EpiMark® Methylated DNA Enrichment Kit

NEB #E2600S

25 reactions

Version 5.0_4/25

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The EpiMark Methylated DNA Enrichment Kit Includes:

The volumes provided are sufficient for the preparation of up to 25 reactions. Store at 4°C. For long-term storage greater than 6 months, MBD2a-Fc protein should be stored at –20°C.

MBD2a-Fc Protein (2 mg/ml)

Protein A Magnetic Beads

Bind/Wash Reaction Buffer (5X)

NaCl High-Salt Elution Buffer (5M)

LINE Primers for Methylated Controls (100 µM each, supplied as a mix)

RPL30 Primers for Non-methylated Controls (100 µM each, supplied as a mix)

MirA Primers for Input Control (100 µM each, supplied as a mix)

Required Materials Not Included:

- 50 bp DNA Ladder (NEB #N3236)
- Nuclease-free Water
- 6-tube Magnetic Separation Rack (NEB #S1506)
- Thermal cycler
- *Taq* DNA Polymerase with Standard *Taq* Buffer (NEB #M0273)
- Deoxynucleotide Solution Mix (NEB #N0447)
- DNA LoBind® Tubes (Eppendorf® #022431021)
- DNase-, RNase-free PCR strip tubes
- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables
- NanoDrop® or Lunatic® (Unchained Labs®) and associated consumables

Introduction

The EpiMark Methylated DNA Enrichment Kit will selectively bind and enrich double-stranded methyl-CpG DNA from 5 ng to 10 µg of fragmented genomic DNA, based on assessments using human gDNA.

Methylated DNA is isolated from fragmented genomic DNA by binding to the methyl-CpG binding domain of human MBD2a protein fused to the Fc tail of human IgG1 (MBD2a-Fc), which is coupled to paramagnetic hydrophilic protein A beads (MBC2- Fc/Protein A Magnetic Bead). Two Fc domains can be bound to one site on protein A with high affinity (K_d=10^{-7}). As the Fc fragment is a dimer, four MBD2 domains are exposed to the solvent per molecule of protein A, increasing the relative equilibrium constant 100-fold.
This stable complex will selectively bind double-stranded methylated CpG containing DNA. After simple wash steps followed by magnetic capture, the enriched DNA sample is easily eluted in a small volume of nuclease-free water by incubation at 65°C. The sample is immediately ready for downstream analysis by a variety of methods including:

- Endpoint and real-time PCR assays
- Methylation analysis using NEBNext Enzymatic Methyl-seq workflows or Bisulfite conversion
- Cloning and sequencing
- Library preparation for high-throughput sequencing
- Labeling for DNA microarray analysis
- Methylation-sensitive restriction enzyme-based assays

MBD-Fc and MCIp were originally developed by Michael Rehli at the University of Regensburg to improve the sensitivity and specificity of conventional CpG binding techniques.

Overview

Step I—Fragment Genomic DNA

DNA must be fragmented by sonication, nebulization or enzymatic treatment to an average size of less than 1,000 bp.

Step II—Combine MBD2a-Fc and Protein A Magnetic Beads in 1X Bind/Wash Buffer (Page 4).

Incubate the reaction for 15 minutes at room temperature. Wash beads two times in Bind/Wash Reaction Buffer.

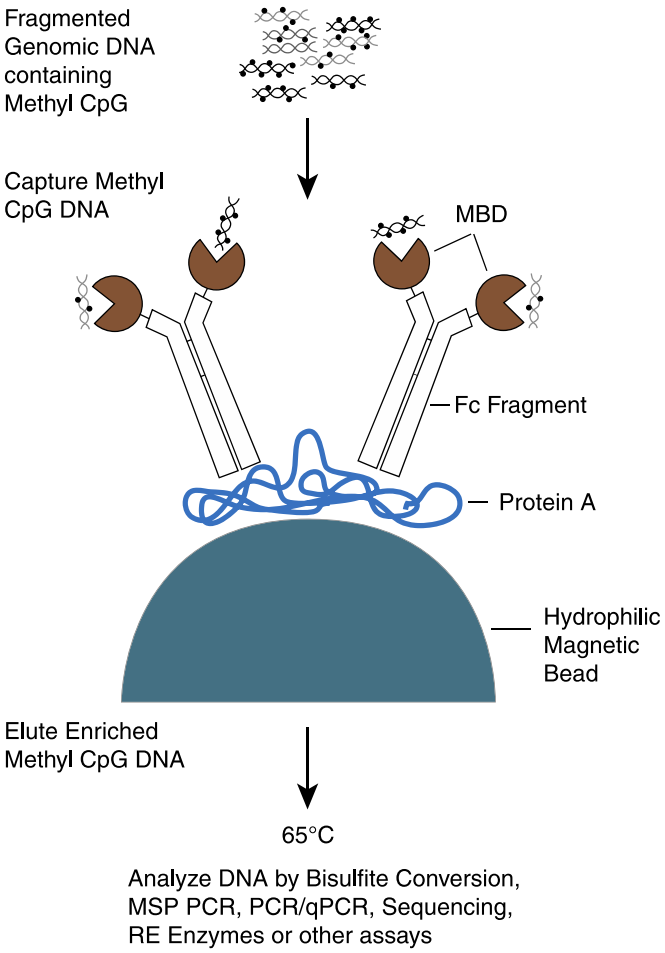
Step III—Add fragmented DNA to MBD2a-Fc / Protein A Magnetic Beads (Page 5).

Incubate the reaction for 20 minutes at room temperature. Wash beads three times at room temperature for five minutes each to remove unbound DNA.

Step IV—Elute enriched methylated CpG DNA from beads (Page 5).

Incubate the sample at 65°C for 15 minutes in DNase-free water.

Figure 1. EpiMark Methylated DNA Enrichment Kit Workflow



Protocol for Enrichment of Methylated DNA using EpiMark Methylated DNA Enrichment Kit

Symbols

This is a point where you can safely stop the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

1. DNA Fragmentation

Note: We recommend running a human control gDNA sample alongside experimental samples.

DNA may be fragmented using enzymatic treatment like NEBNext UltraShear, sonication, nebulization, or other methods that do not alter the methylation status on the DNA. Fragments must average less than 1,000 bp and should be in DNase-free TE buffer (pH 7.5). Determine the approximate size distribution of the DNA by running the fragmented DNA on electrophoresis base instruments (i.e., Bioanalyzer®, TapeStation®, and Lunatic®) or electrophoresis of the sample on an agarose gel alongside a DNA marker (e.g., 50 bp DNA Ladder, NEB #N3236). The fragment size should be appropriate for the desired downstream analysis. For example, DNA fragmented to an average length of ~250 bp is suitable for assay by real-time quantitative PCR (qPCR) or Next Generation Sequencing platforms. It is also important to quantitate the amount of DNA in the experimental sample by A_{260} measurement using a spectrophotometer (i.e., Nanodrop™ and Qubit).

2. Prebind MBD2a-Fc to Protein A Magnetic Beads

Notes:

(1) Before proceeding, determine the number of samples to be analyzed. Use 1 µl of MBD2a-Fc protein and 10 µl Protein A Magnetic Beads for up to 10 µg of input DNA.

(2) The MBD2-Fc/Protein A Magnetic Bead mixture is stable for up to 1 week at 4°C.

2.1. Resuspend Protein A Magnetic Beads by gently pipetting the slurry up and down until suspension is homogeneous or rotating the tube gently for 10 to 30 minutes at room temperature.

2.2. Prepare 1X Bind/Wash Buffer by diluting 1 part of 5X Bind/Wash Buffer with 4 parts of Nuclease-free Water. 3 ml of 1X Buffer is required for preparing the MBD2-Fc / Protein A Magnetic Beads mixture.

2.3. Add the following components to a nuclease-free tube:

COMPONENT	1X REACTION VOLUME (µl)	25X REACTION VOLUME (µl)
1X Bind/Wash Buffer	11 µl	275 µl
Protein A Magnetic Beads	10 µl	250 µl
MBD2-Fc	1 µl	25 µl
Total	**22 µl**	**550 µl**

2.4. Mix the bead-protein mixture by flicking the tube and placing the tube in a rotating mixer for 15 minutes at room temperature.

2.5. Add 1 ml of 1X Bind/Wash Buffer to the bead-protein mixture and place the tube on a rotating mixer for 3 minutes at room temperature to wash and mix the beads.

2.6. Place the tube on the magnetic rack for 2-5 minutes to concentrate all the beads on the inner wall of the tube.

2.7. Remove and discard the supernatant with a pipette without disturbing the beads.

2.8. Repeat steps 2.4 to 2.7 twice, for a total of 3 washes. Make sure all the supernatant is removed.

2.9. Remove the tube from the magnetics rack and add 16 µl of 1X Bind/Wash Reaction Buffer per reaction (for example, 400 µl for 25 reactions). Resuspended the beads by pipetting up and down.

The MBD2-Fc/Protein A Magnetic Bead mixture can be stored at 4°C for up to 1 week.

3. Capture fragmented methylated CpG containing DNA

Note: We recommend running a fragmented human control gDNA sample alongside experimental samples.

3.1. Add the following components to a nuclease-free tube:

COMPONENT	1X REACTION VOLUME
DNA Input (5 ng – 10 µg) *,**	65 µl
5X Bind/Wash Buffer	20 µl
MBD2-Fc / Protein A Magnetic Beads	15 µl
Total Volume	**100 µl**

* If DNA input volume is less than 65 µl, add Nuclease-free water to bring up the volume to 65 µl.
** The DNA input amount should be appropriate for the input type and desired downstream analysis. For example, greater than 5 ng of human gDNA input is recommended for use in downstream DNA sequencing applications.

3.2. Mix by pipetting up and down.

3.3. Incubate the reaction for 20 minutes at room temperature with rotation.

4. Wash Off Unbound DNA

4.1. Prepare 1X Bind/Wash Buffer by diluting 1 part of 5X Bind/Wash Buffer with 4 parts of Nuclease-free Water. 3 ml of 1X Buffer is required for each sample.

4.2. After incubating the DNA and MBD2a-Fc/ Protein A Magnetic Beads, place the tube on the magnetic rack for 2–5 minutes to concentrate the beads on the inner wall of the tube.

4.3. Carefully remove the supernatant with a pipette without disturbing the beads. **Save supernatant in a clean microcentrifuge tube**. This saved supernatant is the Unbound DNA fraction. Store the Unbound DNA sample(s) on ice or at -20°C.

4.4. Add 1 ml of 1X Bind/Wash Buffer to the tube to remove residual non-specifically bound DNA.

4.5. Mix the beads on a rotating mixer for 3 minutes at room temperature.

4.6. Place the tube on the magnetic rack for 2–5 minutes to concentrate the beads on the inner wall of the tube. Remove and discard the supernatant.

4.7. Repeat steps 4.4 to 4.6 two more times for a total of 3 washes

4.8. Proceed immediately to elution of the captured methylated CpG DNA, do not allow the beads to over-dry. Follow Step 5.1-5.4 for heat-based elution (recommended for most down-stream applications) or the Appendix for NaCl-based elution.

5. Elute Captured Methylated CpG DNA

Note: **For all downstream techniques, including endpoint and real-time PCR assays, enzymatic conversion, bisulfite conversion, cloning and sequencing, direct sequencing, library preparation for high-throughput sequencing, labeling for DNA microarray analysis or methylation-sensitive restriction enzyme-based assays, heating the sample at 65°C for 15 minutes is the recommended method of choice for elution of DNA. The appendix section also includes a protocol for NaCl multi-fraction elution series, followed by Monarch spin column cleanup if necessary.**

5.1. Add 50–100 µl of DNase-free water to the sample of magnetic beads pellet. Mix beads by flicking the tube. Incubate the slurry in a heat block or thermomixer set at 65°C for 15 minutes, with frequent mixing.

5.2. Briefly centrifuge the sample.

5.3. Place the tube on the magnetic rack for 2–5 minutes to concentrate the beads on the wall of the tube.

5.4. Transfer the supernatant to a fresh nuclease-free tube. This eluted supernatant contains enriched methyl CpG containing DNA (Bound fraction). Proceed directly to downstream analysis or store the eluted DNA sample at –20°C.

DNA fractions can be placed at –20°C for long-term storage.

6. Downstream Analysis

Notes:

(1) This section describes quantitation approaches by standard or real-time qPCR using the supplied Methylated (LINE), Non-methylated (RPL30), and control (MirA) primer pairs.

(2) We recommend running human control Input DNA, Unbound DNA, and Bound DNA samples alongside experimental samples.

(3) Several downstream techniques may be applied to Input DNA samples, Unbound DNA samples, and Bound DNA samples. These include endpoint and real-time PCR assays, enzymatic conversion, bisulfite conversion, cloning and sequencing, direct sequencing, library preparation for high-throughput sequencing, labeling for DNA microarray analysis, or methylation-sensitive restriction enzyme-based assays. Follow reagent and equipment manufacturer recommendations for these assays.

6.1. Quantitation of DNA by Standard PCR Reaction (for fragmented DNA)

6.1.A. Perform capture experiment as described above. Elute Bound DNA samples by incubating at 65°C in 50 µl of nuclease-free water for 15 minutes.

6.1.B. Prepare dilutions of the Input DNA samples (1:2000 dilution) and Unbound DNA samples (1:1000 dilution) with nuclease-free water.

6.1.C. Add the following components to a nuclease-free PCR tube on ice:

REAGENT	1X REACTION VOLUME
DNA Sample*	13.5 µl
Standard *Taq* Buffer (10X)	2.0 µl
dNTP Mix (4 mM)	1.0 µl
Primers (LINE, RPL30, or MirA, 10 µM)	1.0 µl
Taq DNA Polymerase	0.5 µl

*1:2000 diluted Input DNA; 1:1000 diluted Unbound DNA; undiluted Bound DNA.
** PCR master mix for each control primer pair can be prepared immediately prior to use.

6.1.D. Set a 20 µl pipette to 10 µl and then pipette the entire reaction volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

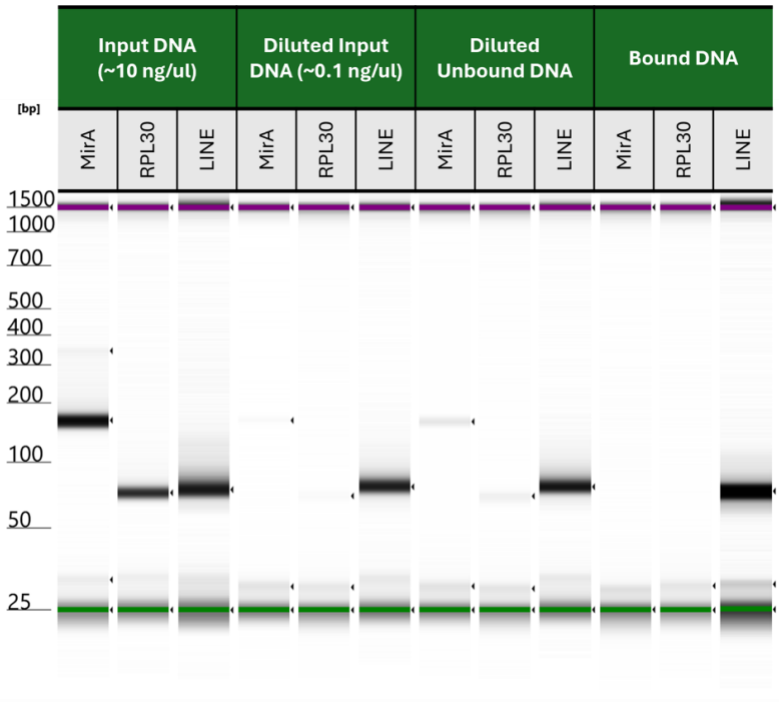
6.1.E. Place the tube in a thermal cycler with the heated lid set to 105°C or on and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Initial Denaturation	95°C	5 Seconds	1
Denaturation	95°C	15 Seconds	25*
Annealing	60°C	15 Seconds	
Extension	72°C	30 Seconds	
Final Extension	72°C	5 Minutes	1
Hold	4°C	∞	

*PCR cycles may need to be optimized based on DNA input amount and primers.

6.1.F. Remove 10 µl of each PCR product for analysis by 2% agarose gel or 10% polyacrylamide gel electrophoresis using the 50 bp DNA Ladder (NEB #N3236) as a standard. Alternatively, PCR products can be assessed with a *TapeStation® HS D1000 ScreenTape®*. The expected size of the amplicons from human gDNA is ~150 bp for MirA and ~75 bp for RPL30 and LINE.

Figure 6.1: PCR Analysis of MirA, RPL30, and LINE (methylated locus) targets in Input DNA, Unbound DNA, and Bound DNA



TapeStation® HS D1000 ScreenTape® traces of endpoint MirA, RPL30, and LINE PCR products. Human genomic DNA used as input for EpiMark Methylated DNA Enrichment Kit workflow.

6.2. Quantitation of DNA by qPCR (for control fragmented gDNA)

6.2.A. Perform capture experiment as described above. We recommend including a control human gDNA sample in parallel with experimental samples. Elute Bound DNA samples by incubating at 65°C in 100 µl of nuclease-free water for 15 minutes.

6.2.B. Prepare 1:1000 dilutions of the Input DNA samples and Unbound DNA samples with nuclease-free water.

6.2.C. Aliquot 10 µl of each DNA sample (Input DNA, Unbound DNA, and Bound DNA samples) in triplicate for each primer pair (methylated [LINE], unmethylated [RPL30], and input control [MirA] primers) into a qPCR plate.

6.2.D. Add the appropriate volume of qPCR primers for your qPCR kit. Dilute as needed. Primers are supplied as 100 µM each.

6.2.E. Perform qPCR and analyze results as appropriate for your qPCR reagents and instrument.

6.2.F. Perform qPCR standard reaction program using an annealing temperature of 60°C.

6.2.G. Analyze quantitative PCR results using software provided with the real-time PCR machine.

Note: For a control human gDNA sample enriched using the EpiMark Methylated DNA Enrichment Kit, more than 100-fold enrichment of the methylated locus (LINE) versus the active gene (RPL30) within the methylated eluant sample as compared to the input DNA should be observed. Conversely, no enrichment of the methylated locus should be observed in the saved supernatant of the non-captured DNA fraction.

Troubleshooting Guide:

PROBLEM	POSSIBLE CAUSE(S)	SOLUTION(S)
No or poor DNA target detection by PCR-based method in Unbound and/or Bound DNA fraction(s)	DNA is degraded	• Take precautions to prevent DNA degradation by maintaining a nuclease-free environment. • Increase EDTA concentration of sample to 10 mM
	Not enough target DNA	• Verify target DNA concentration by nanodrop instrument, or other sensitive DNA detection system • Run target DNA on agarose gel or TapeStation to determine quantity, quality and size
	DNA did not elute from the MBD2a-Fc beads	• Raise the temperature of the elution to 98°C, mindful that this will render the sample single- stranded
Unable to clone eluted DNA fragments	DNA ends are frayed due to sonication or nebulization, or DNA has been rendered single-stranded	• Repair DNA ends using a blunt-end repair kit (e.g., Quick Blunting Kit, NEB #E1201)
Controls did not work, did not see bands as expected on gel	DNA is degraded	• See above for DNA precautions
	DNA did not elute from the MBD2a-Fc beads	• See above for elution at 98°C
	PCR did not work	• Verify that all the components have been added to the PCR reaction mix. • Lower the annealing temperature of the reaction to 55°C
Controls did not work, did not see my gene of interest	DNA target does not contain enough CpG methylation	• Raise input DNA concentration to at least 1 µg
	PCR did not work	• Optimize PCR conditions for your target sequence

Appendix A: NaCl Elution Protocol

The standard protocol described elsewhere in this Instruction Manual is designed to allow for heat-based elution of captured DNA for downstream applications. However, Appendix A describes an alternate NaCl-based elution approach for the captured methylated CpG containing DNA, either as a single fraction using 2 M NaCl, or multiple fractions.

Note: Before proceeding, determine the stepwise elution series to be used to elute the DNA based on the number of methylation sites per molecule. The Bind/Wash Buffer contains 150 mM NaCl. To elute captured CpG-methylated DNA into distinct fractions, start with the lowest NaCl concentration (e.g., 200 mM NaCl). Follow this with the next higher NaCl concentration (e.g., 350 mM NaCl). Continue this process until all the desired fractions have been collected.

A.1. For each capture reaction, prepare 1 ml elution buffers using the ratios below:

ELUTION BUFFER 1 ml	NaCl CONCENTRATION	AMOUNT BIND/WASH	AMOUNT 5 M NaCl
Wash	150 mM	1 ml	0 ml
Elution 1	200 mM	990 µl	10 µl
Elution 2	350 mM	960 µl	40 µl
Elution 3	450 mM	940 µl	60 µl
Elution 4	600 mM	910 µl	90 µl
Elution 5	1,000 mM	830 µl	170 µl
Elution 6	2,000 mM	630 µl	370 µl

A.2. Immediately following Step 4.6., elute the captured DNA by resuspending the beads in 200 µl of Elution 1 Buffer.

A.3. Place resuspended beads on a rotating mixer for 3 minutes.

A.4. Place the mixed beads on a magnetic rack for 2–5 minutes to allow the solution to clear.

A.5. Carefully remove the supernatant to a fresh microcentrifuge tube labeled Elution 1 and store on ice.

A.6. Resuspend the beads in 200 µl of the next Elution buffer and repeat Steps A3-A5 until all the elution steps are completed.

A.7. Desalt and purify samples using Monarch or other PCR clean up kits following manufacturer recommendations.

Kit Components

NEB #E2600S Table of Components

NEB #	PRODUCT	VOLUME
B1032AVIAL	Bind/Wash Reaction Buffer (5X)	2 x 25 ml
B1033AVIAL	NaCl High-Salt Elution Buffer (5M)	15 ml
N0485AVIAL	LINE Primers for Methylated Controls	0.02 ml
N0486AVIAL	RPL30 Primers for Non-Methylated Controls	0.02 ml
N0487AVIAL	MirA Primers for Input Control	0.02 ml
M0358AVIAL	MBD2a-Fc Protein	0.025 ml
S1426AVIAL	Protein A Magnetic Beads	0.25 ml

Control Primer Sequences

PRODUCT	INDEX PRIMER SEQUENCE	HUMAN DNA AMPLICON SIZE
LINE Primers for Methylated Controls (100 µM)	5′- GAAATGCAGAAATCA CCCGTCTT forward 5′- CGGAGGCCGAATAGG AACAGCTCCG reverse	77 bp
RPL30 Primers for Non-methylated Controls (100 µM)	5′- GCCCGTTCAGTCTCTTCGATT forward 5′- CAAGGCAAAGCGAAATTGGT reverse	73 bp
MirA Primers for Input Control (100 µM)	5′- TTTAAACAGGATATTTACGTTCTGC forward 5′- GAGGAAATCTTCACATCCACG reverse	154 bp

References

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Revision History

REVISION #	DESCRIPTION	DATE
1.3	N/A	6/14
2.0		7/17
3.0		2/18
4.0	Apply new manual format.	8/20
5.0	Kit component update and manual format update.	4/25