illumina®

Illumina 5-Base DNA Prep with Enrichment

Product Documentation

ILLUMINA PROPRIETARY
Document # 200073498 v02
August 2026

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

Table of Contents

1. Overview

This section includes the following information:

1.1 Introduction

The Illumina 5-Base DNA Prep with Enrichment enables library preparation from high quality genomic DNA (gDNA) or cell-free DNA (cfDNA) input.

Illumina 5-Base DNA Prep with Enrichment:

- 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 or 1 ng cfDNA.
- Supports automated preparation of gDNA input and cfDNA input. For automation information, refer to *1.3 Library Prep Automation (Optional)* on page 2.
- Enables the detection of both methylation and genomic variants from a single library prep using the DRAGEN Enrichment application available on BaseSpace Sequence Hub.
- Enables enrichment of methylation-converted libraries. Design an enrichment panel using Illumina Custom Enrichment Panel v2 through Illumina DesignStudio. Illumina optimizes the panel design for the unique methylation conversion chemistry specific to Illumina 5-Base DNA Prep with Enrichment. To use DesignStudio, refer to the Illumina support website.
- Includes the option to prepare a control panel which provides methylation conversion quality control metrics.

1.2 DNA Input Recommendations

The Illumina 5-Base DNA Prep with Enrichment protocol enables library preparation from gDNA or cfDNA input. For more information, refer to the section appropriate for your input type.

gDNA Input

The Illumina 5-Base DNA Prep with Enrichment 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.

cfDNA Input

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

4.3 Prepare Size-Select cfDNA on page 20 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 with Enrichment 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 with Enrichment is engineered for fully automated, high-capacity library preparation and target enrichment workflows using third-party liquid-handling platforms. To use on an automated liquid-handling system, the Illumina 5-Base DNA Prep with Enrichment requires the following kit: Illumina 5-Base DNA Prep with Enrichment (96 samples).

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

- Supports automated preparation of gDNA input and cfDNA input.
- Supports the preparation of up to 96 libraries per automation run and subsequent enrichment, up to 24, four-plex enrichment reactions on automation, 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 3.

2. Consumables & Equipment

The Illumina 5-Base DNA Prep with Enrichment 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 with Enrichment protocol requires library prep reagents, an Illumina Custom Enrichment Panel v2, and indexes. For Illumina Custom Enrichment Panel v2 information, refer to *Illumina Custom Enrichment Panel v2 (Required)* on page 6.

Product Contents includes the following information:

- *Reagent Kit Contents—24 Samples* on page 3.
- *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 with Enrichment (24 samples)	20140366
	Illumina 5-Base DNA Prep with Enrichment (96 samples)*	20140367

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

In the reagent kit contents, Genomic DNA Methylation Control (GDMC) and Short Fragment Methylation Control (SFMC) contain the control genomes for methylation conversion quality control. Methylation conversion quality control assessment requires the inclusion of the **[Optional]** Methylation Control Panel. For information about the **[Optional]** Methylation Control Panel, refer to *Methylation Control Panel (Optional)* on page 7.

Reagent Kit Contents—24 Samples

The following tables list the contents of each box and bag included in the 24 sample library prep reagent kit. Additional Illumina components must be included to perform the Illumina 5-Base DNA Prep with Enrichment. Refer to *Additional Illumina Components* on page 6 for information.

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

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

Illumina 5-Base DNA Prep with Enrichment Bag 1, Store at -25°C to -15°C

24 Sample Kit	Reagent	Description
1	EEW	Enhanced Enrichment Wash
1	EPM4	Enhanced PCR Mix 4
1	NHB2	Hyb Buffer 2 + IDT NXT Blockers
1	PPC	PCR Primer Cocktail

Illumina 5-Base DNA Prep with Enrichment Bag 2, Store at 4°C

24 Sample Kit	Reagent	Description
1	EHB2	Enrich Hyb Buffer 2
1	SMB4	Streptavidin Magnetic Beads 4

Reagent Kit Contents—96 Samples

The following tables list the contents of each box and bag included in the 96 sample library prep reagent kit. Additional Illumina components must be included to perform the Illumina 5-Base DNA Prep with Enrichment. 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

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

96 Sample Kit	Reagent	Description
1	EEW	Enhanced Enrichment Wash
1	EPM4	Enhanced PCR Mix 4
1	NHB2	Hyb Buffer 2 + IDT NXT Blockers
1	PPC	PCR Primer Cocktail

Illumina 5-Base DNA Prep with Enrichment-High Throughput Box 2, Store at 4°C

96 Sample Kit	Reagent	Description
1	EHB2	Enrich Hyb Buffer 2
1	SMB4	Streptavidin Magnetic Beads 4
1	IPB	Illumina Purification Beads
1	RSB	Resuspension Buffer

Additional Illumina Components

Illumina Custom Enrichment Panel v2 (Required)

Use the required Illumina Custom Enrichment Panel v2 designed for Illumina 5-Base DNA Prep with Enrichment through Illumina DesignStudio.

- 75 kb–1 Mb panel size

For more information about using DesignStudio to create Illumina custom enrichment panels, refer to the DesignStudio support page on the Illumina support website.

Description	Number of Samples Supported
Illumina Custom Enrichment Panel v2	Prepare up to 32 samples using 4-plex enrichment (32 µl, 120 bp)
	Prepare up to 384 samples using 4-plex enrichment (384 µl, 120 bp)
	Prepare up to 1536 samples using 4-plex enrichment (1536 µl, 120 bp)

Methylation Control Panel (Optional)

The **[Optional]** Methylation Control Panel provides methylation conversion quality control metrics. Use the **[Optional]** Methylation Control Panel with the required Illumina Custom Enrichment Panel v2. To prepare the **[Optional]** Methylation Control Panel for the protocol, refer to *7.1 Prepare Reagents for Enrichment* on page 37, and then *7.2 Hybridize Probes* on page 38.

The **[Optional]** Methylation Control Panel is available from DesignStudio. For more information, refer to the DesignStudio support page on the Illumina support website.

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] 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
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

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

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] Covaris LE220 Plus[3], or other sonication instrument	Covaris, part number # 500569, or equivalent supplier
[gDNA] Rack 12 place 8 microTUBE Strip, or equivalent for other sonication instrument	Covaris, part number # 500191, or equivalent supplier
Microcentrifuge	General lab supplier
One of the following systems, depending on your quantification method: • Agilent Bioanalyzer 2100 System[1] • Agilent TapeStation 4200 System	One of the following, depending on qualification method: • Agilent, catalog # G2939BA[1] • Agilent, catalog # G2991BA[2]
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.

3. Before You Begin

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

- *3.1 Illumina 5-Base DNA Prep with Enrichment Kit Workflow* on page 11.
- *3.2 Tips and Techniques* on page 12.
- *3.3 Sonication Guidelines for gDNA Input* on page 15.

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 3.
- 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 with Enrichment Kit Workflow

The following diagram illustrates the Illumina 5-Base DNA Prep with Enrichment 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
- To prevent amplification product or probe carryover, avoid returning to the pre-amplification area after beginning work in the post-amplification area.
- Change gloves if gloves come in contact with indexing primers, samples, or probes.
- 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 × 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 × 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 µl pipette for volumes 20 µl to 200 µl.
  - 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.

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 with Enrichment 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.

4. Prepare Input DNA

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

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

- *4.1 Prepare Reagents for Input DNA* on page 16.

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

- *4.2 Prepare gDNA* on page 18.
- *4.3 Prepare Size-Select cfDNA* on page 20.

After preparing your input type, navigate to *5.1 Prepare Reagents for Library Prep Steps* on page 24. At *5.1 Prepare Reagents for Library Prep Steps* on page 24, 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 24.

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 18 *4.3 Prepare Size-Select cfDNA* on page 20
RSB*	All input types	Use at room temperature.	*4.2 Prepare gDNA* on page 18 *4.3 Prepare Size-Select cfDNA* on page 20

* 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]	Thaw on ice.	*4.2 Prepare gDNA* on page 18
SFMC	[cfDNA]	Thaw on ice.	*4.3 Prepare Size-Select cfDNA* on page 20

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 µl 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 µl.
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 µl.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive, and then centrifuge briefly at 280 × 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 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.
4. Remove from the magnetic stand.
5. Add 47 µl 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 µl 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 × 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 24.

4.3 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 µl 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 10–20 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 24.

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 25.

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 µl from the SS2 plate and add directly to 198 µl 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/µl 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

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.

   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 27

Table 4 -25°C to -15°C Storage

Reagent	Instructions	Step Where Used
ERP6	Thaw on ice.	*5.2 Perform End Repair* on page 25
ATL4	Bring to room temperature to thaw completely, and then keep on ice.	*5.3 Perform A-Tailing* on page 25
LIGX	Thaw on ice.	*5.4 Ligate Adapters* on page 26
MRR	Bring to room temperature to thaw.	*5.4 Ligate Adapters* on page 26 *5.5 Clean Up Ligation* on page 27
UMI3	Bring to room temperature to thaw.	*5.4 Ligate Adapters* on page 26

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 30.
- *6.2 Denaturation* on page 31.
- *6.3 Conversion* on page 32.
- *6.4 Index PCR* on page 33.
- *6.5 Clean Up Index PCR* on page 34.

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 34
RSB*	Use at room temperature.	*6.5 Clean Up Index PCR* on page 34

* 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 31
IME	Thaw on ice.	*6.2 Denaturation* on page 31
MCB	Bring to room temperature.	*6.2 Denaturation* on page 31
MAM	Thaw on ice.	*6.2 Denaturation* on page 31 *6.4 Index PCR* on page 33
Illumina DNA/RNA UD Indexes plate	Bring to room temperature to thaw.	*6.4 Index PCR* on page 33

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 PCR10 program on the thermal cycler:
   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
10 cycles for [gDNA] and [cfDNA]	98°C	10 seconds
	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 PCR10 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 90 µl IPB to each well of the 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 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 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 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 µl 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 µl 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 × g. Store at -25°C to -15°C for up to 30 days.

7. Perform Enrichment Steps

Use the following workflow in the order shown to perform enrichment steps:

- *7.1 Prepare Reagents for Enrichment* on page 37.
- *7.2 Hybridize Probes* on page 38.
- *7.3 Capture Hybridized Probes* on page 41.
- *7.4 Amplify Enriched Libraries* on page 44.
- *7.5 Clean Up Amplified Enriched Libraries* on page 46.
- *7.6 Quantify Enriched Libraries* on page 48.

After following these steps, continue with *8. Prepare for Sequencing* on page 49.

7.1 Prepare Reagents for Enrichment

In the post-amplification area, prepare reagents to perform enrichment steps. This table identifies reagents in the order that they are used.

This section ends at a safe stopping point.

1. Remove the Libraries plate from storage and bring to room temperature.
2. Remove the reagents from the box and prepare as follows.

Table 7 Room Temperature Storage

Reagent	Instructions	Step Where Used
RSB[1]	Bring to room temperature.	*7.2 Hybridize Probes* on page 38[2] *7.4 Amplify Enriched Libraries* on page 44 *7.5 Clean Up Amplified Enriched Libraries* on page 46
IPB	Use at room temperature.	*7.5 Clean Up Amplified Enriched Libraries* on page 46

[1] Reagent can also be stored in 2°C to 8°C Storage.
[2] Reagent used at step with **[Optional]** Methylation Control Panel.

Table 8 2°C to 8°C Storage

Reagent	Instructions	Step Where Used
EHB2	Bring to room temperature.	*7.2 Hybridize Probes* on page 38
SMB4	Bring to room temperature for 30 minutes before use.	*7.3 Capture Hybridized Probes* on page 41 *7.5 Clean Up Amplified Enriched Libraries* on page 46

Table 9 -25°C to -15°C Storage

Reagent	Instructions	Step Where Used
Illumina Custom Enrichment Panel v2	Thaw at room temperature.	*7.2 Hybridize Probes* on page 38
NHB2	Thaw at room temperature.	*7.2 Hybridize Probes* on page 38
[Optional] Methylation Control Panel	Thaw at room temperature.	*7.2 Hybridize Probes* on page 38
EEW	Thaw at room temperature for at least 30 minutes before use.	*7.3 Capture Hybridized Probes* on page 41
EPM4	Thaw on ice.	*7.4 Amplify Enriched Libraries* on page 44
PPC	Thaw on ice.	*7.4 Amplify Enriched Libraries* on page 44

7.2 Hybridize Probes

This step binds customer-supplied enrichment probe oligonucleotides to targeted regions of the libraries.

This step includes the **[Optional]** Methylation Control Panel and the instructions to dilute the panel for use. The diluted **[Optional]** Methylation Control Panel enables monitoring of the methylation conversion using the spike-in control genomes included in the kit (GDMC and SFMC). The **[Optional]** Methylation Control Panel must be diluted to avoid over-enrichment of the small genome controls. The dilution factor is dependent on the input sample type (cfDNA or gDNA) and the size of the required Illumina Custom Enrichment Panel v2.

Consumables

- Illumina Custom Enrichment Panel v2
- EHB2 (Enrich Hyb Buffer 2)
- NHB2 (Hyb Buffer 2 + IDT NXT Blockers)
- [Optional] Methylation Control Panel
- 1.7 ml microcentrifuge tube
- 96-well PCR plate
- 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.

About Reagents

- NHB2:
  - Precipitates and separates during storage.
  - Must be at room temperature before use.
- [Optional] Methylation Control Panel:
  - To obtain methylation conversion quality control metrics, the HYB Master Mix must include the control panel.

Preparation

1. Prepare the following consumables:
   - Illumina Custom Enrichment Panel v2—Vortex to mix, and then centrifuge briefly.
   - EHB2:
     a. Vortex for 30 seconds.
     b. If crystals and cloudiness are visible, repeat vortex, or pipette up and down to mix until the solution is clear.
   - NHB2:
     a. Vortex three times at maximum speed for 10 seconds each time.
     b. Centrifuge briefly.
     c. Pipette up and down from the bottom of the tube.

d. If crystals and cloudiness are visible, repeat vortex, or pipette up and down to mix until the solution is clear.

e. If needed, warm tube in hand and repeat vortex.

2. Prepare the Libraries plate—Pipette to mix, and then centrifuge briefly.
3. **[Optional]** Prepare the Methylation Control Panel:
   a. Calculate the dilution factor using the following dilution constants and equation:
      - 0.01 for typical cfDNA applications
      - 0.05 for typical gDNA applications

$$\text{Dilution factor} = \frac{50 + \text{Custom panel size in kb}}{550 \times \text{Dilution Constant}}$$

   b. Vortex to mix, and then centrifuge briefly.
   c. Dilute the Methylation Control Panel in RSB according to the calculated dilution factor.
4. Label a new 96-well PCR plate HYB.
5. Save the following HYB program on the thermal cycler:
   a. Choose the preheat lid option and set to 100°C.
   b. Set the reaction volume to 27 µl.
   c. Set the temperature and time for each set of cycles.

Cycles	Temperature	Time
1	98°C	5 minutes
19 cycles	From 96°C, decrease by 2°C per cycle until reaching 58°C	1 minute each
1	58°C	30 minutes
1	58°C	Hold

Procedure

1. To prepare four-plex enrichment pools, combine the libraries as follows.
   a. Transfer 2 µl from each of the four libraries of the Libraries plate into a single well of the HYB plate.
   Make sure that the indexes do not overlap.
   b. Repeat transfer from each of the four libraries to new, single wells of the HYB plate.
2. Adjust pipette to 5 µl, and then pipette each enrichment pool five times to mix.
3. Apply Microseal 'B' to the Libraries plate and store at -25°C to -15°C.
4. Combine the following volumes in the order listed to prepare the HYB Master Mix. Multiply each volume by the number of reactions. After adding EHB2, the master mix can become cloudy over time.

- NHB2 (13.75 µl)
- Illumina Custom Enrichment Panel v2 (4 µl)
- One of the following options:
  - Diluted Methylation Control Panel (0.4 µl)
  - RSB (0.4 µl)
- EHB2 (2.75 µl)

These volumes produce 20.9 µl HYB Master Mix per sample, which includes extra volume for accurate pipetting.

5. Vortex HYB Master Mix to mix, and then centrifuge briefly.
   If the master mix appears phase-separated, repeat the vortex.
6. Add 19 µl HYB Master Mix to each well of the HYB plate, and then pipette 10 times to mix.
7. Apply Microseal 'B' to the HYB plate, and then centrifuge briefly.
8. Place on the preprogrammed thermal cycler and run the HYB program.
9. When the program reaches the 58°C hold, ***immediately*** proceed to *7.3 Capture Hybridized Probes* on page 41.

7.3 Capture Hybridized Probes

This step uses SMB4 (Streptavidin Magnetic Beads 4) to capture probes hybridized to the targeted regions of interest. Heated washes remove nonspecific DNA binding from the beads. The enriched library is then eluted from the beads and prepared for amplification.

Consumables

- EEW (Enhanced Enrichment Wash)
- SMB4 (Streptavidin Magnetic Beads 4)
- 96-well PCR plate
- 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.

About Reagents

- EEW must be at room temperature before use.
- SMB4:
  - Make sure to use SMB4 and not IPB for this procedure.
  - Make sure that SMB4 are at room temperature before use. If SMB4 are not at room temperature, salt crystals can form. If salt crystals are visible, bring SMB4 to room temperature and vortex thoroughly before use.
  - Steps involving the centrifuge can cause SMB4 to form a bead pellet. If pelleting occurs, pipette to mix until fully resuspended.

Preparation

1. Prepare the following consumables:
   - EEW—Vortex to mix. If precipitates are visible, vortex until dissolved.
   - SMB4:
     a. Vortex to resuspend. If salt crystals are visible, bring SMB4 to room temperature and vortex thoroughly before use.
     b. If the bead pellet is still visible, pipette up and down to release the pellet, and then repeat the vortex.
2. Label a new 96-well PCR plate ELU.
3. Save the following INC58 program on the thermal cycler:
   a. Choose the preheat lid option and set to 70°C.
   b. Set the reaction volume to 100 µl.
   c. Hold at 58°C.

Procedure

Bind

1. Remove the HYB plate from the thermal cycler and centrifuge briefly.
2. Vortex SMB4 for 1 minute.
3. Add 62 µl SMB4 to each well of the HYB plate.
4. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
5. If splashing occurs, centrifuge briefly (~5 seconds). Make sure that the beads do not form a pellet.
6. Place on the preprogrammed thermal cycler and run the INC58 program. Incubate at 58°C for 15 minutes.

   Keep the thermal cycler program running until after the final incubation of the Elute step. Terminating the thermal cycler program early can affect assay performance.

7. If splashing occurs, centrifuge briefly.

8. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
9. Without disturbing the beads, remove and discard all supernatant from each well.
10. Using a 100 µl pipette, remove residual supernatant.

Wash

1. Vortex EEW for 1 minute.
2. Wash the beads as follows.
    a. Remove the HYB plate from the magnetic stand.
    b. Add 75 µl EEW to each well.
    c. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
    d. Centrifuge briefly.
    e. Place on the preprogrammed thermal cycler and run the INC58 program. Incubate at 58°C for 10 minutes.
    f. Remove from the thermal cycler and centrifuge briefly.
    g. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
    h. Without disturbing the beads, use a pipette to remove and discard all supernatant from each well.
3. Wash the beads a **second** time.
4. Using a 20 µl pipette, remove residual supernatant from each well.
5. Centrifuge briefly.
6. Place on the magnetic stand (~30 seconds).
7. Using a 20 µl pipette, remove all residual supernatant from each well.

Elute

1. Remove the HYB plate from the magnetic stand.
2. Add 100 µl EEW to each well.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. If splashing occurs, centrifuge briefly.
5. Transfer the entire volume of the resuspended bead solution to the ELU plate.

    Transferring to the new plate is critical. This step minimizes carryover of residual reagents that can inhibit downstream PCR.

6. Discard the HYB plate.
7. Apply Microseal 'B' to the ELU plate.
8. Place on the preprogrammed thermal cycler and run the INC58 program. Incubate at 58°C for 5 minutes.
9. During incubation, ***immediately*** proceed to next step.

7.4 Amplify Enriched Libraries

This step uses primers to amplify enriched libraries.

Consumables

- EPM4 (Enhanced PCR Mix 4)
- PPC (PCR Primer Cocktail)
- RSB (Resuspension 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:
   - EPM4—Vortex to mix, and then centrifuge briefly.
   - PPC—Vortex to mix, and then centrifuge briefly.
   - RSB—Vortex briefly.
2. Combine the following volumes in the order listed to prepare the PCR Elution Mix. Multiply each volume by the number of samples.
   - RSB (27.5 µl)
   - EPM4 (22 µl)
   - PPC (5.5 µl)

   These volumes produce 55 µl PCR Elution Mix per sample, which includes extra volume for accurate pipetting.
3. Vortex PCR Elution Mix to mix, and then place on ice.

4. Save the following EL-PCR program on the thermal cycler. The PCR cycles listed in the table are recommendations based on panel size.
   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	98°C	30 seconds
(X) cycles*	98°C	10 seconds
	60°C	30 seconds
	72°C	30 seconds
1	72°C	5 minutes
1	10°C	Hold

Panel Size Range (kb)	Number of PCR Cycles (X)*
75–200	19
>200–750	17
>750–1,000	15

Procedure

1. Remove the ELU plate from the thermal cycler and centrifuge briefly.
2. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
3. Without disturbing the beads, use a pipette to remove and discard all supernatant from each well.
4. Apply Microseal 'B' and centrifuge briefly.
5. Place on the magnetic stand for 30 seconds.
6. Using a 20 µl pipette, remove residual supernatant from each well.
7. Vortex PCR Elution Mix briefly to mix.
8. Remove the ELU plate from the magnetic stand.
9. Add 50 µl PCR Elution Mix to each well of the ELU plate.
10. Apply Microseal 'B' and shake at 1800 rpm for 1 minute.
11. Inspect each well. If a bead pellet is still visible, pipette up and down to release the pellet.
12. Centrifuge briefly.
13. Place on the preprogrammed thermal cycler and run the EL-PCR program.
14. When the program reaches the 10°C hold, ***immediately*** proceed to the next step.

7.5 Clean Up Amplified Enriched Libraries

This step uses SMB4 (Streptavidin Magnetic Beads 4) and IPB (Illumina Purification Beads) to purify the enriched libraries.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- SMB4 (Streptavidin Magnetic Beads 4)
- Absolute ethanol (EtOH)
- Nuclease-free water
- 96-well PCR plates, LoBind
- Microseal 'B' adhesive seals

About Reagents

- SMB4:
  - Make sure that SMB4 are at room temperature before use. If SMB4 are not at room temperature, salt crystals can form. If salt crystals are visible, bring SMB4 to room temperature and vortex thoroughly before use.
  - Steps involving the centrifuge can cause SMB4 to form a bead pellet. If pelleting occurs, pipette to mix until fully resuspended.

Preparation

1. Prepare 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.
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 Probe Clean Up.
4. Label a new 96-well PCR plate PCR Clean Up.
5. Label a new 96-well PCR plate PL.

Procedure

Probe cleanup

1. Remove the ELU plate from the thermal cycler.
2. Centrifuge briefly.

3. Place on the magnetic stand for 1 minute.
4. Transfer 45 µl from each well of the ELU plate into the corresponding well of the Probe Clean Up plate.
5. Add 10 µl SMB4 to each well.
6. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
7. Centrifuge briefly.
8. Place on the preprogrammed thermal cycler and run the INC58 program. Incubate at 58°C for 5 minutes.
9. During incubation, proceed to next step.

Bind

1. Vortex IPB briefly to resuspend the beads.
2. Add 45 µl IPB to each well of the PCR Clean Up plate.
3. Remove the Probe Clean Up plate from the thermal cycler and centrifuge briefly.
4. Place the Probe Clean Up plate on the magnetic stand for 1 minute.
5. Transfer 50 µl from each well of the Probe Clean Up plate to the corresponding well of the PCR Clean Up plate. Pipette 10 times to mix.
6. Discard the Probe Clean Up plate.
7. Incubate the PCR Clean Up plate at room temperature for 5 minutes.

Wash

1. Place the PCR Clean Up plate on the magnetic stand for 2 minutes.
2. Without disturbing the beads, remove and discard all supernatant from each well.
3. Wash the beads as follows.
   a. Keep on the magnetic stand and add 150 µl 80% EtOH to each well.
   b. Wait 30 seconds.
   c. Without disturbing the beads, remove and discard all supernatant from each well.
4. Wash the beads a **second** time.
5. Centrifuge briefly.
6. Using a 20 µl pipette, remove residual supernatant from each well.

Elute

1. Remove the plate from the magnetic stand.
2. Add 35 µl RSB to each well.
3. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
4. Inspect each well. If a bead pellet is visible, pipette up and down to release the pellet.
5. Centrifuge briefly.
6. Incubate at room temperature for 2 minutes.
7. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

8. Transfer 30 µl from each well of the PCR Clean Up plate to the corresponding well of the PL plate.
9. Discard the PCR Clean Up plate.

SAFE STOPPING POINT

If you are stopping, seal the PL plate with Microseal 'B' adhesive, and then centrifuge briefly at 280 × g. Store at 2°C to 8°C overnight or at -25°C to -15°C for up to 30 days.

7.6 Quantify Enriched Libraries

Perform the following to check the quantity and quality of the purified enriched 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 µl library from each well using a Qubit dsDNA BR Assay Kit.
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 µl using TapeStation 4200 with D1000 Kit.
   - Analyze 1 µl using Bioanalyzer DNA 1000 Kit.

   For cfDNA, expect a distribution of templates with an average size range of ~310 bp to ~350 bp.
   For gDNA, expect a distribution of templates with an average size range of ~600 bp to ~700 bp.

8. 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.
- NextSeq 1000 and NextSeq 2000 Sequencing System P2 or P3 flow cells using the NextSeq 1000 and NextSeq 2000 Sequencing System XLEAP-SBS Onboard protocol.

Refer to *8.1 Dilute Libraries to the Starting Concentration* on page 49 for instructions.

8.1 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 and 350 bp for cfDNA.

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

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
NextSeq 1000/2000 P2 Flow Cell XLEAP-SBS Onboard	2	750
NextSeq 1000/2000 P3 Flow Cell XLEAP-SBS Onboard[2]	2	750

[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.
[2] Use XLEAP-SBS reagents with NextSeq 1000/2000. Standard reagents have not been evaluated.

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.

9. Resources & References

The Illumina 5-Base DNA Prep with Enrichment 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.

9.1 Revision History

Document	Date	Description of Change
Document # 200073498 v02	August 2026	Added: • Automation information. Updated: • Product Contents labeling and description information. • Dilute Libraries to the Starting Concentration to include NextSeq 1000/2000 P2 Flow Cell. • Document structure and formatting.
Document # 200073498 v01	November 2025	Updated: • Clean Up Index PCR to consolidate steps. • Hybridize Probes to simplify dilution equation and calculation. • Clean Up Amplified Enriched Libraries to specify EtOH volume for preparation. • Dilute Libraries to the Starting Concentration to remove using standard SBS reagents with NextSeq 1000/2000. Updated document formatting.
Document # 200073498 v00	October 2025	Initial Release.

10. 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.

illumina®