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Illumina DNA Methylation Prep

Product Documentation

ILLUMINA PROPRIETARY
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Overview

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

Illumina DNA Methylation Prep:

- Uses a proprietary enzymatic reaction to convert methylated cytosines to thymidine in a single step, while retaining the identity of unmethylated cytosines.
- Generates sequencing-ready libraries from a minimum of 50 ng gDNA or 1 ng cfDNA in ~7 hours.
- 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.

DNA Input Recommendations

The Illumina DNA Methylation Prep protocol enables library preparation from gDNA or cfDNA input.

gDNA Input

The Illumina DNA Methylation Prep protocol enables library preparation from 50–100 ng gDNA.

The input preparation procedure requires sonication of gDNA samples. Optimize sonication for the sample type, sonication equipment, and sonication consumables. Use a sonication method that produces an average fragment size containing ~450 bp. Overfragmenting can reduce sample yield, introduce GC bias, and affect assay performance. Illumina recommends using Covaris LE220 Plus with the 8 microTUBE Strip for sonication.

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

cfDNA Input

The Illumina DNA Methylation Prep protocol enables library preparation from 1–20 ng cfDNA input.

Prepare Size-Select cfDNA on page 14 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 DNA Methylation 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.

Consumables & Equipment

The Illumina DNA Methylation Prep protocol requires the following consumables and equipment.

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

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

Product Contents

Completing the Illumina DNA Methylation Prep protocol requires library prep reagents and indexes.

Component	Kit Options	Catalog #
Library prep reagents	Illumina DNA Methylation Prep, (24 samples) with Cloud Analysis	20134144
	Illumina DNA Methylation Prep, (24 samples) with Server Analysis	20140364

Reagent Kit Contents

Each library prep reagent kit comprises two boxes. The following tables list the contents of each box.

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

Quantity	Reagent	Description
1	ATL4	A-Tailing Mix
1	DDR	DNA Denaturation Reagent
1	ERP6	End Repair Mix
1	GDMC	Genomic DNA Methylation Control
1	IME	Illumina Methylation Enzyme
1	LIGX	DNA Ligase X
1	MAM	Methylation Amplification Mix
1	MCB	Methylation Conversion Buffer
1	MRR	Methylation Resuspension Reagent
1	SFMC	Short Fragment Methylation Control
1	UMI3-A	Unique Molecular Identifier

Illumina DNA Methylation Prep Box 2, Store at 15°C to 30°C

Quantity	Reagent	Description
1	IPB	Illumina Purification Beads
1	RSB	Resuspension Buffer

Illumina Unique Dual Indexes

Store at -25°C to -15°C

Component	Description	Catalog #
Indexes	Illumina Unique Dual Indexes Set A (96 Indexes, 96 Samples)	20140368
	Illumina Unique Dual Indexes Set B (96 Indexes, 96 Samples)	20140369
	Illumina Unique Dual Indexes Set C (96 Indexes, 96 Samples)	20140370
	Illumina Unique Dual Indexes Set D (96 Indexes, 96 Samples)	20140371

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
[cfDNA] Eppendorf twin.tec 96-well PCR plates, LoBind, thin wall, semi-skirted (250 µl minimum volume)	Eppendorf, catalog # 0030129504
Hard-shell 96-well PCR plates, thin wall, semi-skirted (250 µl minimum volume)	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001

Consumable	Supplier
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	One of the following, depending on quantification method: • Thermo Fisher Scientific, catalog # Q32851 • Thermo Fisher Scientific, catalog # Q32854

[Optional] 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

Equipment	Supplier
Adapter for PCR Plate, 96 well	Q Instruments, model # 1808-1041
BioShake iQ High-Speed Thermal Mixer	Q Instruments, model # 1808-0506
BioShake XP High-Speed Thermal Mixer	Q Instruments, model # 1808-0505
[gDNA] Covaris LE220 Plus[3], or other sonication instrument	Covaris, part number # 500569, or equivalent supplier
Microcentrifuge	General lab supplier
One of the following systems, depending on your qualification 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
Rack 12 place 8 microTUBE Strip	Covaris, part number # 500191
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.

Protocol

This section describes the Illumina DNA Methylation Prep protocol and provides instructions for preparing gDNA and cfDNA input. Use the procedures and reagents appropriate for your input type. After completing input preparation, continue the protocol with *Prepare for Protocol* on page 17.

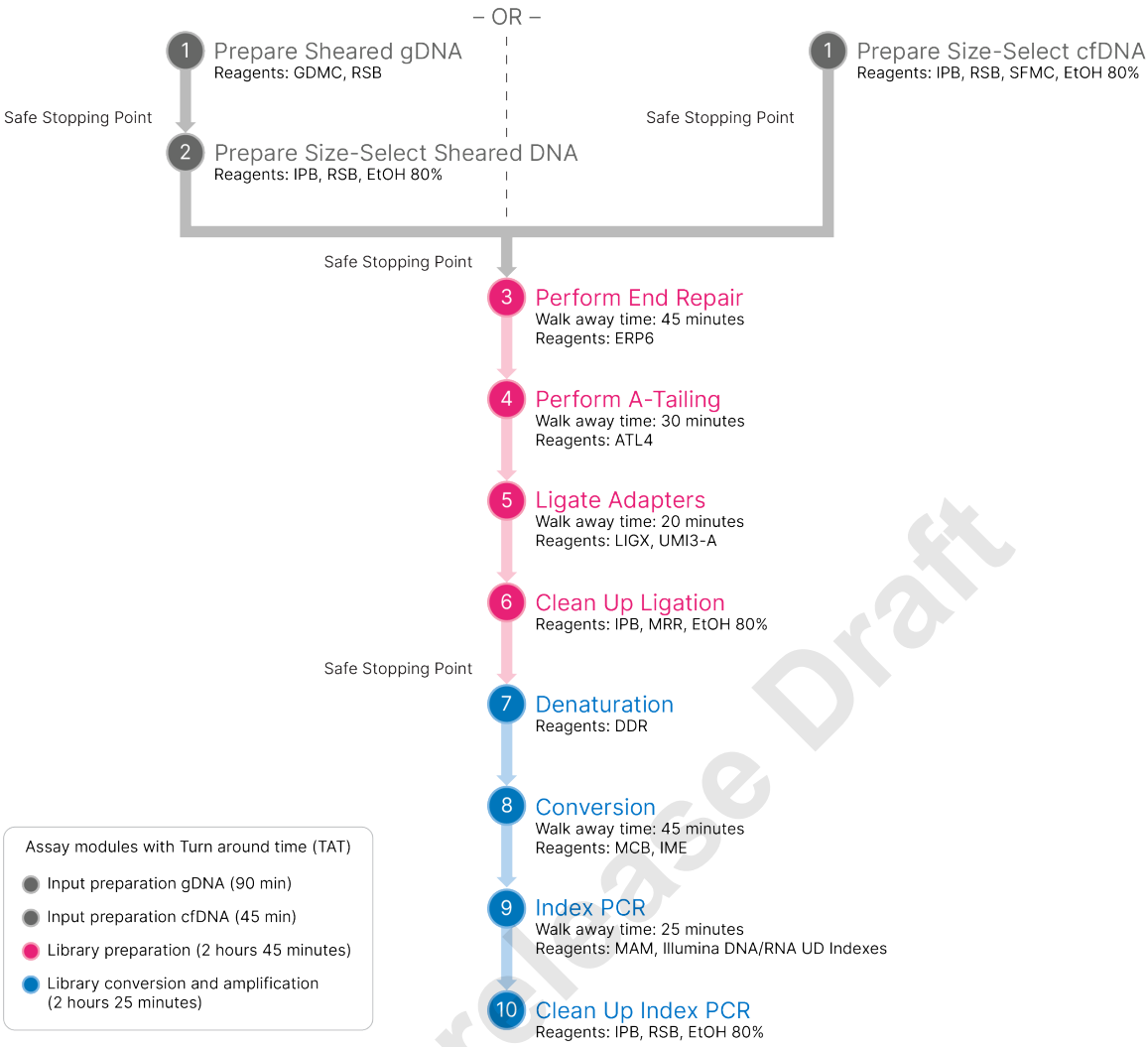
Before you begin, do as follows.

- Review the entire protocol.
- Review the preparation sections appropriate for your input type.
- 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 *Product Contents* on page 3.
- 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.

Illumina DNA Methylation Prep Kit Workflow

The following diagram illustrates the Illumina DNA Methylation Prep kit workflow.

- Safe stopping points are marked between steps.
- Time estimates are based on preparing 24 samples using a multichannel pipette.



Tips and Techniques

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

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

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, 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 Microseal 'B' 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 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 LoBind plates. Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or 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.

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 bead solution:
  - 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.

Prepare for Protocol

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	Instructions
IPB	Use at room temperature.
RSB	Use at room temperature.

Table 2 -25°C to -15°C Storage

Reagent	Instructions
[gDNA] GDMC	Thaw on ice.
[cfDNA] SFMC	Thaw on ice.

Prepare Sheared 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. To prepare each sample, combine gDNA with 2.5 µl GDMC.
   Make sure that the total volume does not exceed 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.

Prepare 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 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 to the corresponding well of the SS2 plate.

Remove Small DNA Fragments

1. Vortex the IPB for 10 seconds immediately before use.
2. Add 19 µl IPB to each well.
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 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.

2. Wash the beads a **second** time.
3. Using a 20 µl pipette, remove residual EtOH from each well.
4. Air-dry for at least 30 seconds and no more than 2 minutes.
5. Remove from the magnetic stand.
6. 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.
7. Apply Microseal 'B' and shake at 2200 rpm for 1 minute.
8. If pellets are visible, pulse vortex 3 times for 3 seconds at maximum speed.
9. Incubate at room temperature for 2 minutes.
10. Centrifuge briefly.
11. Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
    Retain the supernatant.
12. Transfer 45 µl from each well into the corresponding well of the the LP 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 *Prepare for Protocol* on page 17.

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 LoBind 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 fresh 80% EtOH from absolute EtOH. Vortex to mix.
3. Label a new LoBind 96-well PCR plate LP.
4. Label a new 96-well PCR plate cfDNA Elution plate.

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 LoBind 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.
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 to the corresponding well of the cfDNA Elution plate.

Purify Small DNA Fragments

1. Vortex the IPB for 10 seconds immediately before use.
2. Add 96 µl IPB to each well.
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 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.
2. Wash the beads a **second** time.
3. Using a 20 µl pipette, remove residual EtOH from each well.
4. 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. Incubate at room temperature for 2 minutes.
5. Centrifuge briefly.
6. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
   Retain the supernatant.
7. Transfer 42.5 µl 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 cfDNA Elution plate with the remaining volume (2.5 µl). Seal the plate with Microseal 'B'. Use this plate for cfDNA quantification after size-selection clean-up.
   - 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.

Quantify Size-Selected cfDNA (Optional)

Preparation

1. Prepare the Qubit dsDNA HS Kit per manufacturers instructions.
2. Prepare the cfDNA Elution 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 cfDNA Elution 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 cfDNA Elution plate and add directly to 198 µl Qubit reagent.
3. Measure cfDNA elution according to manufacturers instructions.

Prepare for Protocol

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

Table 3 Room Temperature Storage

Reagent	Instructions
IPB	Use at room temperature.

Table 4 -25°C to -15°C Storage

Reagent	Instructions
ATL4	Bring to room temperature to thaw completely, and then keep on ice.
ERP6	Thaw on ice.
LIGX	Thaw on ice.
MRR	Bring to room temperature to thaw.
UMI3-A	Bring to room temperature to thaw.

Perform End Repair

Consumables

- ERP6 (End Repair Mix)
- 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:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 55 µl
   - 30°C for 30 minutes
   - 70°C for 10 minutes
   - 4°C for 5 minutes

- Hold at 4°C

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.

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:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 90 µl
   - 37°C for 20 minutes
   - 70°C for 5 minutes
   - 4°C for 5 minutes
   - Hold at 4°C

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.

Ligate Adapters

Consumables

- LIGX (DNA Ligase X)
- MRR (Methylation Resuspension Reagent)
- UMI3-A (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-A—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:
    - Choose the preheat lid option and set to 42°C
    - Set the reaction volume to 100 µl
    - 30°C for 20 minutes
    - 4°C for 1 minute
    - Hold at 4°C

Procedure

1. Add 5 µl LIGX to each well of the LP plate.
2. Add 5 µl UMI3-A 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.

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 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 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.
2. Wash the beads a **second** time.
3. Apply Microseal 'B' and centrifuge briefly to bring any residual EtOH to the bottom of well.
4. Place on the magnetic stand (~10 seconds).

5. 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.

6. 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).
   Retain the supernatant.
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.

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.

Prepare for Protocol

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
IPB	Use at room temperature.
RSB	Use at room temperature.

Table 6 -25°C to -15°C Storage

Reagent	Instructions
DDR	Bring to room temperature to thaw.
Illumina DNA/RNA UD index plate	Bring to room temperature to thaw.
IME	Thaw on ice.
MAM	Thaw on ice.
MCB	Bring to room temperature.

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—Pipette mix 10 times with a 200 µl pipette, and then centrifuge briefly.
   - 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:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 25 µl

- 70°C for 10 minutes
- 4°C for 3 minutes
- Hold at 4°C

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.

Conversion

Consumables

- Microseal 'B' adhesive seal

Preparation

1. Save the following CONV program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 57°C for 45 minutes
   - Hold at 4°C

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.

Index PCR

Consumables

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

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Preparation

1. Prepare the following consumables:
   - Illumina DNA/RNA UD Index 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:
     a. Pulse vortex 3 times to mix, and then centrifuge briefly.
     b. Centrifuge briefly before use.
2. Save the following PCR6 program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C

- Set the reaction volume to 100 µl
- 98°C for 30 seconds
- 6 cycles of:
  - 98°C for 10 seconds
  - 59°C for 30 seconds
  - 65°C for 60 seconds
- 65°C for 5 minutes
- Hold at 4°

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.

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 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 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.
2. Wash the beads a **second** time.
3. Using a 20 µl pipette, remove residual supernatant.
4. 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).
   Retain the supernatant.
7. Transfer 18 µl 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.

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 µl library from each well using a Qubit dsDNA BR Assay Kit.
   - Typical yield for 50 ng gDNA input is 4 to 20 ng/µl
   - Typical yield for 10 ng cfDNA input is 10 to 30 ng/µl
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.

Dilute Libraries to the Starting Concentration

Illumina recommends setting up a paired-end run with 151 cycles per read (2 x 151) and 2 x 10 cycles per Index Read 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.

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.

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

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system. 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)*
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

* 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.
   For both NovaSeq 6000 and NovaSeq X Series, Illumina recommends the 1% PhiX spike-in instructions.

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.

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