



Illumina DNA PCR-Free Library Prep, Tagmentation

Reference Guide

ILLUMINA PROPRIETARY

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Overview

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. Due to the lower yields from this protocol, there will not be sufficient library to sequence across all platforms or configurations. Confirm the minimum library input required for successful sequencing on your sequencing platform of choice.

100–299 ng DNA—Quantification is required. Use a thermal cycler and follow the standard input procedure for tagmentation and cleanup. The use of an incubator is not supported for ≤ 299 ng input. Final libraries are not normalized. Inputs ≤ 250 ng could affect the generated libraries. Confirm the minimum library input required for successful sequencing on your sequencing platform of choice.

300–2000 ng DNA—Initial quantification is not required, but quantification of the final library pool is still required. Use a thermal cycler or incubator 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. Refer to 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)

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

* For more information on blood and saliva input requirements, refer to [Blood and Saliva Input Recommendations](#).

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, refer to [Supplementary Information on page 42](#).

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.

Assess Purity

Assess purity to make sure that 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, refer to *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.

Custom Primer Considerations

Sequencing of Illumina DNA PCR-Free libraries on the iSeq 100, MiniSeq, MiSeq, NextSeq 500/550, and standard kits on the NextSeq 1000/2000 instruments requires the additional kit, Illumina DNA PCR-Free Prep Sequencing and Indexing Primer (part # 20041797, sold separately). These primers are compatible with Illumina DNA PCR-Free libraries, the PhiX control library (Catalog # FC-110-3001), and all currently-supported Illumina libraries. The custom sequencing primers are provided at final loading concentration and require no further dilution. Follow the instructions for custom primers in the System Reference Documentation or Custom Primers Loading Documentation to load and set up the run. Sequencing on MiSeq only requires the Custom Read 1 Primer VP10.

Sequencing on MiSeq i100 Series, NextSeq 1000/2000 XLEAP kits, NovaSeq 6000, and NovaSeq X Series instruments does not require custom primers.

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 [**Incubator 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).

Consumables & Equipment

The Illumina DNA PCR-Free protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

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

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

Product Contents

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

- Library prep reagents
- Index adapters
- Custom sequencing primers
- **[Optional]** Lysis reagents

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	Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20091654
	Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	20091656
	Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20091658
	Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20091660
Custom primers [If required for your sequencing platform]	Illumina DNA PCR-Free Prep Sequencing and Indexing Primer	20041797
[Optional] Lysis reagents	Illumina Lysis Kit	20042221

Reagent Kit Contents

The following tables list the contents of each box.

Illumina DNA PCR-Free Prep Purification Beads + Buffers, 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 Prep Tagmentation Beads + Buffers, 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

Index Adapters Contents

The following tables list the contents of each box.

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

Quantity	Description	Catalog #
1	Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20091654
1	Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	20091656
1	Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20091658

Quantity	Description	Catalog #
1	Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20091660

Custom Primers Contents

The following tables list the contents of each box.

Illumina DNA PCR-Free Prep Sequencing and Indexing Primer (catalog 20041797), Store at -25C to -15C

For NextSeq 1000/2000 standard reagents, NextSeq 500/550, MiSeq, MiniSeq, and iSeq 100, follow the instructions for custom primers in the System Reference Guide or Custom Primers Loading Guide to load and set up the run. MiSeq only requires the Custom Read 1 Primer VP10.

Quantity	Acronym	Description
2	VP10	VP10 Custom Read 1 Primer (7.5 ml each)
2	VP14	VP14 Custom Index 2 Primer (10.5 ml each)

[Optional] Lysis Reagents

The following tables list the contents of each box.

Illumina Lysis Reagent Kit Box 1 (Catalog 20042221), 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

User-Supplied Consumables & Equipment

Ancillary Consumables

Consumables	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
[Incubator] Abgene 96 well 0.8 ml polypropylene deepwell storage plates (MIDI plate)	ThermoFisher Scientific, catalog # AB-0859
1.5 or 1.7 ml microcentrifuge tubes	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB1001B
[Optional] Microseal 'F' foil seals	Bio-Rad, catalog # MSF1001B
RNase/DNase-free multichannel reagent reservoirs, disposable	General lab supplier
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 # 07960140001 or catalog # 07960336001
Qubit dsDNA HS Assay Kit	ThermoFisher Scientific, catalog # Q32851 or # Q32854
[Dependent on workflow] Qubit ssDNA assay kit	ThermoFisher Scientific, catalog # Q10212

Consumables	Supplier
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 # OGR-600
[Dried blood] Whatman® 903 protein saver cards	Cytiva Lifesciences, catalog # 10531018
[Blood] Blood collection tubes, EDTA	Becton Dickinson
[Whole blood, dried blood, saliva] 2.0 ml microcentrifuge tubes	General lab supplier
[Whole blood or saliva] 15 or 50 ml conical centrifuge tubes	General lab supplier

Ancillary Equipment

Equipment	Supplier
[Incubator] One of the following mixers: Bioshake XP tube and plate mixer Bioshake iQ thermomixer	VWR, catalog # 102407-832 or # 102407-834
Microplate centrifuge	General lab supplier
Microcentrifuge	General lab supplier
Sealing roller or wedge	General lab supplier
Vortexer	General lab supplier
Qubit® Fluorometer v2.0 or higher	ThermoFisher Scientific, [v3.0] catalog # Q33216 [v4.0] catalog # Q33238
[Low input] Real-time PCR detection system	Refer to qPCR library quant kit recommendations
[Incubator] Heat block for 96 well plate <ul style="list-style-type: none"> • Temperature range: Ambient +5°C to 99°C • Temperature regulation: ±0.1°C • Heated lid • Compatible with the Illumina MIDI heat block insert 	General lab supplier or Illumina, catalog # SC-60-503 or # SC-60-504

Equipment	Supplier
Illumina MIDI heat block insert	Illumina, catalog # BD-60-601
[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
[Incubator] Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
[Dried blood spot and whole blood] ThermoMixer C	Eppendorf, catalog # 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, refer to product pages.

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. Refer to [DNA Input Recommendations on page 1](#) for information on choosing the correct protocol for your input. Refer to [Protocol for Thermal Cycler, Standard Input on page 11](#) for the standard input thermal cycler protocol and [Protocol for Thermal Cycler, Low Input on page 22](#) for the low input thermal cycler protocol.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required components, equipment, and consumables. Refer to [Consumables & Equipment on page 5](#). The Illumina DNA PCR-Free protocol requires the following components:
 - Library prep reagents
 - Index adapters (sold separately)
 - Custom sequencing primers (sold separately)

Protocol for Thermal Cycler, Standard Input

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 (Illumina DNA/RNA UD Indexes)
 - ST2 (Stop Tagment Buffer 2)
 - TWB (Tagment Wash Buffer)

! | **This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations.** For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

About Reagents

- Always use the specified magnetic stand to prevent failure.
- BLT-PF
 - Store frozen and upright to make sure that 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	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.



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 μ l
 - 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 μ l
 - 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 μ l 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 μ l, bring the total volume to 25 μ l 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 μ l BLT-PF to each well.
5. **[Blood/Saliva input]** Add 10 μ l BLT-PF to each well containing 30 μ l extracted DNA.
6. Using a P200 pipette set to 35 μ l, pipette to mix.
7. Add 10 μ l TB1 to each well.
8. Using a P200 pipette set to 40 μ l, 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 on page 26](#).

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 μ l ST2 to each well.
2. Using a P200 pipette set to 50 μ l, 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 μ l, remove and discard all supernatant from each well.
6. Remove the plate from the magnetic stand.
7. Add 150 μ l 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 (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 supernatant from each well.
2. Remove the plate from the magnetic stand.
3. Add 45 μ l 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 that you intend to use. Use a new pipette tip for each well.

- **[96 samples]** Align a new semiskirted 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 μ l index adapters to each well.
 7. Using a P200 pipette set to 45 μ l, 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 μ l 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 $280 \times g$ for 10 seconds.
 17. Place on the magnetic stand.
 18. Without disturbing the bead pellet, remove and discard supernatant from each well.
 19. Remove the plate from the magnetic stand.
 20. Add 45 μ l 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 on page 16](#).

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 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 μ l 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, label a new 96-well PCR plate LP2.
7. Vortex and then invert IPB until fully resuspended.
8. Add 42 μ l IPB to each well of LP2.
9. Without disturbing the bead pellet, **transfer** 76 μ l 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 μ l 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 \times g for 10 seconds.
18. Place on the magnetic stand and wait until the liquid is clear (~5 minutes)
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 μ l 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 × 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 on page 18](#).

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 such as Bioanalyzer. Only Qubit ssDNA assay kit or qPCR library quantification methods can be used.

For pooling guidelines, refer to *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 each library in a 1.5 or 1.7 ml microcentrifuge tube.
2. Vortex to mix, and then centrifuge at 280 × 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 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 $280 \times g$ for 1 minute.
5. Proceed immediately to [Dilute Libraries to the Starting Concentration and Sequence on page 20](#).

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.

- 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.
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.

$$\text{Molarity (nM)} = \text{Yield} \left(\frac{\text{ng}}{\mu\text{l}} \right) \times 3.36$$

- Dilute the pool to the starting concentration for your system.

The following table provides verified loading concentrations for Illumina sequencing systems. Limited testing has been performed on other platforms. For further details, review the [Optimal loading concentrations for Illumina DNA PCR-Free libraries Tech Note](#) and the [Denature and Dilute Protocol Generator](#) for the appropriate Illumina sequencing system.

Sequencing Platform	Starting Concentration	Final Loading Concentration
iSeq 100	1 nM	120 pM
NextSeq 1000/2000 Standard SBS Kits with Onboard Denature	2 nM	1000 pM
NextSeq 1000/2000 Standard SBS Kits with Manual Denature	500 pM	100 pM
NovaSeq 6000 Standard Loading	2–3 nM	400–600 pM
NovaSeq 6000 XP Loading	1.5–2 nM	300–400 pM
NovaSeq X/X Plus 1.5B and 10B Kits*	2 nM	140 pM
NovaSeq X/X Plus 25B Kits*	2 nM	150 pM

*Refer to the [Optimal variant calling with Illumina DNA PCR-Free Prep on the NovaSeq X Series Tech Note](#).

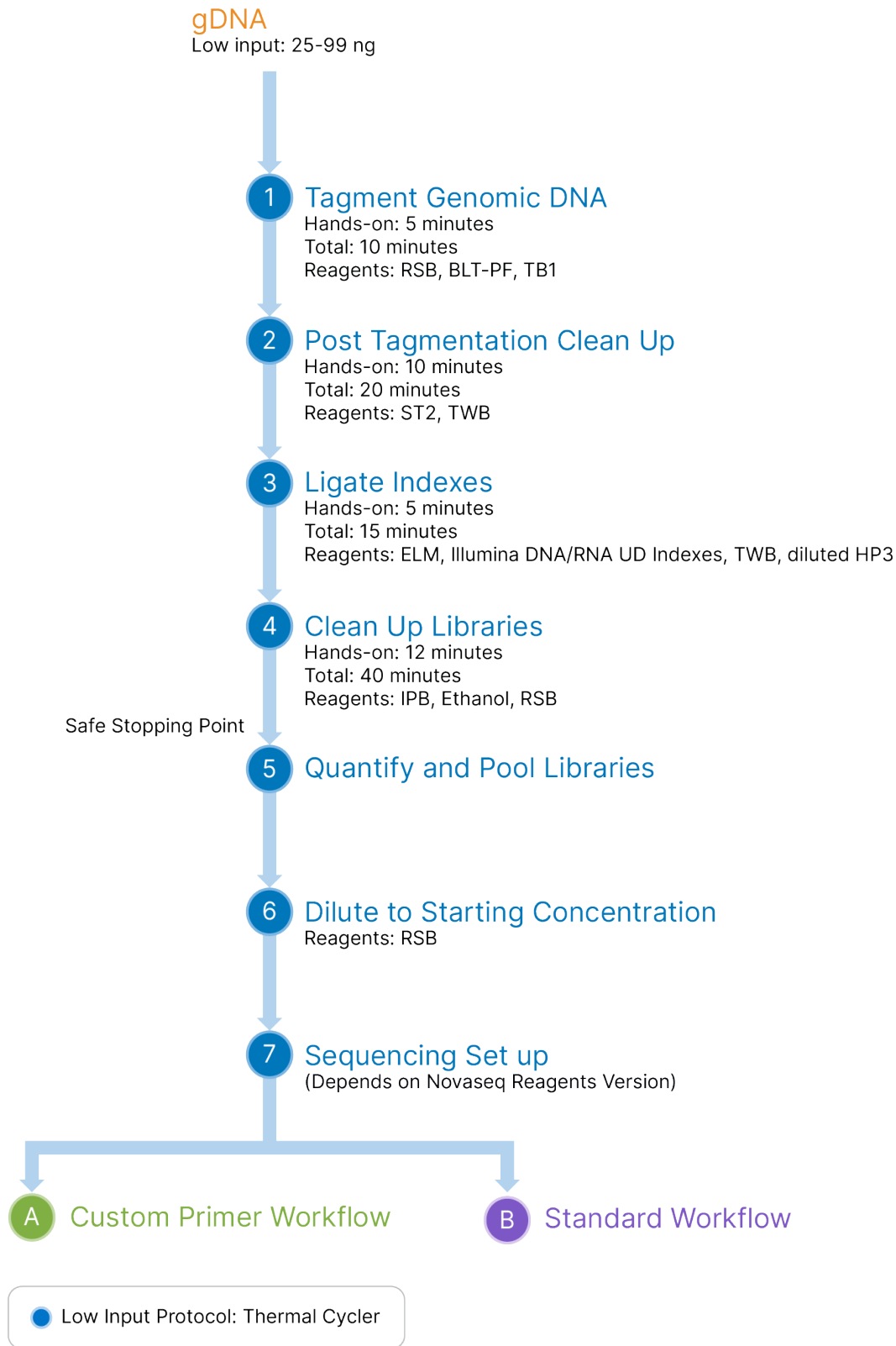
3. If you are using a system that requires custom primers, prepare the VP10 Custom Read 1 Primer and VP14 Custom Index 2 Primer. Refer to [Custom Primer Considerations on page 3](#) for more information on using custom sequencing primers with Illumina DNA PCR-Free.
4. Follow the standard or manual workflow instructions to denature and dilute libraries to the final loading concentration. Refer to the appropriate section of the [Denature and Dilute Protocol Generator](#) or the Sequencer System Guide for your sequencing workflow.
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). Refer to the Sequencing System Guide for your system for additional sequencing information.
Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

Protocol for Thermal Cycler, Low Input

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. Due to the lower yields from this protocol, there will not be sufficient library to sequence across all platforms or configurations. Confirm the minimum library input required for successful sequencing on your sequencing platform of choice.

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 (Illumina DNA/RNA UD Indexes)
 - ST2 (Stop Tagment Buffer 2)
 - TWB (Tagment Wash Buffer)

! | **This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations.** For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

About Reagents

- Use the specified magnetic stand.
- BLT-PF
 - Store frozen and upright to make sure that 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.

i | 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 × 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, pipette to mix and then seal.
10. Place on the preprogrammed thermal cycler and run the TAG program.
11. Proceed Immediately to [Post Tagmentation Cleanup on page 26](#).

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 μ l ST2 to each well.
2. Using a P200 pipette set to 50 μ l, 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 μ l, remove and discard all supernatant from each well.
6. Remove the plate from the magnetic stand.
7. Add 150 μ l 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 (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 supernatant from each well.
2. Remove the plate from the magnetic stand.
3. Add 45 μ l 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 that you intend to use. Use a new pipette tip for each well.

- **[96 samples]** Align a new semiskirted 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 μ l index adapters to each well.
 7. Using a P200 pipette set to 45 μ l, 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 μ l 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 \times g for 10 seconds.
 17. Place on the magnetic stand.
 18. Without disturbing the bead pellet, remove and discard supernatant from each well.
 19. Remove the plate from the magnetic stand.
 20. Add 45 μ l 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 on page 16](#).

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 μ l 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 μ l 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 \times 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 μ l 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 \times g for 10 seconds.
18. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
19. Transfer 14 μ l supernatant from each well of the plate to the corresponding well of a new PCR plate.
20. Proceed immediately to [Quantify and Pool Libraries on page 30](#).

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 before 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, refer to *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 μ l 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 the final concentration.
4. Vortex to mix, and then centrifuge at $280 \times g$ 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.

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

2. Dilute the pool to the starting concentration for your system.
3. If you are using a system that requires custom primers, prepare the VP10 Custom Read 1 Primer and VP14 Custom Index 2 Primer. Refer to [Custom Primer Considerations on page 3](#) for more information on using custom sequencing primers with Illumina DNA PCR-Free.
4. Follow the standard or manual workflow instructions to denature and dilute libraries to the final loading concentration. Refer to the appropriate section of the [Denature and Dilute Protocol Generator](#) or the Sequencer System Guide for your sequencing workflow.

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). Refer to your sequencer's Sequencing System Guide for additional sequencing information.

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

Protocol for Incubator

This chapter describes the Illumina DNA PCR-Free Library Prep protocol when using a sample incubator and gDNA inputs ≥ 300 ng. If using whole blood, dried blood spots, saliva, or inputs 100–299 ng, refer to [Protocol for Thermal Cycler, Standard Input on page 11](#). If using inputs of < 99 ng, refer to [Protocol for Thermal Cycler, Low Input on page 22](#).

- 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. Refer to [Consumables & Equipment on page 5](#). The Illumina DNA PCR-Free protocol requires the following components:
 - Library prep reagents
 - Index adapters (sold separately)
 - Custom sequencing primers (sold separately)

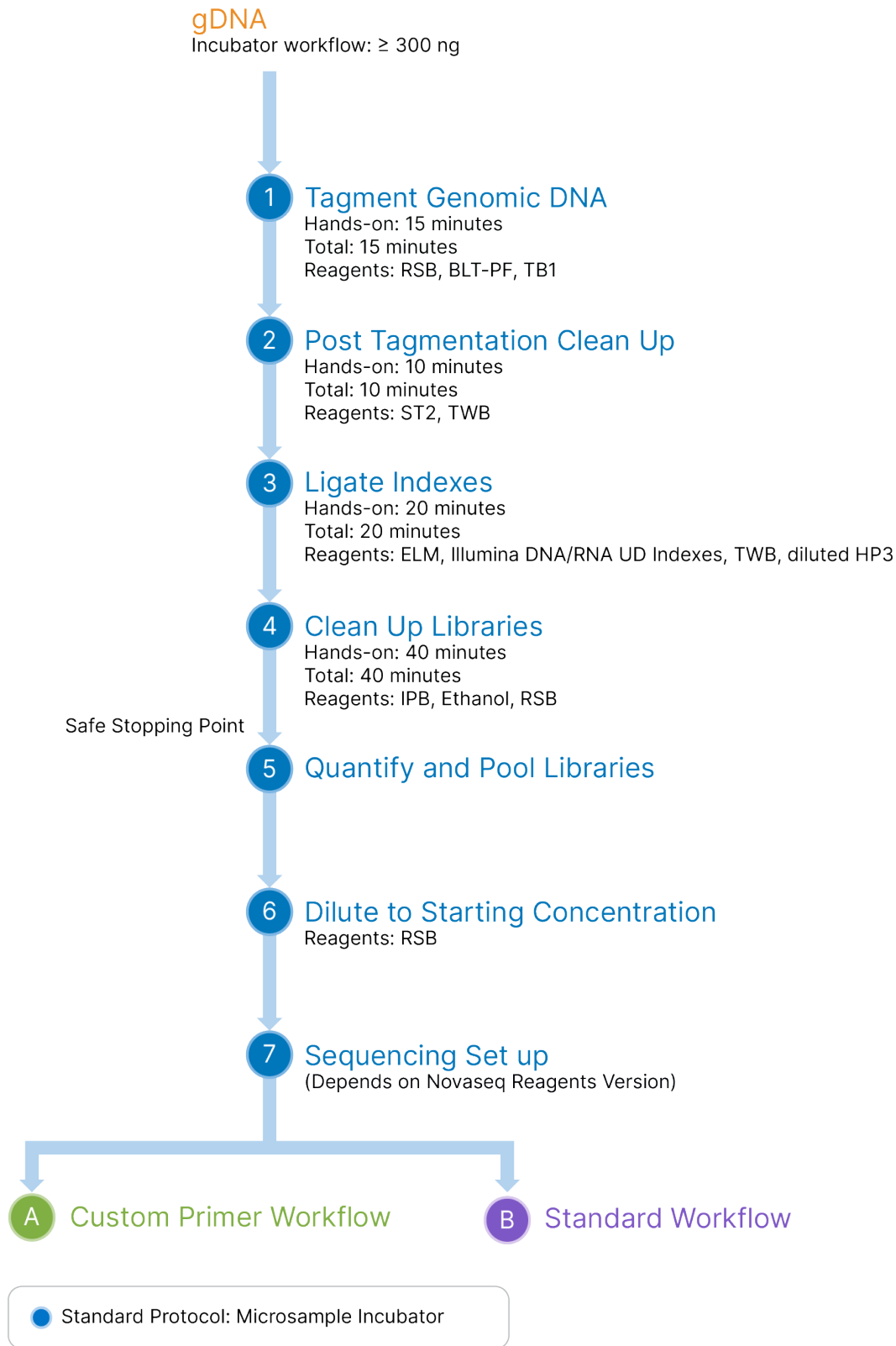
This protocol also requires a Sample Incubator. The incubator must meet the following performance specifications.

- Temperature range: Ambient +5°C to 99°C
- Temperature regulation: $\pm 0.1^\circ\text{C}$
- The incubator must have a heated lid.
- The incubator must be compatible with the Illumina MIDI heat block insert (catalog # BD-60-503).

Library Prep Workflow

The following diagram illustrates the Illumina DNA PCR-Free Library Prep Incubator 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 (Illumina DNA/RNA UD Indexes)
 - ST2 (Stop Tagment Buffer 2)
 - TWB (Tagment Wash Buffer)

! **This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Ventilation should be appropriate for handling of hazardous materials in reagents. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations.** For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

About Reagents

- Use the specified magnet.
- BLT-PF
 - Store frozen and upright to make sure that 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.

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

- 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.
- 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.
- Preheat the incubator to 45°C.

Procedure

1. Label a new 96-well MIDI plate LP1.
2. Add 2–25 μ l DNA to each well, so that the total input amount is within the desired range.
3. If sample volume is < 25 μ l, bring the total volume to 25 μ l using RSB.
4. Add 10 μ l TB1 to each well.
5. Vortex BLT-PF vigorously for 1 minute to resuspend. Repeat as necessary.
6. Add 15 μ l BLT-PF to each well.
7. Seal and shake at 1800 rpm for 1 minute.
8. Incubate in preheated sample incubator for 8 minutes. Proceed immediately to [Post Tagmentation Cleanup on page 36](#).

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 μ l 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 μ l 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 (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 that you intend to use. Use a new pipette tip for each well.
 - [**96 samples**] Align a new semiskirted 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 in the preheated sample incubator for 8 minutes.
The incubator 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.

- Without disturbing the bead pellet, use a 20 μ l pipette to remove and discard residual TWB from each well.
- Add 45 μ l diluted HP3 to each well.
- 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 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

- Vortex IPB, and then invert until fully resuspended.
- Add 36 μ l IPB to each well containing BLT-PF beads.
- Seal and shake at 1800 rpm for 1 minute.
- Incubate at room temperature for 2 minutes.
- Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- While the plate incubates, label a new 96-well MIDI plate LP2.
- Add 42 μ l IPB to each well of LP2.
- Without disturbing the bead pellet, **transfer** 76 μ l supernatant from each well of LP1 to the corresponding well of the LP2.
- Seal and shake at 1800 rpm for 1 minute.
- 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 μ l 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 μ l pipette, remove residual EtOH from each well.
17. Discard unused 80% EtOH.
18. Air-dry on the magnetic stand (~2 minutes).
19. Add 22 μ l 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 μ l supernatant from each well of LP2 to the corresponding well of FLP.
25. Proceed immediately to [Quantify and Pool Libraries on page 39](#).

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, refer to *Index Adapter Pooling Guide (document # 1000000041074)*.

1. Combine 9 μ l of each library in a 1.5 or 1.7 ml microcentrifuge tube.
2. Vortex to mix, and then centrifuge at 280 \times g for 1 minute.
3. Quantify the library pool:
 - Analyze 2 μ l 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.

- 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.
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.

$$\text{Molarity (nM)} = \text{Yield} \left(\frac{\text{ng}}{\mu\text{l}} \right) \times 3.36$$

- Dilute the pool to the starting concentration for your system.

The following table provides verified loading concentrations for Illumina sequencing systems. Limited testing has been performed on other platforms. For further details, review the [Optimal loading concentrations for Illumina DNA PCR-Free libraries Tech Note](#) and the [Denature and Dilute Protocol Generator](#) for the appropriate Illumina sequencing system.

Sequencing Platform	Starting Concentration	Final Loading Concentration
iSeq 100	1 nM	120 pM
NextSeq 1000/2000 Standard SBS Kits with Onboard Denature	2 nM	1000 pM
NextSeq 1000/2000 Standard SBS Kits with Manual Denature	500 pM	100 pM
NovaSeq 6000 Standard Loading	2–3 nM	400–600 pM
NovaSeq 6000 XP Loading	1.5–2 nM	300–400 pM
NovaSeq X/X Plus 1.5B and 10B Kits*	2 nM	140 pM
NovaSeq X/X Plus 25B Kits*	2 nM	150 pM

*Refer to the [Optimal variant calling with Illumina DNA PCR-Free Prep on the NovaSeq X Series Tech Note](#).

3. If you are using a system that requires custom primers, prepare the VP10 Custom Read 1 Primer and VP14 Custom Index 2 Primer. Refer to [Custom Primer Considerations on page 3](#) for more information on using custom sequencing primers with Illumina DNA PCR-Free.
4. Refer to [Custom Primer Considerations on page 3](#) for more information on using custom sequencing primers with Illumina DNA PCR-Free.
5. Follow the standard or manual workflow instructions to denature and dilute libraries to the final loading concentration. Refer to the appropriate section of the [Denature and Dilute Protocol Generator](#) or the Sequencer System Guide for your sequencing workflow.

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). Refer to your sequencer's Sequencing System Guide for additional sequencing information.


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

Supplementary Information

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

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.

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

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.

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

Procedure

- Label a 15 ml tube Lysis Master Mix.
- 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.
- Vortex or invert the master mix thoroughly to mix, and then centrifuge.
- For each sample, add 280 µl master mix to a new 2 ml tube.
- Invert each EDTA tube five times to mix.
- Add 20 µl blood to each 2 ml tube.
- Pipette to mix.
- Vortex briefly, and then centrifuge.
- Incubate and shake at 1000 rpm for 15 minutes in ThermoMixer C.
- Centrifuge briefly.
- Make sure that the liquid is brown. If the liquid is not brown, shake at 1000 rpm for 5 minutes. The brown color indicates that the sample is fully lysed.
- Vortex IPB, and then invert until fully resuspended.
- Add 135 µl IPB to each tube.
- Vortex for 15 seconds, and then pipette to mix.
- Incubate at room temperature for 5 minutes.
- 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 that 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 12](#).

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.

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

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 μ l)
 - PK1 (2 μ l)
 - Nuclease-free water (268 μ l)These volumes produce 300 μ l 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 μ l master mix to a new 2 ml tube.
5. Add 6 x 3 mm² punches to 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 that the liquid is brown. If the liquid is not brown, shake at 1000 rpm for 5 minutes. The brown color indicates that 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 μ l 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 that all lysis material is removed.
18. Wash beads as follows.
 - a. Keep on the magnetic stand and add 500 μ l 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 μ l 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 (\geq 2 minutes).
28. Label a new PCR plate LP1.
29. Transfer as follows.
 - If you are not quantifying before beginning library prep, transfer 30 μ l supernatant from each tube to the LP1 plate.
 - If you are quantifying before beginning library prep, transfer 32 μ l 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 12](#).

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.

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

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 instructions from the manufacturer for saliva extraction.
After incubation, you can use the saliva immediately or store it at room temperature. For long-term storage, refer to 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 that 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 μ l 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 (\geq 2 minutes).
23. Label a new PCR plate LP1.
24. Transfer as follows.
 - If you are not quantifying before beginning library prep, transfer 30 μ l supernatant from each tube to the LP1 plate.
 - If you are quantifying before beginning library prep, transfer 32 μ l 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 12](#).

SAFE STOPPING POINT

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

Resources & References

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

Additional Resources

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

Revision History

Document	Date	Description of Change
Document # 1000000086922 v04	June 2026	<p>Updated:</p> <ul style="list-style-type: none"> • Index Reagent kits to Illumina DNA/RNA UD Indexes and removed IDT for Illumina kits • Custom Primers kit contents • Sequencing Primers • Custom Primer Considerations to give more guidance around using custom primers • Hybex procedure to Incubator procedure • Catalog numbers for consumables and equipment <p>Removed:</p> <ul style="list-style-type: none"> • Acronyms • Loading concentrations for NovaSeq procedures <p>Added:</p> <ul style="list-style-type: none"> • Disclaimer about low input resulting in lower performance • Units across document <p>Clarified wait times for library clean-up steps Generalized dilution steps across platforms</p>
Document # 1000000086922 v03	February 2021	<p>Corrected formula for calculating molarity. Updated final library storage temperature to 30 days.</p>
Document # 1000000086922 v02	February 2021	<p>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.</p>
Document # 1000000086922 v01	July 2020	<p>Corrected plate name from "PCR" to "MIDI" in Clean Up Libraries for Hybex protocol.</p>
Document # 1000000086922 v00	July 2020	<p>Initial release.</p>



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

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