagmentation, 16 Samples	20025519
	Illumina DNA Prep - (S) Tagmentation, 96 Samples	20025520

Component	Kit Options	Illumina Catalog #
Enrichment probe panel Alternatively, use a third-party probe panel that meets specifications. See *Third-Party Panel Requirements* on page 49.	Illumina Exome Panel (8 Enrichment Reactions) (CEX)	20020183
	TruSight One (6 Enrichment Reactions) (TOO)	20029227
	TruSight One Expanded (6 Enrichment Reactions) (TOE)	20029226
	TruSight Cancer (8 Enrichment Reactions)	FC-121-0202
	TruSight Hereditary Cancer (8 Enrichment Reactions)	20029551
	TruSight Cardio (8 Enrichment Reactions)	20029229
	TruSeq Neurodegeneration (8 Enrichment Reactions)	20029550
	Illumina Custom Enrichment Panel via DesignStudio (8 Enrichment Reactions)	20025371
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 B, Tagmentation (96 Indexes, 96 Samples)	20027214
	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
	IDT for Illumina Nextera DNA UD Indexes Sets A–D (384 Indexes, 384 Samples)	20027217
[Optional] FFPE QC[2]	Infinium FFPE QC Kit (384 reactions)	WG-321-1001
[Optional] Blood lysis[3]	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] Illumina DNA Prep with Enrichment Reagents must be ordered with Illumina DNA Prep - (S) Tagmentation Library Prep Reagents.

[2] Required when starting the protocol from extracted FFPE.

[3] Required when starting the protocol from fresh whole blood samples.

Third-Party Panel Requirements

If using third-party biotinylated DNA probes, (fixed or custom panels), make sure they meet the following specifications:

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

For information on performing the Illumina DNA Prep with Enrichment protocol with third-party RNA-based probes, see Illumina DNA Prep with Enrichment with RNA Probes (document # 1000000070581).

Non-Standard Enrichment Plexity Reagents

To run enrichment plexities between 1-plex and 12-plex, additional pre-enrichment library prep reagents are required. Additional enrichment probe panel reagents can also be required depending on the number of enrichment reactions.

The following table provides information on additional library prep reagents needed for nonstandard enrichment plexities based on enrichment plexity, number of samples, and number of required reactions using the 16 sample configuration.

Table 6 Reagents for Non-Standard Plexities

			Quantity	
Enrichment Plexity	**Number of Samples**	**Enrichment Reactions Required**	**Catalog #20025523 (Library Prep and Enrichment)**	**Catalog # 20025519 (Library Prep Only)**
3	24	8	1	1
3	48	16	1	2
4	24	6	1	1
4	48	12	1	2
6	24	4	1	1
6	48	8	1	2
8	24	3	1	1
8	48	6	1	2

The number of enrichment reactions provided for each enrichment probe panel must meet the number of enrichment reactions required for your desired enrichment plexity and number of samples.

To determine the number of enrichment probe panel reagents that are needed, divide the number of enrichment reactions required by the enrichment reactions listed per enrichment probe panel and round up to the nearest whole number.

Illumina DNA Prep - (S) Tagmentation Kit Contents

Illumina DNA/RNA Prep - 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		**Acronym**	**Reagent Name**	**Tube Cap Color**
16 Samples	**96 Samples**			
1	4	ST2	Stop Tagment Buffer 2	Red
1	1	TWB	Tagment Wash Buffer	Clear

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		**Acronym**	**Reagent Name**	**Tube Cap Color**
16 Samples	**96 Samples**			
1	4	eBLT	Enrichment BLT	Yellow
1	2	RSB	Resuspension Buffer	Clear

Illumina Purification Beads are not included in this kit and must be purchased separately.

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		**Acronym**	**Reagent Name**	**Tube Cap Color**
16 Samples	**96 Samples**			
1	4	TB1	Tagmentation Buffer 1	Clear
2	4	EPM	Enhanced PCR Mix	Clear

Illumina DNA Prep with Enrichment - (S) Tagmentation Contents

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

Tube Quantity		Acronym	Reagent Name	Tube Cap Color
16 Samples	96 Samples			
4	2	SMB3	Streptavidin Magnetic Beads	Clear
1	1	RSB	Resuspension Buffer	Clear
1	1	EHB2	Enrich Hyb Buffer 2	Clear
1	1	ET2	Elute Target Buffer 2	Clear

ℹ Illumina Purification Beads are not included in this kit and must be purchased separately.

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

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

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

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

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

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

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

Description
IDT for Illumina Nextera DNA UD Indexes Set C (96 Indexes, 96 Samples)
IDT for Illumina Nextera DNA UD Indexes Set D (96 Indexes, 96 Samples)
IDT for Illumina Nextera DNA UD Indexes Sets A–D (384 Indexes, 384 Samples)

[Optional] Infinium FFPE QC Kit, Store at -25°C to -15°C

Quantity	Acronym	Reagent Name	Tube Cap Color
1	QCP	QC Primer Reagent	Clear
1	QCT	QC Template Reagent	Clear

[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	Room temperature
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.
🍷	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).
RUO	The intended use is Research Use Only (RUO).
REF	Indicates the part number so that the consumable can be identified.[2]
LOT	Indicates the batch code to identify the manufacturing batch or lot of the consumable.[1]
⚠	Indicates that caution is necessary.
◆	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

Make sure that you have the required consumables 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.

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 Deepwell 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 LoBind PCR Plates, Skirted	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, catalog # 89094-658
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, product # E7023
Nuclease-free water	General lab supplier
Illumina Purification Beads	Illumina, 1 x 100 ml, catalog # 20060057 Illumina, 4 x 100 ml, catalog # 20060058
Qubit dsDNA BR Assay Kit	Thermo Fisher Scientific, catalog # Q32850 or Q32853

Consumable	Supplier
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 Deepwell Storage (MIDID 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
1.5 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	Becton Dickinson

Consumable	Supplier
[Salivia] Oragene DNA Collection Kit for Saliva	Genotek, catalog # OGR-500 or OGD-510

Consumables for FFPE Input

Consumable	Supplier
KAPA qPCR Master Mix (Universal) and Primer Premix	KAPA Biosystems, catalog # KK4923- 07960441001
Qiagen AllPrep DNA/RNA FFPE Kit	Qiagen, Catalog # 80234
Infinium FFPE QC Kit	Illumina, catalog # WG-321-1001
qPCR plates compatible with your sequencing system	General lab supplier

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
One of the following thermal cyclers: • Bio-Rad C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module • Bio-Rad DNA Engine Tetrad 2	Bio-Rad, part # • 1851197 • PTC-0240G* *No longer available for purchase.
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
Microcentrifuge	General lab supplier
Microplate centrifuge	General lab supplier
Microheating System-Hybex System for Illumina	SciGene, catalog # • 1057-30-0 (115 V) or • 1057-30-2 (230 V)

Equipment	Supplier
MIDI Heat Block Insert for SciGene Hybex System	Illumina, catalog # BD-60-601
Qubit Fluorometer 3.0	ThermoFisher 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, see web product pages for catalog numbers Agilent Technologies: • Part # G2940CA
[Saliva] Water or air incubator capable of reaching 50°C	See DNA Genotek product pages
[FFPE] Bio-Rad CFX96 Touch Real-Time PCR Detection System or similar qPCR system for FFPE qualification	Bio-Rad, part # 1855196
[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 cycler. 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

Acronyms

Acronym	Definition
BLB	Blood Lysis Buffer
BLT	Bead-Linked Transposome
eBLT	Enrichment BLT
CEX	Coding Exome Oligos
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
IEM	Illumina Experiment Manager
IPB	Illumina Purification Bead
NHB2	Hyb Buffer 2 + IDT NXT Blockers
NXT	Nextera
PK1	Proteinase K
PPC	PCR Primer Cocktail

Acronym	Definition
QCP	QC Primer Reagent
QCT	QC Template Reagent
RSB	Resuspension Buffer
SMB3	Streptavidin Magnetic Beads
ST2	Stop Tagment Buffer 2
TB1	Tagmentation Buffer 1
TOO	TruSight One Oligos
TOE	TruSight One Expanded
TSHC	TruSight Hereditary Cancer
TWB	Tagment Wash Buffer
UD	Unique Dual

Revision History

Document	Date	Description of Change
Document #1000000048041 v07	August 2021	Add IPB bead resuspension section to tips and techniques. Clarified the average library size for qualification method other than bioanalyzer. Replaced AMPure XP with IPB Bead.
Document #1000000048041 v06	April 2021	Replaced SMB with SMB3.
Document #1000000048041 v05	June 2020	Added dilution information for the NextSeq 2000 Sequencing System. Added information for IDT for Illumina Nextera Indexes.
Document #1000000048041 v04	April 2020	Changed product name from Nextera Flex for Enrichment to Illumina DNA Prep with Enrichment. Renamed index and reagent kits. Updated catalog numbers for UD Index kits sets C and D. Removed obsolete UD Indexes sets A-D kit option.

Document	Date	Description of Change
Document #1000000048041 v03	November 2019	Added Illumina catalog # 20029551 for the TruSight Hereditary Cancer panel. Added TruSight Hereditary Cancer (TSCH) to the acronyms list. Added a chemical warning with a link to the safety data sheet (SDS) to the tagment genomic DNA step. Replaced the reagent Hyb Buffer + IDT NXT Blockers (NHB1) with Hyb Buffer 2 + IDT NXT Blockers (NHB2). Clarified that low minor allele frequency detection when using one-plex enrichment is for germline or somatic variant calling. Removed inaccurate references to Nextera XT v2 and Nextera XT indexes.
Document #1000000048041 v02	May 2019	Added information on IDT for Illumina Nextera UD Indexes. Updated language and relocated information previously in Pool Pre-Enriched Libraries to new sections in protocol introduction. Updated documentation introduction to provide more concise information on the Nextera Flex for Enrichment workflow. Updated the Additional Resources section to provide new language and removed extraneous resources. Updated language in Prepare for Pooling to provide more consistent organization.

Document	Date	Description of Change
Document #1000000048041 v01	January 2019	Added a recommended method to quantify FFPE reference materials. Added a hybridization temperature for somatic variant calling. Added symbol descriptions for packaging and consumables. Updated and relocated the recommended gDNA input quantification to the gDNA input < 50 ng section. Updated the sample input recommendations for genomic DNA and extracted FFPE. Updated the final loading concentration recommendations for MiSeq and NovaSeq.
Document #1000000048041 v00	October 2018	Initial release.

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	

Region	Toll Free	International
Spain	+34 800 300 143	+34 911 899 417
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.

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