# Single Cell Methylation Kit v1.1 Large

## Protocol

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# Legal Notices

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## Table of Contents

*Legal Notices* ..... 2
*Required Materials* ..... 4
*Best Practices* ..... 7
*Workflow Diagram* ..... 8
*Assay Introduction* ..... 9
*Sample Plating Recommendations for Indexed Tagmentation* ..... 12
*Step 1: Nuclei Fixation* ..... 14
*Step 2: Nucleosome Disruption* ..... 17
*Step 3: Indexed Tagmentation and Nuclei Sorting* ..... 19
*Step 4: Bisulfite Conversion* ..... 24
*Step 5: Post-Bisulfite Conversion Cleanup and Elution* ..... 27
*Step 6: Ligation* ..... 30
*Step 7: Indexed PCR* ..... 32
*Step 8: Purification of Index PCR Products* ..... 34
*Step 9: Library Quality Control* ..... 37
*Step 10: Sequencing Parameters* ..... 38
*Appendix A: Fluorescence Activated Cell Sorting (FACS) Settings* ..... 39
*Appendix B: Purification for QC Sequencing* ..... 40
*Appendix C: Post-Fixation Nuclei Storage in Nuclei Storage Buffer* ..... 42
*Appendix D: Purification of Index PCR Products using only SPRIselect* ..... 43
*Document Revision History* ..... 45

## Required Materials

ScaleBio™ Single Cell Methylation Nuclei Resuspension Kit (PN 1064637) Contents:

| Kit Module | Consumable | Part Number | Qty | Cap Color | Storage Temp |
|---|---|---|---|---|---|
| Single Cell Methylation Nuclei Resuspension Kit (PN 1066591) | Met Wash Buffer | 940564 | 2 | - | -20°C |
| | Met Wash Buffer Additive | 940563 | 2 | - | -20°C |
| | Nuclei Storage Buffer | 1064649 | 1 | - | -20°C |

ScaleBio™ Single Cell Methylation Kit v1.1 – Large (PN 1064640) Contents:

| Kit Module | Consumable | Part Number | Qty | Cap Color | Storage Temp |
|---|---|---|---|---|---|
| Met Nuclei Tagment Module A (PN 937587) | Met Tagment Barcode Plate 1 | 941770 | 1 | - | -20°C |
| | Met Tagment Barcode Plate 2 | 941779 | 1 | - | -20°C |
| | Met Tagment Barcode Plate 3 | 941782 | 1 | - | -20°C |
| | Met Nuclei Tagment Buffer | 941788 | 1 | Blue | -20°C |
| Met Nuclei Tagment Module B – Large (PN 943619) | Nucleosome Disruption Solution | 941785 | 1 | Blue | RT |
| | Lysis Buffer – Large | 943626 | 1 | Blue | RT |
| | Lysis Enzyme – Large | 943628 | 1 | Blue | RT |
| | ScaleBio Sample Collection Funnel | 202100005 | 2 | - | RT |
| Met Bisulfite Conversion Module (PN 943631) | Bisulfite Conv. Reagent | 943637 | 4 | - | RT |
| | Rehydration Buffer | 943640 | 4 | - | RT |
| | Dilution Buffer | 943645 | 4 | - | RT |
| | Reaction Buffer | 943650 | 4 | Black | RT |
| Met Conversion Clean-up Module (PN 944302) | Wash Buffer (Pure) | 944314 | 4 | - | RT |
| | Bisulfite Clean-up Buffer | 944321 | 4 | - | RT |
| | DNA Spin Plate | 944329 | 8 | - | RT |
| | Elution Buffer | 937121 | 4 | - | RT |
| | DNA Binding Buffer | 944305 | 4 | - | RT |
| Met Ligation and Index PCR Module v1.1 – Large (PN 1064644) | Ligation Master Mix | 1094665 | 4 | Clear | -20°C |
| | Met Adaptor Primers | 1064655 | 4 | Clear | -20°C |
| | Met Index PCR Master Mix | 1096656 | 4 | - | -20°C |
| | Met Index PCR Plate 1 | 1064657 | 1 | - | -20°C |
| | Met Index PCR Plate 2 | 1064659 | 1 | - | -20°C |
| | Met Index PCR Plate 3 | 1064663 | 1 | - | -20°C |
| | Met Index PCR Plate 4 | 1064647 | 1 | - | -20°C |
| | Met Index PCR Plate 5 | 1064653 | 1 | - | -20°C |
| | Met Index PCR Plate 6 | 1064664 | 1 | - | -20°C |
| | Met Index PCR Plate 7 | 1064656 | 1 | - | -20°C |
| | Met Index PCR Plate 8 | 1064654 | 1 | - | -20°C |

Consumables and reagents manufactured by other vendors:

| Consumable or reagent | Supplier | Part Number |
|---|---|---|
| Nuclease-free water | Various | Various |
| Ethanol (pure) | Various | Various |
| 16% Formaldehyde (w/v) (methanol free) | Thermo Fisher | 28908 |
| UltraPure Glycine (powder) | Thermo Fisher | 15527013 |
| SPRIselect | Beckman Coulter | B23317 |
| Nuclei counting dye | Various | Various |
| DNA staining dye (e.g. DAPI, DRAQ5™) for nuclei sorting | Various | Various |
| Elution Buffer (10 mM Tris-Cl, pH 8.5) | Qiagen | 19086 |
| Pipette tips (nuclease-free, filtered, low retention for P1000, P200, P20, P2) | Various | Various |
| Pipette tips (wide-bore, nuclease-free, filtered, low retention for P1000) | Various | Various |
| 0.2-mL PCR tube strips (nuclease-free) | Various | Various |
| 1.5-mL DNA LoBind tubes | Eppendorf | 0030108418 |
| 5-mL DNA LoBind tubes | Eppendorf | 0030108310 |
| 15-mL conical tubes | Various | Various |
| FACS tubes | Various | Various |
| 96-well LoBind semi-skirted PCR plates | Eppendorf | 0030129504 |
| 2.0-mL 96-well deep well plates | Various | Various |
| Microseal 'B' PCR Plate Sealing Film | Bio-Rad | MSB1001 |
| 10 mL, Disposable Reservoirs, 1 Sleeve of 50, Sterile, Polystyrene, SureFlo™ anti-sealing array | INTEGRA | 4373 |
| 25 mL, Disposable Reservoirs, 1 Sleeve of 50, Sterile, Polystyrene, SureFlo™ anti-sealing array | INTEGRA | 4383 |
| Qubit dsDNA HS Assay Kit * | Thermo Fisher | Q33230 |
| NucleoSpin Gel and PCR Clean-up Mini Kit (or equivalent) | Macherey-Nagel | 740609.50 |
| Agilent High Sensitivity D5000 ScreenTape System (or equivalent kit) * | Agilent | 5067-5592, 5067-5593, 5067-5594 |
| NEBNext Library Quant Kit for Illumina * | NEB | E7630 |

* Required for quality control of library.

Equipment List:

| Item | Supplier | Part Number |
|---|---|---|
| Pipettes (P1000, P200, P20, P10, P2) | Various | Various |
| Multi-channel pipettes (P100, P20, P10) | Various | Various |
| Magnetic stand for 0.2-mL tube strips | Various | Various |
| 96-well aluminum cooler blocks | Various | Various |
| Vortex mixer | Various | Various |
| Mini centrifuge for 1.5-mL tubes or PCR strip-tubes | Various | Various |
| Shaker for semi-skirted 96-well plates and 5-mL tubes (300-2000 rpm, e.g. ThermoMixer) | Various | Various |
| Cell counter | Various | Various |
| Cell sorter with 100µm nozzle, and filter appropriate for used DNA staining dye | Various | Various |
| Centrifuge with temperature control and swing-bucket rotor (5-mL and 15-mL tubes, 96-well deep well plates) | Various | Various |
| Thermocycler with lid temperature control (0-105°C) for semi-skirted 96-well plates * | Various | Various |
| qPCR machine for 96-well plates ** | Various | Various |
| Qubit 4 Fluorometer ** | Thermo Fisher | Q33238 |
| 4200 TapeStation System (or equivalent system) ** | Agilent | G2991BA |
| Chemical fume hood | Various | Various |
| Rotator or Hula Mixer (optional) | Various | Various |
| PCR plate rack/adapter | Various | Various |

* Minimum of two Thermocyclers are needed.

** Required for quality control of library.

## Best Practices

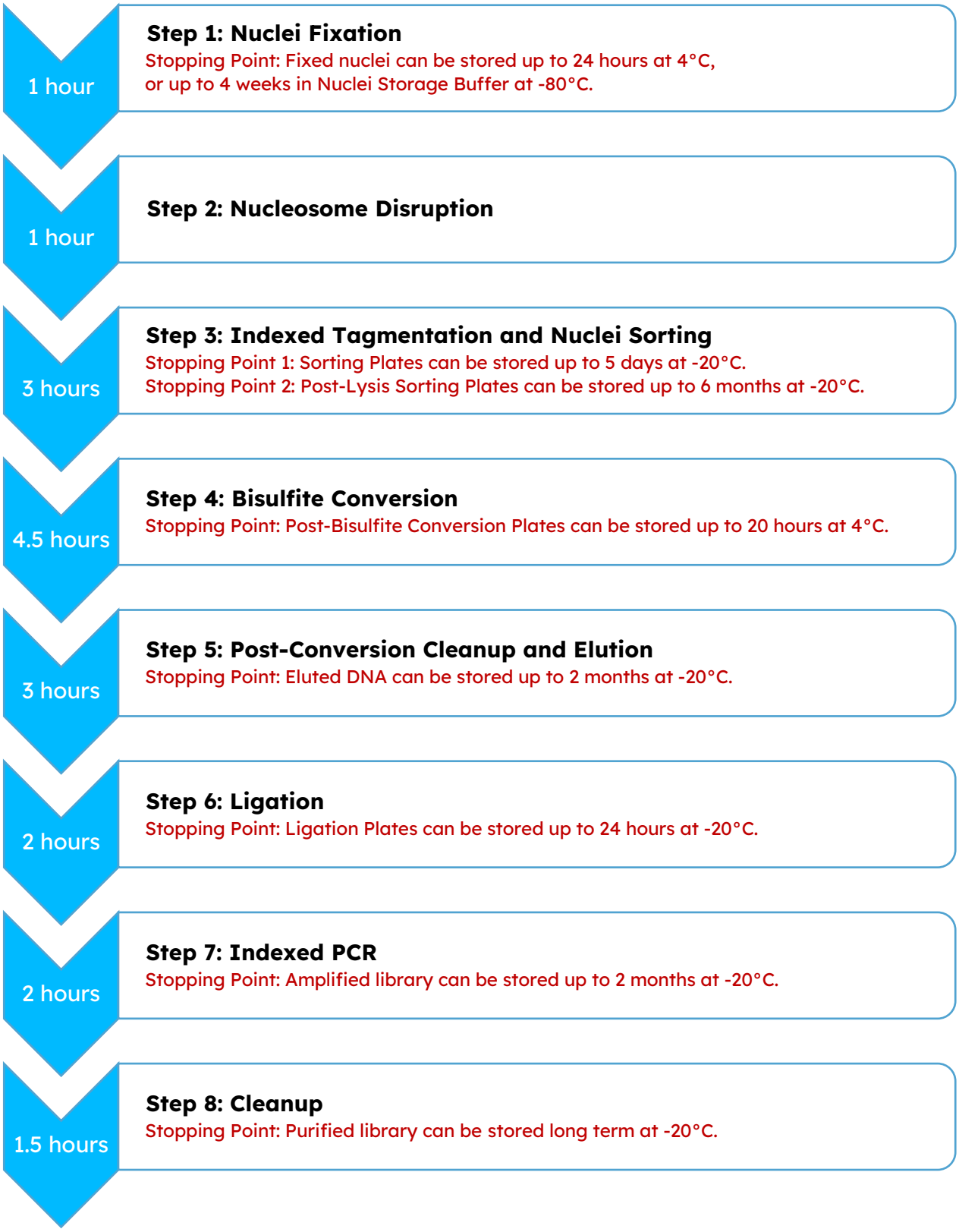
For general laboratory best practices:

- Calibrate and service pipettes every 12 months to ensure accurate sample volume transfer at each step.
- Store all reagents at the storage conditions recommended by the supplier.
- Thaw all reagents on ice, unless otherwise specified.
- Never reuse pipette tips or tubes.
- Keep pipette tip boxes, reagent containers, and sample tubes closed when not in use.
- Wear suitable protective clothing, eyewear, and gloves.
- Dedicate separate laboratory workspaces to pre-amplification process and post-amplification process.
- Routinely wipe work surfaces with a 10% bleach solution to remove DNA amplicon contaminants.

For prevention of amplicon cross-contamination in sequencing libraries:

- Thaw and prepare reagent mixes in pre-amplification workspaces.
- Perform amplification in post-amplification workspaces.
- Perform PCR purification steps in post-amplification workspace.
- Never bring material or equipment from post-amplification workspaces into pre-amplification workspaces.

## Workflow Diagram



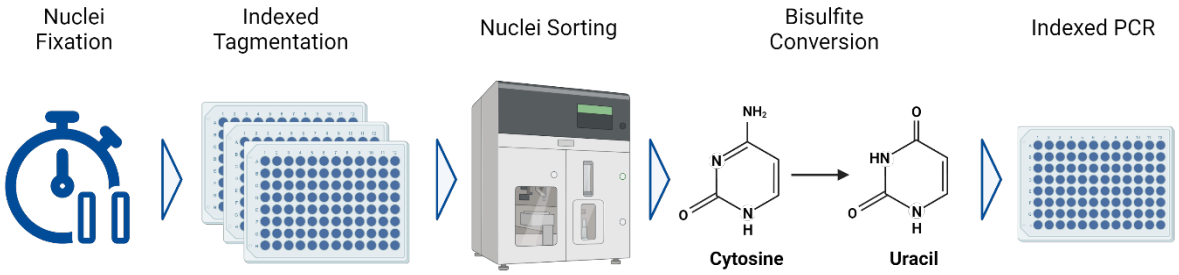
## Assay Introduction

The ScaleBio™ Single Cell Methylation Kit enables single cell resolution into DNA methylation states through a high-throughput sequencing readout for up to 6 samples simultaneously (up to 8 samples if an additional Nuclei Preparation Kit is purchased). The workflow starts with isolated and purified nuclei, and generates a sequencing ready library for the Illumina platform. It is provided in two kit sizes depending on the desired number of nuclei to be processed:

- Small kit for ~4,600 nuclei
- Large kit for ~18,400 nuclei

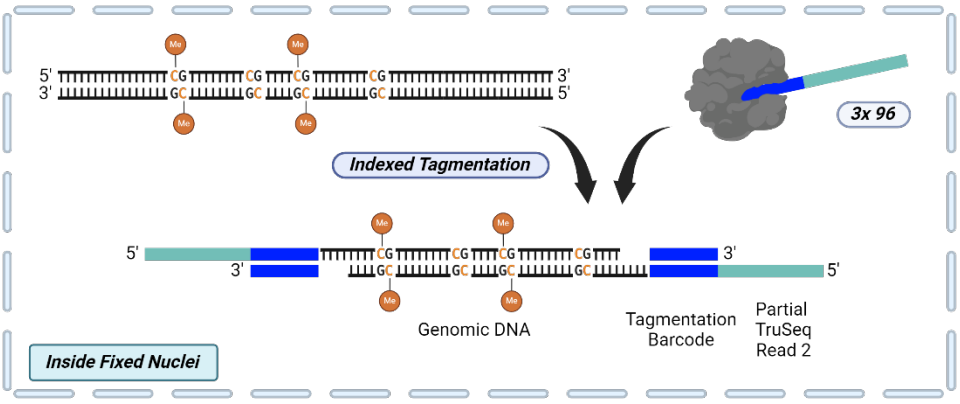
**This user guide is written specifically for the application of the large kit. When processing samples with the small kit, a separate user guide is provided.**

*Figure 1: Overall procedure*



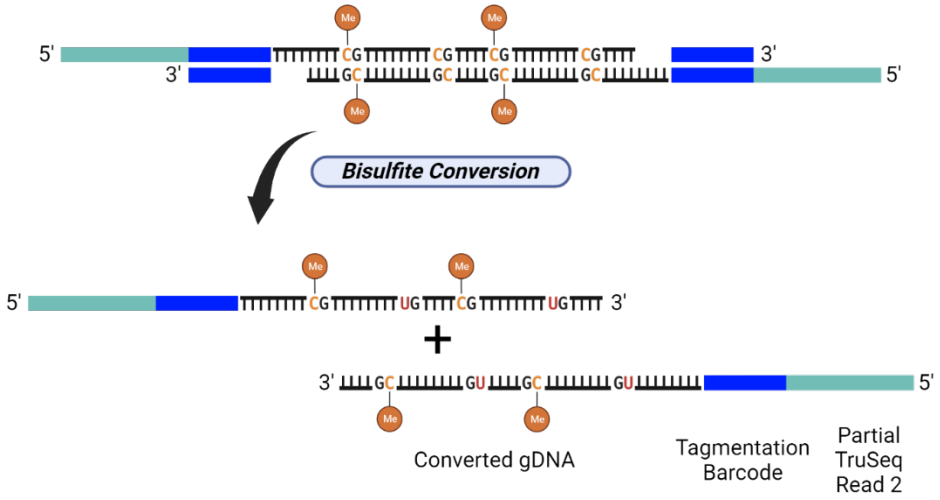

The single cell methylation workflow begins with nuclei fixation. Fixed nuclei samples are then barcoded in an indexed tagmentation reaction. Afterwards, 24 nuclei are deposited per well across 96-well plates using a cell sorter.

*Figure 2: Indexed Tagmentation*



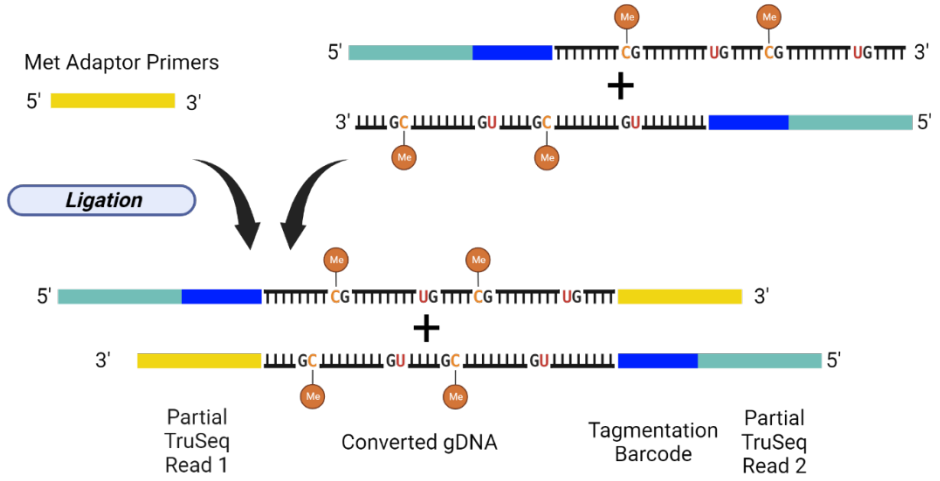

Bisulfite conversion is then performed to convert unmethylated cytosines into uracils. Methylated cytosines are protected by their methylated state and will remain cytosines.

*Figure 3: Bisulfite Conversion*



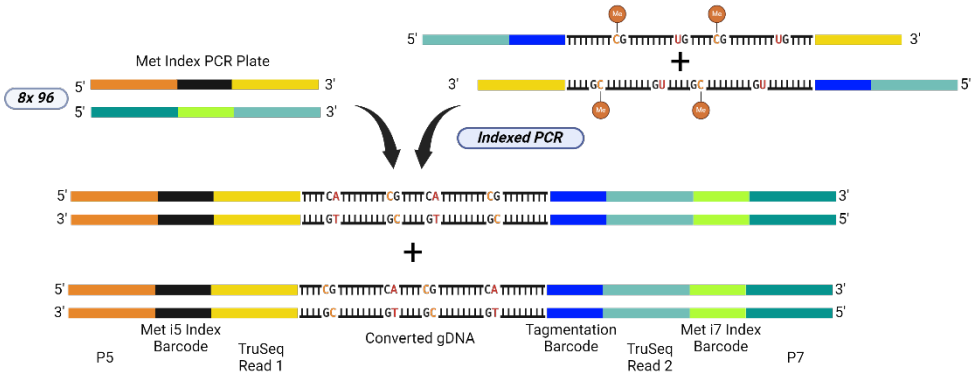

The bisulfite conversion is followed by sample cleanup and the addition of amplification adaptors through a ligation reaction.

*Figure 4: Ligation*



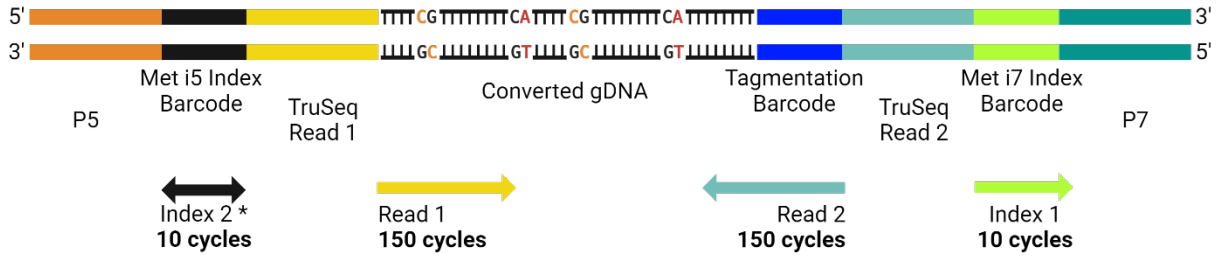

Finally, a second barcode is added across the plates in an Indexed PCR reaction to complete the library construction.

*Figure 5: Indexed PCR*




ScaleBio single cell methylation libraries are compatible with standard Illumina sequencing. The ScaleBio bioinformatics pipeline (ScaleBio Seq Suite Methylation v1.0 or later) can be used to demultiplex the raw data and assign the reads to individual nuclei to produce a single cell methylation matrix for further downstream analysis.

*Figure 6: Final Library Structure*




* orientation depends on sequencer and sequencing chemistry

## Sample Plating Recommendations for Indexed Tagmentation

*Step 3: Indexed Tagmentation* is performed in a total of three 96-well Met Tagment Barcode Plates with one unique Tagment Barcode per well (288 unique barcodes total). The Tagment Barcodes are used to assign the sequenced reads to the individual samples. It is critical to note which plate and wells are loaded with which sample for the correct downstream sample identification during data analysis in ScaleBio SeqSuite.

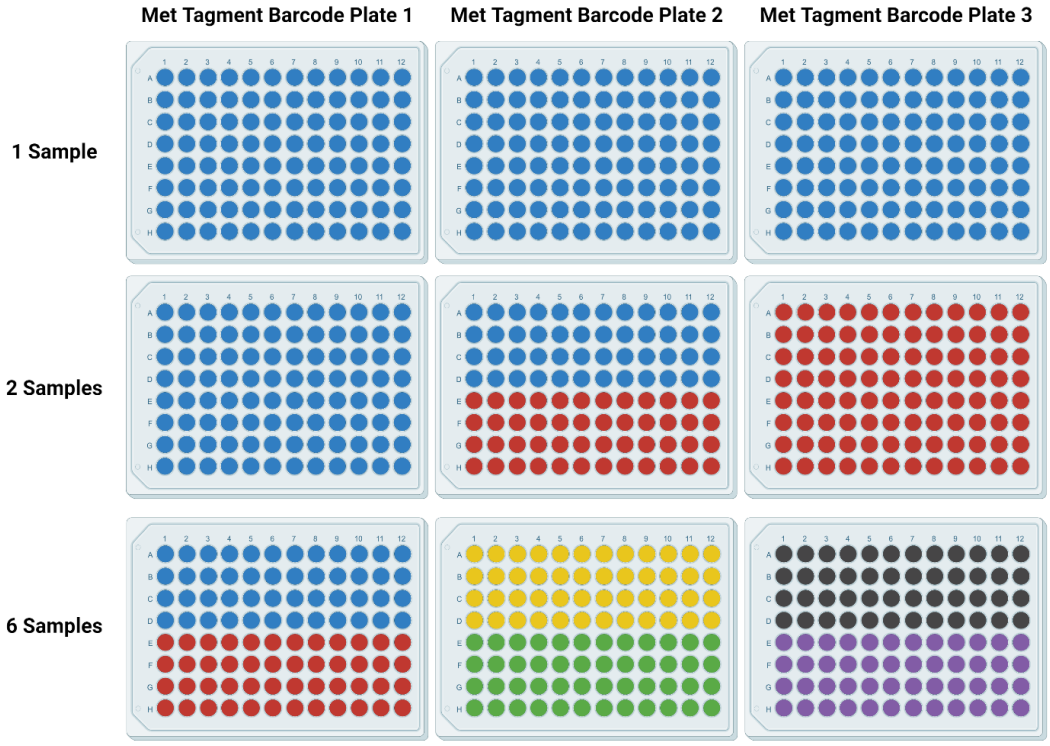
The supplied material allows fixation of up to 6 nuclei samples (up to 8 samples if an additional Nuclei Preparation Kit is purchased). Fixed nuclei suspensions are loaded onto the Met Tagment Barcode Plates with 1,000 to 5,000 nuclei per well. Table 1 shows the required number of wells when loading the samples in an even by-row distribution across all three plates. See Figure 7 for examples on sample plating strategies across the three plates.

*Table 1: Guidance on Met Tagment Barcode Plate Loading*

| Number of Samples | Total Number of Loaded Wells of 3x 96 Well-Plates | Number of 12-Well Rows to be filled | Total Nuclei Loaded per Sample at 5000 Nuclei per Well (+15%) | Total Nuclei Loaded per Sample at 1000 Nuclei per Well (+15%) |
|---|---|---|---|---|
| 1 | 288 | 24 | 1,656,000 | 331,200 |
| 2 | 144 | 12 | 828,000 | 165,600 |
| 3 | 96 | 8 | 552,000 | 110,400 |
| 4 | 72 | 6 | 414,000 | 82,800 |
| 5 | ~57 | ~4 | 331,200 | 66,240 |
| 6 | 48 | 4 | 276,000 | 55,200 |
| 7 * | ~41 | ~3 | 236,000 | 47,320 |
| 8 * | 36 | 3 | 207,000 | 41,400 |

** Additional Methylation Nuclei Preparation Kit required.*

*Figure 7: Example Loading Maps for the Met Tagment Barcode Plates*



## Step 1: Nuclei Fixation

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Nuclei Resuspension Kit | Met Wash Buffer | - | -20°C | On ice | ⊗ | ⊗ |
| | Met Wash Buffer Additive | - | -20°C | RT | ⊗ | ⊗ |
| | Nuclei Storage Buffer * | - | -20°C | On ice | ⊗ | ⊗ |
| Other Vendors | 16% Formaldehyde (w/v) (methanol free)<br>UltraPure Glycine<br>Wide-bore P1000 pipette tips<br>5-mL DNA LoBind tubes<br>Nuclei counting dye<br>Chemical fume hood | | | | | |

* Optional: only required if storing nuclei post-fixation at -80°C

**Before you begin:**

**Caution**: Timing of the first four steps of the workflow is critical. Fixed nuclei must be tagmented within 24 hours, either when freshly fixed or when thawed after storage. Nucleosome disruption, tagmentation and nuclei sorting must be completed on the same day.

- Start with nuclei resuspended in MetWashBuffer+. See instructions below on how to prepare MetWashBuffer+. Process the nuclei immediately after nuclei isolation.
- Prepare fresh 2.5 M Glycine:
  - Dissolve 1.88 grams of glycine in 8 mL of nuclease free water, and then add further nuclease free water to a total volume of 10 mL.
  - Vortex until fully dissolved. 37°C heat bath can be used to help dissolve the glycine.
- Prepare MetWashBuffer+:
  - Add the Met Wash Buffer Additive tablet to the bottle of Met Wash Buffer.
  - Vortex intermittently until completely dissolved.
  - Place on ice. MetWashBuffer+ is stable at 4°C for up to 1 month.
- Pre-cool swing bucket centrifuge compatible with 5-mL tubes to 4°C.
- If frozen, thaw nuclei on ice. Do not vortex. Review Table 2 for recommended numbers of nuclei to begin the workflow.

*Table 2: Recommended Number of Nuclei per Sample for Fixation*

| Processed Samples | Minimum Required Nuclei | Recommended Nuclei | Recommended Aliquots | Processed Nuclei for Sequencing |
|---|---|---|---|---|
| 1 | 650,000 | 1 million | 1-3 | 18,400 |
| 2 | 325,000 | 1 million | 1-3 | 9,200 |
| 3 | 250,000 | 1 million | 1-2 | 6,100 |
| 4 | 250,000 | 650,000 | 1 | 4,600 |
| 5 | 250,000 | 650,000 | 1 | 3,600 |
| 6 | 250,000 | 650,000 | 1 | 3,000 |
| 7 * | 250,000 | 650,000 | 1 | 2,600 |
| 8 * | 250,000 | 650,000 | 1 | 2,300 |

** Additional Methylation Nuclei Preparation Kit required.*

**Note:** It is highly recommended to follow the "Recommended Nuclei" column from Table 2 as the lower "Minimum Required Nuclei" numbers can make it challenging to see a pellet during the workflow.

**Procedure:**

1. Place each nuclei sample **on ice** in its own **5-mL DNA LoBind** tube.
2. Add MetWashBuffer+ to each nuclei sample **on ice** to a total volume of **1 mL** with a wide-bore P1000 pipette tip and gently pipette mix to resuspend nuclei.
3. Determine the concentration of the nuclei suspension(s) with cell counting equipment.
4. Aliquot **1 million nuclei** per tube into new 5-mL DNA LoBind tubes based on the recommended number of aliquots required per biological sample (Table 2). Processing less than 1 million nuclei can be utilized, but nuclei recovery may vary.

**Note:** In total, six 1-million nuclei fixations can be processed, irrespective of the number of samples.

**Caution**: Allow nuclei to warm up to room temperature before adding formaldehyde. Proceed through nuclei fixation quickly until quenching the reaction with glycine.

5. In a chemical fume hood, adjust the volume in each aliquot tube with MetWashBuffer+ to a total final volume of **953 µL**.
6. Add **47 µL** of 16% formaldehyde per aliquot for a final concentration of 0.75% formaldehyde in a total volume of **1 mL**, gently invert the tubes five times.
7. Incubate nuclei at **room temperature** for **10 minutes**, gently inverting the tubes every **2 minutes**.
8. In a chemical fume hood at **room temperature**, add **47 µL** of 2.5 M glycine per aliquot.

9. At **room temperature**, gently invert the tubes five times, then place **on ice** for **5 minutes**. Invert the tubes every **2 minutes**.
10. Centrifuge nuclei at **500 x g** for **5 minutes** at **4°C**.
11. In a chemical fume hood, carefully remove supernatant without disturbing the pellet.

**Note:** If storing fixed nuclei at -80°C, proceed with the instructions in *Appendix C: Post-Fixation Nuclei Storage in Nuclei Storage Buffer* at this step.

12. **On ice**, gently add **970 µL** of MetWashBuffer+ without disturbing the pellet.
13. Store the fixed nuclei, or proceed directly to *Step 2: Nucleosome Disruption*.


**Safe stopping point**. Fixed nuclei can be stored up to 24 hours at 4°C, or up to 4 weeks at -80°C in Nuclei Storage Buffer by following *Appendix C: Post-Fixation Nuclei Storage in Nuclei Storage Buffer*.


**Caution**: If storing fixed nuclei at 4°C, resuspend nuclei gently with a wide-bore P1000 pipette tip. Perform tagmentation within 24 hours.

## Step 2: Nucleosome Disruption

**Caution**: Nucleosome Disruption, Tagmentation and Sorting must be completed on the same day.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Step 1: Nuclei Fixation | MetWashBuffer+ | - | 4°C | On ice | ✗ | ✗ |
| Met Nuclei Tagment Module B - Large | Nucleosome Disruption Solution | Blue | RT | RT | ✓ | ✓ |
| Other Vendors | Wide-bore P1000 pipette tips<br>1.5-mL DNA LoBind tubes<br>Nuclei counting dye | | | | | |

**Before you begin:**

- Pre-cool a swing bucket centrifuge compatible with 5-mL tubes and 96-well plates to 4°C.
- Pre-heat a ThermoMixer with a 5-mL tube adapter to 37°C.
- If the Nucleosome Disruption Solution shows visible precipitate, incubate at 37°C for 30 minutes, or until precipitate is fully dissolved.

**Procedure:**

1. Follow the instructions below based on where the fixed nuclei were stored:

| Freshly Fixed | Stored at 4°C Overnight | Stored at -80°C |
|---|---|---|
| • The fixed nuclei from *Step 1: Nuclei Fixation* should still contain an undisturbed pellet in MetWashBuffer+<br><br>• At room temperature, add 30 µL of Nucleosome Disruption Solution on the surface of the 970 µL MetWashBuffer+ for a total volume of 1 mL; DO NOT mix until step 2 | • The fixed nuclei should form a pellet in MetWashBuffer+ after storing overnight at 4°C<br><br>• At room temperature, add 30 µL of Nucleosome Disruption Solution on the surface of the 970 µL MetWashBuffer+ for a total volume of 1 mL; DO NOT mix until step 2 | • Follow *Appendix C: Post-Fixation Nuclei Storage in Nuclei Storage Buffer* to thaw and wash nuclei<br><br>• At room temperature, add 30 µL of Nucleosome Disruption Solution on the surface of the 970 µL MetWashBuffer+ for a total volume of 1 mL; DO NOT mix until step 2 |

**Caution**: DO NOT place Nucleosome Disruption Solution on ice.

2. Gently resuspend nuclei by pipette mixing with a wide-bore P1000 pipette tip.
3. Incubate nuclei on a ThermoMixer according to Program 1.

*Program 1: Nucleosome Disruption*

| Temperature | Time | Speed (rpm) |
|---|---|---|
| 37°C | 20 min | 300 |

4. Centrifuge nuclei at **500 x g** for **5 minutes** at **4°C** or at **room temperature**.
5. At **room temperature**, carefully remove supernatant without disturbing the pellet.

**Caution:** The nuclei pellet may not be visible after centrifugation. Keep supernatant in case pellet was accidentally resuspended in the supernatant.

6. Add **room temperature,** add **350 µL** of MetWashBuffer+ with a wide-bore P1000 pipette tip. With the same pipette tip, resuspend all nuclei aliquots belonging to the **same** biological sample by gently pipette mixing and pooling the aliquots into **one** 1.5-mL DNA LoBind tube for a total of **350 µL** per sample.

**Caution**: DO NOT pool different biological samples. Only pool aliquots belonging to the same biological sample.

7. Determine the concentration of the nuclei suspension(s) with cell counting equipment.
8. Dilute nuclei with MetWashBuffer+ to a concentration range of **182-910 nuclei per µL** depending on the number of nuclei loaded per well of the Met Tagment Barcode Plates (see Table 3). Review *Sample Plating Recommendations for Indexed Tagmentation* for the total number of nuclei required per sample, and review Table 5 for the total volume of diluted nuclei required per sample.
9. Proceed directly to *Step 3: Indexed Tagmentation and Nuclei Sorting*.

*Table 3: Target Nuclei Concentration Based on Met Tagment Barcode Plate Loading*

| Nuclei per well | 1,000 | 2,000 | 3,000 | 4,000 | 5,000 |
|---|---|---|---|---|---|
| Target concentration (nuclei per µL) | 182 | 364 | 545 | 727 | 910 |

## Step 3: Indexed Tagmentation and Nuclei Sorting

This step is performed in a total of three 96-well Met Tagment Barcode Plates with one unique Tagment Barcode per well (288 unique barcodes total). The Tagment Barcodes are used to assign the sequenced reads to the individual samples. It is critical to note which plate and wells are loaded with which sample for the correct downstream sample identification during data analysis in ScaleBio SeqSuite.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Step 2: Nucleosome Disruption | Nuclei in MetWashBuffer+ | - | On ice | On ice | - | - |
| Met Nuclei Tagment Module A | Met Nuclei Tagment Buffer | Blue | -20°C | On ice | ✓ | ✓ |
| | Met Tagment Barcode Plates 1/2/3 | - | -20°C | On ice | ✗ | ✓ |
| Met Nuclei Tagment Module B – Large | ScaleBio Sample Collection Funnel | - | RT | RT | - | - |
| | Lysis Buffer | Blue | RT | RT | ✓ | ✓ |
| | Lysis Enzyme | Blue | RT | RT | ✗ | ✓ |
| Other Vendors | Nuclease-free water<br>1.5-mL DNA LoBind tubes<br>96-well LoBind semi-skirted PCR plates<br>Wide-bore P1000 pipette tips<br>FACS tubes<br>DNA staining dye (e.g. DAPI, DRAQ5™) for nuclei sorting<br>Cell Sorter (100 µm nozzle, and appropriate filter for used DNA staining dye) | | | | | |

**Before you begin:**

- Pre-cool a swing bucket centrifuge compatible with 5-mL tubes and 96-well plates to 4°C.
- Pre-heat three thermocyclers (Program 2) and hold at 55°C.
- Keep nuclei on ice.
- If Lysis Buffer shows visible precipitate, incubate at 37°C for 30 minutes, or until precipitate is fully dissolved. A little remaining precipitation is acceptable.
- Prepare cell sorter instrument per manufacturer's instructions. Review *Appendix A: Fluorescence Activated Cell Sorting (FACS) Settings*.

**Note:** Sorting Plates can be prepared ahead of time and used within 8 hours. Follow the instructions below (steps a-g) before starting the procedure with the Indexed Tagmentation step.

a. At room temperature, prepare Sorting Buffer in a 5-mL DNA LoBind tube according to Table 4.

*Table 4: Sorting Buffer*

| Reagent | 8x Sorting Plates (µL) |
|---|---|
| Nuclease-free water | 818.4 |
| Lysis Buffer | 880 |
| Lysis Enzyme | 61.6 |
| **Total volume** | **1760** |

b. Briefly vortex the Sorting Buffer.
c. At **room temperature,** aliquot Sorting Buffer into each tube of a tube strip (enough for eight plates):
    a. For an **8-tube strip**, aliquot **216 µL** in each tube.
    b. For a **12-tube strip**, aliquot **144 µL** in each tube.
d. To prepare the Sorting Plates, use a multichannel pipette to distribute **2 µL** of Sorting Buffer into each well of eight 96-well LoBind PCR plates.
e. Seal the Sorting Plates.
f. Centrifuge the Sorting Plates at **1000 x g** for **30 seconds** at **4°C**.
g. Keep Sorting Plates at **room temperature** until ready to sort the tagmented nuclei in step 23.

**Procedure:**

1. Centrifuge the three Met Tagment Barcode Plates at **200 x g** for **1 minute** at **4°C**.
2. Place **on ice**.
3. **On ice**, prepare one Tagmentation-Nuclei Mix per sample (Table 5). The indicated volumes assume an even distribution of the samples across the three Met Tagment Barcode Plates.

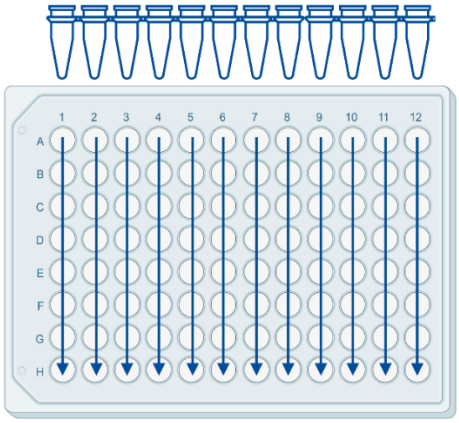
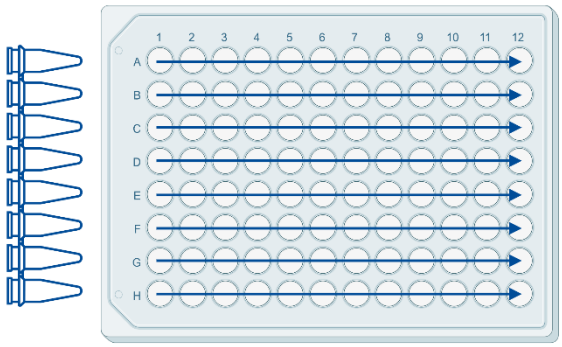
*Table 5: Tagmentation-Nuclei Mix*

| Number of Processed Samples | – | 1 | 2 | 3 | 4 | 5 | 6 | 7 * | 8 * |
|---|---|---|---|---|---|---|---|---|---|
| **Number of Reactions** | 1X | 330X | 165X | 110X | 82X | 66X | 55X | 47X | 41X |
| **Diluted Nuclei (µL)** | 5.5 | 1815 | 908 | 605 | 451 | 363 | 303 | 259 | 226 |
| **Met Nuclei Tagment Buffer (µL)** | 2.5 | 825 | 413 | 275 | 205 | 165 | 138 | 118 | 103 |
| **Total Volume (µL)** | **8.0** | **2640** | **1321** | **880** | **656** | **528** | **441** | **377** | **329** |

** Additional Methylation Nuclei Preparation Kit required.*

4. Gently invert the tubes five times to mix nuclei homogenously.
5. **On ice**, aliquot the Tagmentation-Nuclei Mix into tube strips according to the planned sample plating layout (review *Sample Plating Recommendations for Indexed Tagmentation*). Use Table 6 as example for distribution when processing a single sample.

*Table 6: Guidelines for distribution of one sample into Met Tagment Barcode Plates*

| **For distribution into columns, using a 12-channel multichannel pipette and a 12-tube strip** | |
|---|---|
| • For **one sample**, prepare Tagmentation-Nuclei Mix in a 12-tube strip by adding **215 µL** of the diluted Tagmentation-Nuclei Mix to each tube. |  |
| **For distribution into rows using an 8-channel multichannel pipette and an 8-tube strip** | |
| • For **one sample** and **EACH** Met Tagment Barcode Plate, prepare Tagmentation-Nuclei Mix in an 8-tube strip by adding **108 µL** of the diluted Tagmentation-Nuclei Mix to each tube. |  |

6. Using a multichannel pipette, add **8 µL** of Tagmentation-Nuclei Mix into the bottom of each well of the three Met Tagment Barcode Plates.

**Caution**: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

**Note:** Write down the used well ID and Met Tagment Barcode Plate ID for each biological sample to demultiplex samples during data analysis.

7. Seal the Met Tagment Barcode Plates and place on a plate shaker.
8. Shake the plate at **2000 rpm** for **30 seconds**.
9. Centrifuge the Met Tagment Barcode Plates at **100 x g** for **30 seconds** at **4°C**.

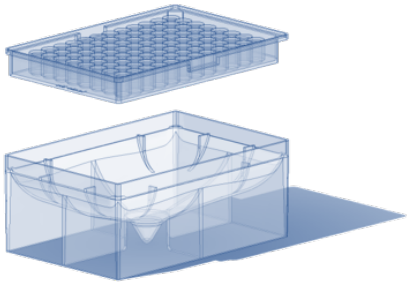
10. Incubate the Met Tagment Barcode Plates in three **pre-heated** thermocyclers according to Program 2.

> **Caution**: If the plates cannot be incubated at the same time, keep the remaining prepared Met Tagment Barcode Plates on ice until a thermocycler is available.

*Program 2: Met Nuclei Tagmentation*

| Lid Temperature | Reaction Volume |
|---|---|
| 65°C | 10 µL |
| **Temperature** | **Time** |
| 55°C | 15 min |

11. Immediately place the Met Tagment Barcode Plates **on ice** for **5 minutes.**
12. Unpack the ScaleBio Sample Collection Funnel and place **on ice**. **Do not touch the inside of the collection funnel**.
13. Shake one of the Met Tagment Barcode Plates at **2000 rpm** for **30 seconds**.
14. Check the bottom of the Met Tagment Barcode Plate for debris and moisture. If debris or moisture is present, wipe the bottom of the plate with a clean Kimwipe coated in 70% ethanol, followed by a dry Kimwipe until the plate bottom is fully dry.
15. Unseal the Met Tagment Barcode Plate, place the collection funnel on top, and quickly invert the assembly according to the figure below.



16. Centrifuge the collection funnel with an appropriately weighted balance at **400 x g** for **3 minutes** at **4°C**.
17. Transfer pooled nuclei (~1 mL) from the collection funnel into a **5-mL DNA LoBind** tube and place **on ice**.
18. Repeat steps 13-17 for the other two Met Tagment Barcode Plates, respectively, using the same collection funnel and 5-mL DNA LoBind tube.
19. Centrifuge the pooled nuclei at **500 x g** for **5 minutes** at **4°C**.
20. Carefully remove supernatant without disturbing the pellet, leaving ~100 µL of residual volume behind.

> **Caution**: Depending on nuclei type and the total number of nuclei loaded into the Met Tagment Barcode Plates, the nuclei pellet might not be visible.

21. Resuspend the nuclei in **1 mL** of MetWashBuffer+.

22. Add DNA staining dye (DAPI, DRAQ5™, or equivalent) to the pooled nuclei and transfer the pooled nuclei to a FACS tube.
23. Keep FACS tube **on ice** until sorting.

**Caution**: Nuclei sorting must begin within 2 hours of completing Indexed Tagmentation. Once sorting begins, sorting must be finished within 2 hours. Keep nuclei on ice until ready to sort.

**Nuclei Sorting:** In a 4°C sorting chamber and using a 100 µm nozzle, gate nuclei that are **positive** for DNA staining dye and sort **24 nuclei per well** into **every Sorting Plate**.

24. Seal the Sorting Plates containing nuclei.
25. Centrifuge the Sorting Plates at **1000 x g** for **30 seconds** at **4°C**.
26. Place the Sorting Plates **on ice**.

**Safe stopping point.** Sorting Plates can be stored up to 5 days at -20°C.

27. Pre-heat two thermocyclers (Program 3) and hold at 50°C.
28. Incubate the Sorting Plates in **pre-heated** thermocyclers according to Program 3. If the Sorting Plates are previously frozen, thaw the Sorting Plates on ice and then centrifuge the Sorting Plates at **1000 x g** for **30 seconds** at 4**°C** before starting Program 3.

**Caution**: If plates cannot be incubated at the same time, keep the second plate on ice until the thermocycler is available.

*Program 3: Nuclei Lysis*

| Lid Temperature | Reaction Volume |
|---|---|
| 55°C | 10 µL |
| **Temperature** | **Time** |
| 50°C | 20 min |
| 4°C | ∞ |

29. Centrifuge the Post-Lysis Sorting Plates at **1000 x g** for **1 minute** at **RT**, then place **on ice**.
30. Store the lysed Sorting Plates or proceed to *Step 4: Bisulfite Conversion*.

**Safe stopping point**. Post-Lysis Sorting Plates can be stored up to 6 months at -20°C.

## Step 4: Bisulfite Conversion

**Caution**: Post-Bisulfite Conversion Plates must be processed through cleanup within 20 hours after conversion.

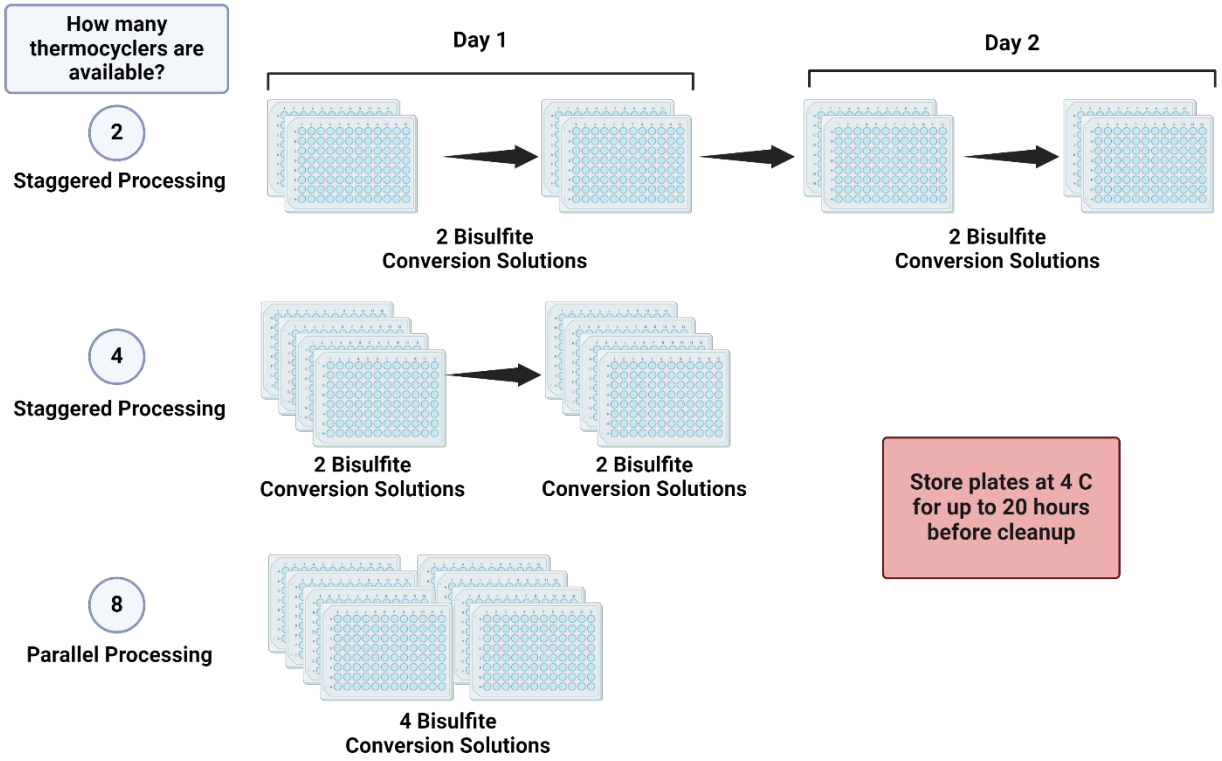
Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Met Bisulfite Conversion Module | Bisulfite Conv. Reagent | - | RT | RT | - | - |
| | Rehydration Buffer | - | RT | RT | - | - |
| | Dilution Buffer | - | RT | RT | - | - |
| | Reaction Buffer | Black | RT | RT | - | - |

**Before you begin:**

- Process **two to four** Sorting Plates **per** Bisulfite Conversion Solution. Reagents in the kit are enough for 4 Bisulfite Conversion Solutions.
- Plates must be processed in parallel and/or staggered on multiple thermocyclers (see Figure 8).
- Pre-heat two - eight thermocyclers (Program 4) and hold at 98°C.
- Bring Sorting Plates to **room temperature** before processing.
- Set swing-bucket centrifuge for 96-well plates to room temperature.

*Figure 8: Plate Logistics for Bisulfite Conversion*



**Procedure:** Bisulfite Conversion of **Two** Post-Lysis Sorting Plates in Parallel:

1. Dissolve the Bisulfite Conv. Reagent at **room temperature** by adding reagents from Table 7 **directly** to the Bisulfite Conv. Reagent bottle.

*Table 7: Dissolving the Bisulfite Conv. Reagent*

| Reagent | Volume (mL) |
|---|---|
| Bisulfite Conv. Reagent * | 1 bottle |
| Rehydration Buffer | 7.9 |
| Dilution Buffer | 3 |

2. For **20 minutes**, rotate at a minimum of 20 rotations per minute or vortex at maximum speed.
3. Using a P1000 pipette with a P1000 pipette tip, verify that the Bisulfite Conv. Reagent is fully dissolved.
4. To prepare the Bisulfite Conversion Solution, use a P1000 pipette tip to slowly add **1.6 mL** of Reaction Buffer to the dissolved Bisulfite Conv. Reagent (Note: Reaction Buffer is viscous).
5. Vortex Bisulfite Conversion Solution for **10 seconds**.

**Caution**: If plate incubation is staggered, keep Bisulfite Conversion Solution at 4°C away from light, and the next batch of Sorting Plates at -20°C. Use Bisulfite Conversion Solution within 4 hours.

6. If Sorting Plates had been stored at -20°C, centrifuge both Sorting Plates at **1000 x g** for **1 minute** at **RT**.
7. Unseal the Sorting Plates.
8. At **room temperature**, aliquot **7 mL** Bisulfite Conversion Solution into a reservoir.
9. Using a multichannel pipette, distribute **28 µL** of Bisulfite Conversion Solution into the bottom of each well of the Sorting Plates.

**Caution**: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

10. Seal the Sorting Plates and place on a plate shaker.
11. Shake the Sorting Plates at **2000 rpm** for **30 seconds**.
12. Centrifuge the Sorting Plates at **1000 x g** for **30 seconds** at **RT**.

13. Place the Sorting Plates in **pre-heated** thermocyclers and skip the holding step to continue Program 4.

*Program 4: Bisulfite Conversion*

| Lid Temperature | Reaction Volume |
|---|---|
| 105°C | 30 µL |
| **Temperature** | **Time** |
| 98°C | ∞ |
| *Skip the HOLD step after placing the plate in the thermocycler.* | |
| 98°C | 8 min |
| 64°C | 3.5 hours |
| 4°C | ∞ |

14. After bisulfite conversion, store the Post-Bisulfite Conversion Plates or proceed to *Step 5: Post-Bisulfite Conversion Cleanup and Elution*.


**Safe stopping point.** Post-Bisulfite Conversion Plates can be stored up to 20 hours at 4°C.

## Step 5: Post-Bisulfite Conversion Cleanup and Elution

**Caution**: Post-Bisulfite Conversion Plates must be processed through cleanup within 20 hours after conversion. Process two plates in parallel.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Met Conversion Clean-up Module | Wash Buffer (Pure) | - | RT | RT | - | - |
| | Bisulfite Clean-up Buffer | - | RT | RT | - | - |
| | DNA Spin Plate | - | RT | RT | - | - |
| | Elution Buffer | - | RT | RT | - | - |
| | DNA Binding Buffer | - | RT | RT | - | - |
| Other Vendors | Ethanol (pure)<br>2.0-mL 96-well deep well plates<br>96-well semi-skirted PCR plates<br>Reservoirs | | | | | |

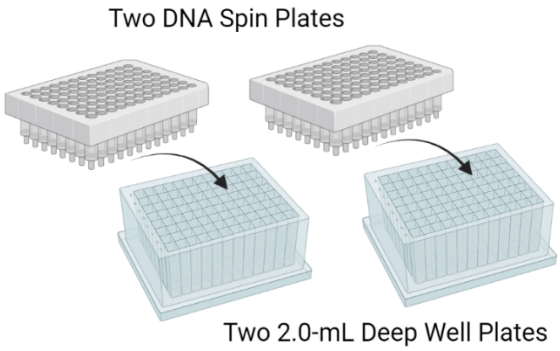
**Before you begin:**

- Set swing-bucket centrifuge for 96-well deep well plates to room temperature.

**Procedure:** Cleanup of **Two** Post-Bisulfite Conversion Plates in Parallel

1. Centrifuge two Post-Bisulfite Conversion Plates at **1000 x g** for **1 minute** at **RT**.
2. Unseal the plates.
3. Place two DNA Spin Plates on top of two clean 2.0-mL 96-well deep well plates (Figure 9). Keep the plate assembly until step 19.

*Figure 9: DNA Spin Plates on top of 2.0-mL Deep Well Plates*




4. Distribute **25 mL** of DNA Binding Buffer into a clean reservoir.

5. Using a multichannel pipette, add **120 µL** of DNA Binding Buffer into the bottom of each well of the Post-Bisulfite Conversion Plates. Using the same pipette tip, pipette mix **six times** and transfer the total volume **(~150 µL)** to the DNA Spin Plates without touching the column membrane.

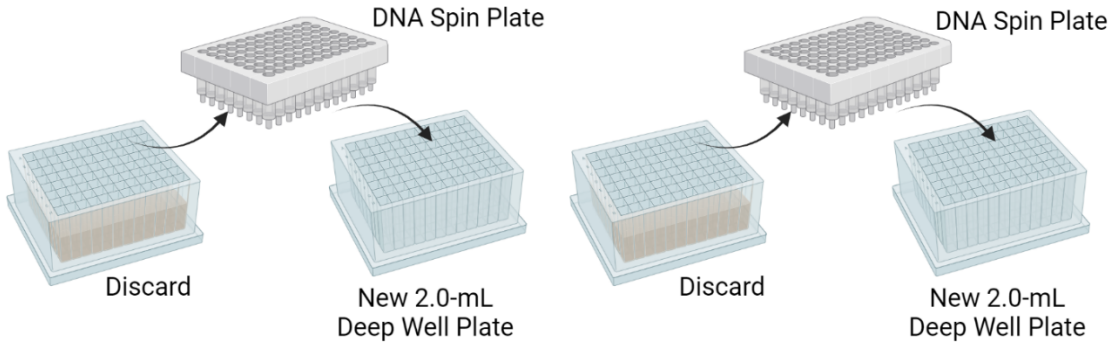
**Caution**: Change pipette tips between wells to avoid cross-contamination.

6. Centrifuge the plate assembly at **2200 x g** for **8 minutes** at **RT**.

**Note:** During spinning, prepare Wash Buffer (step 7).

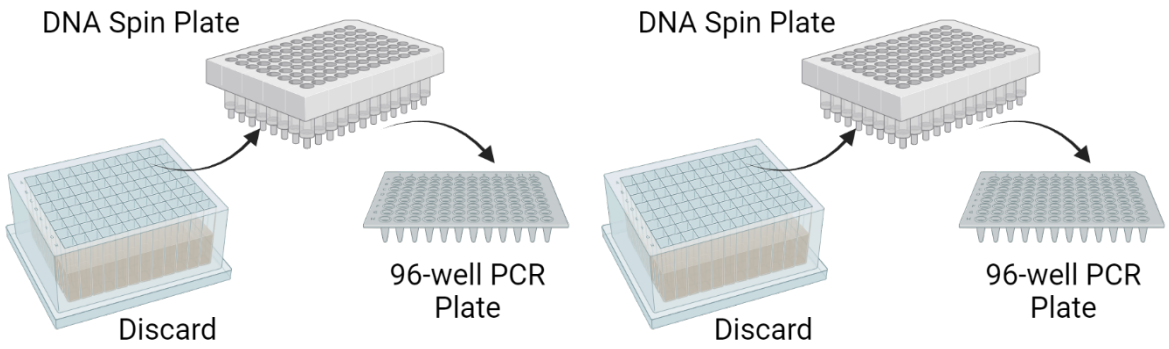
7. At **room temperature**, add **52 mL** of 100% ethanol to one Wash Buffer bottle. Invert bottle to mix.
8. Aliquot **21 mL** Wash Buffer into a clean reservoir to be used in step 9.
9. Using a multichannel pipette, add **100 µL** of Wash Buffer into the bottom of each well of the DNA Spin Plates without touching the column membrane.
10. Centrifuge the plate assembly at **2200 x g** for **8 minutes** at **RT**.
11. Aliquot **11 mL** Bisulfite Clean-up Buffer into a clean reservoir.
12. Using a multichannel pipette, add **50 µL** of Bisulfite Clean-up Buffer to the bottom of each well of the DNA Spin Plates without touching the column membrane.
13. Incubate the plate assembly for **15-20 minutes** at **room temperature**.
14. Centrifuge the plate assembly at **2200 x g** for **8 minutes** at **RT**.
15. Aliquot **42 mL** Wash Buffer into a clean reservoir to be used in step 16.
16. Using a multichannel pipette, add **100 µL** of Wash Buffer into the bottom of each well of the DNA Spin Plates without touching the column membrane.
17. Centrifuge the plate assembly at **2200 x g** for **8 minutes** at **RT**.
18. Repeat steps 16-17 for a total of two washes.
19. Transfer the two DNA Spin Plates on top of two clean 2.0-mL 96-well deep well plates (Figure 10); discard the used 2.0-mL 96-well deep well plates.

*Figure 10: Transfer DNA Spin Plates to new 2.0-mL Deep Well Plates*



20. Centrifuge the new plate assembly at **2200 x g** for **5 minutes** at **RT**.
21. Transfer the two DNA Spin Plates on top of two clean 96-well LoBind semi-skirted PCR plates (Figure 11); discard the used 2.0-mL 96-well deep well plates.

*Figure 11: Transfer DNA Spin Plates to new 96-well LoBind semi-skirted PCR Plates*




22. At **room temperature**, aliquot Elution Buffer into each tube of a tube strip:
    a. For an 8-tube strip, aliquot **210 µL** into each tube.
    b. For a 12-tube strip, aliquot **160 µL** into each tube.
23. Using a multichannel pipette, transfer **8 µL** of Elution Buffer from the tube strips to the bottom of each well of the DNA Spin Plates without touching the column membrane.
24. Carefully tap the plate assembly on the bench to ensure the Elution Buffer covers the column membrane in the bottom of the well completely.
25. Incubate for **1 minute**.
26. Centrifuge the plate assembly at **2200 x g** for **8 minutes** at **RT**.
27. Discard the used DNA Spin Plates.
28. Seal the Elution Plates for storage, or proceed to *Step 6: Ligation*.


**Safe stopping point**. Eluted DNA can be stored for up to 2 months at -20°C.

## Step 6: Ligation

**Note:** Process two plates in parallel.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Met Ligation and Index PCR Module v1.1 – Large | Ligation Master Mix | Clear | -20°C | On ice | ✓ | ✓ |
| | Met Adaptor Primers | Clear | -20°C | On ice | ✗ | ✓ |

**Before you begin:**

- If frozen, thaw Elution Plates on ice.
- Pre-cool swing-bucket centrifuge for 96-well plates to 4°C.
- Pre-heat two thermocyclers (Program 5) and hold at 95°C.

**Procedure:**

1. If Elution Plates had been stored at -20°C, centrifuge the Elution Plates at **1000 x g** for **1 minute** at **4°C**.
2. Incubate the Elution Plates in **pre-heated** thermocyclers according to Program 5:

**Caution**: If plates cannot be incubated at the same time, keep the second plate on ice until the thermocycler is available.

*Program 5: DNA Denaturation*

| Lid Temperature | Reaction Volume |
|---|---|
| 105°C | 10 µL |
| **Temperature** | **Time** |
| 95°C | 3 min |

3. Immediately move the Elution Plates **on ice** for **2 minutes**.
4. Start Program 6 on two thermocyclers and hold at 37°C.
5. Centrifuge Elution Plates at **1000 x g** for **1 minute** at **4°C**.
6. **On ice**, distribute Met Adaptor Primers to each tube of a tube strip (enough for two plates):
    a. For an **8-tube strip**, aliquot **30 µL** into each tube.
    b. For a **12-tube strip**, aliquot **20 µL** into each tube.

7. Using a multichannel pipette, add **1 µL** of Met Adaptor Primers to the bottom of each well of two Elution Plates **on ice**.

**Caution**: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

8. Vortex the Ligation Master Mix for **30 seconds**, spin down briefly and place **on ice**.
9. **On ice**, distribute the Ligation Master Mix into each tube of a tube strip (enough for two plates):
    a. For an **8-tube strip**, aliquot **132 µL** into each tube.
    b. For a **12-tube strip**, aliquot **88 µL** into each tube.
10. To prepare the Ligation Plates use a multichannel pipette to add **5 µL** of Ligation Master Mix to the bottom of each well of the Elution Plates.

**Caution**: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

11. Seal the Ligation Plates and shake on a plate shaker at **2000 rpm** for **30 seconds**.
12. Centrifuge the Ligation Plates at **1000 x g** for **30 seconds** at **4°C**.
13. Incubate the Ligation Plates in two **pre-heated** thermocyclers according to Program 6.

**Caution**: If plates cannot be incubated at the same time, keep the second plate on ice until the thermocycler is available.

*Program 6: Ligation*

| **Lid Temperature** | **Reaction Volume** |
|---|---|
| 75°C | 15 µL |
| **Temperature** | **Time** |
| 37°C | ∞ |
| *Skip the HOLD step after placing the plate in the thermocycler.* | |
| 37°C | 45 min |
| 65°C | 20 min |
| 4°C | ∞ |

14. Store the Ligation Plates or proceed to *Step 7: Indexed PCR*.


**Safe stopping point**. Ligation Plates can be stored for up to 24 hours at -20°C.

## Step 7: Indexed PCR

**Note:** Process two plates in parallel.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Met Ligation and Index PCR Module v1.1 – Large | Met Index PCR Master Mix | - | -20°C | On ice | ✓ | ✗ |
| | Met Index PCR Plate 1 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 2 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 3 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 4 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 5 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 6 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 7 | - | -20°C | On ice | ✗ | ✓ |
| | Met Index PCR Plate 8 | - | -20°C | On ice | ✗ | ✓ |
| Other Vendors | Nuclease-free water<br>15-mL conical tube | | | | | |

**Before you begin:**

- Thaw Ligation Plates on ice if frozen.
- Pre-cool a swing-bucket centrifuge for 96-well plates to 4°C.

**Procedure:**

1. Centrifuge two Ligation Plates at **1000 x g** for **1 minute** at **4°C**.
2. **On ice**, prepare the Amplification Master Mix by mixing **6 mL** of Met Index PCR Master Mix with **2.4 mL** of nuclease-free water in a 15-mL conical tube.
3. Vortex until the Amplification Master Mix is homogeneous, then transfer to a reagent reservoir **on ice**.
4. **On ice**, using a multichannel pipette, distribute **35 µL** of Amplification Master Mix into the bottom of each well of the Ligation Plates.

**Caution**: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

5. Centrifuge the Met Index PCR Plates at **200 x g** for **1 minute** at **4°C** and remove the seals.

6. To prepare the Index PCR Plates, use a multichannel pipette to transfer **4 µL from corresponding wells** from one of the **Met Index PCR Plates** to the Ligation Plates.

**Caution**: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

**Note:** Write down the Met Index PCR Plate name used for each Ligation Plate for downstream data analysis.

7. Seal the Index PCR Plates and place on a plate shaker.
8. Shake the plates at **2000 rpm** for **30 seconds**.
9. Briefly spin down the plates at **1000 x g** for **30 seconds** at **4°C**.
10. Incubate the Index PCR Plates in two thermocyclers according to Program 7.

**Caution**: If the plates cannot be incubated at the same time, keep the second plate on ice until the thermocycler is available.

*Program 7: Indexed PCR*

| **Lid Temperature** | | **Reaction Volume** | |
|---|---|---|---|
| 105°C | | 50 µL | |
| **Step** | **Temperature** | **Time** | **Cycles** |
| 1 | 98°C | 3 min | 1 |
| 2 | 98°C | 10 sec | 15 (steps 2-4) |
| 3 | 58°C | 30 sec | |
| 4 | 72°C | 30 sec | |
| 5 | 72°C | 1 min | 1 |
| 6 | 4°C | ∞ | 1 |

11. Store the Index PCR Plates or proceed to *Step 8: Purification of Index PCR Products*.

**Safe stopping point**. Amplified library can be stored for up to 2 months at -20°C.

## Step 8: Purification of Index PCR Products

**Note:** Process two plates in parallel.

Instructions below are for purification of **two individual libraries**, one per Index PCR Plate, pooling **all wells** per plate, respectively.

Users may wish to perform a quality control (QC) check by sequencing one column from each Index PCR Plate (representing ~384 nuclei) before proceeding to sequencing the entire plate. For purification for QC sequencing, proceed directly to *Appendix B: Purification for QC Sequencing*. For purification using SPRIselect beads only, proceed directly to *Appendix D: Purification of Index PCR Products using only SPRIselect*.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material |
|---|---|
| Other Vendors | Elution Buffer (10 mM Tris-Cl, pH 8.5)<br>Nuclease-free water<br>Ethanol, pure<br>SPRIselect beads<br>5-mL DNA LoBind tubes<br>NucleoSpin Gel and PCR Clean-up Kit |

**Before you begin:**

- Prepare fresh 80% ethanol (1 mL per library, or per Index PCR Plate).
- Perform cleanup at room temperature.

**Procedure:**

NucleoSpin® Gel and PCR Cleanup

1. Bring two Index PCR Plates to **room temperature** and briefly spin down.
2. Pool **10 µL** from each well of the Index PCR Plate into a **5.0-mL DNA LoBind** tube (**960 µL** total volume) following the guidance below:
   a. For an **8-tube strip**, transfer **10 µL** with a multi-channel pipette from each plate column into the tube strip. Using a single channel pipette, pipette mix the pool and transfer **120 µL** from each strip tube to the 5.0-mL tube.
   b. For a **12-tube strip**, transfer **10 µL** with a multi-channel pipette from each plate row into the tube strip. Using a single channel pipette, pipette mix the pool and transfer **80 µL** from each strip tube to the 5.0-mL tube.

**Caution**: Change pipette tips between wells to avoid cross-contamination.

3. Purify the library with the NucleoSpin Gel and PCR Clean-up Kit. Follow the manufacturer's PCR clean-up protocol to concentrate the library.
    a. Add **1920 µL** of NT1 Binding Buffer to **960 µL** pooled library.
    b. Vortex briefly.
    c. Bind the DNA to one NucleoSpin Gel and PCR Clean-up Column in four successive centrifugation steps with **720 µL** each.
4. For the final elution step, elute the library in **50 µL** of Elution Buffer (NucleoSpin Gel and PCR Clean-up Kit).

First SPRIselect Cleanup

1. Transfer **50 µL** of the eluted library to a new 0.2-mL PCR tube.
2. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
3. Transfer **40 µL** of SPRIselect beads (0.8X) to the 0.2-mL tube containing eluted library.
4. Vortex to mix.
5. Incubate at **room temperature** for **5 minutes**.
6. Briefly spin the tube and place on the magnetic stand.
7. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
8. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
9. Incubate the tube on the magnetic stand for **30 seconds**.
10. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
11. Repeat steps 8-10 for a total of two washes.
12. Briefly spin the tube and place on the magnetic stand.
13. Remove residual 80% ethanol without disturbing the beads.
14. Air dry the beads for **1 minute**.
15. Remove the tube from the magnetic stand and add **50 µL** Elution Buffer.
16. Vortex to mix.
17. Incubate the tube **off** the magnetic stand for **5 minutes**.
18. Briefly spin the tube and place on the magnet stand until the solution is clear.
19. Transfer the supernatant to a new 0.2-mL PCR tube and discard the beads.

Second SPRIselect Cleanup

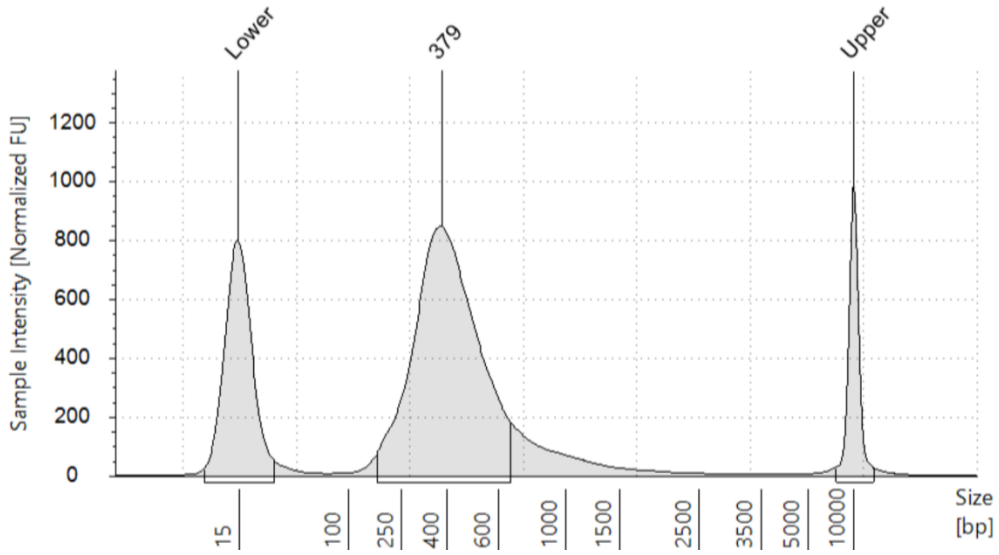
1. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
2. Add **40 µL** of SPRIselect beads (0.8X) into the 0.2-mL tube.
3. Vortex to mix.
4. Incubate at **room temperature** for **5 minutes**.
5. Briefly spin the tube and place on the magnetic stand.
6. Once the solution is clear, carefully remove the supernatant without disturbing the beads.
7. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
8. Incubate the tube on the magnetic stand for **30 seconds**.

9. Once the solution is clear, carefully remove the supernatant without disturbing the beads.
10. Repeat steps 7-9 for a total of two washes.
11. Briefly spin the tube and place on the magnetic stand.
12. Remove residual 80% ethanol without disturbing the beads.
13. Air dry the beads for **1 minute**.
14. Remove the tube from the magnetic stand and add **20 µL** Elution Buffer to the beads.
15. Vortex to mix.
16. Incubate the tube **off** the magnetic stand for **5 minutes**.
17. Briefly spin the tube and place on the magnet stand until the solution is clear.
18. Transfer the supernatant to a new 0.2-mL PCR tube and discard the beads.
19. Proceed to *Step 9: Library Quality Control*.

## Step 9: Library Quality Control

1. Determine the average fragment size of the library using Agilent High Sensitivity D5000 ScreenTape System for the 4200 TapeStation instrument (or equivalent reagent kit for high sensitivity DNA fragment analysis for an alternative system). Example library traces are shown in Figure 12. The library was diluted to 1 ng/µL and loaded with 2 µL on TapeStation; it represents an experiment consisting of human PBMC samples.

*Figure 12: Representative Library Traces on TapeStation*




2. Determine library concentration for sequencing with a commercial qPCR kit for Illumina libraries according to manufacturer's protocol (for example, NEBNext Library Quant Kit for Illumina or equivalent kit). Perform library dilution and clustering according to sequencing manufacturers parameters.

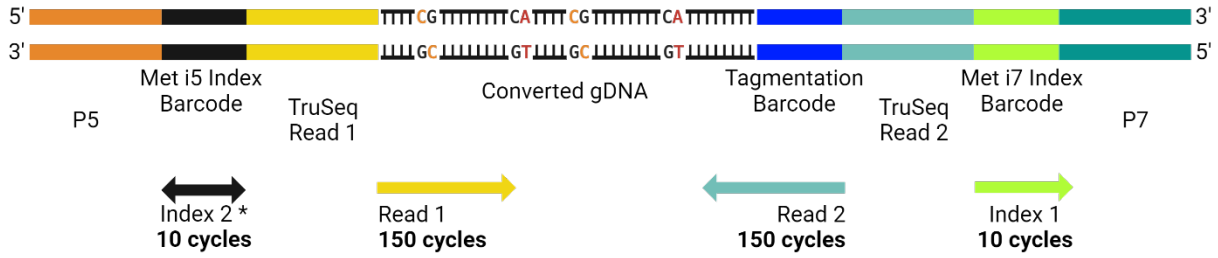
**Caution**: It is strongly recommended to quantify the library by qPCR. Failing to do so may lead to under-clustering on sequencing flow cell.

3. Pool the purified libraries from each plate together in equimolar quantities to create one pooled library.
4. Store the purified library or proceed to *Step 10: Sequencing Parameters.*

**Note:** (Optional) Purified libraries can be used directly as input DNA for Target Enrichment workflows (any hybridization-based workflow may be compatible). The Twist Human Methylome Panel (PNs 105517, 105520, 105521) from Twist Bioscience has been demonstrated. Please follow the manufacturer's protocol.

**Safe stopping point**. Purified library can be stored long term at -20°C.

## Step 10: Sequencing Parameters




* orientation depends on sequencer and sequencing chemistry

| Read | Length | Purpose |
|---|---|---|
| Read 1 | 150 cycles | Converted gDNA |
| Read 2 | 150 cycles | Tagmentation Barcode and Converted gDNA |
| Index 1 | 10 cycles | Met i7 Index Barcode |
| Index 2 | 10 cycles | Met i5 Index Barcode |

| Sequencing Depth |
|---|
| We recommend a minimum sequencing depth of **1 million read pairs per nuclei**. Expected nuclei recovery is ~85-90% of the processed ~18,400 nuclei. |

| Recommended Final Loading Concentrations | |
|---|---|
| NovaSeq X | 150 pM |
| NovaSeq 6000 | 150 pM |
| NextSeq 2000 (XLEAP SBS) | 0.5 nM (on-board denaturation) |
| NextSeq 2000 (Standard SBS) | 0.5 nM (on-board denaturation) |
| PhiX | 5% |

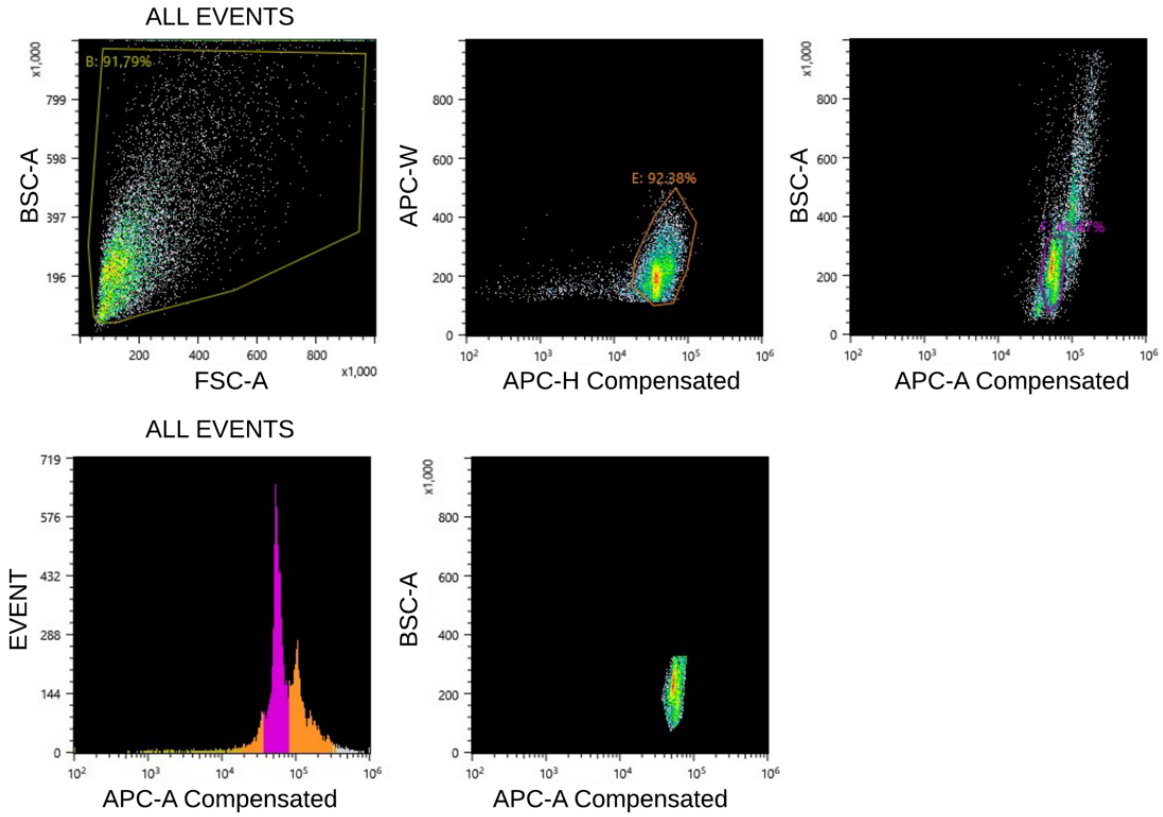
# Appendix A: Fluorescence Activated Cell Sorting (FACS) Settings

- Tested Sorter Models: Aria II, Influx, Sony SH800, Aria Fusion, Aria IIu and Symphony S6 Sorter.
- Recommended conditions:
  - Nozzle Size: 100 µm
  - Sample chamber: 4-5°C
- DNA Staining Dye Recommendations:
  - DRAQ5™: 5 µL of 5 mM per pool of the tagmented nuclei
  - DAPI: 10 µL of 100 µM per pool of the tagmented nuclei

Example sorting times for different amounts of PBMC nuclei per well used for tagmentation:

| Nuclei/well during tagmentation | Expected sorting time for each plate (minutes) |
|---|---|
| 5,000 | 3 – 5 |
| 2,500 | 5 – 10 |
| 1,000 | 15 – 20 |

*Figure 13: Gate Examples of PBMC Nuclei Sorting (stained by DRAQ5™ using Sony SH800 Sorter)*



## Appendix B: Purification for QC Sequencing

Instructions below are for purification of a **single library** consisting of **one column from two Index PCR Plates** (representing ~384 nuclei).

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material |
|---|---|
| Other Vendors | Elution Buffer (10 mM Tris-Cl, pH 8.5)<br>Nuclease-free water<br>Ethanol, pure<br>SPRIselect beads<br>1.5-mL DNA LoBind tubes<br>NucleoSpin Gel and PCR Clean-up Kit |

**Before you begin:**

- Prepare fresh 80% ethanol (1 mL per library).
- Perform cleanup at room temperature.

**Procedure:**

NucleoSpin Gel and PCR Cleanup

1. Bring two Index PCR Plates to **room temperature**.
2. Pool **10 µL** from each well of **one column from each Index PCR Plate** into a 1.5-mL DNA LoBind tube (**160 µL** total volume from 16 wells). When pooling, select a different column in each plate, ensuring that **no two plates share the same column for pooling** (e.g. if pooling from column 1 from one plate, do not pool from column 1 from the other plate).

**Caution**: Change pipette tips between wells to avoid cross-contamination.

**Caution**: Pooling from the same column from both plates may lead to index hopping during sequencing.

3. Purify the library with the NucleoSpin Gel and PCR Clean-up Kit. Follow the manufacturer's PCR clean-up protocol to concentrate the library.
4. For the final elution step, elute the library in **50 µL** of Elution Buffer (NucleoSpin Gel and PCR Clean-up Kit).

First SPRIselect Cleanup

1. Transfer **50 µL** of the eluted library to a new 0.2-mL PCR tube.
2. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
3. Transfer **40 µL** of SPRIselect beads (0.8X) to the 0.2-mL tube containing the library.

4. Vortex to mix.
5. Incubate at **room temperature** for **5 minutes**.
6. Briefly spin the tube and place on the magnetic stand.
7. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
8. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
9. Incubate the tube on the magnetic stand for **30 seconds**.
10. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
11. Repeat steps 8-10 for a total of two washes.
12. Briefly spin the tube and place on the magnetic stand.
13. Remove residual 80% ethanol without disturbing the beads.
14. Air dry the beads for **1 minute**.
15. Remove the tube from the magnetic stand and add **50 µL** Elution Buffer.
16. Vortex to mix.
17. Incubate the tube **off** the magnetic stand for **5 minutes**.
18. Briefly spin the tube and place on the magnet stand until the solution is clear.
19. Transfer the supernatant to a new 0.2-mL PCR tube and discard the beads.

Second SPRIselect Cleanup

1. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
2. Add **40 µL** of SPRIselect beads (0.8X) into the 0.2-mL tube.
3. Vortex to mix.
4. Incubate at **room temperature** for **5 minutes**.
5. Briefly spin the tube and place on the magnetic stand.
6. Once the solution is clear, carefully remove the supernatant without disturbing the beads.
7. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
8. Incubate the tube on the magnetic stand for **30 seconds**.
9. Once the solution is clear, carefully remove the supernatant without disturbing the beads.
10. Repeat steps 7-9 for a total of two washes.
11. Briefly spin the tube and place on the magnetic stand.
12. Remove residual 80% ethanol without disturbing the beads.
13. Air dry the beads for **1 minute**.
14. Remove the tube from the magnetic stand and add **20 µL** Elution Buffer to the beads.
15. Vortex to mix.
16. Incubate the tube **off** the magnetic stand for **5 minutes**.
17. Briefly spin the tube and place on the magnet stand until the solution is clear.
18. Transfer the supernatant to a new 0.2-mL PCR tube and discard the beads.
19. Proceed to *Step 9: Library Quality Control*.

## Appendix C: Post-Fixation Nuclei Storage in Nuclei Storage Buffer

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material | Cap Color | Take from: | Place at: | Brief Vortex | Brief Spin |
|---|---|---|---|---|---|---|
| Nuclei Resuspension Kit | Met Wash Buffer | - | -20°C | On ice | - | - |
| | Met Wash Buffer Additive | - | -20°C | RT | - | - |
| | Nuclei Storage Buffer | - | -20°C | On ice | - | - |

1. After **5 minutes** incubation on ice with glycine, centrifuge the nuclei at **500 x g** for **5 minutes** at **4°C**.
2. In a chemical fume hood, carefully remove supernatant without disturbing the pellet.
3. Resuspend in **500 µL** of Nuclei Storage Buffer to make the nuclei suspension 2 million nuclei per mL.
4. Store nuclei directly at **-80°C**.


**Safe Stopping Point**. Fixed nuclei can be stored up to 4 weeks at -80°C.

5. Thaw the frozen nuclei on ice.
6. Centrifuge the nuclei at **500 x g** for **5 minutes** at **4°C**.
7. Carefully remove supernatant without disturbing the pellet.
8. Resuspend nuclei in **1 mL** MetWashBuffer+.
9. Centrifuge the nuclei at **500 x g** for **5 minutes** at **4°C**.
10. Carefully remove supernatant without disturbing the pellet.
11. **On ice**, gently add **970 µL** of MetWashBuffer+ without disturbing the pellet.
12. Proceed directly to *Step 2: Nucleosome Disruption*.

# Appendix D: Purification of Index PCR Products using only SPRIselect

**Note:** Process two plates in parallel.

Instructions below are for purification of **two individual libraries**, one per Index PCR Plate, pooling **all wells** per plate, respectively, using SPRIselect only.

Please review the table below to prepare reagents before starting this protocol section:

| Source | Material |
|---|---|
| Other Vendors | Elution Buffer (10 mM Tris-Cl, pH 8.5)<br>Nuclease-free water<br>Ethanol, pure<br>SPRIselect beads<br>2-mL DNA LoBind tubes<br>0.2-mL PCR tube strips (nuclease-free)<br>Magnetic stand for 2-mL tubes<br>Magnetic stand for 0.2-mL strip tubes |

**Before you begin:**

- Prepare fresh 80% ethanol (5 mL per library, or per Index PCR Plate).
- Perform cleanup at room temperature.

**Procedure:**

First SPRIselect Cleanup

1. Bring two Index PCR Plates to **room temperature** and briefly spin down.
2. Pool **10 µL** from each well of an Index PCR Plate into a **2.0-mL DNA LoBind** tube (**960 µL** total volume) following the guidance below:
   a. For an **8-tube strip**, transfer **10 µL** with a multi-channel pipette from each plate column into the tube strip. Using a single channel pipette, pipette mix the pool and transfer **120 µL** from each strip tube to the 2.0-mL tube.
   b. For a **12-tube strip**, transfer **10 µL** with a multi-channel pipette from each plate row into the tube strip. Using a single channel pipette, pipette mix the pool and transfer **80 µL** from each strip tube to the 2.0-mL tube.
3. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
4. Transfer **768 µL** of SPRIselect beads (0.8X) to the 2.0-mL tube containing Indexed PCR products.
5. Vortex to mix.
6. Incubate at **room temperature** for **5 minutes**.
7. Briefly spin the tube and place on the magnetic stand for **5 minutes**.
8. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.

9. Keep the tube on the magnetic stand and add **2 mL** of 80% ethanol to the beads.
10. Incubate the tube on the magnetic stand for **1 minute**.
11. Carefully remove and discard the supernatant without disturbing the beads.
12. Repeat steps 9-11 for a total of two washes.
13. Briefly spin the tube and place on the magnetic stand.
14. Remove residual 80% ethanol without disturbing the beads.
15. Air dry the beads for **4 minutes**.
16. Remove the tube from the magnetic stand and add **50 µL** Elution Buffer.
17. Vortex to mix.
18. Incubate the tube **off** the magnetic stand for **5 minutes**.
19. Briefly spin the tube and place on the magnet stand until the solution is clear.
20. Transfer the supernatant to a new 0.2-mL PCR tube and discard the beads.

Second SPRIselect Cleanup

1. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
2. Add **40 µL** of SPRIselect beads (0.8X) into the 0.2-mL tube.
3. Vortex to mix.
4. Incubate at **room temperature** for **5 minutes**.
5. Briefly spin the tube and place on the magnetic stand.
6. Once the solution is clear, carefully remove the supernatant without disturbing the beads.
7. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
8. Incubate the tube on the magnetic stand for **30 seconds**.
9. Carefully remove the supernatant without disturbing the beads.
10. Repeat steps 7-9 for a total of two washes.
11. Briefly spin the tube and place on the magnetic stand.
12. Remove residual 80% ethanol without disturbing the beads.
13. Air dry the beads for **1 minute**.
14. Remove the tube from the magnetic stand and add **20 µL** Elution Buffer to the beads.
15. Vortex to mix.
16. Incubate the tube **off** the magnetic stand for **5 minutes**.
17. Briefly spin the tube and place on the magnet stand until the solution is clear.
18. Transfer the supernatant to a new 0.2-mL PCR tube and discard the beads.
19. Proceed to *Step 9: Library Quality Control*.

## Document Revision History

| Revision | Revision Date | Document ID | Changes |
|---|---|---|---|
| Rev A | Sep 2024 | 1102479 | Initial release. |
| Rev B | Oct 2024 | 1102479 | Changed nuclei resuspension buffer requirements.<br>Updated pooling instructions for *Appendix B: Purification for QC Sequencing*. |
| Rev C | Jan 2025 | 1102479 | Added *Appendix D: Purification of Index PCR Products using only SPRIselect*. |