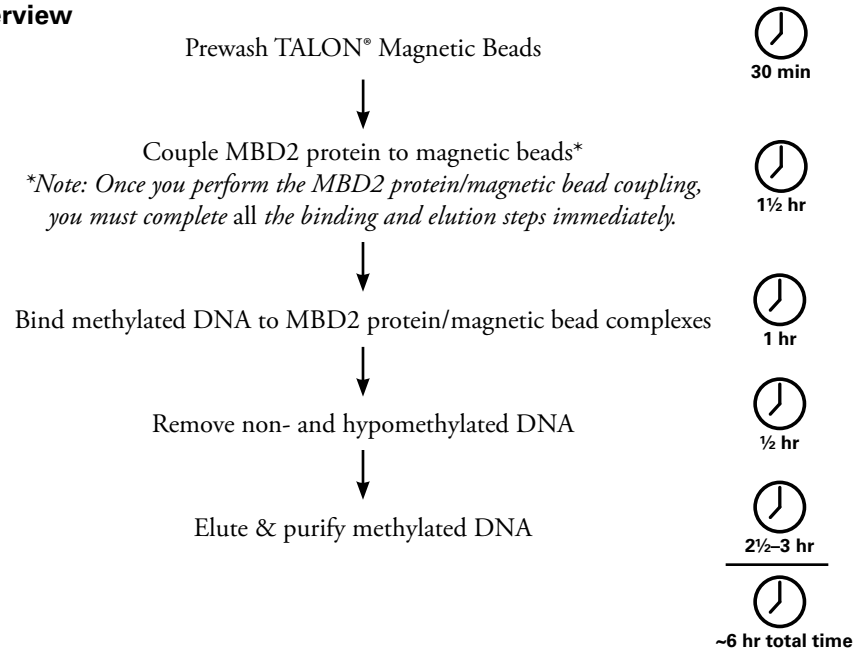
Protocol-at-a-Glance

EpiXplore™ Methylated DNA Enrichment Kit Protocol-at-a-Glance

(PT5034-2)

This protocol is provided for use with the **EpiXplore Methylated DNA Enrichment Kit** (Cat. No. 631963). The kit provides a simple magnetic bead-based method for enriching methylated DNA fragments from the whole genome. Enrichment leads to superior results in downstream applications: the methylation status of specific regions of interest can be easily evaluated by real-time or end-point PCR, or the methylation status of a large number of genes or the entire genome can be analyzed using microarray- or sequencing-based DNA profiling methods.

Protocol Overview



Sample Preparation

1. Make 1X Binding/Washing Buffer:
   Dilute 1 volume of 4X Binding/Washing Buffer in 3 volumes of nuclease-free water.
   Each enrichment run requires ~1.25 ml of 1X Binding/Wash Buffer and 25 µl of 4X Binding/Wash Buffer.
2. Prepare genomic DNA samples:
   Each enrichment run requires 10 ng–1 µg DNA.
   a. Isolate and purify genomic DNA using your method of choice. We recommend purifying genomic DNA samples with the **NucleoSpin Tissue Kit** (Cat. No. 740952.50).
   b. Shear your DNA by your method of choice to a size of 100–1,000 bp.
   The optimum fragment size depends on your downstream application.
3. Additional materials required:
   - Magnetic separator stand
   - Nuclease-free microcentrifuge tubes
   - 3 M sodium acetate, pH 5.2
   - 96–100% ethanol
   - Cold 70% ethanol

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A. Prewash TALON Magnetic Beads

1. Vortex the TALON Magnetic Beads gently to resuspend.
2. For each methylated DNA enrichment reaction, transfer 100 µl of TALON Magnetic Bead suspension to a nuclease-free, 1.5 ml microcentrifuge tube.
3. Wash beads with 100 µl of 1X Binding/Wash Buffer. Vortex gently and spin down briefly.
4. Use a magnetic separator for microfuge tubes to pull the magnetic beads to the wall of the tube. Remove and dispose of the supernatant.
5. Repeat step A.4 one more time.
6. After removing the supernatant for the second time, add 100 µl of 1X Binding/Wash Buffer to the magnetic beads and store them at 4°C until you are ready to perform the MBD2/bead coupling and the methylated DNA enrichment (Protocols B–D).

B. Couple the MBD2 Protein to the TALON Magnetic Beads

NOTE: Do not begin this protocol until you are also ready to perform the complete methylated DNA enrichment process. You must proceed *immediately* from Protocol B to Protocols C–D, to prevent the MBD2 protein/magnetic bead complexes from drying out.

1. For each methylated DNA enrichment reaction, add 10 µl of MBD2 protein and 90 µl of 1X Binding/Wash Buffer to a microcentrifuge tube.
2. Transfer the diluted MBD2 protein to the tube containing 100 µl of resuspended magnetic beads from Step A.6.
3. Mix the MBD2 protein/magnetic bead mixture on a rocking shaker (or platform rotator) for 1 hr at room temperature to allow coupling.
4. Spin down briefly, then place the microcentrifuge tube on a magnetic separator and allow the magnet to pull the magnetic beads to the wall of the tube. Remove and dispose of the supernatant.
5. Wash the MBD2 protein/magnetic bead mixture with 100 µl of 1X Binding/Washing Buffer. Vortex gently and spin down briefly. Place the microcentrifuge tube on a magnetic separator and allow the magnet to pull the magnetic beads to the wall of the tube. Remove and dispose of the supernatant. Make sure to completely separate the supernatant from the magnetic beads.
6. Repeat step B.5 two more times.
7. Proceed immediately to Protocol C (Methylated DNA Capture) to prevent MBD2 protein/magnetic bead complexes from drying out.

C. Bind the Methylated DNA to the MBD2 Protein/TALON Magnetic Bead Complexes

1. For each methylated DNA enrichment reaction, add 25 µl of 4X Binding/Washing Buffer to a clean microcentrifuge tube.
2. Add 10 ng–1 µg sheared DNA to the tube.
3. Add nuclease-free H_2O to a final volume of 100 µl.
4. Transfer the diluted DNA sample to the tube containing the MBD2 protein/magnetic beads from step B.7.
5. Incubate on a rocking shaker (or platform rotator) for 1 hr at room temperature.
6. Spin down briefly, then place the microcentrifuge tube on a magnetic separator and allow the magnet to pull the magnetic beads to the wall of the tube. Make sure to completely separate the supernatant from the magnetic beads.
7. Remove supernatant and transfer to a nuclease-free microcentrifuge tube, and save as Fraction I (Flowthrough). Non-methylated and hypomethylated DNA is enriched in this fraction.
8. Wash the DNA/MBD2 protein/magnetic bead complexes with 200 µl of 1X Binding/Wash Buffer. Vortex gently and spin down briefly. Place the microcentrifuge tube on a magnetic separator and allow the magnet to pull the magnetic beads to the wall of the tube. Make sure to completely separate the supernatant from the magnetic beads. Remove the supernatant and transfer to a microcentrifuge tube.
9. Repeat step C.8 one more time. Pool the supernatants from steps C.8 and C.9 and save as Fraction II (Wash).
10. Proceed immediately to Protocol D (Methylated DNA Elution).

NOTE: If desired, the DNA in Fractions I and II can be purified as directed in Protocol E.

D. Elute the Enriched Methylated DNA

There are two ways to elute enriched methylated DNA from the MBD2 protein/magnetic bead complexes: as a single fraction [using Elution Buffer (High)] or as multiple fractions [using all three Elution Buffers].

Enrich for total methylated DNA

This method is suitable for most applications, because you can easily separate methylated DNA from non- and hypomethylated DNA, using a single elution buffer.

1. Wash the DNA/MBD2/magnetic bead complexes with 200 µl of Elution Buffer (High). Vortex gently and spin down briefly.
2. Place the microcentrifuge tube on a magnetic separator and allow the magnet to pull the magnetic beads to the wall of the tube. Make sure to completely separate the supernatant from the magnetic beads.
3. Remove the supernatant and transfer to a nuclease-free microcentrifuge tube.
4. Repeat steps 1–3 once. Pool the two eluted samples and save as Fraction III.

OR

Fractionate hypo- and hypermethylated DNA

Use this method if you require a more detailed profile of the DNA's methylation status.

1. Wash the DNA/MBD2/magnetic bead complexes with 200 µl of Elution Buffer (Low). Vortex gently and spin down briefly.
2. Place the microcentrifuge tube on a magnetic separator and allow the magnet to pull the magnetic beads to the wall of the tube. Make sure to completely separate the supernatant from the magnetic beads.
3. Remove the supernatant and transfer to a nuclease-free microcentrifuge tube.
4. Repeat steps 1–3 once. Pool the two eluted samples and save as Fraction III.
5. Repeat steps 1–4 with Elution Buffer (Middle) and then with Elution Buffer (High). Label the pooled elution samples as Fractions IV and V, respectively.

E. Purify the Enriched Methylated DNA

1. Add the following to each fraction from Protocol D.
   - 0.1 volume of 3 M sodium acetate, pH 5.2 (e.g., 40 µl per 400 µl of sample).
   - 4 µl* of Dr. GenTLE Precipitation Carrier.
     *If the sample volume is > 400 µl, add an additional 1 µl of Dr. GenTLE Precipitation Carrier per 100 µl of sample solution.
   - 2 volumes of 96–100% ethanol.
2. Mix well and incubate at –80°C for at least 2 hr.
3. Centrifuge at ≥12,000 × g at 4°C for 15 min.
4. Carefully discard the supernatant without disturbing the pellet.
5. Add 500 µl of cold 70% ethanol.
6. Centrifuge the tube at ≥12,000 × g at 4°C or at room temperature for 5 min.
7. Carefully discard the supernatant without disturbing the pellet.
8. Repeat Steps E.5–E.7 one more time and remove any residual supernatant.
9. Air-dry the pellet for ~5 min (do not completely dry the pellet).
10. Resuspend the DNA pellet in 60 µl of DNase-free water (or other appropriate volume of buffer or water; as needed for your specific downstream application).

NOTE: If desired, the DNA in Fractions I and II (from protocol C) can be purified in the same manner.

Appendix A. Example Enrichment Results

Three enrichments were performed according to this protocol, using the Labeled DNA Duplex controls and the multiple fraction purification method (Table I; Figures 1–2).

- Methylated DNA enrichment efficiency was determined by comparing the fluorescence of each flowthrough, wash, and elution fraction (Figure 1; Table I).
- DNA integrity was determined by PAGE. The DNA was the same size before and after enrichment (Figure 2).

Table I. Control Methylated DNA Enrichment Experiment

Experimental Setup					Results
	Control (Labeled DNA Duplex)		TALON Magnetic Beads	MBD2 Protein	% DNA recovered in methylated DNA elution fractions (III-V)
	Methylated DNA	Non-Methylated DNA			
Tube 1	100 µl (50 nM)	-	100 µl	-	0.7%
Tube 2	100 µl (50 nM)	-	100 µl	10 µl	95.1%
Tube 3	-	100 µl (50 nM)	100 µl	10 µl	0%

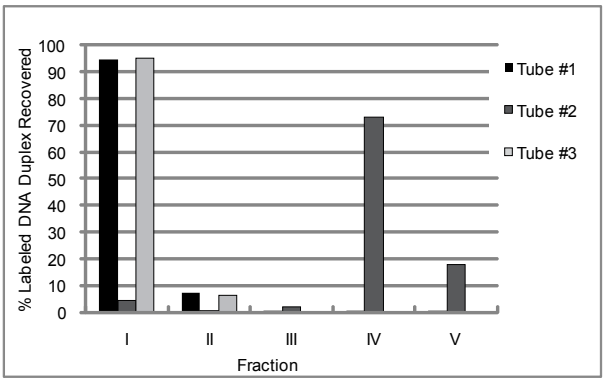

Figure 1. Methylated DNA enrichment is highly specific. Methylated DNA binds to the magnetic beads via the MBD2 protein (Tube 2, Fractions IV–V). Methylated DNA does not bind in the absence of MBD2 Protein (Tube 1), and non-methylated DNA does not bind (Tube 3). Fraction I: Flow-through containing unbound (non- or hypomethlyated) DNA. Fraction II: Wash. Fraction III: Elution Buffer (Low). Fraction IV: Elution Buffer (Medium). Fraction V: Elution Buffer (High).

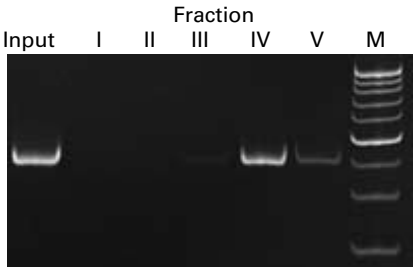

Figure 2. DNA integrity is maintained during enrichment. The size of Labeled Methylated DNA (Tube 2) was the same before and after enrichment, as measured by PAGE (12.5% polyacrylamide gel). M: 20 bp DNA ladder. Input: Labeled Methylated DNA prior to enrichment. Fractions I–V: see Figure 1 above.