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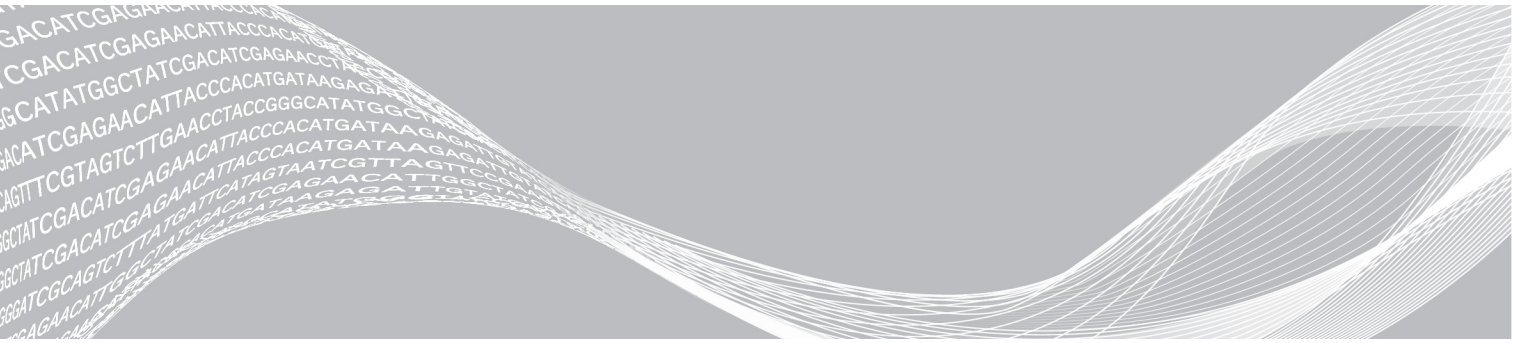
Illumina DNA PCR-Free Library Prep, Tagmentation

Reference Guide



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Revision History

Document	Date	Description of Change
Document # 1000000086922 v03	February 2021	Corrected formula for calculating molarity. Updated final library storage temperature to 30 days.
Document # 1000000086922 v02	February 2021	Clarified that low input libraries are sequenced using the NovaSeq 6000 XP workflow. Clarified quantification requirements for 300–2000 ng DNA input type. Updated the catalog number for IDT for Illumina DNA/RNA UD Indexes Set D, Tagmentation. Updated the equipment required for saliva lysis. Corrected MLB storage temperature to -25°C to -15°C.
Document # 1000000086922 v01	July 2020	Corrected plate name from "PCR" to "MIDI" in Clean Up Libraries for Hybex protocol.
Document # 1000000086922 v00	July 2020	Initial release.

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Introduction

This protocol explains how to prepare up to 96 dual-indexed paired-end single-stranded libraries from DNA using the Illumina DNA PCR-Free Library Prep workflow.

Illumina DNA PCR-Free Library Prep :

- Simultaneously performs fragmentation and adds adapter sequences.
- Normalizes samples at inputs ≥ 300 ng.
- Streamlines sample pooling and sequencing at inputs ≥ 300 ng.
- Generates libraries from ≥ 25 ng input using a low input protocol.
- Offers compatibility with whole blood, dried blood spots, or saliva.

DNA Input Recommendations

The Illumina DNA PCR-Free Library Prep protocol accepts purified DNA inputs of 25–2000 ng. Follow the protocol appropriate for your input. Acceptable input types are as follows:

25-99 ng DNA—Quantification is required. Use a thermal cycler and follow the low input procedures for tagmentation and clean up. Final libraries are not normalized. Sequence low input libraries using the NovaSeq 6000 XP workflow.

100-299 ng DNA—Quantification is required. Use a thermal cycler and follow the standard input procedure for tagmentation and cleanup. The use of a Hybex incubator is not supported for ≤ 299 ng input. Final libraries are not normalized.

300-2000 ng DNA—Initial quantification is not required, but quantification of the final library pool is still required. Use a thermal cycler or Hybex and follow the standard input procedure for tagmentation and cleanup. Final libraries are normalized.

Blood/saliva—Quantification is not required. Use a thermal cycler. Final libraries are normalized. See the following section for more information on blood and saliva input requirements.

Input Type (ng)	Initial Quantification Required	Final Libraries Normalization	Protocol Compatibility
25–99 DNA	Yes	No	Thermal cycler (low input)
100–299 DNA	Yes	No	Thermal cycler (standard input)
300–2000 DNA	No	Yes	Thermal cycler (standard input) or Hybex
Blood/Saliva*	No	Yes	Thermal cycler

* For more information on blood and saliva input requirements, see the following section.

Blood and Saliva Input Recommendations

The Illumina DNA PCR-Free protocol is compatible with fresh whole blood, dried blood spots, and saliva sample inputs. To extract gDNA from blood or saliva inputs, the Illumina Lysis Reagent Kit is required.

For information about protocols specific to whole blood, dried blood spots, and saliva, see on page 37.

When starting with inputs of 20 µl liquid whole blood from EDTA tubes, 50 µl saliva from Oragene tubes, or 6 x 3 mm² dried blood spot punches in Whatman 903 protein saver cards, expect normalization of libraries equal to that observed when using 300 ng gDNA input. Blood and saliva are heterogeneous sample types, therefore the ability of Illumina DNA PCR-Free 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

Input DNA Quantification

- Quantify the starting DNA using a fluorescence-based quantification method, such as Qubit dsDNA HS. Do not use a UV spectrometer method.
  - Fluorescence-based methods employ a dye specific to double-stranded DNA (dsDNA) and specifically and accurately quantify dsDNA, even when many common contaminants are present.
  - Use 2 µl for quantification.

- In contrast, UV spectrometer methods based on 260 OD readings can overestimate DNA concentrations. The overestimation is due to the presence of RNA and other contaminants common to DNA preparations.

Custom Primer Considerations

The Illumina DNA PCR-Free Library Prep protocol requires the use of the VP10 primer for the Illumina DNA PCR-Free Kit, unless you are using a v1.5 or newer NovaSeq reagent kit. Follow the read 1 instructions in the *NovaSeq Series Custom Primers Guide (document # 1000000022266)* for information on loading and setting up runs with custom sequencing primers.

Assess Purity

Assess purity to ensure the DNA sample contains < 1 mM EDTA and is free of phenol, ethanol, and other organic contaminants. These contaminants can interfere with the tagmentation reaction, causing library prep failure, suboptimal clustering, or low-quality sequencing data. For a complete list of contaminants with sources, prevention, and effects, see *Nextera XT Library Prep: Tips and Troubleshooting (Pub. No. 770-2015-015)*.

Using a UV absorbance method, a ratio of absorbance at 260 nm to absorbance at 280 nm indicates DNA purity. The Illumina DNA PCR-Free protocol is optimized for 260/280 absorbance ratio values of 1.8–2, which indicates a pure DNA sample. For a secondary indication of purity, use the ratio of absorbance at 260 nm to absorbance at 230 nm and target a 260/230 ratio of 2–2.2. Values outside this range indicate the presence of contaminants.

Tips and Techniques

Protocol Continuity

- Follow the protocol in the order described using the specified parameters.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocols:
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps **[Thermal Cycler Protocols]**

  - Shaking steps **[Hybex Protocol]**
- Apply the adhesive seal to cover the plate, and seal with a rubber roller or wedge.
- Use the appropriate seal:
  - Use Microseal 'B' adhesive seals for shaking, centrifuging, and long-term storage. The seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates.
  - Microseal 'F' adhesive foils are effective at temperatures as low as -70°C and suitable for long-term storage of the 96-well plate containing final libraries.
- After each use, discard all seals from plates.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well to prevent sample loss.

Handling Beads

- When washing beads:
  - Use the DynaMag-96 Side Skirted Magnet for all PCR plates. Use of another magnet may result in suboptimal library yield.
  - Use Magnetic Stand-96 for all MIDI plates.
  - Dispense liquid so that no beads remain adhered to the side of the well.
  - Keep the plate on the magnetic stand until the instructions specify to remove it. Always remove the plate from the magnetic stand before centrifuging.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.
- Pipette bead suspensions slowly.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex throughout protocol as necessary to keep homogenous.
- If beads are aspirated into pipette tips when they are not intended to be, dispense reactions back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).

Protocols for Thermal Cycler

Illumina DNA PCR-Free Library Prep offers separate standard DNA input and low DNA input thermal cycler protocols. Make sure you are using the appropriate procedure for your input amount. See *DNA Input Recommendations* on page 1 for information on choosing the correct protocol for your input. See

Protocol for Thermal Cycler, Standard Input on page 6 for the standard input thermal cycler protocol and *Protocol for Thermal Cycler, Low Input* on page 17 for the low input thermal cycler protocol.

- Review the 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. See *Supporting Information* on page 45. The Illumina DNA PCR-Free protocol requires the following components.
  - Library prep reagents.
  - Index adapters (sold separately).
  - Custom sequencing primers (sold separately).

Chapter 2 Protocol for Thermal Cycler, Standard Input

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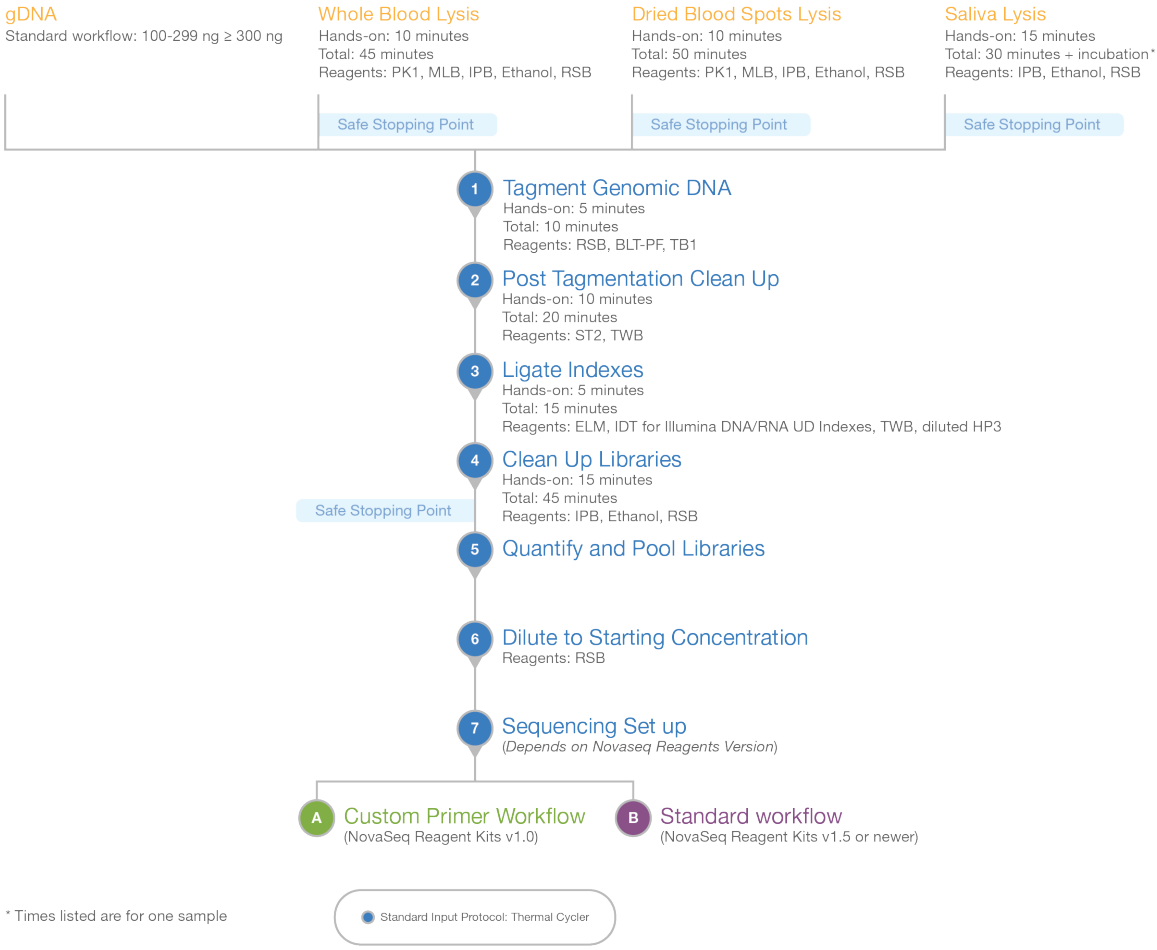
Library Prep Workflow, Standard Input

The following diagram illustrates the Illumina DNA PCR-Free Library Prep thermal cycler workflow for inputs of 100-299 and ≥300 ng DNA.

Figure 1 Illumina DNA PCR-Free Library Prep Standard Input Workflow



Tagment Genomic DNA

This step uses the Bead-Linked Transposomes PCR-Free (BLT-PF) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences. Use the preparation steps in this section to prepare reagents in advance.

Consumables

- BLT-PF (Bead-Linked Transposomes PCR-Free)
- HP3 (2N NaOH)
- RSB (Resuspension Buffer)
- TB1 (Tagmentation Buffer 1)
- Freshly prepared 80% ethanol (EtOH)

- Nuclease-free water
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Prepare for a later section:
  - ELM (Extension Ligation Mix)
  - IPB (Illumina Purification Beads)
  - Index adapters (IDT for Illumina DNA/RNA UD Indexes)
  - ST2 (Stop Tagment Buffer 2)
  - TWB (Tagment Wash Buffer)

Warning

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

- Always use the specified magnetic stand to prevent failure.
- BLT-PF
  - Store frozen and upright to make sure beads are submerged in the buffer.
  - Aspirate and dispense slowly due to viscosity of the solution

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
IPB*	15°C to 30°C	Vortex 1 minute. Invert 2–5 times, and then vortex thoroughly to resuspend.
RSB	15°C to 30°C	Label the tube cap RSB. Vortex or invert to mix.
ST2 *	15°C to 30°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Vortex thoroughly, and then centrifuge.
TWB*	15°C to 30°C	Label the tube cap TWB. Invert thoroughly to mix.

Item	Storage	Instructions
BLT-PF	-25°C to -15° C	Thaw at room temperature until ambient. Vortex to resuspend. Do not centrifuge before pipetting.
ELM*	-25°C to -15°C	Thaw at room temperature. Keep on ice until needed. Invert to mix before use.
HP3	-25°C to -15°C	Thaw at room temperature until ambient. Vortex, and then centrifuge briefly.
Index adapters*	-25°C to -15°C	Thaw at room temperature until ambient. Vortex, and then centrifuge briefly.
TB1	-25°C to -15°C	Thaw at room temperature until ambient. Vortex, and then centrifuge.

* Reagent is used in a later section.

NOTE

RSB and TWB are shipped in similar tubes. Label each cap before beginning the protocol.

2. For each reaction, combine the following volumes to prepare diluted HP3:
   - HP3 (6 µl)
   - Nuclease-free water (54 µl)

   These volumes produce 60 µl diluted HP3 per reaction, including overage.
   Vortex and centrifuge briefly.
3. For each reaction, combine the following volumes to prepare 80% EtOH:
   - EtOH (400 µl)
   - Nuclease-free water (100 µl)

   These volumes produce 500 µl 80% EtOH per reaction, including overage.
   Vortex to mix.
4. 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
   - 41°C for 5 minutes
5. Save the following ELM program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 37°C for 5 minutes
   - 50°C for 5 minutes

Procedure

1. If using gDNA input, do as follows.
   a. Label a new 96-well PCR plate LP1.
   b. Add 2–25 µl DNA to each well, so that the total input amount is within the desired range (100-299 or 300-2000 ng).
   c. If sample volume is < 25 µl, bring the total volume to 25 µl using RSB.
2. Vortex BLT-PF vigorously for 1 minute to resuspend. Repeat as necessary.
3. If beads adhere to the sides or top of the tube, briefly spin down and then pipette to resuspend.
4. **[gDNA input]** Add 15 µl BLT-PF to each well.
5. **[Blood/Saliva input]** Add 10 µl BLT-PF to each well containing 30 µl extracted DNA.
6. Using a P200 pipette set to 35 µl, pipette to mix.
7. Add 10 µl TB1 to each well.
8. Using a P200 pipette set to 40 µl, pipette to mix, and then seal.
9. Place on the preprogrammed thermal cycler and run the TAG program.
10. Proceed immediately to *Post Tagmentation Cleanup*.

Post Tagmentation Cleanup

This step stops the tagmentation reaction and washes the adapter-tagged DNA on the BLT-PF.

Consumables

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

About Reagents

- Pipette TWB and ST2 slowly to minimize foaming.
- Dispense TWB directly onto beads.

Procedure

1. Add 10 µl ST2 to each well.
2. Using a P200 pipette set to 50 µl, pipette to mix.
3. Incubate at room temperature for 2 minutes.
4. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
5. Using a P200 pipette set to 60 µl, remove and discard all supernatant from each well.

6. Remove the plate from the magnetic stand.
7. Add 150 µl TWB to each well.
8. Pipette to mix ten times or until beads are fully resuspended.
9. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

Ligate Indexes

This step ligates Index 1 (i7) and Index 2 (i5) adapters to each sample.

Consumables

- ELM (Extension Ligation Mix)
- Index adapters (IDT for Illumina DNA/RNA UD Indexes)
- TWB (Tagment Wash Buffer)
- Diluted HP3
- Microseal 'B' adhesive seals

About Reagents

- The index plate wells cannot be reused.
- Aspirate and dispense ELM slowly due to the viscosity of the solution.

Procedure

1. Without disturbing the bead pellet, remove and discard residual supernatant from each well.
2. Remove the plate from the magnetic stand.
3. Add 45 µl ELM to each well.
4. Pipette to mix until beads are fully resuspended and not on the side of the well.
5. Pierce the foil seal covering the index adapter plate as follows.
    - **[< 96 samples]** Pierce the wells you intend to use. Use a new pipette tip for each well.
    - **[96 samples]** Align a new semi-skirted 96-well PCR plate over the index adapter plate and slowly press down to puncture all 96 wells. Discard the PCR plate.
6. Add 5 µl index adapters to each well.
7. Using a P200 pipette set to 45 µl, pipette to mix, and then seal.
8. Place on the preprogrammed thermal cycler and run the ELM program.
9. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
10. Remove and discard all supernatant from each well.
11. Remove the plate from the magnetic stand.
12. Add 75 µl TWB onto the beads in each well.

13. Pipette to mix until beads are fully resuspended and not on the side of the well.
14. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. Remove and discard all supernatant from each well.
16. Seal, and then centrifuge at 280 x g for 10 seconds.
17. Place on the magnetic stand.
18. Without disturbing the bead pellet, remove and discard residual supernatant from each well.
19. Remove the plate from the magnetic stand.
20. Add 45 µl diluted HP3 to each well.
21. Pipette to mix until beads are fully resuspended and not on the side of the well.
22. Incubate at room temperature for 2 minutes.
23. Proceed immediately to *Clean Up Libraries*.

Clean Up Libraries

This step uses a double-sided bead purification procedure to purify the libraries. In the first side of the bead purification procedure, Illumina purification beads are added to the sample containing BLT-PF beads. Then the supernatant is transferred to a new plate containing Illumina purification beads for the second side of the double-sided bead purification procedure.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate (2)
- Microseal 'B' adhesive seal
- **[Optional]** Microseal 'F' adhesive foil

About Reagents

- IPB
  - 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

Procedure

1. Vortex IPB.
2. Add 36 µl IPB to each well containing sample with BLT-PF beads.
3. Pipette 10 times to mix.

4. Incubate at room temperature for 2 minutes.
5. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
6. While the plate incubates, labela new 96-well PCR plate LP2.
7. Vortex and then invert IPB until fully resuspended.
8. Add 42 µl IPB to each well of LP2.
9. Without disturbing the bead pellet, **transfer** 76 µl supernatant from each well of LP1 to the corresponding well of the LP2.
10. Pipette to mix until all beads are in solution.
11. Remove LP1 from the magnetic stand, and then discard.
12. Incubate LP2 at room temperature for 2 minutes.
13. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
14. Without disturbing the bead pellet, remove and discard all supernatant from each well.
15. Wash beads as follows.
    a. Keep on magnetic stand and add 180 µl fresh 80% ethanol to each well.
    b. Wait 30 seconds.
    c. Remove and discard all supernatant from each well.
16. Wash beads a **second** time.
17. Apply Microseal 'B' and then centrifuge 280 x g for 10 seconds.
18. Place on the magnetic stand, and then wait 10 seconds.
19. Remove residual EtOH from each well.
20. Discard unused 80% EtOH.
21. Air-dry on the magnetic stand (~2 minutes).
22. Remove from the magnetic stand.
23. Add 22 µl RSB onto the beads in each well.
24. Pipette to mix until beads are fully resuspended and not on the side of the well.
25. Incubate at room temperature for 2 minutes.
26. Centrifuge 280 x g for 10 seconds.
27. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
28. Label a new PCR plate FLP.
29. Transfer 20 µl supernatant from each well of LP2 to the corresponding well of FLP.
30. Proceed to *Quantify and Pool Libraries*.

SAFE STOPPING POINT

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

Quantify and Pool Libraries

Pool equal library volumes and quantify the pool before sequencing to ensure optimal cluster density (for gDNA inputs >300 ng and whole blood, dried blood spots, and saliva sample input protocols only).

Illumina DNA PCR-Free Library Prep libraries are single stranded. It is not possible to view library size distribution on any capillary electrophoresis instrument.

For pooling guidelines, see *Index Adapter Pooling Guide (document # 1000000041074)*.

≥ 300 ng DNA Input

For DNA inputs ≥ 300 ng, quantifying and normalizing libraries from the same experiment is not required. If using libraries from separate experiments, the final yields can vary.

1. For each sequencing library pool, combine 9 µl of each library in a 1.5 or 1.7 ml microcentrifuge tube.
2. Vortex to mix, and then centrifuge at 280 x g for 1 minute.
3. Quantify the library pool:
   - Analyze 2 µl pooled library using the Qubit ssDNA (single-stranded) assay kit or a KAPA qPCR Library Quantification Kit.

100-299 ng DNA Inputs

For DNA inputs 100-299 ng, quantify the library before pooling.

1. Analyze 2 µl of each library using KAPA qPCR library quantification kit.
2. Use 450 bp as the library length.
3. In a 1.5 or 1.7 ml microcentrifuge tube, combine libraries equimolarly as follows.
   - **[XP loading]** Combine libraries equimolarly to between 0.75–1nM final concentration.
   - **[Standard loading]** Combine libraries equimolarly to between 1–1.5nM final concentration.
4. Vortex to mix, and then centrifuge at 280 x g for 1 minute.
5. Proceed immediately to *Dilute Libraries to the Starting Concentration and Sequence*.

Dilute Libraries to the Starting Concentration and Sequence

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.

1. Calculate the volumes of RSB and library pool (for inputs ≥ 300 ng) or single libraries (for inputs 100-299 ng) to dilute libraries to the starting concentration for your system.
   - When using a qPCR method—Use the molarity value determined by the KAPA qPCR protocol. Use 450 bp as the average library size and 660 g/mol as the DNA mass. Illumina recommends diluting libraries by a 1:10000x dilution when using the KAPA method.
   - When using Qubit method—Calculate the molarity value of the pooled libraries using the formula below.

     The formula uses 450 bp as the average library size and 660 g/mol as the DNA mass. This equation will output the double stranded DNA equivalent.

     $$Molarity\ (nM) = Yield\ (\frac{ng}{ul}) \times 3.36$$

Sequencing System	KAPA qPCR Quantification		Qubit ssDNA Quantification	
	Starting Concentration (nM)	Final Loading Concentration (pM)	Starting Concentration (nM)	Final Loading Concentration (pM)
NovaSeq 6000 standard workflow	1–1.5	200–300	2–3	400–600
NovaSeq 6000 Xp workflow	0.75–1	150–200	1.5–2	300–400

2. Dilute the pool to the starting concentration for your system.
3. If you are using a v1.0 NovaSeq reagent kit, prepare the VP10 custom sequencing primer. See *Custom Primer Considerations* on page 3 for more information on using custom sequencing primers with Illumina DNA PCR-Free.
4. Follow the standard workflow instructions in Protocol A or the XP workflow instructions in Protocol B of the the *NovaSeq Denature and Dilute Libraries Guide (document # 1000000106351)* to dilute to the final loading concentration.

   For sequencing, Illumina recommends setting up a paired-end run of 151 cycles per read using 10 bp index reads: (Read 1, i5 index read, i7 index read, Read 2), (151, 10, 10, 151). See the *NovaSeq 6000 Sequencing System Guide (document # 1000000019358)* for additional sequencing information.

Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Chapter 3 Protocol for Thermal Cycler, Low Input

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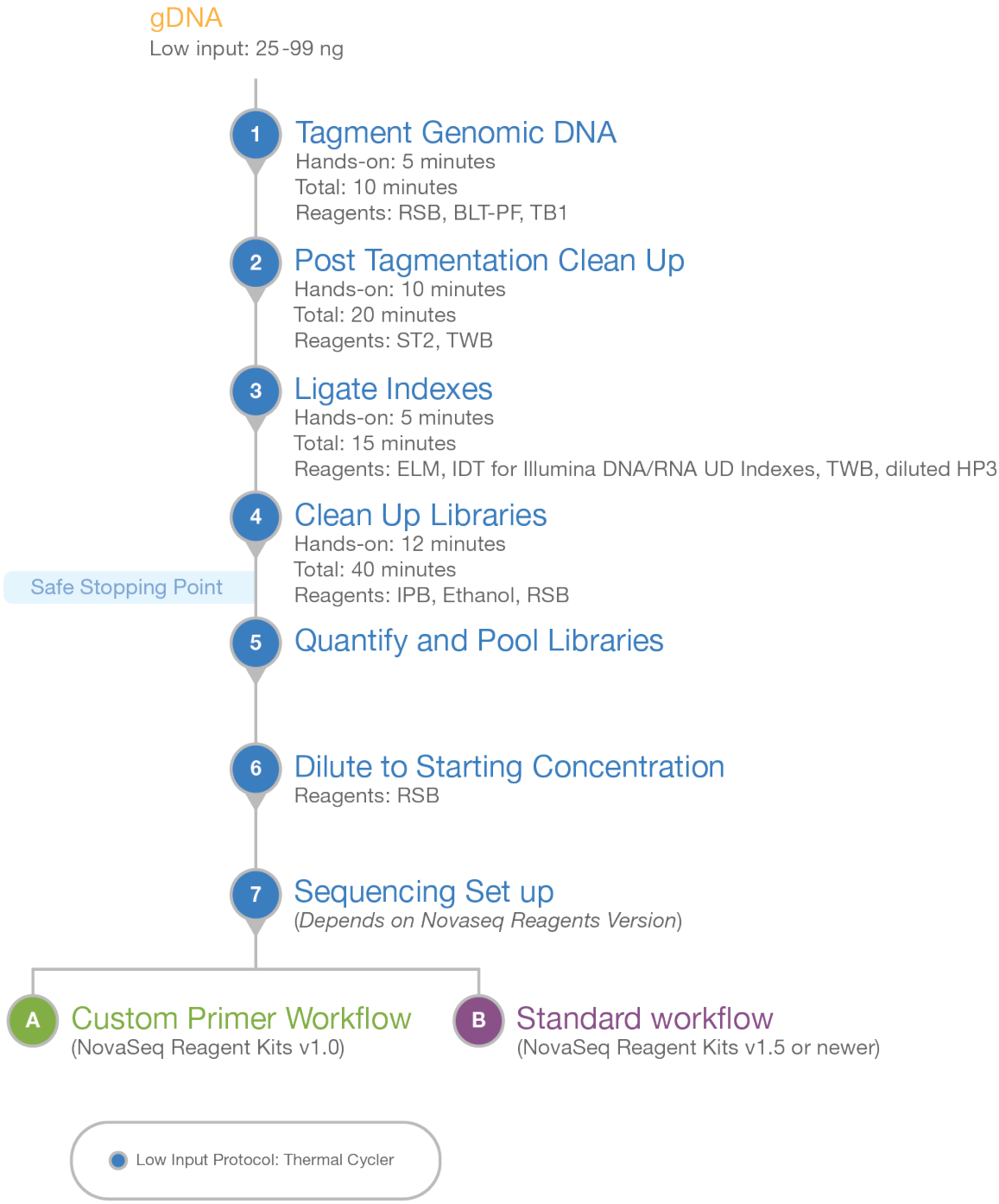
Library Prep Workflow, Low Input

The following diagram illustrates the Illumina DNA PCR-Free Library Prep thermal cycler workflow for inputs of 25-99 ng DNA.

Figure 2 Illumina DNA PCR-Free Library Prep Workflow



Tagment Genomic DNA

This step uses the Bead-Linked Transposomes PCR-Free (BLT-PF) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences. Use the preparation steps in section to prepare all reagents in advance.

Consumables

- BLT-PF (Bead-Linked Transposomes PCR-Free)
- HP3 (2N NaOH)
- RSB (Resuspension Buffer)
- TB1 (Tagmentation Buffer 1)
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 1.5 ml tube
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Prepare for a later section:
  - ELM (Extension Ligation Mix)
  - IPB (Illumina Purification Beads)
  - Index adapters (IDT for Illumina DNA/RNA UD Indexes)
  - ST2 (Stop Tagment Buffer 2)
  - TWB (Tagment Wash Buffer)

Warning

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

- Use the specified magnetic stand.
- BLT-PF
  - Store frozen and upright to make sure beads are submerged in the buffer.
  - Aspirate and dispense slowly due to viscosity of the solution

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
IPB*	15°C to 30°C	Vortex 1 minute. Invert 2–5 times, and then vortex thoroughly to resuspend.
RSB	15°C to 30°C	Label the tube cap RSB. Vortex or invert to mix.
ST2*	15°C to 30°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Vortex thoroughly, and then centrifuge.
TWB*	15°C to 30°C	Label the tube cap TWB. Invert thoroughly to mix.
BLT-PF	-25°C to -15° C	Bring to room temperature. Vortex to resuspend. Do not centrifuge before pipetting.
ELM*	-25°C to -15°C	Thaw at room temperature. Keep on ice until needed. Invert to mix before use.
HP3	-25°C to -15°C	Bring to room temperature. Vortex, and then centrifuge briefly.
Index adapters*	-25°C to -15°C	Bring to room temperature. Vortex, and then centrifuge briefly.
TB1	-25°C to -15°C	Bring to room temperature. Vortex, and then centrifuge.

* Reagent is used in a later section.

NOTE

RSB and TWB are shipped in similar tubes. Label each cap before beginning the protocol.

2. For each reaction, combine the following volumes to prepare diluted HP3:
   - HP3 (6 µl)
   - Nuclease-free water (54 µl)

   These volumes produce 60 µl diluted HP3 per reaction. Overage is included.
   Vortex and centrifuge briefly.
3. For each reaction, combine the following volumes to prepare 80% EtOH:
   - EtOH (400 µl)
   - Nuclease-free water (100 µl)

   These volumes produce 500 µl 80% EtOH per reaction. Overage is included.
   Vortex to mix.
4. 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
- 41°C for 5 minutes

5. Save the following ELM program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 37°C for 5 minutes
   - 50°C for 5 minutes

Procedure

1. Label a new 96-well PCR plate LP1.
2. Add 2–30 µl DNA to each well, so that the total input amount is within the desired range (25-99 ng).
3. If sample volume is < 30 µl, bring the total volume to 30 µl using RSB.
4. Vortex BLT-PF vigorously for 1 minute to resuspend. Repeat as necessary.
5. If beads adhere to the sides or top of the tube, centrifuge at 280 x g for 3 seconds, and then pipette to resuspend.
6. For each reaction, ***combine*** the following volumes in a 1.5 ml tube to prepare the Tagmentation Master Mix:
   - BLT-PF (11 µl)
   - TB1 (11 µl)

   These volumes produce 22 µl Tagmentation Master Mix per sample. Overage is included.
7. Pipette to mix.
8. Add 20 µl master mix to each well containing DNA.
9. Using a P200 pipette set to 45 µl, to mix, and then seal.
10. Place on the preprogrammed thermal cycler and run the TAG program.
11. Proceed Immediately to *Post Tagmentation Cleanup*

Post Tagmentation Cleanup

This step stops the tagmentation reaction and washes the adapter-tagged DNA on the BLT-PF.

Consumables

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

About Reagents

- Pipette TWB and ST2 slowly to minimize foaming.
- Dispense TWB directly onto beads.

Procedure

1. Add 10 µl ST2 to each well.
2. Using a P200 pipette set to 50 µl, pipette to mix.
3. Incubate at room temperature for 2 minutes.
4. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
5. Using a P200 pipette set to 60 µl, remove and discard all supernatant from each well.
6. Remove the plate from the magnetic stand.
7. Add 150 µl TWB to each well.
8. Pipette to mix ten times or until beads are fully resuspended.
9. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

Ligate Indexes

This step ligates Index 1 (i7) and Index 2 (i5) adapters to each sample.

Consumables

- ELM (Extension Ligation Mix)
- Index adapters (IDT for Illumina DNA/RNA UD Indexes)
- TWB (Tagment Wash Buffer)
- Diluted HP3
- Microseal 'B' adhesive seals

About Reagents

- The index plate wells cannot be reused.
- Aspirate and dispense ELM slowly due to the viscosity of the solution.

Procedure

1. Without disturbing the bead pellet, remove and discard residual supernatant from each well.
2. Remove the plate from the magnetic stand.
3. Add 45 µl ELM to each well.

4. Pipette to mix until beads are fully resuspended and not on the side of the well.
5. Pierce the foil seal covering the index adapter plate as follows.
   - **[< 96 samples]** Pierce the wells you intend to use. Use a new pipette tip for each well.
   - **[96 samples]** Align a new semi-skirted 96-well PCR plate over the index adapter plate and slowly press down to puncture all 96 wells. Discard the PCR plate.
6. Add 5 µl index adapters to each well.
7. Using a P200 pipette set to 45 µl, pipette to mix, and then seal.
8. Place on the preprogrammed thermal cycler and run the ELM program.
9. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
10. Remove and discard all supernatant from each well.
11. Remove the plate from the magnetic stand.
12. Add 75 µl TWB onto the beads in each well.
13. Pipette to mix until beads are fully resuspended and not on the side of the well.
14. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. Remove and discard all supernatant from each well.
16. Seal, and then centrifuge at 280 x g for 10 seconds.
17. Place on the magnetic stand.
18. Without disturbing the bead pellet, remove and discard residual supernatant from each well.
19. Remove the plate from the magnetic stand.
20. Add 45 µl diluted HP3 to each well.
21. Pipette to mix until beads are fully resuspended and not on the side of the well.
22. Incubate at room temperature for 2 minutes.
23. Proceed immediately to *Clean Up Libraries*.

Clean Up Libraries

This step uses a single-sided bead purification procedure to purify the libraries. Illumina purification beads are added to the sample containing BLT-PF beads. Then, the supernatant is discarded and the bead pellet is washed with 80% Ethanol before being eluted with RSB.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well PCR plate (2)
- Microseal 'B' adhesive seal

- **[Optional]** Microseal 'F' adhesive foil

About Reagents

- IPB
  - 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

Procedure

1. Add 81 µl IPB to each well containing sample with BLT-PF beads.
2. Pipette 10 times to mix.
3. Incubate at room temperature for 5 minutes.
4. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
5. Remove and discard all supernatant from each well.
6. Wash beads as follows.
   a. Keep on magnetic stand and add 180 µl fresh 80% ethanol to each well.
   b. Wait 30 seconds.
   c. Remove and discard all supernatant from each well.
7. Wash beads a **second** time.
8. Apply Microseal 'B', and then centrifuge 280 x g for 10 seconds.
9. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
10. Remove residual EtOH from each well.
11. Discard unused 80% EtOH.
12. Air-dry on the magnetic stand (~2 minutes).
13. Remove the plate from the magnetic stand.
14. Add 15 µl RSB onto the beads in each well.
15. Pipette to mix until beads are fully resuspended and not on the side of the well.
16. Incubate at room temperature for 2 minutes.
17. Centrifuge 280 x g for 10 seconds.
18. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
19. Transfer 14 µl supernatant from each well of the plate to the corresponding well of a new PCR plate.
20. Proceed immediately to *Quantify and Pool Libraries*.

SAFE STOPPING POINT

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

Quantify and Pool Libraries

We recommend quantifying all samples for normalization prior to sequencing when following the low input protocol. Illumina DNA PCR-Free Library Prep libraries are single stranded. It is not possible to view library size distribution on any capillary electrophoresis instrument. For pooling guidelines, see *Index Adapter Pooling Guide (document # 1000000041074)*.

DNA Inputs 25-99 ng

For DNA inputs 25-99 ng, quantify the library before pooling.

1. Analyze 2 µl of each library using KAPA qPCR library quantification kit.
2. Use 450 bp as the library length.
3. In a 1.5 or 1.7 ml microcentrifuge tubes, combine libraries equimolarly to between 0.75-1nM final concentration.
4. Vortex to mix, and then centrifuge at 280 x g for 1 minute.

Dilute Libraries to the Starting Concentration and Sequence

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.

1. Use the molarity value determined by the KAPA qPCR protocol to calculate the volumes of RSB and library pool to dilute libraries to the starting concentration for your system. Use 450 bp as the average library size and 660 g/mol as the DNA mass. Illumina recommends a 1:1000x dilution for the sample setting a qPCR reaction.

NOTE

Successful use of Illumina DNA PCR-Free for 25-99 ng DNA inputs requires accurate quantification of the starting material once libraries are generated. The existing KAPA qPCR workflow may introduce variability at the lower range of this input. Illumina recommends the user carefully evaluate samples through appropriate use of qPCR replicates and management of outliers.

Sequencing System	KAPA qPCR Quantification	
	Starting Concentration (nM)	Final Loading Concentration (pM)
NovaSeq 6000 Xp workflow	0.75–1	150–200

2. Dilute the pool to the starting concentration for your system.
3. If you are using a v1.0 NovaSeq reagent kit, prepare the VP10 custom sequencing primer. See *Custom Primer Considerations* on page 3 for more information on using custom sequencing primers with Illumina DNA PCR-Free.
4. Follow the XP workflow instructions in Protocol B of *NovaSeq Denature and Dilute Libraries Guide (document # 1000000106351)* to dilute to the final loading concentration.
   For sequencing, Illumina recommends setting up a paired-end run of 151 cycles per read using 10 bp index reads: (Read 1, i5 index read, i7 index read, Read 2), (151, 10, 10, 151). See the *NovaSeq 6000 Sequencing System Guide (document # 1000000019358)* for further sequencing information.
   Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Chapter 4 Protocol for Hybex

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Introduction

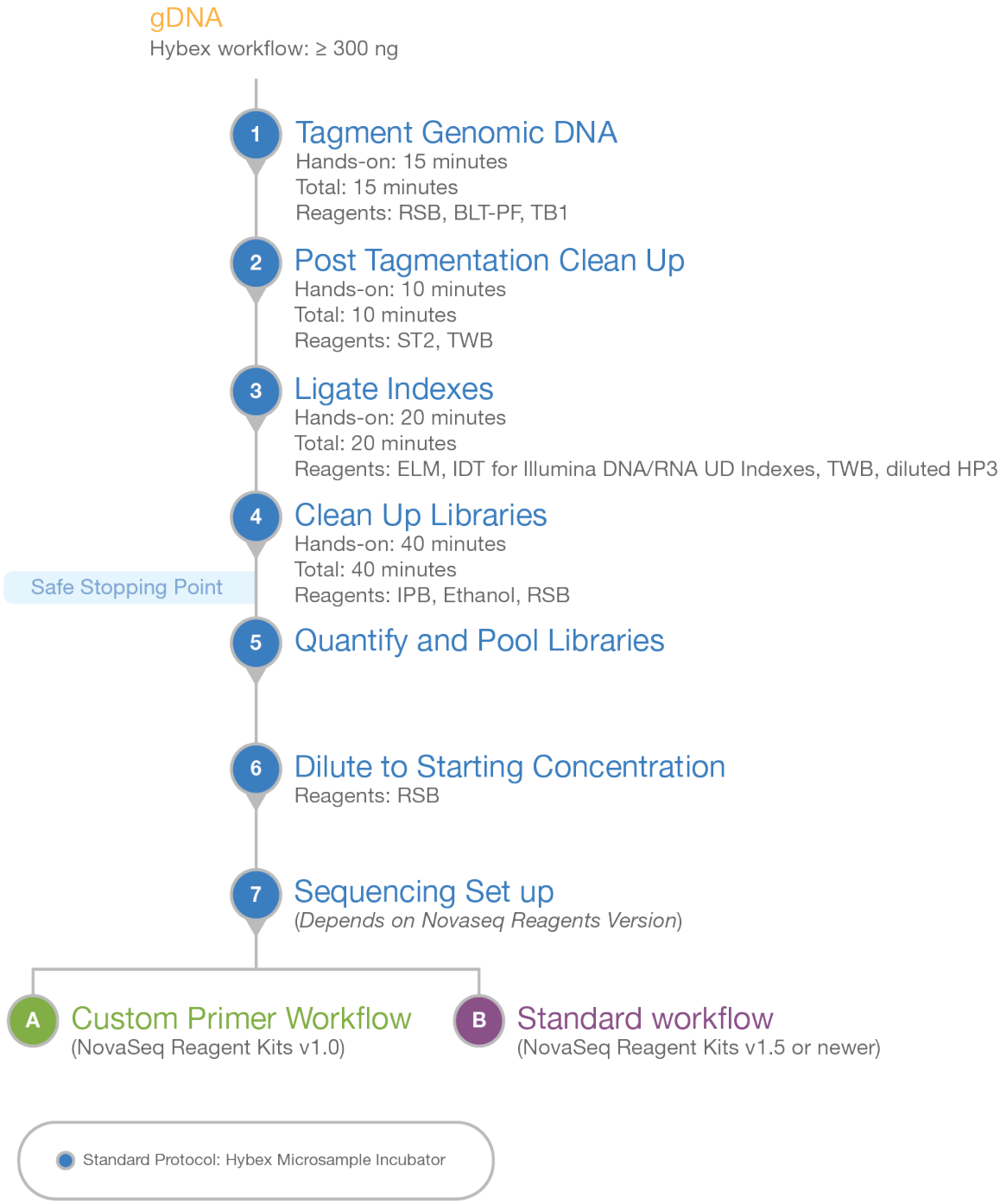
This chapter describes the Illumina DNA PCR-Free Library Prep protocol when using a Hybex incubator and gDNA inputs ≥ 300 ng. If using whole blood, dried blood spots, saliva, or inputs 100-299 ng, see *Protocol for Thermal Cycler, Standard Input* on page 6. If using inputs of <99, see *Protocol for Thermal Cycler, Low Input* on page 17.

- 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. See *Supporting Information* on page 1. The Illumina DNA PCR-Free protocol requires the following components.
- Library prep reagents.
- Index adapters (sold separately).
- Custom sequencing primers (sold separately).

Library Prep Workflow

The following diagram illustrates the Illumina DNA PCR-Free Library Prep Hybex workflow, with inputs of ≥300 ng DNA.

Figure 3 Illumina DNA PCR-Free Library Prep Workflow



Tagment Genomic DNA

This step uses the Bead-Linked Transposomes PCR-Free (BLT-PF) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences. Use the preparation steps in this section to prepare reagents in advance.

Consumables

- BLT-PF (Bead-Linked Transposomes PCR-Free)
- RSB (Resuspension Buffer)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 96-well MIDI plate
- Microseal 'B' adhesive seal
- Prepare for a later procedure:
  - ELM (Extension Ligation Mix)
  - HP3 (2N NaOH)
  - IPB (Illumina Purification Beads)
  - Index adapters (IDT for Illumina DNA/RNA UD Indexes)
  - ST2 (Stop Tagment Buffer 2)
  - TWB (Tagment Wash Buffer)

Warning

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

- Use the specified magnet.
- BLT-PF
  - Store frozen and upright to make sure beads are submerged in the buffer.
  - Aspirate and dispense slowly due to viscosity of the solution

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
IPB*	15°C to 30°C	Vortex 1 minute. Invert 2–5 times, and then vortex thoroughly to resuspend.
RSB	15°C to 30°C	Label the tube cap RSB. Vortex or invert to mix.
ST2 *	15°C to 30°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Vortex thoroughly, and then centrifuge.
TWB*	15°C to 30°C	Label the tube cap TWB. Invert thoroughly to mix.
BLT-PF	-25°C to -15° C	Bring to room temperature. Vortex to resuspend. Do not centrifuge before pipetting.
ELM*	-25°C to -15°C	Thaw at room temperature. Keep on ice until needed. Invert to mix before use.
HP3	-25°C to -15°C	Bring to room temperature. Vortex, and then centrifuge briefly.
Index adapters*	-25°C to -15°C	Bring to room temperature. Vortex, and then centrifuge briefly.
TB1	-25°C to -15°C	Bring to room temperature. Vortex, and then centrifuge.

* Reagent is used in a later section.

NOTE

RSB and TWB are shipped in similar tubes. Label each cap before beginning the protocol.

2. For each reaction, combine the following volumes to prepare diluted HP3:
   - HP3 (6 µl)
   - Nuclease-free water (54 µl)

   These volumes produce 60 µl diluted HP3 per reaction, including overage.
   Vortex and centrifuge briefly.
3. For each reaction, combine the following volumes to prepare 80% EtOH:
   - EtOH (400 µl)
   - Nuclease-free water (100 µl)

   These volumes produce 500 µl 80% EtOH per reaction, including overage.
   Vortex to mix.
4. Preheat Hybex to 45°C.

Procedure

1. Label a new 96-well MIDI plate LP1.
2. Add 2–25 µl DNA to each well, so that the total input amount is within the desired range.
3. If sample volume is < 25 µl, bring the total volume to 25 µl using RSB.
4. Add 10 µl TB1 to each well.
5. Vortex BLT-PF vigorously for 1 minute to resuspend. Repeat as necessary.
6. Add 15 µl BLT-PF to each well.
7. Seal and shake at 1800 rpm for 1 minute.
8. Incubate in pre-heated Hybex for 8 minutes. Proceed immediately to *Post Tagmentation Cleanup*

Post Tagmentation Cleanup

This step stops the tagmentation reaction and washes the adapter-tagged DNA on the BLT-PF.

Consumables

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

About Reagents

- Pipette TWB and ST2 slowly to minimize foaming.
- Dispense TWB directly onto beads.

Procedure

1. Add 10 µl ST2 to each well.
2. Seal and then shake at 1800 rpm for 1 minute.
3. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
4. Without disturbing the bead pellet, remove and discard all supernatant from each well.
5. Add 150 µl TWB to each well.
6. Seal and shake at 1800 rpm for 1 minute.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

Ligate Indexes

This step ligates Index 1 (i7) and Index 2 (i5) adapters to each sample.

Consumables

- ELM (Extension Ligation Mix)
- Index adapters (IDT for Illumina DNA/RNA UD Indexes)
- TWB (Tagment Wash Buffer)
- Diluted HP3
- Microseal 'B' adhesive seals

About Reagents

- The index plate wells cannot be reused.
- Aspirate and dispense ELM slowly due to the viscosity of the solution

Procedure

1. Remove and discard all supernatant from each well.
2. Without disturbing the bead pellet, use a 20 µl pipette to remove and discard residual TWB from each well.
3. Add 45 µl ELM to each well.
4. Pierce the foil seal covering the index adapter plate as follows.
   - **[< 96 samples]** Pierce the wells you intend to use. Use a new pipette tip for each well.
   - **[96 samples]** Align a new semi-skirted 96-well PCR plate over the index adapter plate and slowly press down to puncture all 96 wells. Discard the PCR plate.
5. Add 5 µl index adapters to each well.
6. Seal and shake at 1800 rpm for 1 minute.
7. Incubate to in the preheated Hybex for 8 minutes.
   The Hybex is preheated to 45°C.
8. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
9. Remove and discard all supernatant from each well.
10. Add 75 µl TWB onto the beads in each well.
11. Seal and shake at 1800 rpm for 1 minute.
12. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
13. Remove and discard all supernatant from each well.
14. Without disturbing the bead pellet, use a 20 µl pipette to remove and discard residual TWB from each well.
15. Add 45 µl diluted HP3 to each well.
16. Seal and shake at 1800 rpm for 1 minute.

Clean Up Libraries

This step uses a double-sided bead purification procedure to purify the libraries. In the first side of the bead purification procedure, Illumina purification beads are added to the sample containing BLT-PF beads. Then the supernatant is transferred to a new plate containing Illumina purification beads for the second side of the double-sided bead purification procedure.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- 96-well MIDI plate
- 96-well PCR plate
- Microseal 'B' adhesive seal

About Reagents

- IPB
  - Vortex before each use
  - Aspirate and dispense slowly due to the viscosity of the solution

Procedure

1. Vortex IPB, and then invert until fully resuspended.
2. Add 36 µl IPB to each well containing BLT-PF beads.
3. Seal and shake at 1800 rpm for 1 minute.
4. Incubate at room temperature for 2 minutes.
5. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
6. While the plate incubates, label a new 96-well MIDI plate LP2.
7. Add 42 µl IPB to each well of LP2.
8. Without disturbing the bead pellet, **transfer** 76 µl supernatant from each well of LP1 to the corresponding well of the LP2.
9. Seal and shake at 1800 rpm for 1 minute.
10. Discard LP1.
11. Incubate LP2 at room temperature for 2 minutes.
12. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
13. Without disturbing the bead pellet, remove and discard all supernatant from each well.
14. Wash beads as follows.

a. Keep on magnetic stand and add 180 µl fresh 80% ethanol to each well.
b. Wait 30 seconds.
c. Remove and discard all supernatant from each well.

15. Wash beads a **second** time.
16. Using a 20 µl pipette, remove residual EtOH from each well.
17. Discard unused 80% EtOH.
18. Air-dry on the magnetic stand (~2 minutes).
19. Add 22 µl RSB onto the beads in each well.
20. Seal and shake at 1800 rpm for 1 minute.
21. Incubate at room temperature for 2 minutes.
22. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
23. Label a new PCR plate FLP.
24. Transfer 20 µl supernatant from each well of LP2 to the corresponding well of FLP.
25. Proceed immediately to *Quantify and Pool Libraries*.

SAFE STOPPING POINT

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

Quantify and Pool Libraries

Quantifying and normalizing libraries from the same experiment is not required. If using libraries from separate experiments, the final yields can vary. Illumina DNA PCR-Free Library Prep libraries are single stranded. It is not possible to view library size distribution on any capillary electrophoresis instrument.

For pooling guidelines, see *Index Adapter Pooling Guide (document # 1000000041074)*.

1. Combine 9 µl of each library in a 1.5 or 1.7 ml microcentrifuge tube.
2. Vortex to mix, and then centrifuge at 280 x g for 1 minute.
3. Quantify the library pool:
   - Analyze 2 µl pooled library using the Qubit ssDNA (single-stranded) assay kit or a KAPA qPCR Library Quantification Kit.

Dilute Libraries to the Starting Concentration and Sequence

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.

1. Calculate the volumes of RSB and library pool to dilute pool to the starting concentration for your system.
   - When using a qPCR method—Use the molarity value determined by the KAPA qPCR protocol. Use 450 bp as the average library size and 660 g/mol as the DNA mass. Illumina recommends diluting libraries by a 1:10000x dilution when using the KAPA method.
   - When using Qubit method—Calculate the molarity value of the pooled libraries using the formula below.

     The formula uses 450 bp as the average library size and 660 g/mol as the DNA mass. This equation will output the double stranded DNA equivalent.

     $$Molarity\ (nM) = Yield\ (\frac{ng}{ul}) \times 3.36$$

Sequencing System	KAPA qPCR Quantification		Qubit ssDNA Quantification	
	Starting Concentration (nM)	Final Loading Concentration (pM)	Starting Concentration (nM)	Final Loading Concentration (pM)
NovaSeq 6000 standard workflow	1–1.5	200–300	2–3	400–600
NovaSeq 6000 Xp workflow	0.75–1	150–200	1.5–2	300–400

2. Dilute the pool to the starting concentration for your system.
3. If you are using a v1.0 NovaSeq reagent kit, prepare the VP10 custom Read 1 sequencing primer. See *Custom Primer Considerations* on page 3 for more information on using custom sequencing primers with Illumina DNA PCR-Free.
4. Follow the standard workflow instructions in Protocol A or the XP workflow instructions in Protocol B of the the *NovaSeq Denature and Dilute Libraries Guide (document # 1000000106351)* to dilute to the final loading concentration.

   For sequencing, Illumina recommends setting up a paired-end run of 151 cycles per read using 10 bp index reads: (Read 1, i5 index read, i7 index read, Read 2), (151, 10, 10, 151). See the *NovaSeq 6000 Sequencing System Guide (document # 1000000019358)* for additional sequencing information.

Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Supplementary Procedures

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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.

Perform Whole Blood Lysis

This step uses the Illumina Lysis Reagent Kit to extract normalized DNA from fresh whole blood in collection tubes. Store blood samples at 4°C to 8°C and perform blood lysis within 3 days of collection.

Warning

Blood is a potential source of infectious diseases. Follow site-specific procedures to safely handle samples.

Consumables

- IPB (Illumina Purification Beads)
- PK1 (Proteinase K)
- MLB (Modified Lysis Buffer)
- RSB (Resuspension Buffer)
- Blood in EDTA collection tubes
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free water
- 15 ml conical tube
- 2 ml microcentrifuge tubes

- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' adhesive foil

About Reagents

- IPB
  - 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:

Reagent	Storage	Instructions
IPB	15°C to 30°C	Vortex 1 minute, and then invert to resuspend beads.
MLB	-25°C to -15°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Invert to mix.
PK1	-25°C to -15°C	Invert to mix. Store on ice until required.
RSB	15°C to 30°C	Vortex or invert to mix.

2. For each reaction, combine the following volumes to prepare 80% EtOH:
   - EtOH (960 µl)
   - Nuclease-free water (240 µl)
3. Heat the ThermoMixer C with a 2 ml heat block to 56°C.

Procedure

1. Label a 15 ml tube Lysis Master Mix.
2. For each sample, combine the following volumes in a 15 ml tube to prepare Lysis Master Mix.
   - MLB (30 µl)
   - PK1 (2 µl)
   - Nuclease-free water (248 µl)

   These volumes produce 280 µl Lysis Master Mix per sample. Overage is not included.
3. Vortex or invert the master mix thoroughly to mix, and then centrifuge.
4. For each sample, add 280 µl master mix to a new 2 ml tube.
5. Invert each EDTA tube 5 times to mix.
6. Add 20 µl blood to each 2 ml tube.

7. Pipette to mix.
8. Vortex briefly, and then centrifuge.
9. Incubate and shake at 1000 rpm for 15 minutes in ThermoMixer C.
10. Centrifuge briefly.
11. Make sure the liquid is brown. If the liquid is not brown, shake at 1000 rpm for 5 minutes.
    The brown color indicates the sample is fully lysed.
12. Vortex IPB, and then invert until fully resuspended.
13. Add 135 µl IPB to each tube.
14. Vortex for 15 seconds, and then pipette to mix.
15. Incubate at room temperature for 5 minutes.
16. Place tube on the magnetic stand and wait 5 minutes.
    The lysis reaction prevents the liquid from becoming clear. Wait until beads separate from the solution (~5 minutes).
17. Without disturbing the bead pellet, remove and discard supernatant.
    If beads aspirate into pipette tips, dispense back into tubes and wait 2–5 minutes.
18. Make sure all lysis material is removed.
19. Wash beads as follows.
    a. Keep on the magnetic stand and add 500 µl fresh 80% EtOH to each well.
    b. Wait 30 seconds.
    c. Remove and discard all supernatant from each well.
20. Wash beads a **second** time.
21. Remove all residual EtOH from each well.
22. Discard unused 80% EtOH.
23. Air-dry on the magnetic stand for 5 minutes.
24. Remove from the magnetic stand.
25. Add 35 µl RSB to each tube.
26. Vortex or pipette to mix.
27. Incubate at room temperature for 2 minutes.
28. Place on the magnetic stand until liquid is clear (≥ 2 minutes).
29. Label a new PCR plate LP1.
30. Transfer as follows.
    - If you are not quantifying before beginning library prep, transfer 30 µl supernatant from each tube to the LP1 plate.
    - If you are quantifying before beginning library prep, transfer 32 µl from each tube to the LP1 plate, and then follow the quantification recommendations in *DNA Input Recommendations* on page 1.

31. If not stopping, proceed to *Tagment Genomic DNA* on page 7.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 3 days.

Perform Dried Blood Spots Lysis

This step uses the Illumina Lysis Reagent Kit to extract normalized DNA from whole blood in collection cards. Store dry blood cards at room temperature. After preparing punches, perform blood lysis.

Warning

Blood is a potential source of infectious diseases. Follow site-specific procedures to safely handle samples.

Consumables

- IPB (Illumina Purification Beads)
- MLB (Modified Lysis Buffer)
- PK1 (Proteinase K)
- RSB (Resuspension Buffer)
- Blood in Whatman 903 protein saver cards
- Freshly prepared 80% ethanol (EtOH)
- Nuclease-free Water
- 15 ml conical tube
- 2 ml microcentrifuge tubes
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' adhesive foil

About Reagents

- IPB
  - 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:

Reagent	Storage	Instructions
MLB	-25°C to -15°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Invert to mix.
PK1	-25°C to -15°C	Store on ice until required.
IPB	15°C to 30°C	Vortex 1 minute, and then invert to resuspend beads.
RSB	15°C to 30°C	Vortex or invert to mix.

2. For each reaction, combine the following volumes to prepare 80% EtOH:
   - EtOH (960 µl)
   - Nuclease-free water (240 µl)
3. Heat the ThermoMixer C with a 2 ml heat block to 56°C.

Procedure

1. Label a 15 ml tube Lysis Master Mix.
2. For each sample, combine the following volumes in a 15 ml tube to prepare Lysis Master Mix.
   - 10x Lysis Buffer (30 µl)
   - PK1 (2 µl)
   - Nuclease-free water (268 µl)

   These volumes produce 300 µl Lysis Master Mix per sample. Overage is not included.
3. Vortex or invert the master mix thoroughly to mix, and then centrifuge.
4. For each sample, add 300 µl master mix to a new 2 ml tube.
5. Add 6 x 3 mm^2 punches to a each 2 ml tube.
6. Vortex briefly, and then centrifuge.
7. Incubate and shake at 1000 rpm for 15 minutes in ThermoMixer C.
8. Centrifuge briefly.
9. Make sure the liquid is brown. If the liquid is not brown, shake at 1000 rpm for 5 minutes.
   The brown color indicates the sample is fully lysed.
10. Without removing the punches, transfer all supernatant from each tube to a new 2 ml tube.
11. Vortex IPB, and then invert until fully resuspended.
12. Add 135 µl IPB to each sample tube.
13. Vortex for 15 seconds, and then pipette to mix.
14. Incubate at room temperature for 5 minutes.
15. Place on the magnetic stand and wait 5 minutes.
    The lysis reaction prevents the liquid from becoming clear. Wait until beads separate from the solution (~5 minutes).

16. Without disturbing the bead pellet, remove and discard supernatant.
    If beads aspirate into pipette tips, dispense back into tubes and wait 2–5 minutes.
17. Make sure all lysis material is removed.
18. Wash beads as follows.
    a. Keep on the magnetic stand and add 500 µl fresh 80% EtOH to each well.
    b. Wait 30 seconds.
    c. Remove and discard all supernatant from each well.
19. Wash beads a **second** time.
20. Remove all residual EtOH from each well.
21. Discard unused 80% EtOH.
22. Air-dry on the magnetic stand for 5 minutes.
23. Remove from the magnetic stand.
24. Add 35 µl RSB to each tube.
25. Vortex or pipette to mix.
26. Incubate at room temperature for 2 minutes.
27. Place on the magnetic stand until liquid is clear (≥ 2 minutes).
28. Label a new PCR plate LP1.
29. Transfer as follows.
    - If you are not quantifying before beginning library prep, transfer 30 µl supernatant from each tube to the LP1 plate.
    - If you are quantifying before beginning library prep, transfer 32 µl from each tube to the LP1 plate, and then follow the quantification recommendations in *DNA Input Recommendations* on page 1.
30. If not stopping, proceed to *Tagment Genomic DNA* on page 7.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 3 days.

Perform Saliva Lysis

This step uses the Illumina Lysis Reagent Kit to extract normalized DNA from saliva collection tubes. Store saliva samples according to manufacturer requirements.

Warning

Saliva is a potential source of infectious diseases. Follow site-specific procedures to safely handle samples.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Saliva in Oragene DNA saliva collection tubes.
- Freshly prepared 80% Ethanol
- Nuclease-free Water
- 2 ml microcentrifuge tubes
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' adhesive foil

About Reagents

- IPB
  - 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. Follow manufacturer's instructions for saliva extraction.
   After incubation, you can use the saliva immediately or store it at room temperature. For long-term storage, see the DNA Genotek website.
2. Prepare the following reagents.

Reagent	Storage	Instructions
IPB	15°C to 30°C	Vortex 1 minute, and then invert to resuspend beads.
RSB	15°C to 30°C	Vortex or invert to mix.

3. For each reaction, combine the following volumes to prepare 80% EtOH:
   - EtOH (960 µl)
   - Nuclease-free water (240 µl)

Procedure

1. For each sample, add 250 µl nuclease-free water to a new 2 ml tube.
2. Invert each heat-treated saliva collection tube 5 times to mix.
3. Add 50 µl saliva from the collection tube to each 2 ml tube.
4. Pipette to mix.

5. Vortex briefly, and then centrifuge.
6. Vortex IPB, and then invert until fully resuspended.
7. Add 135 µl IPB to each sample tube.
8. Vortex for 15 seconds, and then centrifuge.
9. Incubate at room temperature for 5 minutes.
10. Place on the magnetic stand and wait 5 minutes.
11. Without disturbing the bead pellet, remove and discard supernatant.
    If beads aspirate into pipette tips, dispense back into tubes and wait 2–5 minutes.
12. Make sure all lysis material is removed.
13. Wash beads as follows.
    a. Keep on the magnetic stand and add 500 µl fresh 80% EtOH to each well.
    b. Wait 30 seconds.
    c. Remove and discard all supernatant from each well.
14. Wash beads a **second** time.
15. Remove all residual EtOH from each well.
16. Discard unused 80% EtOH.
17. Air-dry on the magnetic stand for 5 minutes.
18. Remove from the magnetic stand.
19. Add 35 µl RSB to each tube.
20. Vortex or pipette to mix.
21. Incubate at room temperature for 2 minutes.
22. Place on the magnetic stand until liquid is clear (≥ 2 minutes).
23. Label a new PCR plate LP1.
24. Transfer as follows.
    - If you are not quantifying before beginning library prep, transfer 30 µl supernatant from each tube to the LP1 plate.
    - If you are quantifying before beginning library prep, transfer 32 µl from each tube to the LP1 plate, and then follow the quantification recommendations in *DNA Input Recommendations* on page 1.
25. If not stopping, proceed to *Tagment Genomic DNA* on page 7.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 3 days.

Supporting Information

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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.

Kit Contents

The Illumina DNA PCR-Free protocol requires the following components. Each component is sold separately.

- Library prep reagents
- Index adapters
- Custom sequencing primers

Component	Kit Options	Catalog #
Library prep reagents	Illumina DNA PCR-Free Library Prep (24 Samples)	20041794
	Illumina DNA PCR-Free Library Prep (96 Samples)	20041795
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
Custom primers	Illumina DNA PCR-Free Primer Kit R1 Sequencing	20041796
[Optional] Lysis reagents	Illumina Lysis Reagent Kit	20042221

Illumina DNA PCR-Free Library Prep Contents

Illumina DNA PCR-Free Library Prep Box 1, Store at Room Temperature

Tube Quantity		Reagent	Description
24 Samples	96 Samples		
1	2	IPB	Illumina Purification Beads
1	1	RSB*	Resuspension Buffer
1	4	ST2	Stop Tagment Buffer 2
1	1	TWB*	Tagment Wash Buffer

* RSB and TWB are shipped in similar tubes. Label each cap before beginning the protocol.

Illumina DNA PCR-Free Library Prep Box 2, Store at -25°C to -15°C

Tube Quantity		Acronym	Description
24 Samples	96 Samples		
1	4	BLT-PF	Bead-Linked Transposomes PCR-Free
1	5	ELM	Extension Ligation Mix
1	4	HP3	2N NaOH
1	4	TB1	Tagmentation Buffer 1

IDT for Illumina DNA/RNA UD Indexes (96 Indexes, 96 Samples) Plates, Store at -25°C to -15°C

Quantity	Description	Catalog #
1	IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20027213
1	IDT for Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	20027214
1	IDT for Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20042666
1	IDT for Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20042667

For optimized index representation, Illumina recommends the use of IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (20027213) or Illumina DNA/RNA UD Indexes Set B (20027214).

Illumina Lysis Reagent Kit Box 1, Store at -25°C to -15°C

	Quantity	Acronym	Description
•	4	PK1	Proteinase K
•	4	MLB	Modified Lysis Buffer

Illumina Lysis Reagent Kit Box 2, Store at Room Temperature

	Quantity	Acronym	Description
•	2	IPB	Illumina Purification Beads

Illumina DNA PCR-Free Prep, Sequencing Primers Read 1, Store at -25°C to -15°C

If using the NovaSeq Reagent Kits, excluding NovaSeq Reagent Kits v 1.5, Illumina DNA PCR-Free Library Prep requires the Illumina DNA PCR-Free Prep, Sequencing Primers, Read 1. Follow the instructions for a custom read 1 primer in the *NovaSeq Series Custom Primers Guide (document # 1000000022266)* to load and set up the run.

	Quantity	Acronym	Description
•	4	VP10	VP10 Custom Read 1 Primer

Consumables and Equipment

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

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
10 µl pipette tips	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
200 µl pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl pipette tips	General lab supplier
1000 µl single channel pipettes	General lab supplier
Hard-Shell 96-well PCR plates	Bio-Rad, catalog # HSP-9601
[Hybex] Abgene 96 well 0.8 ml polypropylene deepwell storage plates (MIDI plate)	ThermoFisher Scientific, catalog # AB-0859
15 or 50 ml conical centrifuge tubes	General lab supplier
1.5 or 1.7 ml microcentrifuge tubes	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001

Consumable	Supplier
[Optional] Microseal 'F' foil seals	Bio-Rad, catalog # MSF-1001
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
[Dependent on workflow] KAPA qPCR Library Quantification Kits	Roche, catalog # KK4824 or # KK4873
Qubit dsDNA HS Assay Kit	• ThermoFisher Scientific, catalog # Q32851 or # Q32854
[Dependent on workflowl] Qubit ssDNA assay kit	ThermoFisher Scientific, catalog # Q10212
Qubit Assay tubes	ThermoFisher Scientific, catalog # Q32856
[Optional] 96-well Semi-Skirted PCR plates	General lab supplier
[Saliva] Oragene Saliva Collection Kit (DNA or Discovery)	DNA Genotek, catalog # OGR-500 or # ODG-510
[Dried blood] Whatman® 903 protein saver cards	GE Lifesciences, catalog # 10531018 or # 10534612
[Blood] Blood collection tubes, EDTA	Becton Dickinson
[Whole blood, dried blood, saliva] 2.0 ml microcentrifuge tubes	General lab supplier

Equipment

Equipment	Supplier
[Hybex] One of the following mixers: Bioshake XP, benchtop mixer Bioshake IQ, benchtop thermal mixer	VWR, catalog # 1808-0505 or # 1808-0506
Microplate centrifuge	General lab supplier
Microcentrifuge	General lab supplier
Sealing roller or wedge	General lab supplier
Vortexer	General lab supplier
Qubit® Fluorometer 2.0 or higher	ThermoFisher Scientific, catalog # Q33216, Q33217 or Q33218
[Low input] Real-time PCR detection system	Bio-Rad CFX 96 Touch or equivalent
[Hybex] Hybex Microsample Incubator Illumina	Illumina, catalog # SC-60-503 or # SC-60-504

Equipment	Supplier
[Hybex] MIDI heat block insert	Illumina, catalog # BD-60-503
[Thermal cycler] Thermal cycler with heated lid	General lab supplier
[Dried blood spot] 3 mm^2 puncher	PerkinElmer, Wallac DBS Puncher or equivalent
[Dried blood spot, whole blood, and saliva] DynaMag -2 Magnet	ThermoFisher Scientific, catalog # 12321D
[Thermal cycler and saliva] dynaMag-96 Side Skirted Magnet	Thermo Fisher Scientific, catalog # 12027
[Hybex] Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
[Dried blood spot and whole blood] ThermoMixer C	Eppendorf, catalog # 2231000667 or # 5382000023
[Dried blood spot and whole blood] 2.0 ml adapter block for ThermoMixer C	Eppendorf, catalog # 5362000035
[Saliva] Water bath or air incubator reaching 50°C	As recommended by DNA Genotek, see product pages.

Acronyms

Acronym	Definition
BLT-PF	Bead Linked Transposome PCR-Free
dsDNA	Double stranded DNA
ELM	Extension Ligation Mix
EtOH	Ethanol
FLP	Final Library Plate
HP3	2 N NaOH
IPB	Illumina Purification Beads
LP1	Library Plate 1
LP2	Library Plate 2
MLB	Modified Lysis Buffer
PK1	Proteinase K
RSB	Resuspension Buffer
ssDNA	Single stranded DNA
ST2	Stop Tagment Buffer 2
TB1	Tagmentation Buffer 1

Acronym	Definition
TWB	Tagment Wash Buffer
UD	Unique Dual

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
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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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