

Low-Input MBD-based Methylated DNA Enrichment Protocol

Optimized MethyMiner Protocol

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Materials Needed

- **MethylMiner™ Kit (Invitrogen)**
 - Low-Salt Elution Buffer (0 M NaCl)
 - High-Salt Elution Buffer (2 M NaCl)
 - 5x Bind/Wash Buffer
 - Dynabeads® M-280 Streptavidin
 - MBD-Biotin Protein (0.5 mg/ml)
 - Glycogen (20 µg/µl)
- **100% ethanol**
- **70% ethanol**
- **3 M Sodium Acetate, pH 5.2**
- **Magnetic Tube-rack**
- **Rotating Mixer**

For high-throughput:

- **ABgene Storage Plate, 96-well, 1.2 mL, square well, U-bottomed (AB1127)**
- **Microplate sealing foil**
- **Orbital shaker**
- **96-well microplate magnet**

Solutions

1x Bind/Wash Buffer (BWB)

- **20% 5x Bind/Wash Buffer + 80% nuclease-free water (v/v)**

Wash Buffer (250 mM NaCl)

- **12.5% High Salt Elution Buffer + 87.5% Low Salt Elution Buffer (v/v)**

Elution Buffer (500 mM NaCl)

- **25% High Salt Elution Buffer + 75% Low Salt Elution Buffer (v/v)**

Ethanol Precipitation Buffer - per 400 uL eluate

- **40 uL 3 M Sodium Acetate, pH 5.2 + 880 uL 100% ethanol + 2 uL glycogen (add glycogen immediately before use)**

Preparing the Beads

* *Prepare the MBD-beads in one tube for all samples*

1. Re-suspend the stock of Dynabeads by gently pipetting up and down to obtain a homogenous suspension. *Never mix the beads by vortexing.*
2. Transfer amount of beads needed for the experiment to a clean microcentrifuge tube:
20 uL of stock bead suspension per 1 ug of input DNA
3. Place the tube(s) on a magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
4. With the tube in place on the magnetic rack, remove the supernatant with a pipette without touching the beads. Discard the liquid.
5. Add a volume 1x BWB equal to the initial bead volume to the tube and resuspend by pipetting gently up and down to wash.
6. Repeat Steps 3–5 once more. Resuspend washed beads in 3 times the original bead volume of 1x BWB. Keep on ice.

Coupling the MBD-Biotin Protein to the Beads

1. Remove the MBD-Biotin Protein (0.5 mg/ml) from -80C and briefly vortex and spin-down after thawing. Immediately return to -80C after use.
2. Add the required amount of MBD-Biotin Protein to the tube containing the washed Dynabeads:
14 uL of MBD-biotin Protein per 1 ug of DNA input
3. Incubate the bead-protein mixture on a rotating mixer at room temperature for 1 hour. Prepare the fragmented DNA for capture during this time.

Prepare Fragmented DNA for Capture

1. Normalize all input DNA samples to exactly 100 uL with nuclease-free water. For high-throughput or automated prep, plate the DNA samples into an AB1127 microplate.
2. To each DNA sample add exactly 25 uL of undiluted 5x Bind/Wash Buffer. This will result in the DNA being suspended in a final volume of 125 uL 1x BWB.
3. Proceed to washing the prepared MBD-beads.

Washing the MBD-beads

1. After the 1 hour incubation, place the tube containing the MBD-beads on a magnetic rack for 1 minute to concentrate the beads on the inner wall of the tube.
2. With the tube in place on the magnetic rack, remove and discard the supernatant with a pipette.

3. Re-suspend the beads with a volume of 1x BWB equal to that of the supernatant removed in the previous step.
4. Wash the beads on a rotating mixer at room temperature for 5 minutes.
5. Repeat steps 1–4 twice more.
6. After the third wash, place the tube on the magnetic rack. Remove and discard the supernatant with a pipette.
7. Proceed to MBD-bead dilution and capture reaction.

MBD-bead Dilution and Capture Reaction

Bead Dilution

For accurate aliquoting of the prepared MBD-beads to each DNA sample, it is suggested that they are first diluted. This ensures that the volumes of aliquots are easy to work with for samples with very low input DNA. Since each capture reaction will have final volume of 200 uL, it is best to use a dilution factor that leads to the highest input sample requiring 75 uL of diluted beads.

For example, with a batch of samples ranging for 200 ng to 25 ng input:

DNA Input (ng)	MBD-Beads Needed (uL)	Dilution Factor	Diluted MBD-Beads Needed (uL)	1x BWB Needed (uL)
200	4	18.75	75	0.0
175	3.5	18.75	65.625	9.375
150	3	18.75	56.25	18.75
125	2.5	18.75	46.875	28.125
100	2	18.75	37.5	37.5
75	1.5	18.75	28.125	46.875
50	1	18.75	18.75	56.25
25	0.5	18.75	9.375	65.625

In this example, beads for a total of 900 ng of DNA have been prepared. This corresponds to 18 uL of stock Dynabeads. However, re-suspending the prepared and washed MBD-beads in a volume of 18 uL will make accurate aliquoting for each sample difficult - especially in the case of the 25 ng sample. Therefore, the volume of the prepared MBD-beads is adjusted according to the highest input sample:

$$\frac{75 \text{ uL Max}}{4 \text{ uL Needed}} = 18.75 \text{ dilution factor}$$

So that the volume to re-suspend the prepared beads becomes:

$$18 \text{ uL original stock volume} \times 18.75 \text{ dilution factor} = 337.5 \text{ uL diluted beads}$$

Thus the beads are re-suspended in 337.5 uL of 1x BWB, and added to each sample according to the volumes in the fourth column. Finally, each capture reaction is normalized to 200 uL final volume with 1x BWB.

Capture Reaction

After addition of diluted MBD-beads to each DNA sample, place on a rotating mixer for 2 hours at room temperature. If using a microplate, seal the plate with adhesive foil and mix on an orbital shaker at 650 rpm for 2 hours at room temperature.

Removing the Non-Captured DNA

1. After incubating the DNA and MBD-beads for 2 hours, place the tube/plate on a magnet for 1 minute.
2. While in place on the magnet, remove the supernatant (200 uL) with a pipette without touching the beads, and save it in a clean 2 ml microcentrifuge tube labeled NC.
3. Add 200 µl of 250 mM Wash Buffer to the beads.
4. Mix the beads on a rotating mixer or orbital shaker at 650 rpm for 3 minutes at room temperature.
5. Return the tubes/plate to the magnet for 1 minute
6. While in place on the magnet, remove the supernatant with a pipette without touching the beads, and save it in a clean 2 ml microcentrifuge tube labeled Wash.
7. Repeat steps 3–6 once more to collect a 400 uL total Wash fraction.

Eluting the Captured DNA

500mM NaCl Elution

This elution will be used for NGS library prep so treat these tubes/plates carefully.

1. Re-suspend the beads in 200 µl of 500mM Elution Buffer.
2. Mix the beads on a rotating mixer or orbital shaker at 650 rpm for 3 minutes at room temperature.
3. Return the tubes/plate to the magnet for 1 minute
4. While in place on the magnet, remove the supernatant with a pipette without touching the beads, and save it in a clean 2 mL microcentrifuge tube.
5. Repeat Steps 1–4 once more, collecting a total of 400 uL for E1.

2 M NaCl Elution

This elution will remove any remaining methylated fragments from the beads. These fragments typically are not sequenced, but may be useful depending on the experiment.

6. Re-suspend the beads in 200 μ l of High-Salt Elution Buffer.
7. Mix the beads on a rotating mixer or orbital shaker at 650 rpm for 3 minutes at room temperature.
8. While in place on the magnet, remove the supernatant with a pipette without touching the beads, and save it in a clean 2 mL microcentrifuge tube labeled E2.
9. Repeat Steps 1–4 once more, collecting a total of 400 μ L for E2.

Ethanol Precipitation

1. Add 920 μ L of freshly prepared Ethanol Precipitation Buffer to the appropriate eluate - typically the 500 mM elution fraction, E1.
2. Mix well and incubate at -80°C for at least 2 hours.
3. Centrifuge the tube for one hour at speed $\geq 12,000 \times g$ at 4°C .
4. Carefully decant the supernatant without disturbing the pellet.
5. Add 500 μ l of ice-cold 70% ethanol. Invert gently to wash pellet and tube walls.
6. Centrifuge the tube for 10 minutes $\geq 12,000 \times g$ at 4°C .
7. Carefully decant the supernatant without disturbing the pellet.
8. Repeat Steps 6–7 once and remove any remaining residual ethanol.
9. Air-dry the pellet for ~ 5 minutes (pellet will change from white to transparent as it dries).
10. Re-suspend the DNA pellet in Low-TE (10 mM Tris , 0.1mM EDTA, pH 8.0). The volume will depend on library prep to be used. For Swift 2S, re-suspend the pellet in 42 μ L Low-TE.
11. Total mass of captured DNA will be very low, but is usually detected by Qubit HS DNA assay.