Nextera Flex for Enrichment with RNA Probes

Demonstrated Protocol

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Introduction

This protocol explains how to prepare up to 384 unique dual-indexed paired-end libraries from DNA using the Nextera™ Flex for Enrichment workflow with RNA-based enrichment probes from a third-party vendor.

The Nextera Flex for 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 recommended for optimal performance.

DNA Input Recommendations

The Nextera Flex for 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 Nextera tagmentation reaction and result in assay failure.

gDNA Input ≥ 50 ng

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

gDNA Input < 50 ng

This protocol does not normalize final pre-enriched library yields from 10–49 ng gDNA 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 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. For more information, see *Sample Input Recommendations* on page 3.

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

- For FFPE samples with ΔCq value of ≤ 5, the recommended DNA input is 50–1000 ng.
- Nextera Flex for 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.

NOTE

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

Blood and Saliva Input Recommendations

The Nextera Flex for 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 *Blood Lysis* on page 29 or *Saliva Lysis* on page 31.

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 Nextera Flex for 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 Nextera Flex for 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 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 11.

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	—	Yes
Blood	No	—	Yes

Additional Resources

The following resources provide instructions and guidelines for using the Nextera Flex for Enrichment kit to prepare libraries. Visit the Nextera Flex for Enrichment support pages 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.

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.
Index Adapters Pooling Guide (document # 1000000041074)	Provides pooling guidelines and dual-index strategies for using the 10 base pair IDT for Illumina Nextera DNA UD Indexes or 8 base pair Nextera XT and Nextera XT v2 Indexes with the Nextera Flex for 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 Nextera DNA UD Indexes support page	Provides information about IDT for Illumina Nextera DNA Unique Dual (UD) indexes.

Protocol

Introduction

This chapter describes the Nextera Flex for Enrichment protocol when using RNA-based enrichment probes from a third-party vendor.

- 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 33.
  - Third-party biotinylated probes must meet specific requirements. See *Third-Party Panel Requirements* on page 35 to ensure your third-party probes meet the requirements.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

Prepare for Pooling

If you plan to pool libraries, record information about your samples before starting library prep using Illumina Experiment Manager (IEM), Local Run Manager, or BaseSpace Sequence Hub. For information on the tools compatible with your sequencing system, visit the Nextera Flex for Enrichment Product Compatibility page.

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

Supported Enrichment Plexities

Nextera DNA Flex 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 35.

- **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, Nextera Flex for Enrichment reagents support the following enrichment reactions and plexity indicated in the table below.

Nextera DNA Flex Enrichment Reagents	Enrichment Reactions	Enrichment Plexity
16-sample kit	16 reactions	1-plex
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 tagment 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 Tagment Wash Buffer (TWB)

- Pipette slowly to minimize foaming.

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

- Each index plate is for single use only.

- IDT® for Illumina® Nextera™ DNA UD Indexes use 10 base pair index codes that differ from other Illumina index adapters, which use 8 base pair index codes. Confirm your sequencing system is configured for 10 base pair index codes.

Prepare IDT for Illumina Nextera DNA UD Indexes as follows.

- Centrifuge at 1000 x 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.

Nextera Flex for Enrichment with RNA Probes Workflow

The following diagram illustrates the Nextera Flex for Enrichment workflow when using RNA-based enrichment probes from a third party vendor. Safe stopping points are marked between steps.

Time estimates are based on processing 12 samples at 12-plex enrichment.

Figure 1 Nextera Flex for Enrichment Workflow

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

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

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 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. Use fresh tips for each sample column. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

10 Seal the plate with Microseal 'B', 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)
- Tagment Wash Buffer (TWB)
- 96-well plate magnet
- Microseal 'B' adhesive seal
- 8-tube strip
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

Preparation

1 Prepare the following consumables:

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

Procedure

1 Let the 96-well PCR plate stand at room temperature for 2 minutes.

2 Add 10 µl ST2 (red cap) to the tagmentation reaction. 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. 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 one time for a total of 2 washes.

7 [> 48 samples] Wash as follows.

a Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
b Immediately add 100 µl TWB directly onto the beads using a deliberately slow pipetting technique. A deliberately slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.
c Repeat steps a and b in 1- or 2-column increments until all columns have been processed.
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 Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
h Immediately add 100 µl TWB directly onto the beads.
i Repeat steps g and h in 1- or 2-column increments until all columns have been processed.
j Repeat steps d–g one time for a total of 2 washes.

8 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.

9 Pipette each well slowly to resuspend the beads. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

10 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 Guide (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 35.

Consumables

- Enhanced PCR Mix (EPM)
- Index adapter plate
- Eppendorf Lo Bind PCR Plate
- Nuclease-free water

- Microseal 'B' adhesive seal
- 1.7 ml microcentrifuge tubes
- Pipette tips
  - 20 µl multichannel pipettes
  - 200 µl multichannel pipettes

About Reagents

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

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw 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
50–1000 ng genomic DNA	9
50–1000 ng extracted FFPE	12
Saliva	9
Blood	9

Procedure

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

Reagent overage is included in the volume 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 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 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

- Agencourt AMPure XP beads (AMPure XP beads), 5 ml
- Resuspension Buffer (RSB)
- Freshly prepared 80% ethanol (EtOH)
- 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
- Nuclease-free water

About Reagents

- Agencourt AMPure XP beads
  - 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
AMPure XP beads	2°C to 8°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.

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 solution 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 AMPure XP beads multiple times to resuspend.

5 If you are using gDNA, blood, or saliva sample inputs, perform the following steps.

   a Add 77 µl nuclease-free water to each well containing supernatant.
   b Add 88 µl AMPure XP beads 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 solution is clear (~5 minutes).
   f During incubation, thoroughly vortex the AMPure XP beads, 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 into the corresponding well of the second plate (containing 20 µl AMPure XP beads).
   h Pipette each well in the second 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 If you are using extracted FFPE sample input, perform the following steps.

   a Add 81 µl AMPure XP beads to each midi plate well 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 the sealed midi plate at room temperature for 5 minutes.

8 Place on the magnetic stand and wait until the solution is clear (~5 minutes).

9 Without disturbing the beads, remove and discard supernatant.

10 Wash two times using the following steps.

   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 and add 17 µl RSB to the beads.

14 Seal the plate and 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 solution 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 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 Run 1 µl of the individual library on one of the following instruments:
   - Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit
     - Add 1 µl of RSB to the library to achieve the 2 µl volume required for the Fragment Analyzer
   - 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 2 and Figure 3.
   For FFPE samples, the mean fragment size can be as low as 250 bp.

Figure 2 Example Fragment Analyzer Trace

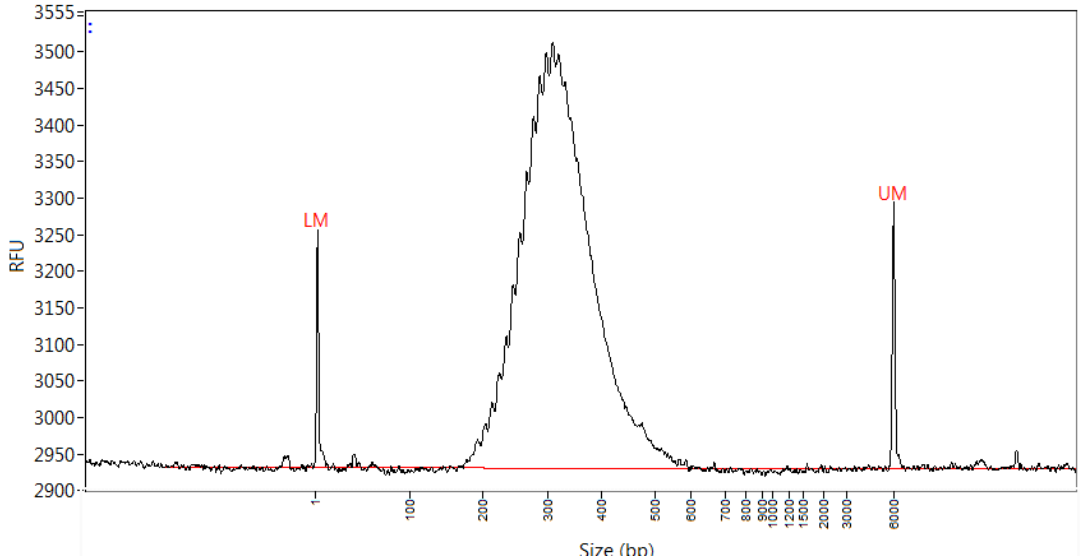
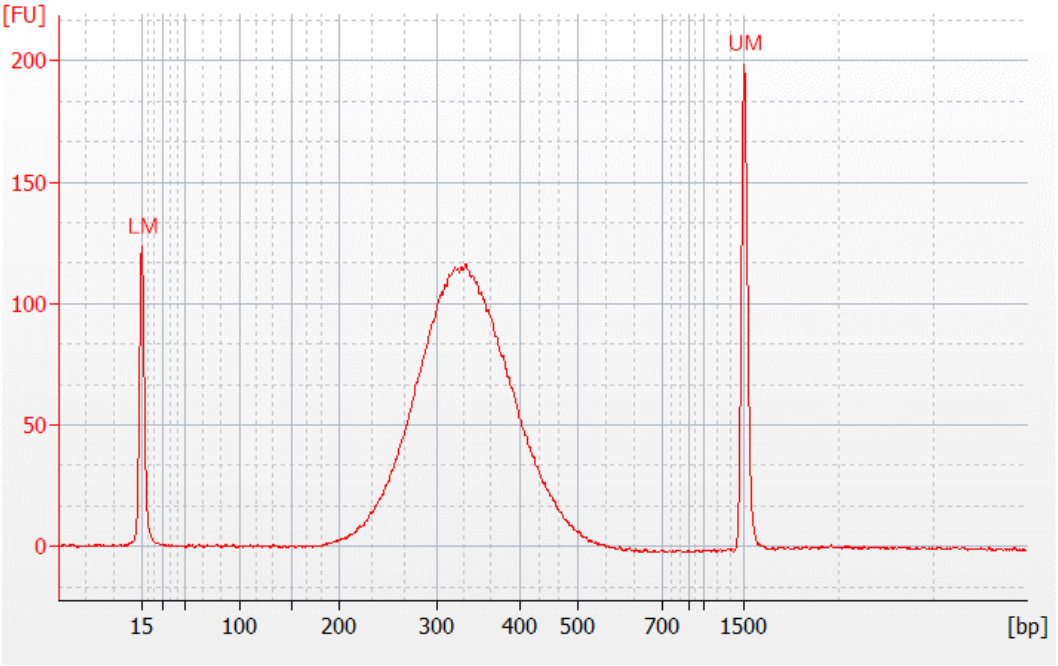


Figure 3 Example Bioanalyzer Trace



Pool Pre-Enriched Libraries

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

Pooling Methods

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

Table 2 Recommended Pooling Methods

Sample Input	Pooling Method
10–49 ng gDNA	Mass 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 enrichemt 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.

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 5, 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

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 3 Expected Pre-Enriched Library Yield

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

NOTE

For other quantification methods with different biases, qualify the quantification method for this workflow. Concentration results may 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 may 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 2–4 in *Pool Pre-Enriched Libraries at Equal Concentration*.

1 Prepare each pre-enriched library to 500 ng. Dilute with RSB as needed.

2 Using the sample tracking method you chose in *Prepare for Pooling* on page 5, record the indexes for the libraries you plan to pool in this step.

3 Combine 500 ng of each library in a 1.5 ml microcentrifuge tube into the plexities shown in the following table.

Library Pool	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 19.
- 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 *Concentrate Pooled Library* on page 28.
  - **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 RNA-based capture probes.

Nextera Flex for Enrichment reagents are compatible with third-party, RNA-based enrichment oligonucleotide panels. For more information on third-party, RNA-based probe requirements, see *Third-Party Panel Requirements* on page 35.

Consumables

- Enrich Hyb Buffer 2 (EHB2)
- RNA-based enrichment probe panel (third-party)
- RNase inhibitor (third-party)
- Hyb Buffer + IDT NXT Blockers (NHB1) (blue cap)
- 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

- NHB1 precipitates and separates during storage.
- RNA-based enrichment probe panel refers to the chosen RNA-based enrichment oligonucleotide panel from 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.
RNA-Based Enrichment Probe Panel	Follow recommendations from third-party vendor	Thaw on ice. 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 35.
RNase Inhibitor	Follow recommendations from third-party vendor	Thaw on ice. Vortex to mix.
NHB1 (blue cap)	-25°C to -15°C	Thaw at room temperature. Once at room temperature, preheat to 50°C on a microheating system for 5 minutes. Vortex at maximum speed 3 times for 10 seconds each to resuspend. Centrifuge briefly. Pipette up and down from the bottom of the tube. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear. Use while warm to avoid precipitates from reforming.

Item	Storage	Instructions
SMB	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-RNA program on the thermal cycler.
- 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
- 65°C for 5 minutes
- 65°C for 1 minute
  - You will need to manually pause the thermal cycler to add an RNA-based enrichment probe panel with RNase inhibitor, as described in step 4 of the *Procedure* on page 20.
- Hold at 58°C for 16 hours
- **[Optional]** Extend the hold to a maximum of 24 hours

Total running time is ~16.5 hours.

Procedure

1 Add the following reagents ***in the order listed*** to each well of a new PCR plate or 8-tube strip. Creating a master mix of NHB1 and EHB2 negatively impacts enrichment performance.
- Pre-enriched library sample or pool (30 µl)
- NHB1 (blue cap) (50 µl)
- EHB2 (10 µl)

2 Using a pipette set to 70 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-RNA program. After starting the thermal cycler, immediately proceed with the following steps. Be prepared to return to the thermal cycler after ~10 minutes with the RNA probe master mix that you create in steps 4a–b.

a Combine the following to prepare the RNA probe master mix.
- RNA-based enrichment probe panel (5 µl or 3 µl, depending on third-party vendor recommendation)
- RNase inhibitor (0.5 µl)

b Add nuclease-free water to the RNA probe master mix to create a final volume of 10 µl. For example, if you added 5 µl of the RNA-based enrichment probe panel, add 4.5 µl nuclease-free water.

c Vortex, and then centrifuge at 280 × g for 10 seconds. Keep at room temperature for use in step 4e.

d After ~10 minutes, select **Pause** on the thermal cycler when the NF-HYB-RNA program reaches step 3. See thermal cycler steps in *Preparation* on page 19 for more information.

e Keep the plate or tube on the thermal cycler and add 10 µl RNA probe master mix to each well.

f Using a pipette set to 70 µl, pipette each well 10 times to mix.

g Seal the plate or cap the tubes.

h Select **Resume** on the thermal cycler to continue the NF-HYB-RNA program.

5 Proceed immediately to the next procedure when the NF-HYB-RNA program hold temperature time ends.

CAUTION

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

Capture Hybridized Probes

This step uses SMB to capture probes hybridized to the targeted regions of interest.

Consumables

- Streptavidin Magnetic Beads (SMB)
- Enhanced Enrichment Wash (EEW) (amber cap)
- Enrichment Elution Buffer 1 (EE1)
- 2N NaOH (HP3)
- Elute Target Buffer 2 (ET2)
- 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
- SMB
  - Make sure to use SMB and not Agencourt AMPure XP beads for this procedure.
  - SMB must be at room temperature before use.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
SMB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert 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 58°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 SMB 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 58°C.
Proceed to step 5 while the sample incubates.

5 Preheat EEW (amber tube) by laying the tube on its side on the MIDI heat block insert on the second microheating system to 58°C. 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 22.

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 58°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.

> **CAUTION**
> Transferring the reagent minimizes carryover of residual reagents that can inhibit downstream PCR.

4 [Tube] Centrifuge briefly.

5 Immediately place on a magnetic stand and wait until the liquid is clear (~2 minutes).

6 Using a pipette set to 200 µl, remove and discard all supernatant from each well or from the tube.

7 Centrifuge the plate or the tube at 280 × g for 30 seconds.

8 Place on a magnetic stand for 10 seconds.

9 Use a 20 µl pipette to remove and discard residual liquid from each well or from the tube.

10 Immediately proceed to *Elute* on page 23 to prevent excessive drying of the beads and library yield loss.

Elute

1 Combine the following volumes to prepare an elution 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 elution mix at 280 × g for 10 seconds.

3 Remove from the magnetic stand.

4 Add 23 µl elution 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-RNA 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 30 seconds
- 12 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.

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-RNA 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 AMPure XP beads to purify the enriched library and remove unwanted products.

Consumables

- Agencourt AMPure XP beads (AMPure XP beads), 5 ml
- 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

- Agencourt AMPure XP beads
  - 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
AMPure XP beads	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB	2°C to 8°C	Bring to room temperature. Vortex to mix.

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 Vortex and invert AMPure XP beads multiple times.

4 Add 45 µl AMPure XP beads 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.

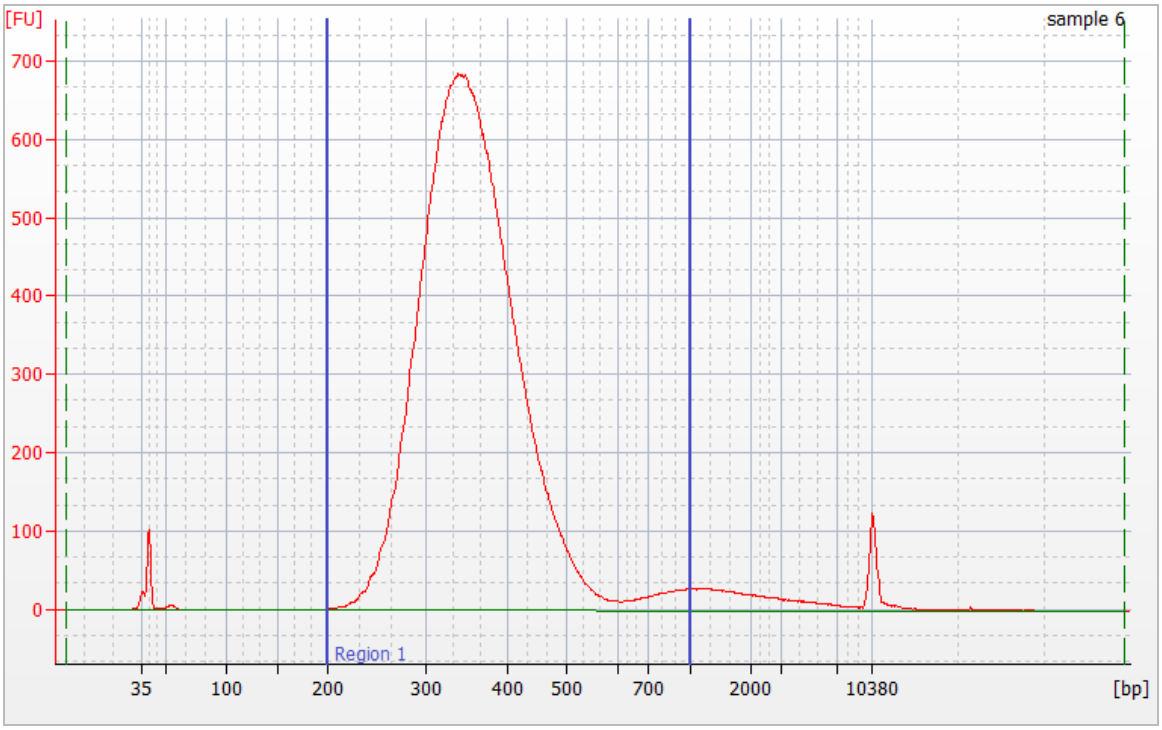
1 Run 1 µl of the enriched libraries using the Qubit dsDNA BR Assay Kit to quantify library concentration.

NOTE

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 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.
- For all other qualification methods, use 350 bp as the average library size.

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

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

Introduction

This section provides instructions for optional procedures within the Nextera Flex for Enrichment workflow.

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 17.

Consumables

- Agencourt AMPure XP beads (AMPure XP beads), 5 ml
- 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

- Agencourt AMPure XP beads
  - 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
AMPure XP beads	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB	2°C to 8°C	Bring to room temperature. Vortex to mix.

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.

NOTE

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

3 Vortex and invert AMPure XP beads multiple times to resuspend.

4 Add 1.8x pool volume of AMPure XP beads 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 liquidis 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 liquidis 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 19.

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.

Blood Lysis

Use this protocol when performing the Nextera Flex for 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 therefore cannot be recommended.

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

CAUTION

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.

Consumables

- Agencourt AMPure XP beads (AMPure XP beads), 5 ml
- 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

- Agencourt AMPure XP beads
  - 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
AMPure XP beads	2°C to 8°C	Let stand at room temperature for 30 minutes. 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.

*BLB is shipped -25°C to -15°C, but stored at 15°C to 30°C.

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 Vortex and invert AMPure XP beads multiple times to resuspend.

7 Add 20 µl AMPure XP beads to the well.

8 Using a pipette set to 50 µl, slowly pipette 10 times to mix.

9 Seal the plate, place on the preprogrammed thermal cycler, and run the BLP program.

10 Place on a magnetic stand and wait 5 minutes.
  The liquid will not become clear due to the dark brown color of the blood from the lysis reaction. 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 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 9 or stop and store the sample bead mixture.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 9, 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.

Saliva Lysis

Use this protocol when performing the Nextera Flex for 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.

WARNING

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

Consumables

- Agencourt AMPure XP beads (AMPure XP beads)
- Oragene DNA collection tubes (for saliva sample collection)
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plate

About Reagents

- Agencourt AMPure XP beads
  - 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
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.
AMPure XP beads	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.

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 Vortex and invert AMPure XP beads multiple times to resuspend.

5 Add 20 µl AMPure XP beads 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 Pipette to remove and discard the EtOH.

14 Use a 20 µl pipette to remove and discard all residual EtOH.

15 Remove the plate from the magnetic stand.

16 Add 30 µl nuclease-free water and pipette to resuspend.

17 Proceed immediately to step 3 of *Tagment Genomic DNA* on page 9 or stop and store the sample bead mixture.

SAFE STOPPING POINT

If you are stopping before proceeding to *Tagment Genomic DNA* on page 9, 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

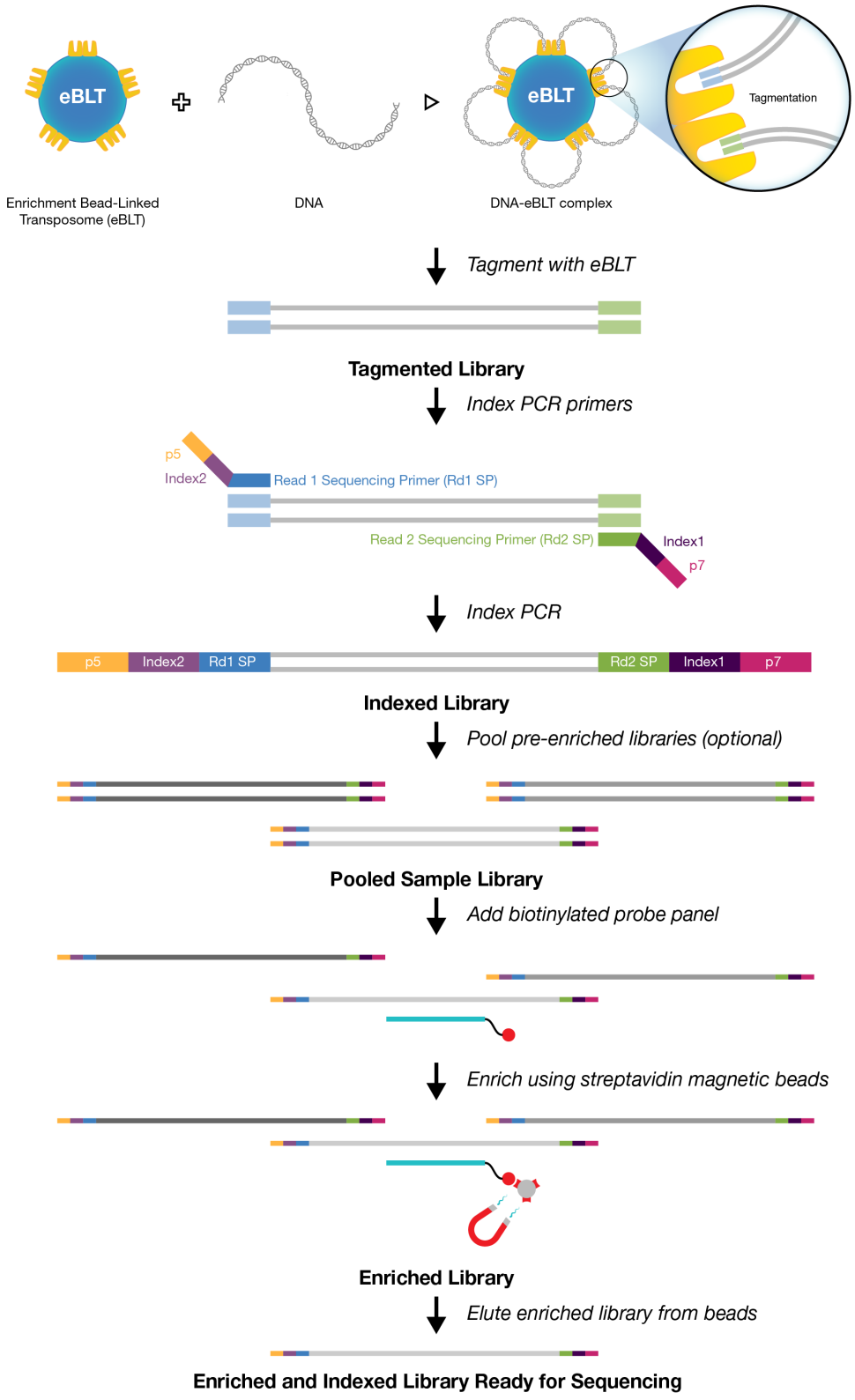
Introduction

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

How the Nextera Flex for Enrichment Assay Works

The Nextera Flex for 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 platforms. 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 (SMB) 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 Nextera Flex for Enrichment Workflow



Kit Contents

Completing the Nextera Flex for 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]	Nextera DNA Flex Pre-Enrichment Library Prep – 16 Samples Nextera DNA Flex Enrichment Reagents – 16 Samples (16, 1-plex Enrichment Reaction)	20025523
	Nextera DNA Flex Pre-Enrichment Library Prep – 96 Samples Nextera DNA Flex Enrichment Reagents – 96 Samples (8, 12-plex Enrichment Reactions)	20025524
Library prep only reagents Use for enrichment plexities between 1-plex and 12-plex. See *Non-Standard Enrichment Plexity Reagents* on page 35.	Nextera DNA Flex Pre-Enrichment Library Prep – 16 Samples	20025519
	Nextera DNA Flex Pre-Enrichment Library Prep – 96 Samples	20025520
Index adapters	IDT for Illumina Nextera DNA UD Indexes Set A (96 Indexes, 96 Samples)	20027213
	IDT for Illumina Nextera DNA UD Indexes Set B (96 Indexes, 96 Samples)	20027214
	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 Set A,B,C,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] Nextera DNA Flex Enrichment Reagents must be ordered with Nextera DNA Flex Pre-Enrichment 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 RNA probes (fixed exome), ensure they meet the following specifications.

- 120 bp probe length
- Between 300,000 to 675,000 probes
- Single-stranded
- Total probe input of ≥ 3 pmols for enrichment at plexities from 1-plex to 12-plex

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 non-standard enrichment plexities based on enrichment plexity, number of samples, and number of required reactions using the 16 sample configuration.

Table 4 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.

Nextera DNA Flex Pre-Enrichment Library Prep Contents

Nextera DNA Flex Pre-Enrichment Library Prep - 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

Nextera DNA Flex Pre-Enrichment Library Prep- 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

NOTE

Agencourt AMPure XP beads are not included in this kit and must be purchased separately.

Nextera DNA Flex Pre-Enrichment Library Prep - 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

Nextera DNA Flex Enrichment Reagents

Nextera DNA Flex Enrichment Reagents - Beads and Buffers, Store at 2°C to 8°C

Tube Quantity		Acronym	Reagent Name	Tube Cap Color
16 Samples	96 Samples			
4	2	SMB	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

NOTE

Agencourt AMPure XP beads are not included in this kit and must be purchased separately.

Nextera DNA Flex Enrichment Reagents - PCR Reagents and 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	2N NaOH	Clear
2	1	NHB1	Hyb Buffer + IDT NXT Blockers	Blue
2	1	EPM	Enhanced PCR Mix	Clear

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

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

Infinium FFPE QC Kit, Store at -25°C to -15°C (Optional)

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

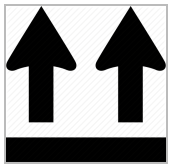
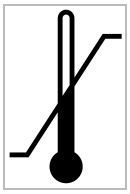
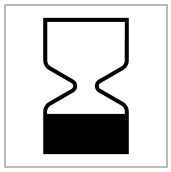
Flex Lysis Reagent Kit (Optional)

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.
	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.

Symbol	Description
	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 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 and Equipment

Confirm that all required user-supplied consumables and equipment are present and available 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 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 block (inhibitor)	Agilent, catalog # 300151 or 300152
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
Agencourt AMPure XP purification beads, 5ml	Beckman Coulter, catalog #A63880
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 Deepwell Storage (midi plate)	Thermo Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier

Consumable	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
Agencourt AMPure XP beads, 5 ml	Beckman Coulter, catalog #A63880
[Blood] Flex Lysis Reagent Kit	Illumina, catalog # 20015884
[Blood] EDTA Blood Collection tubes	Becton Dickinson
[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 instrument	General lab supplier

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)
MIDI Heat Block Insert for SciGene Hybex System	Illumina, catalog # BD-60-601
Qubit Fluorometer 3.0	ThermoFisher Scientific, catalog # Q33216 or Q33217

Equipment	Supplier
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 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

Equipment for Tube Workflow

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

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

Acronym	Definition
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	2N NaOH
IEM	Illumina Experiment Manager
NHB1	Hyb Buffer + IDT NXT Blockers
NXT	Nextera
PK1	Proteinase K
PPC	PCR Primer Cocktail
QCP	QC Primer Reagent
QCT	QC Template Reagent
RSB	Resuspension Buffer
SMB	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 Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
Australia	+1.800.775.688	
Austria	+43 800006249	+43 19286540
Belgium	+32 80077160	+32 34002973
China	400.066.5835	
Denmark	+45 80820183	+45 89871156
Finland	+358 800918363	+358 974790110
France	+33 805102193	+33 170770446
Germany	+49 8001014940	+49 8938035677
Hong Kong	800960230	

Region	Toll Free	Regional
Ireland	+353 1800936608	+353 016950506
Italy	+39 800985513	+39 236003759
Japan	0800.111.5011	
Netherlands	+31 8000222493	+31 207132960
New Zealand	0800.451.650	
Norway	+47 800 16836	+47 21939693
Singapore	+1.800.579.2745	
South Korea	+82 80 234 5300	
Spain	+34 911899417	+34 800300143
Sweden	+46 850619671	+46 200883979
Switzerland	+41 565800000	+41 800200442
Taiwan	00806651752	
United Kingdom	+44 8000126019	+44 2073057197
Other countries	+44.1799.534000	

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

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.