



Illumina 5-Base DNA Prep

Product Documentation

ILLUMINA PROPRIETARY
Document # 200073497 v01
August 2026

For Research Use Only. Not for use in diagnostic procedures.

This document and its contents are proprietary to Illumina, Inc. and its affiliates ("Illumina"), and are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Illumina. Illumina does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s).

FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY, AND WILL VOID ANY WARRANTY APPLICABLE TO THE PRODUCT(S).

ILLUMINA DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN (INCLUDING PARTS THEREOF OR SOFTWARE).

© 2026 Illumina, Inc. All rights reserved.

All trademarks are the property of Illumina, Inc. or their respective owners. For specific trademark information, refer to www.illumina.com/company/legal.html.

Table of Contents

1. Overview	1
1.1 Introduction	1
1.2 DNA Input Recommendations	1
1.3 Library Prep Automation (Optional)	3
2. Consumables & Equipment	4
2.1 Product Contents	4
2.2 User-Supplied Consumables & Equipment	7
3. Before You Begin	10
3.1 Illumina 5-Base DNA Prep Kit Workflow	11
3.2 Tips and Techniques	12
3.3 Sonication Guidelines for gDNA Input	15
3.4 Sonication Guidelines for FFPE DNA Input	16
4. Prepare Input DNA	17
4.1 Prepare Reagents for Input DNA	17
4.2 Prepare gDNA	19
4.3 Prepare FFPE DNA	21
4.4 Prepare Size-Select cfDNA	22
5. Perform Library Prep Steps	26
5.1 Prepare Reagents for Library Prep Steps	26
5.2 Perform End Repair	27
5.3 Perform A-Tailing	27
5.4 Ligate Adapters	28
5.5 Clean Up Ligation	29
6. Perform Conversion & Amplification Steps	32
6.1 Prepare Reagents for Conversion and Amplification	32
6.2 Denaturation	33
6.3 Conversion	34
6.4 Index PCR	35
6.5 Clean Up Index PCR	37
6.6 Quantify Libraries	38
7. Prepare for Sequencing	40
7.1 Dilute Libraries to the Starting Concentration	40

8. Resources & References 41

 8.1 Revision History 41

9. Technical Assistance 42

1. Overview

This section includes the following information:

- [1.1 Introduction on page 1.](#)
- [1.2 DNA Input Recommendations on page 1.](#)
- [1.3 Library Prep Automation \(Optional\) on page 3.](#)

1.1 Introduction

The Illumina 5-Base DNA Prep enables library preparation from high quality genomic DNA (gDNA), cell-free DNA (cfDNA), and formalin-fixed paraffin-embedded (FFPE) DNA input.

Illumina 5-Base DNA Prep:

- Uses a proprietary enzymatic reaction to directly convert methylated cytosines to thymine in a single step, while retaining the identity of unmethylated cytosines.
- Generates sequencing-ready libraries from low input across various sample types, including from a minimum of 50 ng gDNA, 1 ng cfDNA, or 40 ng FFPE DNA.
- Supports automated preparation of gDNA input and cfDNA input. For automation information, refer to [1.3 Library Prep Automation \(Optional\) on page 3.](#)
- Enables the detection of both methylation and genomic variants from a single library prep using the DRAGEN Germline or DRAGEN Somatic applications available on BaseSpace Sequence Hub or on DRAGEN Server.

1.2 DNA Input Recommendations

The Illumina 5-Base DNA Prep protocol enables library preparation from gDNA, cfDNA, and formalin-fixed paraffin-embedded (FFPE) DNA input. For more information, refer to the section appropriate for your input type.

gDNA Input

The Illumina 5-Base DNA Prep protocol enables library preparation from 50–100 ng gDNA.

The input preparation procedure requires sonication of gDNA samples. For sonication guidelines, refer to [3.3 Sonication Guidelines for gDNA Input on page 15.](#)

FFPE DNA Input

The Illumina 5-Base DNA Prep protocol enables library preparation from 40 ng FFPE DNA.

Follow these guidelines for input:

- Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. The Covaris truXTRAC FFPE Total NA Auto 96 Kit, the QIAamp DNA FFPE Tissue Kit for DNA Extraction, and the QIAGEN AllPrep DNA/RNA FFPE Kit - Nucleic Acid Extraction are compatible with the Illumina 5-Base DNA Prep Kit.
- Assess samples for quality. Illumina recommends the TruSight FFPE QC Kit and proceeding with samples of ΔCq value ≤ 5 .
- The input preparation procedure requires sonication of FFPE-extracted DNA samples. For sonication guidelines, refer to [3.4 Sonication Guidelines for FFPE DNA Input on page 16](#).

cfDNA Input

The Illumina 5-Base DNA Prep protocol enables library preparation from 1–20 ng cfDNA.

[4.4 Prepare Size-Select cfDNA on page 22](#) includes bead purification. Bead purification is an essential preparation step. This step is optimized to select the mononucleosomal cfDNA peak and to remove blood-derived components that can interfere with library prep chemistry.

Follow these guidelines for input:

- Use one of the following tested blood collection tubes: the Apostle MiniMax Cell-Free DNA Blood Collection Tube (BCT), QIAGEN PAXgene Blood ccfDNA Tubes, Streck Cell-Free DNA BCT, or BD Vacutainer Plastic Blood Collection Tubes with K2EDTA.
- Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. The QIAGEN QIAamp Circulating Nucleic Acid Kit (without carrier RNA), QIAGEN QIAamp MinElute ccfDNA Midi Kit, and the Zymo MAGicBead cfDNA Isolation Kit are compatible with the Illumina 5-Base DNA Prep Kit.
- Quantify the input of each cfDNA sample using a size-based quantification method such as the Agilent Fragment Analyzer System or Agilent TapeStation. Quantify only the mononucleosomal peak (approximately 75 bp to 250 bp) of the DNA trace to ensure optimal cell-free nucleic acid input. Fluorometric assays are not recommended, as the presence of high molecular weight DNA can lead to an overestimation of cfDNA concentrations.

1.3 Library Prep Automation (Optional)

Illumina 5-Base DNA Prep is engineered for fully automated, high-capacity library preparation using third-party liquid handling platforms. To use on an automated liquid-handling system, the Illumina 5-Base DNA Prep requires the following kit: Illumina 5-Base DNA Prep (96 samples).

The Illumina 5-Base DNA Prep (96 samples):

- Supports automated preparation of gDNA input and cfDNA input.
- Supports the preparation of up to 96 libraries per automation run or manual preparation of up to 120 libraries.
- Is qualified for four freeze–thaw cycles, enabling flexible batching.

This chemistry is qualified for use with the Illumina-ready Automation Protocol on the Hamilton Microlab NGS STAR MOA. For other automation systems, contact your preferred vendor to obtain compatibility details, protocol guidance, or integration requirements. Refer to the [Illumina Library Prep Automation](#) site to learn more. For catalog numbers and component-level specifications, refer to [2.1 Product Contents on page 4](#).

2. Consumables & Equipment

The Illumina 5-Base DNA Prep protocol requires the consumables and equipment identified in this section. 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.

2.1 Product Contents

Completing the Illumina 5-Base DNA Prep protocol requires library prep reagents and indexes.

Product Contents includes the following information:

- [Reagent Kit Contents—24 Samples on page 4.](#)
- [Reagent Kit Contents—96 Samples on page 5.](#)
- [Additional Illumina Components on page 6.](#)

Component	Kit Options	Catalog #
Library prep reagents	Illumina 5-Base DNA Prep (24 samples)	20140364
	Illumina 5-Base DNA Prep (96 samples)*	20140365

* The Illumina 5-Base DNA Prep (96 samples) supports automated preparation of 96 libraries and manual preparation of up to 120 libraries.

Reagent Kit Contents—24 Samples

The following tables list the contents of each box included in the 24 sample library prep reagent kit. Additional Illumina components must be included to perform the Illumina 5-Base DNA Prep. Refer to [Additional Illumina Components on page 6](#) for information.

Illumina 5-Base DNA Prep Box 1, Store at -25°C to -15°C

24 Sample Kit	Reagent	Description
1	ATL4	A-Tailing Mix
1	DDR	DNA Denaturation Reagent
1	ERP6	End Repair Mix 6
1	GDMC	Genomic DNA Methylation Control
1	IME	Illumina Methylation Enzyme

24 Sample Kit	Reagent	Description
1	LIGX	Ligation Mix
1	MAM	Methylation Amplification Mix
1	MCB	Methylation Conversion Buffer
1	MRR	Methylation Resuspension Reagent
1	SFMC	Short Fragment Methylation Control
1	UMI3	Unique Molecular Identifier 3

Illumina 5-Base DNA Prep Box 2, Store at 15°C to 30°C

24 Sample Kit	Reagent	Description
1	IPB	Illumina Purification Beads
1	RSB	Resuspension Buffer

Reagent Kit Contents—96 Samples

The following tables list the contents of each box included in the 96 sample library prep reagent kit. Additional Illumina components must be included to perform the Illumina 5-Base DNA Prep. Refer to [Additional Illumina Components on page 6](#) for information.

Illumina 5-Base DNA Prep High Throughput Box 1, Store at -25°C to -15°C

96 Sample Kit	Reagent	Description
1	ATL4	A-Tailing Mix
1	ERP6	End Repair Mix 6
4	GDMC	Genomic DNA Methylation Control
1	LIGX	Ligation Mix
1	SFMC	Short Fragment Methylation Control
1	UMI3	Unique Molecular Identifier 3

Illumina 5-Base DNA Prep Methylation Conversion Reagents High Throughput, Store at -25°C to -15°C

96 Sample Kit	Reagent	Description
1	DDR	DNA Denaturation Reagent
1	IME	Illumina Methylation Enzyme
1	MAM	Methylation Amplification Mix
1	MCB	Methylation Conversion Buffer
1	MRR	Methylation Resuspension Reagent

Purification Beads and Resuspension Buffer, Store at 2°C to 8°C, Storage Temperatures in Table

96 Sample Kit	Reagent	Description	Storage
3	IPB	Illumina Purification Beads	Room Temperature
2	RSB	Resuspension Buffer	2°C to 8°C

Additional Illumina Components

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

Select at least one set from the following options.

Component	Description	Catalog #
Indexes	Illumina Unique Dual Indexes LT (48 Indexes, 48 Samples)	20098166
	Illumina DNA/RNA UD Indexes Set A (96 Indexes, 96 Samples)	20091654
	Illumina DNA/RNA UD Indexes Set B (96 Indexes, 96 Samples)	20091656
	Illumina DNA/RNA UD Indexes Set C (96 Indexes, 96 Samples)	20091658
	Illumina DNA/RNA UD Indexes Set D (96 Indexes, 96 Samples)	20091660

Indexes, Automation	Illumina DNA/RNA UD Indexes Set A, Auto (96 Indexes, 96 Samples)	20141196
	Illumina DNA/RNA UD Indexes Set B, Auto (96 Indexes, 96 Samples)	20141197
	Illumina DNA/RNA UD Indexes Set C, Auto (96 Indexes, 96 Samples)	20141198
	Illumina DNA/RNA UD Indexes Set D, Auto (96 Indexes, 96 Samples)	20141199

2.2 User-Supplied Consumables & Equipment

Some items are required only for specific workflows.

Consumables

Consumable	Supplier
1.7 ml microcentrifuge tubes	General lab supplier
[gDNA/FFPE] 8 microTUBE Strip (130 µl) or alternative DNA fragmentation instrument and consumables	Covaris, part number # 520053 or # 520109, or supplier appropriate for your fragmentation instrument
[FFPE] 8 AFA-TUBE Fiber TPX Strip	Covaris, part number # 520292*
Absolute ethanol, molecular biology grade	General lab supplier
Hard-shell 96-well PCR plates, thin wall, semi-skirted (250 µl minimum volume)	Eppendorf, catalog # 0030129504 or 951020303
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
Nuclease-free water	General lab supplier
Pipette tips, 10 µl	General lab supplier
Pipette tips, 20 µl	General lab supplier
Pipette tips, 200 µl	General lab supplier
Pipette tips, 1000 µl	General lab supplier
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
Qubit dsDNA Broad Range Assay Kit for quantification	Thermo Fisher Scientific, catalog # Q32850 or Q32853
[Optional] Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific, catalog # Q32851 or Q32854

Consumable	Supplier
The following consumables, depending on quantification method: • [Bioanalyzer] Bioanalyzer DNA 1000 • [TapeStation] D1000 Kit • D1000 Screen Tape • D1000 Reagent	One of the following, depending on the instrument: • Agilent, catalog # 5067-1504 • Agilent, catalog # 5067-5582 • Agilent, catalog # 5067-5583

* Must be paired with Covaris, part number # 500685.

Equipment

Equipment	Supplier
Multichannel pipettes, 10 µl	General lab supplier
Multichannel pipettes, 20 µl	General lab supplier
Multichannel pipettes, 200 µl	General lab supplier
Single channel pipettes, 10 µl	General lab supplier
Single channel pipettes, 20 µl	General lab supplier
Single channel pipettes, 200 µl	General lab supplier
Single channel pipettes, 1000 µl	General lab supplier
96-well plate centrifuge	General lab supplier
96-well plate magnet, either of the following: • DynaMag-96 Side Skirted Magnet • DynaMag-96 Side Magnet	One of the following suppliers: • Thermo Fisher Scientific, catalog # 12027 • Thermo Fisher Scientific, catalog # 12331D
Adapter for PCR Plate, 96 well for use with High-Speed Plate Shaker	Q Instruments, model # 1808-1041
High-Speed Plate Shaker, adjustable up to at least 2200 rpm	One of the following, or equivalent instrument • BioShake iQ, Q Instruments, model # 1808-0506 • BioShake XP, Q Instruments, model # 1808-0505
[gDNA/FFPE] Covaris LE220 Plus ³ , or other sonication instrument	Covaris, part number # 500569, or equivalent supplier
[gDNA/FFPE] Rack 12 place 8 microTUBE Strip, or equivalent for other sonication instrument	Covaris, part number # 500191, or equivalent supplier
[FFPE] Rack 8 AFA-TUBE TPX Strip ⁴	Covaris, part number # 500685

Equipment	Supplier
Microcentrifuge	General lab supplier
One of the following systems, depending on your quantification method: • Agilent Bioanalyzer 2100 System¹ • Agilent TapeStation 4200 System	One of the following, depending on qualification method: • Agilent, catalog # G2939BA¹ • Agilent, catalog # G2991BA²
Qubit 4 Fluorometer	Thermo Fisher Scientific, catalog # Q33238
Thermal cycler (deep well)	General lab supplier
Vortexer	General lab supplier

¹ End of life announced. Refer to vendor site for more information.

² Only one automated electrophoresis tool is required.

³ Fragment size distribution can vary due to differences in the sonication instrument used for fragmentation.

⁴ Must be paired with Covaris, part number # 520292.

3. Before You Begin

Review the following information before starting the Illumina 5-Base DNA Prep:

- [3.1 Illumina 5-Base DNA Prep Kit Workflow on page 11.](#)
- [3.2 Tips and Techniques on page 12.](#)
- [3.3 Sonication Guidelines for gDNA Input on page 15.](#)
- [3.4 Sonication Guidelines for FFPE DNA Input on page 16.](#)

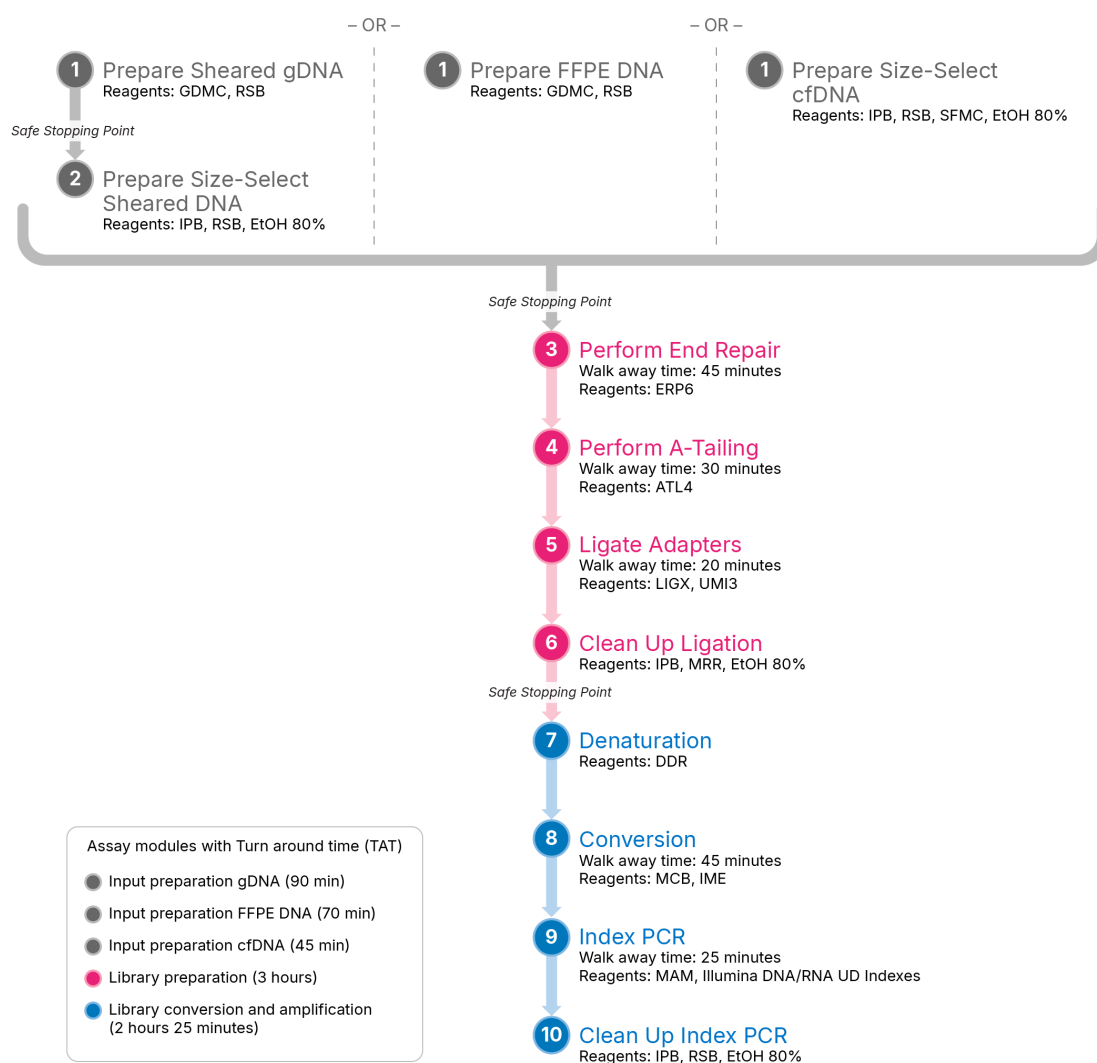
Before starting the protocol, do as follows.

- Review the entire protocol.
- Before proceeding, confirm kit contents and make sure that you have the required equipment and consumables. This protocol requires library prep reagents and indexes. For details, refer to [2.1 Product Contents on page 4.](#)
- Review and complete the preparation sections appropriate for your input type, including sonication, when applicable.
- Have ice available. The library prep PCR plate and many of the consumables must be placed on ice during the protocol.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.
- Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

3.1 Illumina 5-Base DNA Prep Kit Workflow

The following diagram illustrates the Illumina 5-Base DNA Prep Kit workflow.

- Each protocol module starts with a Prepare Reagents section and ends at a safe stopping point.
- Time estimates are based on preparing 24 samples using a multichannel pipette.



3.2 Tips and Techniques

Review *Tips and Techniques* before starting the protocol. Many critical techniques are only listed here and not repeated in the protocol.

Avoiding Cross-Contamination

- Change tips between *each well* for the following steps:
 - Adding or transferring samples
 - Adding indexing primers
 - Pipette mixing samples
- Change gloves if gloves come in contact with indexing primers or samples.
- Clean work surfaces and equipment thoroughly before and after the procedure with an RNase/DNase inhibiting cleaner.
- Do not reuse seals from plates.

Sealing and Unsealing Plates

- Always seal the plate before performing steps with the following actions:
 - Shaking
 - Vortexing
 - Centrifugation
 - Thermal cycling
- When sealing the plate, make sure that the edges and wells are fully sealed. Apply the adhesive cover with a sealing wedge or roller. Use a new seal each time.
- Place the plate on a flat surface before removing the seal to prevent reagent cross-contamination.
- Removing adhesive seals can disturb the beads. After removing the seal, let the plate sit for at least an additional 30 seconds. For steps involving the magnetic stand, remove the seal when first putting the plate onto the magnetic stand.
- Take care when unsealing the plate to prevent material loss.
- If you see droplets inside a sealed plate, to prevent sample loss, centrifuge at $280 \times g$ for 10 seconds.
- For long-term storage, use Microseal 'B' adhesive seals with plates. Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for semiskirted PCR plates.

Plate Transfers

- Label your plates.

- When transferring volumes between plates, note the well position for each sample. Transfer the specified volume from each well of the plate to the corresponding well of the other plate.
- Prepare samples at the same time for more consistent results.

Vortex and Centrifuge Steps

- When instructed to vortex briefly, vortex 3 times for 3 seconds on the maximum setting.
- When instructed to centrifuge briefly, centrifuge at $280 \times g$ for 10 seconds.

Handling Reagents

- Follow the mixing indication for each reagent at each step of the workflow.
- If the reagent sits unused for more than 5 minutes, mix again before use.
- Tightly recap all reagent tubes immediately after use to limit evaporation and prevent contamination.
- Return reagents to the recommended storage conditions when they are no longer needed for the protocol.
- When pipette mixing:
 - Use a suitable pipette and tip size for the volume. For example, use a 200 μ l pipette for volumes 20 μ l to 200 μ l.
 - Adjust the volume setting to ~50–75% of sample volume.
 - Pipette slowly to mix, but avoid splashing or introducing bubbles.
 - If bubbles are introduced or created, centrifuge briefly before resuming pipette-mixing.
- Reference the appropriate supporting material for your given pipette.

Handling Beads

- Immediately before use, inspect the tube and confirm that the tube is a uniform color and that bead pellets are not visible at the bottom of the tube.
- To prepare beads for use, make sure that the beads are fully suspended. To confirm that the beads are fully suspended, invert the tube. Fully suspended beads do not visibly stick to the end of the tube when the tube is inverted. Vortex until beads are fully dispersed. Total vortex time should be less than 1 minute to avoid bead damage.
- During steps that require beads, retain the supernatant until *specifically* instructed to discard. This protocol requires use of the supernatant.
- Do not freeze beads.
- Pipette bead suspensions slowly.
- When aspirating the supernatant from the magnetized beads:

- Inspect before aspirating to confirm that the supernatant is clear.
- Aspirate slowly when removing the supernatant from the beads.
- Check tips to make sure that no beads were aspirated.
- If you disturb the beads or aspirate beads into the pipette tips, dispense into the correct well while the plate is on the magnetic stand. Wait ~2 minutes until the liquid is clear before attempting again.
- When washing beads:
 - Use the specified magnetic stand for the plate.
 - Use fresh 80% ethanol. Make 80% ethanol from absolute ethanol before each procedure requiring its use.
 - Ethanol carryover can affect assay performance. After completing bead-washing steps, make sure to remove all residual ethanol.
 - Dispense liquid so that the beads in the well are covered by the level of wash buffer.
 - Pipette gently against the side of the well, on the opposite side from the beads, and avoid contact with the bead pellet.
 - Keep the plate on the magnetic stand until the instructions specify removal.
 - Do not agitate the plate while it is on the magnetic stand.
 - Do not disturb the bead pellet. Disturbing the bead pellet at wash steps can affect assay performance.
- When air-drying the beads:
 - Use the drying time recommended in the protocol.
 - Avoid over-drying. Pellet should not appear cracked.
- When resuspending beads:
 - Make sure that the bead pellet is fully in solution. For example, when the beads are fully in solution, the solution has a uniform dark brown appearance.
 - If the bead pellet is not fully in solution, seal the plate and pulse-vortex 3 times for 3 seconds. Centrifuge briefly to collect contents.
 - Position the pipette tip above the pellet and dispense directly onto the pellet to release beads from the well or tube.
 - Use the resuspension buffer specified in the protocol.

3.3 Sonication Guidelines for gDNA Input

The input preparation procedure requires sonication of gDNA samples. For sonication information for FFPE DNA input, refer to [3.4 Sonication Guidelines for FFPE DNA Input on page 16](#).

Follow these guidelines for sonication.

- Use a sonication method that produces an average fragment size of ~450 bp. Overfragmenting can reduce sample yield, introduce GC bias, and affect assay performance.
- Sonication reaction must be less than 100 µl to be compatible with the Illumina 5-Base DNA Prep workflow.
- Optimize sonication for the sample type, sonication equipment, and sonication consumables.
- For gDNA, Illumina tested the Covaris LE220 Plus with the 8 microTUBE Strip for sonication with the following settings:

Instrument Settings	Covaris LE220 Plus
Tube Type	8 microTUBE Strip
Tube PN	520053
Volume (µl)	55
Water temperature setpoint (°C)	7°, Range ± 2°
Peak incident power (Watts)	450
Duty factor (%)	18
Cycles per burst (cpb)	200
Total Treatment Time (Seconds)	35

For instructions on how to sonicate gDNA samples, refer to the appropriate supporting material for your sonication equipment.

3.4 Sonication Guidelines for FFPE DNA Input

The input preparation procedure requires sonication of FFPE-extracted DNA samples. For sonication information for gDNA input, refer to [3.3 Sonication Guidelines for gDNA Input on page 15](#).

Follow these guidelines for sonication.

- Use a sonication method that produces an average fragment size of ~200 bp.
- Sonication reaction must be less than 52 µl to be compatible with the Illumina 5-Base DNA Prep workflow.
- Optimize sonication for the sample type, sonication equipment, and sonication consumables.
- For FFPE DNA input, Illumina tested the following sonicator and settings:

Instrument Settings	Covaris LE220 Plus	
Tube Type	8 microTUBE Strip	8 AFA-TUBE Fiber TPX Strip
Tube PN	520053	520292*
Volume (µl)	52	20
Temperature (°C)	7°	10°
Peak incident power (Watts)	450	260
Duty factor (%)	30	26
Cycles per burst (cpb)	200	50
Dithering	N/A	1 mm Y at 20 mm/sec
Repeat Process Duration (Seconds)	N/A	10
Number of Iterations	N/A	11
Delay Time (Seconds)	N/A	10
Total Treatment Time (Seconds)	250	110

* Must be paired with Covaris, part number # 500685.

For instructions on how to sonicate FFPE DNA samples, refer to the appropriate supporting material for your sonication equipment.

4. Prepare Input DNA

This first section of the Illumina 5-Base DNA Prep protocol provides unique instructions for preparing gDNA, FFPE DNA, and cfDNA sample types.

Prepare reagents appropriate to your input type using the following section:

- [4.1 Prepare Reagents for Input DNA on page 17.](#)

After preparing reagents, use the instructions appropriate for your input type:

- [4.2 Prepare gDNA on page 19.](#)
- [4.3 Prepare FFPE DNA on page 21.](#)
- [4.4 Prepare Size-Select cfDNA on page 22.](#)

After preparing your input type, navigate to [5.1 Prepare Reagents for Library Prep Steps on page 26](#). At [5.1 Prepare Reagents for Library Prep Steps on page 26](#), all sample types follow the same workflow for the remainder of the protocol.

4.1 Prepare Reagents for Input DNA

Use the procedures and reagents appropriate for your sample type. After completing input preparation, continue the protocol with [5.1 Prepare Reagents for Library Prep Steps on page 26](#).

Prepare the following reagents appropriate for your input type to perform this section of the protocol. This table identifies reagents in the order that they are used.

This section ends at a safe stopping point.

1. Remove DNA from storage. If frozen, thaw on ice.
 - a. Vortex briefly to mix.
 - b. Centrifuge briefly to collect contents.
2. Prepare reagents as follows.

Table 1 Room Temperature Storage

Reagent	Input Type	Instructions	Step Where Used
IPB	[gDNA] and [cfDNA]	Use at room temperature.	4.2 Prepare gDNA on page 19 4.4 Prepare Size-Select cfDNA on page 22
RSB*	All input types	Use at room temperature.	4.2 Prepare gDNA on page 19 4.3 Prepare FFPE DNA on page 21 4.4 Prepare Size-Select cfDNA on page 22

* Reagent can also be stored in 2°C to 8°C Storage.

Table 2 -25°C to -15°C Storage

Reagent	Input Type	Instructions	Step Where Used
GDMC	[gDNA] and [FFPE]	Thaw on ice.	4.2 Prepare gDNA on page 19 4.3 Prepare FFPE DNA on page 21
SFMC	[cfDNA]	Thaw on ice.	4.4 Prepare Size-Select cfDNA on page 22

4.2 Prepare gDNA

Shear gDNA

Consumables

- GDMC (Genomic DNA Methylation Control)
- RSB (Resuspension Buffer)
- 96-well PCR plate

Preparation

1. Prepare the following consumables:
 - GDMC—Vortex to mix, and then centrifuge briefly.
 - RSB—Vortex to mix.
2. Label a new 96-well PCR plate SS1.

Procedure

1. For each sample, combine 50–100 ng gDNA with 2.5 μ l GDMC in the sonication tube or well. If the volume is less than the recommended volume for your sonication equipment, add RSB accordingly. The total volume must be less than 100 μ l.
2. Sonicate samples to ~450 bp.
3. After sonication, transfer sheared material to the corresponding well of the SS1 plate.
4. Add enough RSB to increase sample volume to 100 μ l.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive, and then centrifuge briefly at 280 $\times$ g. Store at -25°C to -15°C for up to 30 days.

Size-Select Sheared gDNA

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Absolute ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plates

- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:
 - IPB:
 - a. Vortex to mix before use. Inspect for uniform color and make sure that bead pellets are not visible at the bottom of the tube.
 - b. If beads are visible, repeat vortex. Total vortex time should be less than 1 minute to avoid bead damage.
 - RSB—Vortex to mix.
2. Prepare at least 500 µl per sample fresh 80% EtOH from absolute EtOH. Vortex to mix.
3. Label a new 96-well PCR plate SS2.
4. Label a new 96-well PCR plate LP.

Procedure

Remove Large DNA Fragments

1. Vortex the IPB for 10 seconds immediately before use.
2. Add 60 µl IPB to each well of the SS1 plate.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.
5. Centrifuge briefly.
6. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
Retain the supernatant.
7. Transfer 150 µl supernatant to the corresponding well of the SS2 plate.
8. Discard the SS1 plate.

Remove Small DNA Fragments

1. Vortex the IPB for 10 seconds immediately before use.
2. Add 19 µl IPB to each well of the SS2 plate.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.
5. Centrifuge briefly.
6. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
7. Remove and discard all supernatant from each well.

Wash

1. Wash the beads **two** times as follows.
 - a. Keep on the magnetic stand and add 175 μ l fresh 80% EtOH to each well.
 - b. Wait 30 seconds.
 - c. Without disturbing the beads, remove and discard all supernatant from each well.
 - d. Wash the beads a second time (repeat steps a–c), and then proceed to the next step.
2. Using a 20 μ l pipette, remove residual EtOH from each well.
3. Air-dry for at least 30 seconds and no more than 2 minutes.
4. Remove from the magnetic stand.
5. Add 47 μ l RSB to each well. Position the pipette tip above the pellet and dispense directly onto the pellet to release beads from the well.
6. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
7. If pellets are visible, pulse vortex 3 times for 3 seconds at maximum speed.
8. Centrifuge briefly.
9. Incubate at room temperature for 2 minutes.
10. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
11. Transfer 45 μ l supernatant from each well into the corresponding well of the LP plate.
12. Discard the SS2 plate.

SAFE STOPPING POINT

If you are stopping, seal the LP plate with Microseal 'B' adhesive, and then centrifuge briefly at $280 \times g$. Store at -25°C to -15°C for up to 30 days.

To continue the protocol after preparing gDNA input, refer to [5.1 Prepare Reagents for Library Prep Steps on page 26](#).

4.3 Prepare FFPE DNA

Consumables

- GDMC (Genomic DNA Methylation Control)
- RSB (Resuspension Buffer)
- 96-well PCR plates

Preparation

1. Prepare the following consumables:
 - GDMC—Vortex to mix, and then centrifuge briefly.
 - RSB—Vortex to mix.

2. Label a new 96-well PCR plate LP.

Procedure

1. For each sample, combine 40 ng FFPE DNA with 2.5 μ l GDMC in the sonication tube or well.
If the volume is less than the recommended volume for your sonication equipment, add RSB accordingly. The total volume must be $\leq$ 52 μ l.
2. Sonicate samples to $\sim$ 200 bp.
3. After sonication, transfer 45 μ l sheared material to the corresponding well of the LP plate.
If the volume was $\leq$ 45 μ l after sonication, transfer the full volume to the LP plate. Add RSB to make the total volume 45 μ l.

SAFE STOPPING POINT

If you are stopping, seal the SS1 plate with Microseal 'B' adhesive, and then centrifuge briefly at $280 \times g$. Store at -25°C to -15°C for up to 30 days.

To continue the protocol after preparing FFPE DNA input, refer to [5.1 Prepare Reagents for Library Prep Steps on page 26](#).

4.4 Prepare Size-Select cfDNA

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- SFMC (Short Fragment Methylation Control)
- Absolute ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plates
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:
 - IPB:
 - a. Vortex to mix before use. Inspect for uniform color and make sure that bead pellets are not visible at the bottom of the tube.
 - b. If beads are visible, repeat vortex. Total vortex time should be less than 1 minute to avoid bead damage.
 - RSB—Vortex to mix.
2. Prepare at least 500 μ l per sample fresh 80% EtOH from absolute EtOH. Vortex to mix.

3. Label a new 96-well PCR plate SS1.
4. Label a new 96-well PCR plate SS2.
5. Label a new 96-well PCR plate LP.

Procedure

Remove Large DNA Fragments

1. Add 1–20 ng cfDNA mononucleosomal peak in a volume of 80 µl or less to the corresponding well of the SS1 plate. If the cfDNA volume is less than 80 µl, add RSB up to 80 µl precisely.
2. Vortex the IPB for 10 seconds immediately before use.
3. Add 48 µl IPB to each well of the SS1 plate.
4. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
5. Incubate at room temperature for 5 minutes.
6. Centrifuge briefly.
7. Place on the magnetic stand and wait until the liquid is clear (~10 minutes).

Retain the supernatant.

8. Transfer 126 µl supernatant from the SS1 plate to the corresponding well of the SS2 plate.



When aspirating, missing less than 2 µl supernatant can occur and does not impact the subsequent step.

9. Discard the SS1 plate.

Purify Small DNA Fragments

1. Vortex the IPB for 10 seconds immediately before use.
2. Add 96 µl IPB to each well of the SS2 plate.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.
5. Centrifuge briefly.
6. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
7. Remove and discard all supernatant from each well.

Wash

1. Wash the beads **two** times as follows.
 - a. Keep on the magnetic stand and add 175 µl fresh 80% EtOH to each well.
 - b. Wait 30 seconds.
 - c. Without disturbing the beads, remove and discard all supernatant from each well.
 - d. Wash the beads a second time (repeat steps a–c), and then proceed to the next step.
2. Using a 20 µl pipette, remove residual EtOH from each well.
3. Air-dry for at least 30 seconds and no more than 2 minutes.

Elute

1. Remove from the magnetic stand.
2. Add 45 µl RSB to each well. Position the pipette tip above the pellet and dispense directly onto the pellet to release beads from the well.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute. If pellets are visible, pulse vortex 3 times for 3 seconds at maximum speed.
4. Centrifuge briefly.
5. Incubate at room temperature for 2 minutes.
6. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
7. Transfer 42.5 µl supernatant from each well into the corresponding well of the LP plate.
8. Add 2.5 µl SFMC to each well of the LP plate for a total volume of 45 µl.
9. **[Optional]** Save the SS2 plate with the remaining volume (2.5 µl). Seal the plate with Microseal 'B'. Use this plate to perform **[Optional]** Quantify Size-Selected cfDNA to QC cfDNA recovery after size-selection.
 - If processing immediately, store the plate on ice.
 - If processing later, freeze the plate.

SAFE STOPPING POINT

If you are stopping, seal the LP plate with Microseal 'B' adhesive, and then centrifuge briefly at 280 × g. Store at -25°C to -15°C for up to 30 days.

To continue the protocol after preparing cfDNA input, refer to [5.1 Prepare Reagents for Library Prep Steps on page 26](#).

Quantify Size-Selected cfDNA (Optional)

[Optional] This Quality Check step quantifies cfDNA recovery after size-selection.

To perform this step, cfDNA input must be greater than 5 ng prior to size-selection. After performing this step, continue the protocol at [5.2 Perform End Repair on page 27](#).

Preparation

1. Prepare the Qubit dsDNA HS Assay Kit per manufacturers instructions.
2. Prepare the SS2 plate containing the remaining volume as follows.
 - a. If the plate was stored frozen, thaw on ice.
 - b. Briefly vortex and then centrifuge.

Procedure

1. Place the SS2 plate on the bar magnet and wait until the beads fully magnetize on the side of the well (~1 min).

2. Carefully pipette 2 μ l from the SS2 plate and add directly to 198 μ l Qubit reagent.
3. Measure cfDNA elution on the Qubit 4 Fluorometer according to manufacturers instructions.
Typical yields for 10 ng input is in the 0.2 ng/ μ l range.

5. Perform Library Prep Steps

Use the following workflow in the order shown to perform library prep steps:

- [5.1 Prepare Reagents for Library Prep Steps on page 26.](#)
- [5.2 Perform End Repair on page 27.](#)
- [5.3 Perform A-Tailing on page 27.](#)
- [5.4 Ligate Adapters on page 28.](#)
- [5.5 Clean Up Ligation on page 29.](#)

5.1 Prepare Reagents for Library Prep Steps

Prepare reagents to perform the library prep section of the protocol. This table identifies reagents in the order that they are used.

This section ends at a safe stopping point.

1. Remove LP plate from storage. If frozen, thaw on ice.

i | When processing more than one input type, using a single plate suffices for the remainder of the protocol.

- a. Vortex briefly to mix.
 - b. Centrifuge briefly to collect contents.
2. Prepare reagents as follows.

Table 3 Room Temperature Storage

Reagent	Instructions	Step Where Used
IPB	Use at room temperature.	5.5 Clean Up Ligation on page 29

Table 4 -25°C to -15°C Storage

Reagent	Instructions	Step Where Used
ERP6	Thaw on ice.	5.2 Perform End Repair on page 27
ATL4	Bring to room temperature to thaw completely, and then keep on ice.	5.3 Perform A-Tailing on page 27
LIGX	Thaw on ice.	5.4 Ligate Adapters on page 28
MRR	Bring to room temperature to thaw.	5.4 Ligate Adapters on page 28 5.5 Clean Up Ligation on page 29
UMI3	Bring to room temperature to thaw.	5.4 Ligate Adapters on page 28

5.2 Perform End Repair

Consumables

- ERP6 (End Repair Mix 6)
- Microseal 'B' adhesive seal

Preparation

1. Prepare the following consumables:
 - ERP6—Vortex to mix, and then centrifuge briefly.
2. Save the following ERP program on the thermal cycler:
 - a. Choose the preheat lid option and set to 100°C.
 - b. Set the reaction volume to 55 µl.
 - c. Set the temperature and time for each set of cycles.

Cycles	Temperature	Time
1	30°C	30 minutes
1	70°C	10 minutes
1	4°C	5 minutes
1	4°C	Hold

Procedure

1. Remove the LP plate from ice.
2. Add 10 µl ERP6 to each well of the LP plate.
3. Apply Microseal 'B' and shake at 1800 rpm for 1 minute.
4. Centrifuge briefly.
5. Place on the preprogrammed thermal cycler and run the ERP program.
6. When the program reaches the 4°C hold, *immediately* proceed to the next step.

5.3 Perform A-Tailing

Consumables

- ATL4 (A-Tailing Mix)
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:
 - ATL4—Vortex to mix, and then centrifuge briefly. Make sure that ATL4 is dissolved completely.
2. Save the following ATL program on the thermal cycler:
 - a. Choose the preheat lid option and set to 100°C.
 - b. Set the reaction volume to 90 µl.
 - c. Set the temperature and time for each set of cycles.

Cycles	Temperature	Time
1	37°C	20 minutes
1	70°C	5 minutes
1	4°C	5 minutes
1	4°C	Hold

Procedure

1. Add 35 µl ATL4 to each well of the LP plate.
2. Apply Microseal 'B' and shake at 1800 rpm for 1 minute.
3. Centrifuge briefly.
4. Place on the preprogrammed thermal cycler and run the ATL program.
5. When the program reaches the 4°C hold, *immediately* proceed to the next step.

5.4 Ligate Adapters

Consumables

- LIGX (Ligation Mix)
- MRR (Methylation Resuspension Reagent)
- UMI3 (Unique Molecular Identifier)
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:
 - LIGX—Before use, centrifuge briefly, pipette to mix, and then centrifuge briefly again.
 - MRR—Bring to room temperature to prepare for *Clean Up Ligation*.
 - UMI3—Vortex on medium-high speed for at least 10 seconds to mix, and then centrifuge briefly.

2. Save the following LIG program on the thermal cycler:
 - a. Choose the preheat lid option and set to 42°C.
 - b. Set the reaction volume to 100 µl.
 - c. Set the temperature and time for each set of cycles.

Cycles	Temperature	Time
1	30°C	20 minutes
1	4°C	1 minute
1	4°C	Hold

Procedure

1. Add 5 µl LIGX to each well of the LP plate.
2. Add 5 µl UMI3 adapters to each well.
3. Apply Microseal 'B' and shake at 1800 rpm for 1 minute.
4. Centrifuge briefly.
5. Place on the preprogrammed thermal cycler and run the LIG program.
6. When the program reaches the 4°C hold, *immediately* proceed to the next step.

5.5 Clean Up Ligation

Consumables

- IPB (Illumina Purification Beads)
- MRR (Methylation Resuspension Reagent)
- Absolute ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plates
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:
 - IPB:
 - a. Vortex to mix before use. Inspect for uniform color and make sure that bead pellets are not visible at the bottom of the tube.
 - b. If beads are visible, repeat vortex. Total vortex time should be less than 1 minute to avoid bead damage.
 - MRR—Vortex to mix, and then centrifuge briefly.
2. Prepare at least 500 µl per sample fresh 80% EtOH from absolute EtOH. Vortex to mix.
3. Label a new 96-well PCR plate CONV.

Procedure

Bind

1. Vortex the IPB for 10 seconds immediately before use.
2. Add 80 µl IPB to each well in the LP plate.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. Incubate at room temperature for 5 minutes.
5. Centrifuge briefly.
6. Place the LP plate on the magnetic stand until clear (~5 minutes).
7. Without disturbing the beads, remove and discard all supernatant from each well. Use a 20 µl pipette to remove residual supernatant.

Wash

1. Wash the beads **two** times as follows.
 - a. Keep on the magnetic stand and add 175 µl fresh 80% EtOH to each well.
 - b. Wait 30 seconds.
 - c. Without disturbing the beads, remove and discard all supernatant from each well.
 - d. Wash the beads a second time (repeat steps a–c), and then proceed to the next step.
2. Apply Microseal 'B' and centrifuge briefly to bring any residual EtOH to the bottom of well.
3. Place on the magnetic stand (~10 seconds).
4. While the plate is on the magnetic stand, without disturbing the beads, use a 20 µl pipette to remove all residual supernatant from each well.

 Removing residual supernatant without touching the beads is critical. Ethanol carryover can affect assay performance.
5. Air-dry on the magnetic stand for 15 minutes.

Elute

1. Remove the LP plate from the magnetic stand.
2. Add 18 µl MRR to each well. Position the pipette tip above the pellet and dispense directly onto the pellet to release beads from the well.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. If pellets are still visible, pulse vortex 3 times for 3 seconds at maximum speed.
5. Incubate at room temperature for 2 minutes.
6. Centrifuge briefly.
7. Place on the magnetic stand and wait until the liquid is clear (~3 minutes).
8. Without disturbing the beads, transfer 15 µl from each well of the LP plate into the corresponding well of the CONV plate.
If beads are observed in the pipette tips when aspirating, slowly dispense back to the bottom of the originating well. Place on the magnetic stand for 3 minutes before attempting to transfer again.

 | Make sure to only transfer supernatant. Bead carryover can negatively affect conversion.

9. Discard the LP plate.

SAFE STOPPING POINT

If you are stopping, seal the CONV plate with Microseal 'B' adhesive, and then centrifuge briefly at 280 × g. Store at -25°C to -15°C for up to 30 days.

6. Perform Conversion & Amplification Steps

Use the following workflow in the order shown to perform conversion and amplification steps:

- [6.1 Prepare Reagents for Conversion and Amplification on page 32.](#)
- [6.2 Denaturation on page 33.](#)
- [6.3 Conversion on page 34.](#)
- [6.4 Index PCR on page 35.](#)
- [6.5 Clean Up Index PCR on page 37.](#)
- [6.6 Quantify Libraries on page 38.](#)

After completing conversion and amplification, continue with [7. Prepare for Sequencing on page 40.](#)

6.1 Prepare Reagents for Conversion and Amplification

Prepare reagents to perform conversion and amplification steps. This table identifies reagents in the order that they are used.

This section ends at a safe stopping point.

1. If stored at -25°C to -15°C, thaw CONV plate on ice. Vortex briefly, and then centrifuge.
2. Prepare reagents as follows.

Table 5 Room Temperature Storage

Reagent	Instructions	Step Where Used
IPB	Use at room temperature.	6.5 Clean Up Index PCR on page 37
RSB*	Use at room temperature.	6.5 Clean Up Index PCR on page 37

* Reagent can also be stored in 2°C to 8°C Storage.

Table 6 -25°C to -15°C Storage

Reagent	Instructions	Step Where Used
DDR	Bring to room temperature to thaw.	6.2 Denaturation on page 33
IME	Thaw on ice.	6.2 Denaturation on page 33
MCB	Bring to room temperature.	6.2 Denaturation on page 33
MAM	Thaw on ice.	6.2 Denaturation on page 33 6.4 Index PCR on page 35

Reagent	Instructions	Step Where Used
Illumina DNA/RNA UD Indexes plate	Bring to room temperature to thaw.	6.4 Index PCR on page 35

6.2 Denaturation

Consumables

- DDR (DNA Denaturation Reagent)
- IME (Illumina Methylation Enzyme)
- MAM (Methylation Amplification Mix)
- MCB (Methylation Conversion Buffer)
- 1.7 ml microcentrifuge tube
- Microseal 'B' adhesive seals

⚠ | 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.

Preparation

1. Prepare the following consumables:
 - DDR—Pulse vortex 3 times to mix, and then centrifuge briefly.
 - IME:
 - a. Centrifuge briefly.
 - b. Pipette mix 10 times with a 200 µl pipette.
 - c. Centrifuge briefly a second time.
 - MAM—Thaw on ice to prepare for *Index PCR*. Keep on ice.
 - MCB—Pulse vortex 3 times to mix, and then centrifuge briefly.

2. Save the following DEN program on the thermal cycler:
 - a. Choose the preheat lid option and set to 100°C.
 - b. Set the reaction volume to 25 µl.
 - c. Set the temperature and time for each set of cycles.

Cycles	Temperature	Time
1	70°C	10 minutes
1	4°C	3 minutes
1	4°C	Hold

Procedure

1. Add 10 µl DDR to each well of the CONV plate.
2. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
3. Centrifuge briefly.
4. Place on the preprogrammed thermal cycler and run the DEN program.
5. While the DEN program is running, prepare the Conversion Master Mix as follows.
 - a. In a 1.7 ml tube, combine the following volumes. Multiply each volume by the number of reactions. Add volumes in the order listed. Pipette up and down to rinse the pipette tip when dispensing and combining volumes.
 - MCB (29.25 µl)
 - IME (3.25 µl)

These volumes produce 32.5 µl Conversion Master Mix per reaction, which includes extra volume for accurate pipetting.
 - b. Invert the tube 5 times to mix. Flick as needed until the contents are fully mixed.
 - c. Centrifuge briefly and store on ice.
6. When the DEN program reaches the 4°C hold, place the CONV plate on ice, and then *immediately* proceed to the next step.

6.3 Conversion

Consumables

- Microseal 'B' adhesive seal

Preparation

1. Save the following CONV program on the thermal cycler:
 - a. Choose the preheat lid option and set to 100°C.
 - b. Set the reaction volume to 50 µl.
 - c. Set the temperature and time for each set of cycles.

Cycles	Temperature	Time
1	57°C	45 minutes
1	4°C	Hold

Procedure

1. Immediately before use, pipette the Conversion Master Mix 5 times to mix. Avoid introducing bubbles.
2. With the CONV plate on ice, add 25 µl Conversion Master Mix to each well. Pipette up and down to rinse the pipette tip when dispensing.
3. Apply Microseal 'B' and centrifuge briefly.
4. Shake at 2200 rpm for 1 minute.
5. Centrifuge briefly.
6. Place on the preprogrammed thermal cycler and run the CONV program.
7. When the program reaches the 4°C hold, place the CONV plate on ice, and then *immediately* proceed to the next step.

6.4 Index PCR

Consumables

- Illumina DNA/RNA UD Indexes
- MAM (Methylation Amplification Mix)
- Microseal 'B' adhesive seal

⚠ | 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.

Preparation

1. Prepare the following consumables:
 - Illumina DNA/RNA UD Indexes plate—Vortex to mix, and then centrifuge briefly. Assign UD index primers according to your plate layout. Make a note of which index wells are used for each sample.
 - MAM—Pulse vortex 3 times to mix, and then centrifuge briefly before use. Store on ice.
2. Save the following PCR6 program on the thermal cycler:

i | The input type can impact the number of cycles.

- a. Choose the preheat lid option and set to 100°C.
- b. Set the reaction volume to 100 µl.
- c. Set the temperature, time, and number of cycles appropriate for the input type.

Cycles	Temperature	Time
1	98°C	30 seconds
6 cycles for [gDNA] and [cfDNA]	98°C	10 seconds
8 cycles for [FFPE]	59°C	30 seconds
	65°C	60 seconds
1	65°C	5 minutes
1	4°C	Hold

Procedure

1. While keeping the CONV plate on ice, remove Microseal 'B', and do as follows.
 - a. Add 10 µl Illumina DNA/RNA UD Indexes to each well.
 - b. Add 40 µl MAM to each well.
2. Apply Microseal 'B' and shake at 1800 rpm for 1 minute.
3. Centrifuge briefly.
4. Place on the preprogrammed thermal cycler and run the PCR6 program.
5. When the program reaches the 4°C hold, *immediately* proceed to the next step.

6.5 Clean Up Index PCR

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Absolute ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plate
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables:
 - IPB:
 - a. Vortex to mix before use. Inspect for uniform color and make sure that bead pellets are not visible at the bottom of the tube.
 - b. If beads are visible, repeat vortex. Total vortex time should be less than 1 minute to avoid bead damage.
 - RSB—Vortex to mix.
2. Prepare at least 500 µl per sample fresh 80% EtOH from absolute EtOH. Vortex to mix.
3. Label a new 96-well PCR plate Libraries.

Procedure

Bind

1. Vortex the IPB for an additional 10 seconds immediately before use.
2. Centrifuge the CONV plate briefly.
3. Add IPB to each well of the plate.
 - For [gDNA] and [cfDNA], add 90 µl.
 - For [FFPE], add 80 µl.
4. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
5. Incubate at room temperature for 5 minutes.
6. Centrifuge briefly.
7. Place the CONV plate on the magnetic stand and wait until clear (~5 minutes).
8. Without disturbing the beads, remove and discard all supernatant from each well.

Wash

1. Wash the beads **two** times as follows.
 - a. Keep on the magnetic stand and add 175 μ l fresh 80% EtOH to each well.
 - b. Wait 30 seconds.
 - c. Without disturbing the beads, remove and discard all supernatant from each well.
 - d. Wash the beads a second time (repeat steps a–c), and then proceed to the next step.
2. Using a 20 μ l pipette, remove residual supernatant.
3. Air-dry for at least 30 seconds and no more than 2 minutes.

Elute

1. Remove the CONV plate from the magnetic stand and add 20 μ l RSB to each well.
2. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
3. If pellets are observed, mix by pulse vortexing 3 times for 3 seconds at maximum speed.
4. Incubate at room temperature for 2 minutes.
5. Centrifuge briefly.
6. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
7. Avoiding bead carryover, transfer 18 μ l supernatant from each well into the corresponding well of the Libraries plate.

SAFE STOPPING POINT

If you are stopping, seal the Libraries plate with Microseal 'B' adhesive, and then centrifuge briefly at $280 \times g$. Store at -25°C to -15°C for up to 30 days.

6.6 Quantify Libraries

Perform the following to check the quantity and quality of the purified library prep product.

1. If stored at -25°C to -15°C , prepare the plate as follows.
 - a. Remove from storage and thaw on ice.
 - b. Vortex briefly to mix.
 - c. Centrifuge briefly to collect contents.
2. Quantify 2 μ l library from each well using a Qubit dsDNA BR Assay Kit.
 - Typical yield for 50 ng gDNA input is 4 to 20 ng/ μ l.
 - Typical yield for 40 ng FFPE input is 6 to 40 ng/ μ l.
 - Typical yield for 10 ng cfDNA input is 10 to 40 ng/ μ l.
3. **[Optional]** Assess average fragment size using one of the following methods. Use a window setting of 150–1000 bp to calculate average fragment size.
 - Analyze 1 μ l using TapeStation 4200 with D1000 Kit.
 - Analyze 1 μ l using Bioanalyzer DNA 1000 Kit.

For cfDNA, expect a distribution of templates with an average size range of ~310 bp to ~350 bp.

For FFPE DNA, expect a distribution of templates with an average size range of ~330 bp to ~400 bp.

For gDNA, expect a distribution of templates with an average size range of ~600 bp to ~700 bp.

7. Prepare for Sequencing

Illumina recommends setting up a paired-end run with 151 cycles per read (2 x 151) with dual bp indexes for the following configurations:

- NovaSeq 6000 S4 flow cell using the NovaSeq Standard loading and Xp loading protocols.
- NovaSeq X Series 1.5B, 10B, or 25B flow cells using the NovaSeq X Series Standard protocol.

Refer to [7.1 Dilute Libraries to the Starting Concentration on page 40](#) for instructions.

7.1 Dilute Libraries to the Starting Concentration

Use the following instructions to dilute libraries to the starting concentration.

1. Calculate the molarity value of the libraries using the following formula:
 - For libraries assessed on a TapeStation or other automated electrophoresis instrument, use the average fragment size obtained for the library.
 - If fragment traces are not available, use average library sizes of 650 bp for gDNA, 350 bp for cfDNA, and 330 bp for FFPE DNA.

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

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system. When pooling samples, normalize each sample to the starting concentration in the following table, and combine equal volumes of each library to pool.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM) ¹
NovaSeq 6000 S4 Standard	0.500	100
NovaSeq 6000 S4 Xp	0.250	50
NovaSeq X Series 1.5B Flow Cell	2	130
NovaSeq X Series 10B Flow Cell	2	130
NovaSeq X Series 25B Flow Cell	2	150

¹ The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

3. Dilute each library to the starting concentration for your system.
4. Follow the denature and dilute instructions for your system. Refer to the [Denature and Dilute Protocol Generator](#).

Illumina recommends adding the 1% PhiX spike-in to all sequencing runs.

8. Resources & References

The Illumina 5-Base DNA Prep support pages on the [Illumina support site](#) provide additional resources. These resources include training, compatible products, and other considerations. Always check the support pages for the latest versions.

8.1 Revision History

Document	Date	Description of Change
Document # 200073497 v01	August 2026	Added: • FFPE as DNA input type. • Automation information. Updated: • Product Contents labeling and description information. • Clean Up Index PCR to consolidate steps. • Document structure and formatting.
Document # 200073497 v00	October 2025	Initial Release.

9. Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com

Email: techsupport@illumina.com

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

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



Illumina, 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

For Research Use Only. Not for use in diagnostic procedures.

© 2026 Illumina, Inc. All rights reserved.

illumina®