

EpiMark[®] Methylated DNA Enrichment Kit

NEB #E2600S

25 reactions

Version 4.0_8/20

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The EpiMark Methylated DNA Enrichment Kit Includes:

Each kit contains sufficient reagents for the enrichment of methylated DNA from up to 100 µg of fragmented input DNA. If starting with 5 ng to 10 µg of input DNA per experiment, the kit provides sufficient reagents for 25 reactions. Store at 4°C. For long-term storage > 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 (2 M)

Fragmented HeLa DNA (100 µg/ml)

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)

6-tube Magnetic Separation Rack (NEB #S1506)

Taq DNA Polymerase with Standard Taq Buffer (NEB #M0273)

Deoxynucleotide Solution Mix (NEB #N0447)

Nuclease-free Water

Introduction

The EpiMark Methylated DNA Enrichment Kit will selectively bind and enrich double-stranded methyl-CpG DNA from fragmented genomic DNA with as little as 5 ng of input DNA.

Methylated DNA is isolated from fragmented genomic DNA (5 ng–25 µg) 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
- Bisulfite conversion followed by DNA amplification
- Cloning and sequencing
- Direct 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.

Advantages

- High-affinity binding provides greater sensitivity
- Elution in a small volume simplifies downstream applications.
- Easy-to-use protocol yields enriched fractions in less than 2 hours.
- Enriched methylated DNA fractions can be easily ligated to double-stranded adaptors for Next Generation Sequencing.
- Highly pure product from a wide range of input DNA concentrations.

Method 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.

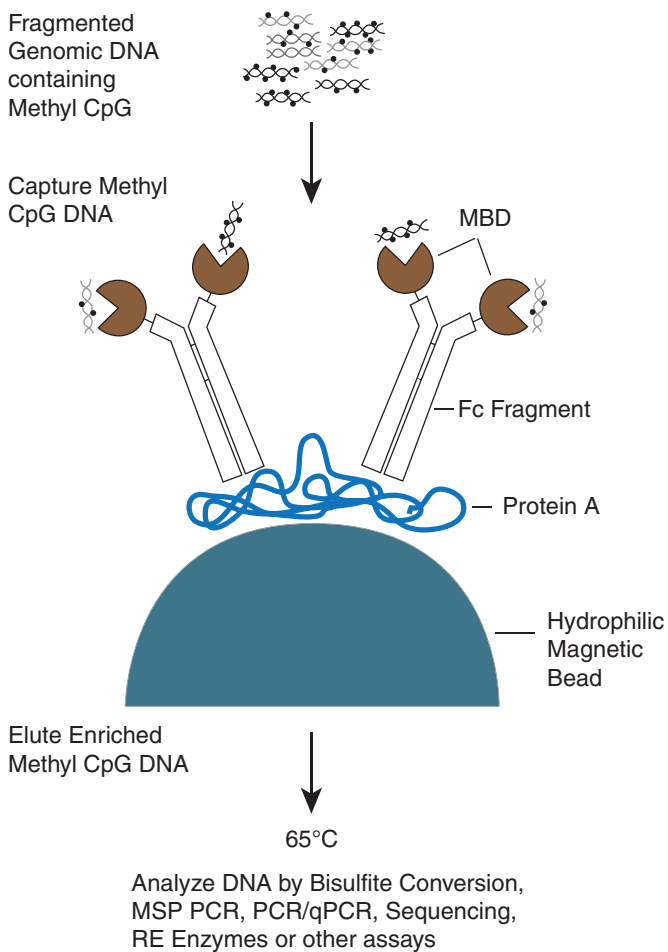
Step III—Add fragmented DNA to MBD2a-Fc / Protein A Magnetic Beads (Page 4).

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. Enrichment Workflow



Protocols

DNA Fragmentation

DNA may be fragmented using sonication, nebulization, enzymatic treatment or by other methods. 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 agarose gel electrophoresis of the sample alongside a DNA marker (e.g., 50 bp DNA Ladder, NEB #N3236). A sample of fragmented HeLa genomic DNA of the size range of 100–1000 bp is included as a control. 4 μ l of this DNA standard (~400 ng) is sufficient to visualize on an agarose gel alongside the experimental sample. The desired 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). Smaller fragments of 150 bp or less are suitable for linker addition in Next Generation Sequencing platforms. It is also important to quantitate the amount of DNA in the experimental sample by A₂₆₀ measurement using a spectrophotometer, Nanodrop™ instrument or Bioanalyzer®.

Prebind MBD2a-Fc to Protein A Magnetic Beads

Before proceeding, determine the number of samples to be analyzed. As a general rule of thumb, use 1 μ l of MBD2a-Fc protein and 10 μ l Protein A Magnetic Beads for up to 10 μ g of input DNA. For larger amounts of input DNA, scale the reaction accordingly (e.g., for an experiment using 100 μ g of input DNA, add 10 μ l MBD2a-Fc protein and 100 μ l Protein A Magnetic Beads).

Resuspend Protein A Magnetic Beads by gently pipetting the slurry up and down until suspension is homogeneous. Alternatively, rotate the tube gently for 30 minutes at room temperature.

Prepare 1X Bind/Wash Buffer by diluting 1 part of 5X Bind/Wash Buffer with 4 parts of DNase-free water. One individual reaction (for up to 10 μ g of input DNA) from start to finish will require ~5 mls of 1X Bind/Wash Buffer.

1. Add 11 μ l of 1X Bind/Wash Buffer, 10 μ l of Protein A Magnetic beads and 1 μ l of MBD2-Fc in one tube. Mix by flicking the tube.
2. Mix the bead-protein mixture by placing the tube in a rotating mixer for 15 minutes at room temperature.
3. Washing
 - a. Add 1000 μ l of 1X Bind/Wash Buffer to the tube to wash the beads.
 - b. Mix the beads on a rotating mixer for 3 minutes at room temperature.
 - c. Place the tube on the magnetic rack for 2–5 minutes to concentrate all of the beads on the inner wall of the tube.
 - d. Carefully remove the supernatant with a pipette without disturbing the beads. Discard the supernatant.
4. Repeat Step 3 two more times. Make sure all the supernatant is removed.
5. Remove the tube from the rack and add 16 μ l of 1X Bind/Wash Reaction Buffer. Resuspended the beads by pipetting up and down.
6. The MBD2-Fc/Protein A Magnetic Bead mixture is stable for up to 1 week at 4°C.

Capture Methylated CpG DNA

1. In a clean tube, add 5 ng–10 μ g of fragmented sample DNA plus DNase free water to 65 μ l.
2. Add 20 μ l of 5X Bind/Wash Buffer.
3. Add 15 μ l of well-mixed MBD2-Fc / Protein A Magnetic Beads.
4. Mix by pipetting up and down.
5. Incubate the reaction for 20 minutes at room temperature with rotation.

Wash Off Unbound DNA

1. 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.
2. Carefully remove the supernatant with a pipette without disturbing the beads. Save supernatant in a clean microcentrifuge tube. This saved supernatant is the non-captured DNA fraction. Store this sample on ice or at -20°C.
3. Add 1000 μ l of 1X Bind/Wash Buffer to the tube to remove the residual non-captured DNA.
4. Mix the beads on a rotating mixer for 3 minutes at room temperature.
5. 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.
6. Repeat steps 3–5 two more times.

Elute Captured Methylated CpG DNA

For all downstream techniques, including endpoint and real-time PCR assays, 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.

Heat Method

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.

Briefly centrifuge the sample.

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

Carefully remove the supernatant to a fresh microcentrifuge tube. This eluted supernatant contains enriched methyl CpG containing DNA. Proceed directly to downstream analysis or store the eluted DNA sample at –20°C.

Capture and Elute Step for Control DNA

Included in the kit are control DNA and primers to detect a methylated region, LINE element, an active gene exon, RPL30, and a micro RNA control region, MirA from a sample of fractionated HeLa DNA. (Sequences of the primers and target DNA are listed in the appendix section). A typical control reaction, that will yield a sufficient quantity of eluted material to perform qPCR reactions in triplicate, is outlined below.

1. Perform capture experiment using 5 µl (0.5 µg) of fractionated HeLa Control DNA and 15 µl MBD2-Fc/Protein A Magnetic Beads as described above.
2. Elute the beads by incubation at 65°C in 100 µl for 15 minutes of DNase-free water.

Downstream Analysis

Quantitation of DNA by qPCR (for control fragmented HeLa DNA)

1. After magnetic capture, aliquot 10 µl each of the supernatant in triplicate for each methylated, unmethylated and control sample.
2. For input control and saved supernatant of the non-captured DNA fraction, dilute 1 µl of these samples in 1 ml water, aliquot 10 µl each of the sample in triplicate for each methylated, unmethylated and control sample.
3. Add 1 µl of the primer mix specific to each control locus to each tube.
4. Add 10 µl of the desired qPCR Master Mix according to manufacturer's recommendations.
5. Perform qPCR standard reaction program using an annealing temperature of 60°C.
6. Analyze quantitative PCR results using software provided with the real-time PCR machine.

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.

Quantitation of DNA by Standard PCR Reaction (for control fragmented DNA)

Perform capture experiment as described previously. Elute sample by incubation at 65°C in 50 µl of DNase-free water. Prepare a master reaction mix as described below:

REAGENT	VOLUME FOR 1 PCR REACTION
DNA Samples (1:1000 diluted non-captured DNA, 1:2000 diluted input DNA or undiluted capture DNA)	13.5 µl
Standard <i>Taq</i> Buffer (10X)	2.0 µl
dNTP Mix (4 mM)	1.0 µl
Primers (LINE, RPL30, MirA, 10 µM)	1.0 µl
<i>Taq</i> DNA Polymerase	0.5 µl

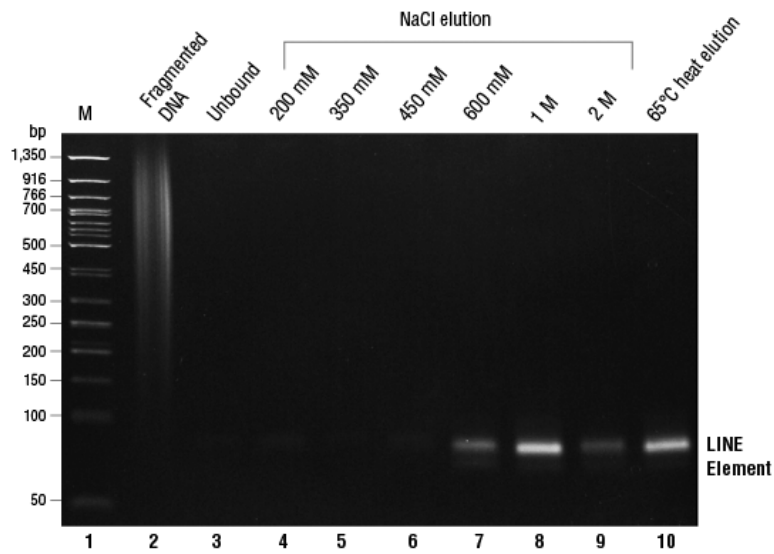
Transfer PCR tubes to a thermocycler:

Thermocycling conditions for a routine PCR:

CYCLE STEP	TEMP	TIME
Initial Denaturation	95°C	5 Seconds
25 cycles	95°C	15 Seconds
	60°C	15 Seconds
	72°C	30 Seconds
Final Extension	72°C	5 Minutes
Hold	4°C	

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. The expected size of all the amplicons is ~75 bp. Alternatively, the eluted sample DNA from the qPCR reaction tube may be used.

Figure 2: Endpoint PCR of a methylated locus (LINE) that has been purified using the EpiMark methylated DNA Enrichment Kit



Analyzed fractions are described above gel.

Troubleshooting Guide:

PROBLEM	POSSIBLE CAUSE(S)	SOLUTION(S)
No or poor DNA target detection by PCR-based method in unbound and/or elution fraction(s)	DNA is degraded	<ul style="list-style-type: none"> • Take precautions to prevent DNA degradation by maintaining a nuclease-free environment. • Increase EDTA concentration of sample to 10 mM
	Not enough target DNA	<ul style="list-style-type: none"> • Verify target DNA concentration by nanodrop instrument, or other sensitive DNA detection system • Run target DNA on agarose gel to determine quantity, quality and size
	DNA did not elute from the MBD2a-Fc beads	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • See above for DNA precautions
	DNA did not elute from the MBD2a-Fc beads	<ul style="list-style-type: none"> • See above for elution at 98°C
	PCR did not work	<ul style="list-style-type: none"> • Verify that all of 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 sufficient amounts of CpG methylation	<ul style="list-style-type: none"> • Raise input DNA concentration to at least 1 µg
	PCR did not work	<ul style="list-style-type: none"> • Optimize PCR conditions for your target sequence

Appendix

NaCl Elution Protocol

In this step, the captured methylated CpG containing DNA will be eluted with NaCl, either as a single fraction using 2 M NaCl, or multiple fractions. Before proceeding, determine the step-wise 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.

Prepare Elution Buffers:

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

2. Immediately following the Wash Off Unbound DNA Protocol, elute the captured DNA by resuspending the beads in 200 µl of Elution 1 Buffer.
3. Incubate the beads on a rotating mixer for 3 minutes. Place the tube on the magnetic rack for 2–5 minutes to allow the solution to clear.
4. Carefully remove the supernatant to a fresh microcentrifuge tube labeled Elution 1 and store on ice.
5. Resuspend the beads in 200 µl of the next Elution buffer and repeat the above process until all of the elution steps are completed.
6. Desalt and purify samples using Monarch or other PCR clean up kit following manufacturer's recommendations.

qPCR Primer Sequences and Amplicons

Methylated Sequence: LINE Element

GAAATGCAGAAATCA CCCGTCTT forward

CGGAGGCCGAATAGG AACAGCTCCG reverse

Amplicon-

GAAATGCAGAAATCACCCGTCTTCTGCATCGCTCACACTGGGAGCTGTAGACCGGAGCTGTTCTTATT

CGGCCTCCG 5 CpG

Unmethylated sequence: RPL30

CAAGGCAAAGCGAAATTGGT forward

GCCCGTTCAGTCTCTTCGATT reverse

Amplicon-

GCCCGTTCAGTCTCTTCGATTACCTCAAAGCTGGGCAGTTGTTAGCGAGAATGACCAATTTTCGCTTTCCTTG 4 CpG

Control Sequence: MirA

TTTAAACAGGATATTTACGTTCTGC forward

GAGGAAATCTTCACATCCACG reverse

Amplicon-

TTTAAACAGGATATTTACGTTCTGCTACAATTGACTGATAACACTTGAAGTGTAGTCTGAACAGTAATTTTGTTAATCATTTC

AACAAGTATTTGCTAAGTGAAGCCAGAAGAGGAGGAAAATGTTTTGCCACGTGGATGTGAAGATTTCTC 2 CpG

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Ordering Information

NEB #	PRODUCT	SIZE
E2600S	EpiMark Methylated DNA Enrichment Kit	25 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
N3236S/L	50 bp DNA Ladder	100-200/500-1,000 gel lanes

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

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

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