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MethylMiner™ Methylated DNA Enrichment Kit

For the enrichment of fragmented DNA based on the degree of methylation

Catalog no. ME10025

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User Manual

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Kit Contents and Storage

Kit Components and Storage

Sufficient components are provided for the enrichment of methylated DNA from up to 25 micrograms of fragmented input DNA. If you are starting with 5 ng to 1 µg of input DNA per reaction, the kit provides reagents for 25 separate capture reactions. See **Reaction Scale and Number of Reactions per Kit**, page 11.

Component	Amount	Shipping	Storage
Dynabeads® M-280 Streptavidin	250 µl	Wet ice	4°C *(Do not freeze)*
5X Bind/Wash Buffer	2 × 5 ml	Wet ice	4°C
Low-Salt Elution Buffer (contains no NaCl)	2 × 50 ml	Wet ice	4°C
High-Salt Elution Buffer (2000 mM NaCl)	2 × 50 ml	Wet ice	4°C
MBD-Biotin Protein (0.5 mg/ml)	200 µl	Dry ice	–80°C
Glycogen (20 µg/µl)	200 µl	Dry ice	–20°C
Primers for Non-Methylated Controls (100 µM each primer, forward and reverse, supplied as a mix)	20 µl	Dry ice	–20°C
Primers for Methylated Controls (100 µM each primer, forward and reverse, supplied as a mix)	20 µl	Dry ice	–20°C
Non-Methylated DNA (1 ng/µl)	20 µl	Dry ice	–20°C
Methylated DNA (1 ng/µl)	20 µl	Dry ice	–20°C
K-562 DNA (50 µg/ml)	100 µl	Dry ice	–20°C

Product Qualification

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Control DNA Sequences

Below are the sequences for the Methylated and Non-Methylated DNA control duplexes and associated primers.

Methylated DNA Control Duplex

```
5′-GCTATACAGGGMGTGTTAAMGATATAAMGTTTTGGCTMGACCAGTGACMGGACTCTMGTTCCTACCAGMGCAAMGCCCCC-3′
3′-CGATATGTCCCGMACAATTGMTATATTGMAAAACCGAGMTGGTCACTGGMCTGAGAGMAAGGATGGTCGMGTTGMGGGGG-5′
```

M = 5-methyl C
Methylated CpG dinucleotides are shaded in gray
Primer sequences are underlined
Amplified product length = 65 bp

Non-Methylated DNA Control Duplex

```
5′-GGCCCGGCGGTCGCCACACCAATTCGTTACTCAGGGACGTTACCACGGCTACTATCGTCGCAATTCAGTCAGGGATCTCG-3′
3′-CCGGGCCGCCAGCGGTGTGGTTAAGCAATGAGTCCCTGCAATGGTGCCGATGATAGCAGCGTTAAGTCAGTCCCTAGAGC-5′
```

Non-methylated CpG dinucleotides are shaded in gray
Primer sequences are underlined
Amplified product length = 69 bp

Forward Primer for Methylated DNA Control (22 bases)

```
5′-ACA GGG CGT GTT AAC GAT ATA A-3′
```

Reverse Primer for Methylated DNA Control (20 bases)

```
5′-CGC TGG TAG GAA CGA GAG TC-3′
```

Forward Primer for Non-Methylated DNA Control (24 bases)

```
5′-GTC GCC ACA CCA ATT CGT TAC TCA-3′
```

Reverse Primer for Non- Methylated DNA Control (24 bases)

```
5′-AGA TCC CTG ACT GAA TTG CGA CGA-3′
```

Materials Supplied by the User

Materials Supplied by the User

In addition to the kit components, you should have the following items on hand before using this kit. Ordering information for Invitrogen products listed below is provided on page 29.

- Magnet or magnetic rack (*e.g.*, the DynaMag™-2, catalog no. 123-21D, or DynaMag™-Spin Magnetic Rack, catalog no. 123-20D)
- Rotating mixer, for end-over-end rotation of tubes containing the Dynabeads® during binding and wash steps
- Vortex mixer
- 1.7-ml DNase-free microcentrifuge tubes
- Pipettes and DNase-free pipette tips
- DNase-free water
- 3 M Sodium Acetate, pH 5.2
- 100% ethanol
- 70% ethanol

Overview of the System

Overview of the MethylMiner™ Kit

The MethylMiner™ Methylated DNA Enrichment Kit is designed for the enrichment and fractionation of methylated double-stranded DNA (dsDNA) based on the degree of methylation. Methylated DNA is isolated from fragmented whole genomic DNA (5 ng–25 µg) via binding to the methyl-CpG binding domain of human MBD2 protein, which is coupled to paramagnetic Dynabeads® M-280 Streptavidin via a biotin linker. The methylated fragments can then be eluted as a single enriched population with a 2000 mM NaCl elution buffer, or into distinct subpopulations based on the degree of methylation by increasing the NaCl concentration of the elution buffer from 200 mM to 2000 mM in a stepwise gradient. In a stepwise gradient elution, the lower salt fractions contain fragments with fewer methyl groups, while higher salt fractions contain more highly methylated DNA.

The high affinity of MethylMiner™ MBD-Biotin Protein for CpG-methylated DNA provides greater sensitivity than antibody binding, while the use of Dynabeads® provides for a simplified, streamlined workflow. The kit provides materials for 25 affinity-based separations when starting with 5 ng–1 µg of fragmented genomic input DNA, and is scalable up to a single separatation using 25 µg of input DNA. The methlyated DNA may be eluted into as many as 8 fractions per separation.

With only minor processing, the methylated dsDNA is ready for downstream analysis by a variety of methods, including endpoint and real-time PCR assays; bisulfite conversion followed by amplification, cloning, and sequencing; direct sequencing; library preparation for high-throughput sequencing; labeling for DNA microarray analysis; and methylation-sensitive restriction enzyme-based assays.

Advantages of the System

- High-affinity binding of the MethylMiner™ MBD-Biotin Protein provides greater sensitivity than antibody-based methods
- Use of MBD-biotin allows fractionation of the sample based on CpG methylation density, allowing you to better focus on regions of interest
- Capture of dsDNA facilitates ligation of double-stranded adaptors for high-throughput sequencing
- Different elution methods support a wide range of downstream applications
- Simple, streamlined protocol can yield enriched fractions in less than 4 hours
- High-quality reagents and materials such as Dynabeads® ensure consistent results

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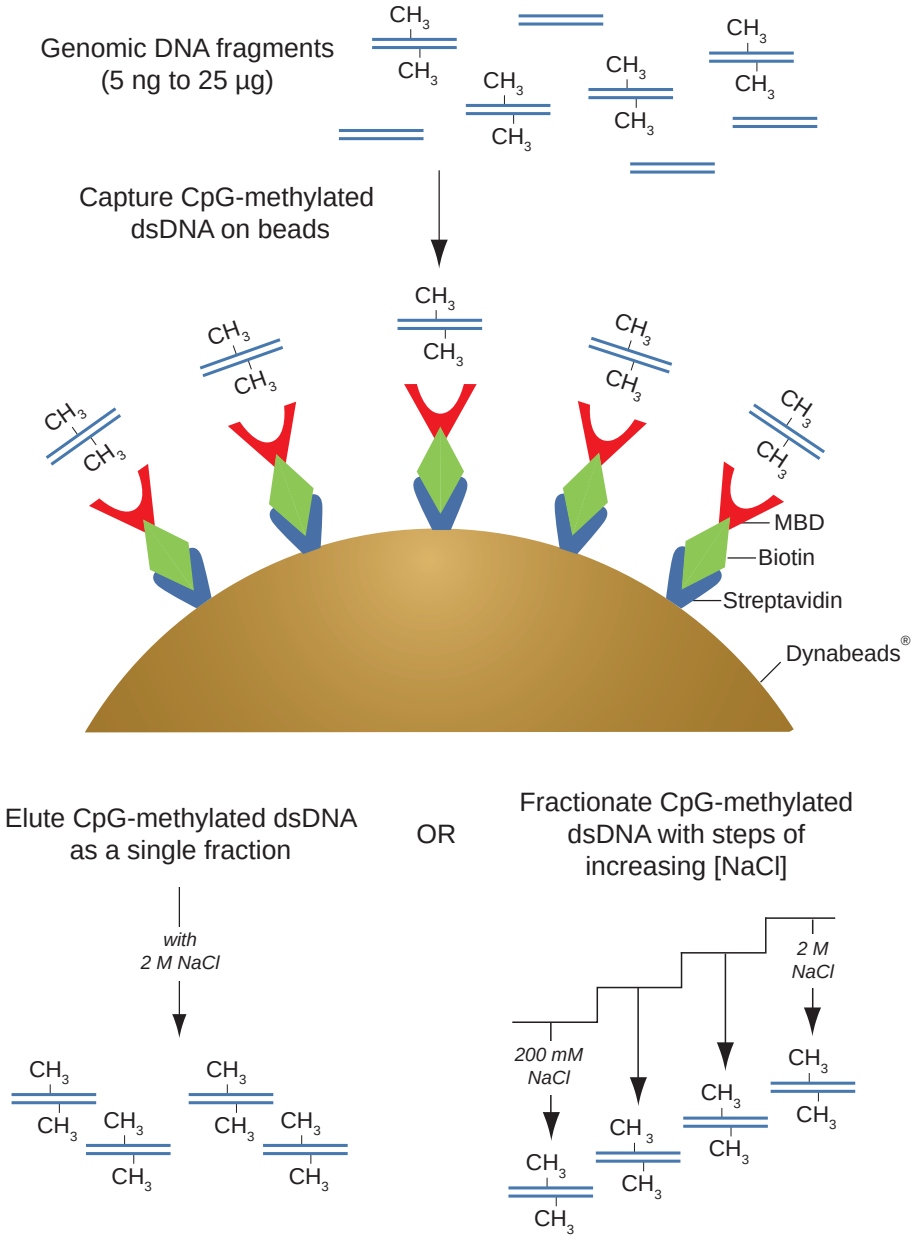
Experimental Illustration

MethylMiner™ allows for different elution strategies, depending on your preferred workflow and downstream application. You can perform:

- A single elution with undiluted High-Salt Elution Buffer containing 2000 mM NaCl
- A series of step-wise elutions with buffers containing successively greater NaCl concentrations, which will fractionate the DNA based on the degree of methylation

These options are illustrated in the figure below.



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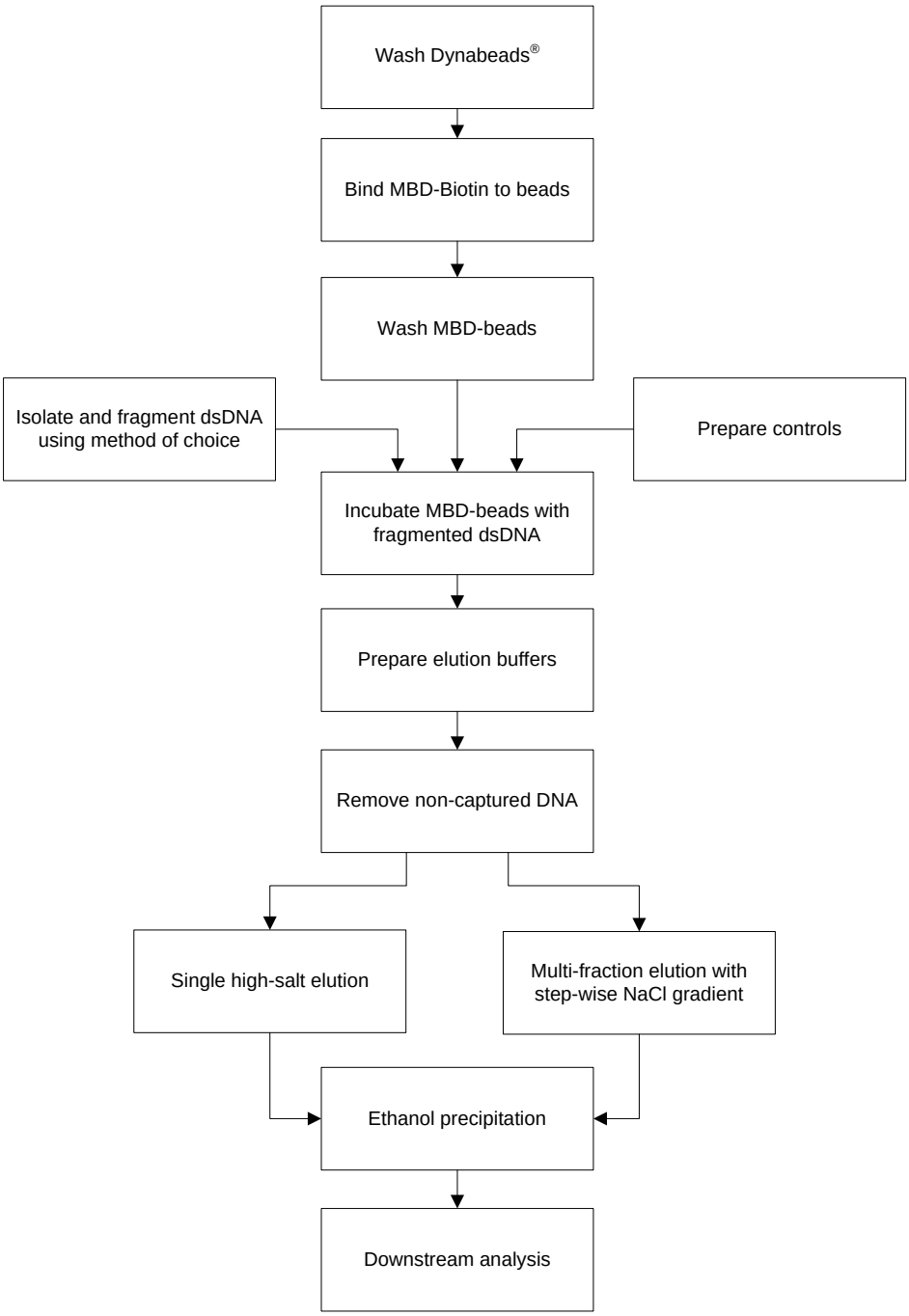
System Workflow Diagram



Scale of Reactions

Reaction Scale and Number of Reactions per Kit

The MethylMiner™ kit is designed to provide reagents for the enrichment of methylated DNA from up to 25 micrograms of fragmented double-stranded input DNA. If you are starting with 5 ng to 1 µg of input DNA per reaction, the kit provides sufficient reagents for 25 separate capture reactions. However, the reagents and protocol are fully scalable; you can use as much as 25 µg of input DNA in a single reaction.

Each capture reaction uses 10 µl of beads per microgram of input DNA. The protocol in this manual provides separate volumes and guidelines for the capture and elution of methylated DNA from the following input ranges:

- 5 ng to 1 µg of input DNA (25 capture reactions per kit)
- >1 µg to 10 µg of input DNA (~2–22 capture reactions per kit.)
- >10 µg to 25 µg of input DNA (1–2 capture reactions per kit)

Reaction Scale and Downstream Applications

For downstream analyses like PCR and qPCR, as little as 5 ng of input DNA can be used. For applications that require larger amounts of methylated DNA, such as library construction for high-throughput sequencing or amplification and labeling for microarray analysis, starting amounts of 10–25 µg of fragmented input DNA are most appropriate, though in some cases as little as 1 µg can be used. Typical total yields of mammalian CpG-methylated DNA are 3–20% of the input mass of DNA, or 0.3–5.0 µg when starting with 10–25 µg.

In many cases, the preferred elution strategy for downstream analysis will be a single fraction with the High-Salt Elution Buffer (2000 mM NaCl) provided in the kit. However, multiple fractions are also possible (see **Elution Strategies**, next page). For example, with MCF-7 cells (a human breast-cancer derived cell-line), we have observed that after washing, approximately equal masses of different populations of captured DNA can be eluted from MBD-beads by successive elutions with a buffer containing 500 mM NaCl followed by a buffer containing 1000 mM NaCl.

Elution Strategies

Elution Strategies

One flexible feature of the MethylMiner™ kit is the different elution strategies that can be used with it:

- **A single elution with High-Salt Elution Buffer that contains 2000 mM NaCl**. This will elute ~70–90% of the mass of CpG-methylated dsDNA that is captured. The eluted DNA can be directly precipitated with ethanol, resuspended in the buffer of choice, and used in most downstream analysis workflows. Note that antibody-based capture methods are not compatible with this elution strategy.
- **A multi-fraction elution with a step-wise series of buffers of increasing NaCl concentration**. CpG-methylated DNA elutes from the beads as a function of the number of methylation sites per molecule and ionic strength; higher salt concentrations release dsDNA molecules that have greater amounts of CpG-methylation. To elute the DNA into distinct populations based on the degree of methylation, you can generate a series of elution buffers of increasing NaCl concentration. The Low-Salt Buffer and High-Salt Buffer are mixed at differing ratios as described in the protocol to create buffers containing from 200 to 2000 mM NaCl. This capacity to easily fractionate the CpG-methylated DNA into sub-populations is another feature that antibody-based methods lack.

Each elution protocol is provided in **Eluting the Captured DNA**, page 22.

DNA Isolation and Fragmentation

Isolating Genomic DNA

Isolate DNA using your method of choice. The PureLink™ Genomic DNA Mini Kit is a complete kit for the isolation of genomic DNA. See page 29 for ordering information. A wide range of ChargeSwitch® Genomic DNA purification kits is also available from Invitrogen.

Important

Be careful to preserve the double-stranded nature of the DNA. MBD-Biotin Protein will not effectively bind single-stranded DNA.

General Handling of DNA

When handling DNA, use sterile conditions to ensure that no DNases are introduced. All equipment that comes into contact with DNA should be sterile and DNase-free, including pipette tips, microcentrifuge tubes, and pipettes. Be sure pipette barrels are clean and treated with ethanol.

DNA Yield

DNA yield can be estimated by UV absorbance at 260 nm or using Quant-iT™ dsDNA Assay Kits.

UV Absorbance

1. Measure the A_{260} of the solution using a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.5-8.5.
2. Calculate the amount of DNA using the formula:

DNA (µg) = [(A_{260} × 50 µg)/(1 A_{260} × 1 ml)] × dilution factor × total sample vol. (ml)

For DNA, A_{260} = 1 for a 50 µg/ml solution measured in a cuvette with an optical path length of 1 cm.

Quant-iT™ dsDNA Assay Kits

Quant-iT™ dsDNA Assay Kits provide a rapid, sensitive, and specific method for dsDNA quantitation with minimal interference from RNA, protein, single-stranded DNA (primers), or other common contaminants that affect UV absorbance.

Each kit contains a state-of-the-art quantitation reagent and a pre-made buffer to allow fluorescent DNA quantitation using standard fluorescent microplate readers/fluorometers or the Qubit™ Fluorometer.

DNA Fragmentation

DNA may be fragmented using your method of choice. DNA must be fragmented to an average size of less than 1,000 bp and should be in DNase-free water, TE buffer, or another low ionic-strength, neutral pH buffer.

The fragment size should be appropriate for your downstream analysis. For example, DNA fragmented to an average length of ~250 bp is suitable for assay by real-time quantitative PCR (qPCR). Similarly, DNA fragmented to an average length of ~100–200 bp is suitable for fragment library construction for short-read high-throughput sequencing.

DNA Length

To determine the size distribution of the DNA, perform gel electrophoresis on an agarose gel.

Preparing the Beads

Introduction

In this step, you couple the MBD-Biotin Protein to the Dynabeads® M-280 Streptavidin.

Materials Needed

The following materials are supplied by the user:

- 1X Bind/Wash Buffer (prepared from 5X Bind/Wash Buffer; see next page)
- 1.7-ml DNase-free microcentrifuge tubes
- Pipettes and DNase-free pipette tips
- Magnet or magnetic rack (*e.g.*, DynaMag™-2 or DynaMag™-Spin Magnetic Rack recommended; see page 29 for ordering information)
- Rotating mixer

Important

Always keep the following in mind when working with Dynabeads®:

- Never mix Dynabeads® by vortexing, as this will damage the beads.
- Never freeze Dynabeads®, as this will damage the beads.
- When removing liquid from Dynabeads®, avoid touching the beads with the pipette tip. This will disturb the bead pellet.
- Do not allow the beads to dry out. Resuspend the beads within 1 minute of removing any liquid from them.

Note

Before proceeding, we recommend reading through the entire protocol to determine the volumes of beads and 1X Bind/Wash Buffer you will need for the number and size of reactions you are performing.

Resuspending Dynabeads®

To resuspend Dynabeads®, use gentle up-and-down pipetting while taking care to avoid creating air bubbles. Never mix the beads by vortexing.

After resuspension, mix the beads by gently inverting the tube using continuous slow rotation.

Removing Liquid from Dynabeads®

To remove liquid from Dynabeads®:

1. Place the microcentrifuge tube containing the beads in a magnetic rack and allow to stand for at least 1 minute. During this time, the beads will concentrate as a pellet along the inner surface of the tube wall.
2. Open the microcentrifuge tube without displacing it from the rack or disturbing the bead pellet and carefully extract the liquid volume with a pipette tip *without touching the bead pellet*.
3. After the liquid has been removed, remove the tube from the rack and quickly and gently resuspend the beads with the volume of appropriate solution. *Do not allow the beads dry out.* Add the next solution within 1 minute.

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Bead Volume

For each microgram (µg) of input DNA, use 10 µl of Dynabeads® M-280 Streptavidin and 3.5 µg (7 µl) of MBD-Biotin Protein. For input amounts of less than 1 µg (*i.e.*, 5 ng to 1 µg), use 10 µl of beads and 7 µl of protein. The reaction size can be scaled up to 25 µg of input DNA.

In general, it is expected that more than one reaction will be carried out simultaneously, so multiple tubes are usually handled in parallel throughout the workflow.

Prepare 1X Bind/Wash Buffer

Prepare 1X Bind/Wash Buffer by diluting 1 part of 5X Bind/Wash Buffer with 4 parts of DNase-free water.

For example, for each 5 ng–1 µg capture reaction, prepare 1.8 ml of 1X Bind/Wash Buffer by diluting 360 µl of 5X Bind/Wash Buffer with 1.44 ml of DNase-free water. Read through the entire protocol and then scale accordingly depending on the number and size of your capture reactions.

Initial Bead Wash

In this step, you wash the Dynabeads® M-280 Streptavidin prior to coupling them with the MBD-Biotin Protein.

1. Resuspend the stock of Dynabeads® M-280 Streptavidin by gently pipetting up and down to obtain a homogenous suspension. *Never mix the beads by vortexing.*
2. For each microgram (µg) of input DNA, add 10 µl of beads to a 1.7-ml DNase-free microcentrifuge tube. For input amounts of 5 ng to 1 µg, add 10 µl of beads.
3. *For bead volumes <100 µl*: Bring the volume up to 100 µl with 1X Bind/Wash Buffer. Mix by gentle pipetting; do not mix by vortexing.

   For bead volumes >100 µl: Proceed to Step 4.
4. Place the tube(s) on a magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
5. With the tube in place on the magnetic rack, remove the liquid with a pipette without touching the beads with the pipette tip. Discard the liquid.
6. Remove the tube from the magnetic rack.
7. Add an equal volume (100–250 µl) of 1X Bind/Wash Buffer to the beads and resuspend by pipetting gently up and down.
8. Repeat Steps 4–7 once more, and then proceed to **Coupling the MBD-Biotin Protein to the Beads**, next page.

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Coupling the MBD-Biotin Protein to the Beads

In this step, you couple the Dynabeads® M-280 Streptavidin with the MBD-Biotin Protein. ***Do not add DNA in this step.***

1. For each microgram (µg) of input DNA, add 7 µl (3.5 µg) of MBD-Biotin Protein to a 1.7-ml DNase-free microcentrifuge tube. For input DNA amounts less than 1 µg, use 7 µl of MBD-Biotin Protein.
2. *If starting with ≤1 µg of input DNA:* Add 1X Bind/Wash Buffer to the protein to a final volume of 100 µl.

   If starting with >1 µg–10 µg of input DNA: Add 1X Bind/Wash Buffer to the protein to a final volume of 200 µl.

   If starting with >10 µg–25 µg of input DNA: Add 1X Bind/Wash Buffer to the protein to a final volume of 500 µl.
3. Transfer the diluted MBD-Biotin Protein to the tube of resuspended beads from Step 8, **Initial Bead Wash**, previous page (total volume = 200–750 µl).
4. Mix the bead-protein mixture on a rotating mixer at room temperature for 1 hour, then proceed to **Wash the MBD-beads**, next page.

Wash the MBD-beads

After mixing the beads and protein for 1 hour, follow the steps below to wash the coupled MBD-beads:

1. 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 the liquid with a pipette without touching the beads with the pipette tip, and discard the liquid. See **Removing Liquid from Dynabeads®**, page 14, for detailed instructions. *Always avoid touching the beads with the pipette tip.*
3. Resuspend the beads with 100–250 µl of 1X Bind/Wash Buffer (the same volume used in **Initial Bead Wash**, Step 7, previous page).
4. Mix the beads on a rotating mixer at room temperature for 5 minutes.
5. Repeat steps 1–4 two more times.
6. Place the tube on the magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
7. With the tube in place on the magnetic rack, remove the liquid with a pipette and discard the liquid.
8. Resuspend the beads in the same volume of 1X Bind/Wash Buffer as used above in Step 3.

The beads are now ready for methylated DNA capture.

Incubating MBD-Beads with Fragmented DNA

Introduction

In this step, you capture the fragmented methylated DNA on the MBD-beads.

Materials Needed

The following materials are supplied by the user:

- Fragmented DNA in DNase-free water, TE buffer, or other low ionic-strength, neutral pH buffer
- 1.7-ml DNase-free microcentrifuge tubes
- Pipettes and DNase-free pipette tips
- DNase-free water
- Rotating mixer
- Ice

Fragmented DNA

DNA must be fragmented to an average size of less than 1,000 bp and should be in DNase-free water, TE buffer, or other low ionic-strength, neutral pH buffer. For input amounts greater than 1 µg, the fragmented DNA should be at a concentration of 25 ng/µl or higher.

The fragment size should be appropriate for your downstream analysis. For example, DNA fragmented to an average length of ~100–200 bp is suitable for fragment library construction for short-read high-throughput sequencing. DNA fragmented to an average length of ~250 bp is suitable for assay by qPCR. In general, for PCR-based assays, the average fragment length should exceed the length of the sequence(s) to be amplified.

Control DNA

The MethylMiner™ kit includes two sets of synthetic duplex DNAs (labeled Methylated DNA and Non-Methylated DNA), two PCR primer sets specific for the duplexes, and one tube of pre-fragmented K-562 DNA for use as controls.

Each synthetic duplex is 80 bp long and is provided at a concentration of 1 ng/µl. See page 6 for each sequence.

- The Methylated DNA control sequence has 8 fully methylated CpG dinucleotides distributed along its length, and is used as a positive control.
- The Non-Methylated DNA control sequence has 9 non-methylated CpG dinucleotides along its length, and is used as a negative control.

To validate the use of the kit, "spike" 10 pg of each duplex DNA (diluted as below) into a control sample of 1 µg of the K-562 DNA, then perform the capture reaction, elute, and detect by PCR or qPCR using the PCR primer sets that are specific for each duplex.

Diluting the Control Duplexes

Immediately before use, dilute 1 µl of each control duplex provided in the kit (Methylated DNA or Non-Methylated DNA) in 99 µl of DNase-free water, to obtain a concentration of 10 pg/µl.

Always make a fresh dilution. Do not store synthetic duplexes at a concentration below 1 ng/µl.

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Note

We recommend using the control DNA duplexes in an external control reaction with the K-562 DNA provided in the kit. Although the primer sets provided for the control DNA are designed to be "alien" to human and mouse genomes and to the sequences that comprise NCBI's non-redundant DNA sequence database (as of September 2008), we do not currently recommend spiking the duplexes into actual test samples. Fragmented human DNA from cultured K-562 cells has been demonstrated to be suitable for use with the control duplexes. We have also successfully tested the duplexes with fragmented DNA from MCF-7 cells.

Input DNA Amounts

Different protocols are provided depending on the amount of input DNA you are starting with (5 ng–1 µg, >1 µg–10 µg, or >10 µg–25 µg). A separate protocol is also provided for a control reaction (using 1 µg of K-562 DNA).

Capture Reaction: 5 ng–1 µg Input DNA

1. To a clean 1.7-ml DNase-free microcentrifuge tube, add 20 µl of 5X Wash/Bind Buffer. (***Note:*** Be sure to use 5X buffer in this step, *not* 1X buffer.)
2. Add 5 ng–1 µg of fragmented sample DNA to the tube (added volume should be ≤ 80 µl).
3. Bring the final volume to 100 µl with DNase-free water.
4. Transfer the DNA/Buffer mixture to the tube containing the MBD-beads (from **Wash the MBD-beads**, page 16, Step 8).
5. Mix the MBD-beads with the DNA on a rotating mixer for 1 hour at room temperature (alternatively, you can mix overnight at 4ºC).

If you will be eluting in a stepwise gradient of increasing NaCl concentration, prepare the buffers while the beads are mixing by proceeding to **Preparing Buffers for a Step-wise Elution Series**, page 20.

Otherwise, continue to **Removing the Non-Captured DNA**, page 21.

Capture Reaction: >1 µg–10 µg Input DNA

1. To a clean 1.7-ml DNase-free microcentrifuge tube, add 100 µl of 5X Wash/Bind Buffer. (***Note:*** Be sure to use 5X buffer in this step, *not* 1X buffer.)
2. Add >1–10 µg of fragmented sample DNA at a concentration of 25 ng/µl to the tube (added volume will be 40–400 µl).
3. Bring the final volume to 500 µl with DNase-free water.
4. Transfer the DNA/buffer mixture to the tube containing the MBD-beads (from **Wash the MBD-beads**, page 16, Step 8).
5. Mix the MBD-beads with the DNA on a rotating mixer for 1 hour at room temperature (alternatively, you can mix overnight at 4ºC).

If you will be eluting in a stepwise gradient of increasing NaCl concentration, prepare the buffers while the beads are mixing by proceeding to **Preparing Buffers for a Step-wise Elution Series**, page 20.

Otherwise, continue to **Removing the Non-Captured DNA**, page 21.

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Capture Reaction: >10 µg–25 µg Input DNA

1. To a clean 1.7-ml DNase-free microcentrifuge tube, add 250 µl of 5X Wash/Bind Buffer. (***Note:*** Be sure to use 5X buffer in this step, *not* 1X buffer.)
2. Add >10–25 µg of fragmented sample DNA at a concentration of 25 ng/µl to the tube (added volume will be 400–1000 µl).
3. Bring the volume to 1,250 µl with DNase-free water.
4. Transfer the DNA/Buffer mixture to the tube containing the MBD-beads (from **Wash the MBD-beads**, page 16, Step 8).
5. Mix the MBD-beads with the DNA on a rotating mixer for 1 hour at room temperature (alternatively, you can mix overnight at 4ºC).

If you will be eluting in a stepwise gradient of increasing NaCl concentration, prepare the buffers while the beads are mixing by proceeding to **Preparing Buffers for a Step-wise Elution Series**, next page.

Otherwise, continue to **Removing the Non-Captured DNA**, page 21.

Control Capture Reaction with 1 µg of K-562 DNA

The following control reaction uses 1 µg of K-562 DNA, supplied in the kit. Note that the K-562 DNA is already fragmented.

1. To a clean 1.7-ml DNase-free microcentrifuge tube, add 20 µl of 5X Wash/Bind Buffer. (***Note:*** Be sure to use 5X buffer in this step, *not* 1X buffer.)
2. Thaw and briefly vortex the K-562 DNA (50 µg/ml) provided in the kit, and add 20 µl (1 µg) to the tube.
3. Add 1 µl of the diluted Methylated DNA control (10 pg/µl) and 1 µl of the diluted Non-Methylated DNA control (10 pg/µl) to the tube.
4. Bring the final volume to 100 µl with DNase-free water
5. Transfer the DNA mixture to the tube containing the MBD-beads (from **Wash the MBD-beads**, page 16, Step 8).
6. Mix the MBD-beads with the DNA on a rotating mixer for 1 hour at room temperature (alternatively, you can mix overnight at 4ºC).

If you will be eluting in a stepwise gradient of increasing NaCl concentration, prepare the buffers while the beads are mixing by proceeding to **Preparing Buffers for a Step-wise Elution Series**, next page.

Otherwise, continue to **Removing the Non-Captured DNA**, page 21.

Preparing Buffers for a Multi-Fraction Elution Series

Step-wise Elution Buffers

CpG-Methylated DNA that has been captured on the MBD-beads can be eluted from the beads in a single or multiple fractions, as described in **Elution Strategies** on page 12.

To elute the CpG-methylated DNA as a single fraction, use the undiluted High-Salt Elution Buffer (2000 mM NaCl).

To fractionate the CpG-methylated DNA into distinct populations based on the number of methylation sites per molecule, you can use a step-wise elution with a series of buffers of increasing NaCl concentration. Two stock buffers are provided to prepare such a series of buffers:

- Low-Salt Elution Buffer contains no NaCl (0 mM)
- High-Salt Elution Buffer contains 2000 mM NaCl

These two stock buffers differ only in their NaCl concentrations. To achieve any desired concentration of NaCl up to 2000 mM, simply mix the two buffers in the appropriate ratio. In practice, buffers ranging from 200 mM NaCl to 2000 mM NaCl work best, since the Bind/Wash buffer contains approximately 160 mM NaCl.

We recommend testing a few different fractionation schemes to determine what works best for your particular workflow, number of samples to analyze, and downstream applications. The following table shows an example of a series of Elution Buffers and the appropriate ratios of Low-Salt and High-Salt Buffers to prepare them.

Example Multi-Fraction Elution Series

Elution Buffer/Fraction (from first to last)	NaCl Concentration	Percent Low-Salt Buffer	Percent High-Salt Buffer
Non-captured DNA supernatant	~160 mM NaCl (1X Bind/Wash Buffer alone)	—	—
Non-captured DNA wash	~160 mM NaCl (1X Bind/Wash Buffer alone)	—	—
Elution 1	200 mM	90%	10%
Elution 2	350 mM	82.5%	17.5%
Elution 3	450 mM	77.5%	22.5%
Elution 4	600 mM	70%	30%
Elution 5	1000 mM	50%	50%
Elution 6	2000 mM	0%	100%

Preparing Each Buffer for a Multi-Fraction Elution Series

For each capture reaction, prepare 1,400 µl of each elution buffer desired (~20% more than is minimally needed). For example, 1,400 µl of the Elution 3 buffer is composed of a mixture of 1,085 µl (77.5% of 1,400 µl) of Low-Salt Elution Buffer and 315 µl (22.5% of 1,400 µl) of High-Salt Elution Buffer.

Removing the Non-Captured DNA

Introduction

In this step, you collect the non-captured DNA from the bead solution.

Materials Needed

In addition to the kit components, you will need the following:

- 1X Bind/Wash Buffer
- Magnet or magnetic rack
- Rotating mixer
- 1.7-ml DNase-free microcentrifuge tubes
- Pipettes and DNase-free pipette tips
- DNase-free water
- Ice
- Prepared Elution Buffers

Important

Be sure to have your elution buffer(s) prepared and ready to add to the beads before you perform the protocol below. After performing the final wash step, *immediately* proceed to **Eluting the Captured DNA**, starting on page 18, and add elution buffer to prevent the beads from drying out.

Removing Non-Captured DNA from the Beads

1. After mixing the DNA and MBD-beads (**Methylated DNA Capture**, pages 18–19), place the tube on the magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
2. With the tube in place on the magnetic rack, remove the supernatant liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. This saved supernatant is the non-captured DNA supernatant fraction. Store this sample on ice.
3. Add 200 µl of 1X Bind/Wash Buffer to the beads to wash the beads of residual non-captured DNA.
4. Mix the beads on a rotating mixer for 3 minutes.
5. Place the tube on the magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
6. With the tube in place on the magnetic rack, remove the liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. This saved liquid is a non-captured DNA wash fraction. Store this sample on ice.
7. *For capture reactions of ≤1 µg of input DNA:* Repeat steps 3–6 once more to obtain two wash fractions in total. Store each sample on ice.

   For capture reactions of >1 µg – 25 µg input DNA: Repeat steps 3–6 three more times to obtain four wash fractions in total. Store each sample on ice.

   Important: After collecting the final wash fraction, immediately proceed to **Eluting the Captured DNA**, starting on page 18, and resuspend the beads in elution buffer to prevent the beads from drying out.

Pool the first and second wash fractions together and label **"Wash A"**. If applicable, pool the third and fourth wash fractions and label **"Wash B"**. ***Note:*** Pooling the wash fractions is not mandatory, but is suggested as a matter of convenience prior to ethanol precipitation.

Eluting the Captured DNA

Introduction

This section provides protocols for eluting with NaCl, either as a single fraction or multiple fractions.

Materials Needed

In addition to the kit components, you will need the following:

- Prepared Elution Buffers
- Magnet or magnetic rack
- Rotating mixer
- 1.7-ml DNase-free microcentrifuge tubes
- Pipettes and DNase-free pipette tips
- DNase-free water

Note

- For ≤1 µg input DNA, perform each NaCl elution at each ionic strength twice to ensure complete (>95%) removal of DNA from beads. For >1 µg input DNA, perform each elution three times.
- To elute captured CpG-methylated DNA into distinct fractions, start with the lowest NaCl concentration Elution Buffer (*e.g.*, 200 mM NaCl). Follow this with the next higher NaCl concentration (*e.g.*, 350 mM NaCl). Continue this process in order until all the desired fractions have been collected.

Single Fraction Elution with 2000 mM NaCl

Immediately after removing the non-captured DNA from the beads (**Removing Non-Captured DNA from the Beads**, Step 7, page 21), follow the protocol below to elute the captured DNA as a single fraction using the High-Salt Elution Buffer.

1. *For ≤1 µg of input DNA:* Resuspend the beads in 200 µl of the High-Salt Elution Buffer (2000 mM NaCl) provided in the kit.

   For >1 µg–25 µg of input DNA: Resuspend the beads in 400 µl of the High-Salt Elution Buffer (2000 mM NaCl) provided in the kit.
2. Incubate the beads on a rotating mixer for 3 minutes.
3. Place the tube on the 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 liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. Store this sample on ice.
5. *For ≤1 µg of input DNA:* Repeat Steps 1–4 once, collecting the second sample in a different tube. Pool these two elution samples (the total volume will be 400 µl). Store the pooled sample on ice.

   For >1 µg–25 µg of input DNA: Repeat Steps 1–4 twice, collecting each sample in a different tube. This ensures complete (>95%) elution of the DNA that can be eluted at this ionic strength. Store each sample on ice.

Proceed to **Ethanol Precipitation**, page 25.

Continued on next page

Multi-Fraction Elution— ≤1 µg Input DNA

Use the following protocol if you started with ≤1 µg input DNA.

Immediately after removing the non-captured DNA from the beads (**Removing Non-Captured DNA from the Beads**, Step 7, page 21), elute the captured DNA as multiple fractions with step-wise increases in NaCl concentration as follows:

1. Resuspend the beads in 200 µl of the lowest NaCl-concentration Elution Buffer that you have prepared (*e.g.,* 200 mM NaCl in the example table on page 20).
2. Incubate the beads on a rotating mixer for 3 minutes.
3. Place the tube on the 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 liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. Label this tube "Elution 1a" and store on ice.
5. Repeat Steps 1–4 once, collecting the second sample in a different tube. Pool these two elution samples (Elution 1a and 1b) into one tube and label the tube "Elution 1" (the total volume will be 400 µl).
6. Resuspend the beads in 200 µl of the next higher NaCl-concentration Elution Buffer that you have prepared (*e.g.,* 350 mM NaCl in the example table on page 20).
7. Incubate the beads on a rotating mixer for 3 minutes.
8. Place the tube on the magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
9. With the tube in place on the magnetic rack, remove the liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. Label this tube "Elution 2a" and store on ice.
10. Repeat Steps 6–9 once, collecting second sample in a different tube. Pool the two elution samples (Elution 2a and 2b) into one tube and label the tube "Elution 2" (the total volume will be 400 µl). Store this sample on ice.
11. Repeat Steps 6–10 using each successively higher NaCl concentration buffer and numbering the collected samples accordingly (Elution 3, 4, etc.) until all elutions have been completed.

Proceed to **Ethanol Precipitation**, page 25.

Continued on next page

Multi-Fraction Elution— >1–25 µg Input DNA

Use the following protocol if you started with >1–25 µg input DNA.

Immediately after removing the non-captured DNA from the beads (**Removing Non-Captured DNA from the Beads**, Step 7, page 21), elute the captured DNA as multiple fractions with step-wise increases in NaCl concentration as follows:

1. Resuspend the beads in 400 µl of the lowest NaCl-concentration Elution Buffer that you have prepared (*e.g.,* 200 mM NaCl in the example table on page 20).
2. Incubate the beads on a rotating mixer for 3 minutes.
3. Place the tube on the 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 liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. Label this tube "Elution 1a" and store on ice.
5. Repeat Steps 1–4 twice, collecting each sample in a different tube. Label these tubes "Elution 1b" and "Elution 1c" and place on ice.
6. Resuspend the beads in 400 µl of the next higher NaCl-concentration Elution Buffer that you have prepared (*e.g.,* 350 mM NaCl in the example table on page 20).
7. Incubate the beads on a rotating mixer for 3 minutes.
8. Place the tube on the magnetic rack for 1 minute to concentrate all of the beads on the inner wall of the tube.
9. With the tube in place on the magnetic rack, remove the liquid with a pipette without touching the beads with the pipette tip, and save it in a clean DNase-free microcentrifuge tube. Label this tube "Elution 2a" and store on ice.
10. Repeat Steps 6–9 twice, collecting each sample in a different tube. Label these tubes "Elution 2b" and "Elution 2c" and place on ice.
11. Repeat Steps 6–10 using each successively higher NaCl concentration buffer and numbering the collected samples accordingly (Elution 3a, 3b, 3c, 4a, 4b, 4c, etc.) until all elutions have been completed.

Proceed to **Ethanol Precipitation**, page 25.

Ethanol Precipitation

Introduction

In this step, you clean up the DNA from all non-captured, wash, and elution fractions via ethanol precipitation.

Materials Needed

In addition to the kit components, you will need the following:

- 3 M Sodium Acetate, pH 5.2
- 100% ethanol
- 70% ethanol
- Ice

DNA Cleanup and Concentration by Ethanol Precipitation

1. To each non-captured, wash, and elution fraction from the previous steps, add the following components:
   - 1 µl Glycogen (20 µg/µl) (included in kit)
   - 1/10th sample volume of 3 M sodium acetate, pH 5.2 (*e.g.*, 40 µl per 400 µl of sample)
   - 2 sample volumes of 100% ethanol (*e.g.*, 800 µl per 400 µl of sample)
2. Mix well and incubate at –80°C for at least 2 hours.
3. Centrifuge the tube for 15 minutes ≥12,000 × *g* at 4°C or room temperature.
4. Carefully discard the supernatant without disturbing the pellet.
5. Add 500 µl of cold 70% ethanol.
6. Centrifuge the tube for 5 minutes ≥12,000 × *g* at 4°C or room temperature.
7. Carefully discard the supernatant without disturbing the pellet.
8. Repeat Steps 6–7 once and remove any remaining residual supernatant.
9. Air-dry the pellet for ~5 minutes (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 specific downstream applications).
11. Place the DNA on ice and proceed to any desired downstream applications, or store the DNA at –20°C or below until further use.

Downstream Analysis

Types of Analysis

The recovered DNA in each fraction is largely double-stranded and is ready for analysis by:

- Cloning and Sanger sequencing
- Bisulfite conversion, followed by PCR amplification, cloning, and sequencing. The MethylCode™ Bisulfite Conversion Kit is available separately from Invitrogen; see page 29 for ordering information.
- Methylation-sensitive assays
- Locus detection by endpoint PCR
- Locus detection and quantification by real-time PCR
- Library construction for high-throughput sequencing
- Amplification and labeling for microarray-based profiling

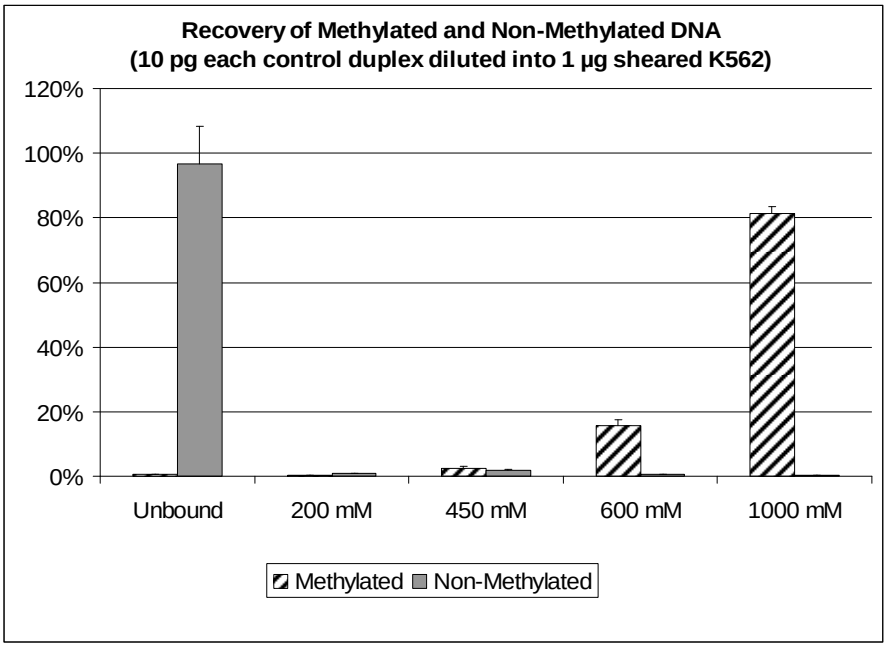
Expected Endpoint PCR and qPCR Results Using the Controls

You should observe the following results for the control reaction, based on detection by endpoint PCR or qPCR using the PCR primer sets specific for each control DNA duplex:

- The Non-Methylated DNA duplex will not be captured by the beads (*i.e.*, >70% will remain in the supernatant)
- The Methylated DNA duplex will be tightly bound by the beads and require a high ionic-strength elution buffer (*e.g.*, NaCl at 1000 mM) to release >70% from the beads.

Example qPCR Results for a Control Reaction

The percent recovery in the graph below was determined by qPCR. The elution pattern as determined by 27 cycles of PCR is shown in the 4% agarose gel images.



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Example Endpoint PCR Results for a Control Reaction

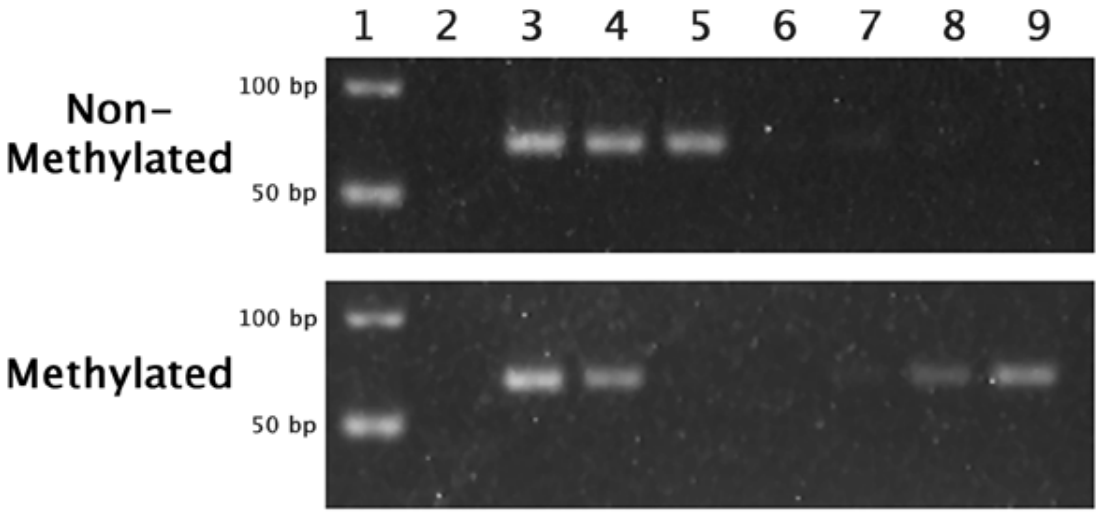
One microliter each of input, unbound, and eluted DNA (1/60th of ethanol precipitation reaction) from a control reaction were subjected to 27 cycles of PCR with Platinum® PCR SuperMix High Fidelity (Catalog no. 12532-016) and the appropriate primer mix. Twenty percent (10 µl) of each reaction was loaded onto E-Gel® 4% High Resolution Agarose Gels (Catalog no. G018-04) in parallel with a 50-bp DNA Ladder (Catalog no. 10416-014).

The lanes are as follows:

1. 50-bp ladder
2. Fragmented K-562 human genomic DNA
3. Mix of Non-Methylated and Methylated DNA
4. Input DNA (K-562 DNA + mix of Non-Methylated and Methylated DNA)
5. Non-captured (unbound) DNA fraction
6. 200 mM NaCl elution fraction
7. 450 mM NaCl elution fraction
8. 600 mM NaCl elution fraction
9. 1000 mM NaCl elution fraction

Thermocycling conditions were:

- Step 1: 94°C for 2 min.
- Step 2: 94°C for 15 sec.
- Step 3: 55°C for 15 sec.
- Step 4: 68°C for 30 sec.
- Step 5: Repeat steps 2–4 for 26 times
- Step 6: 68°C for 5 min.
- Step 7: 4°C hold



Appendix

Troubleshooting

Problem	Cause	Solution
No or poor DNA detection by spectrophotometry of the unbound or elution fraction(s)	Yield is too low to detect by spectrophotometry	Use a more sensitive method to quantitate DNA (*e.g.*, a Quant-iT Assay or PCR/qPCR).
	Interference from elution buffer components	Clean up DNA by ethanol precipitation.
No or poor DNA target detection by PCR-based method in unbound and/or elution fraction(s)	PCR conditions are sub-optimal	Optimize PCR conditions for your target sequence.
	Average DNA fragment size is smaller than expected amplicon size	Design PCR primers to complement average DNA fragment size, or fragment DNA to a larger average size to suit your amplicon size.
	DNA is degraded	Take proper precautions to maintain a nuclease-free environment.
	Some or all of the DNA sample was lost during cleanup by ethanol precipitation	Repeat the experiment using 2 µl of glycogen during ethanol precipitation to obtain a more visible pellet.
	DNA is highly methylated and did not elute from the MBD-beads	Elute DNA with a high ionic strength buffer: either the High-Salt Buffer (2000 mM NaCl) provided in the kit, or a 50:50 mixture of this buffer with 5 M NaCl to yield an elution buffer containing 3.5 M NaCl.
No or poor DNA target detection by PCR-based method in elution fraction, but DNA is detected in unbound fraction	DNA is denatured and is not captured by MBD-Biotin Protein	Maintain the double-stranded nature of the DNA. MBD-biotin will not bind single-stranded DNA efficiently.
	DNA target does not contain sufficient amounts of CpG methylation	Verify the CpG methylation status of your target sequence by a bisulfite sequencing method. Refer to our website at www.invitrogen.com/epigenetics for available products and methods.
	Kit components or procedure may have been compromised (capture reaction failed)	Perform a control reaction with the control DNA included in the kit, followed by PCR-based detection as described on pages 26–27.

Accessory Products

Additional Products

Additional products are available separately from Invitrogen. Ordering information is provided below. For more information, visit our website at www.invitrogen.com or contact Technical Support (page 29).

Product	Quantity	Catalog no.
MethylCode™ Bisulfite Conversion Kit	50 rxns	MECOV-50
PureLink™ Genomic DNA Mini Kit	10 preps 50 preps 250 preps	K1820-00 K1820-01 K1820-02
PureLink™ Genomic Plant DNA Purification Kit	50 preps	K1830-01
Quant-iT™ dsDNA Assay Kit, Broad Range, 2–100 ng	1000 assays	Q-33130
Quant-iT™ dsDNA Assay Kit, High Sensitivity, 0.2–100 ng	1000 assays	Q-33120
Quant-iT™ PicoGreen® dsDNA Assay Kit	2000 assays	P7589
Qubit™ Fluorometer	1 unit	Q32857
DynaMag™-2	1 magnet	123-21D
DynaMag™-Spin	1 magnet	123-20D
Dynal® Sample Mixer	1 mixer	947-01
Dynal® MX1 Mixer	1 mixer	159-07
E-Gel® 4% High-Resolution Agarose	18 gels	G5018-04
E-Gel® 2% General Purpose Agarose Gels	Starter pack with base 18 gels	G6000-01 G5018-02
E-Gel® 2% with SYBR Safe™	Starter pack with base 18 gels	G6206-02 G5218-02
TrackIt™ 50 bp DNA Ladder	100 applications	10488-043
EXPRESS SYBR® GreenER™ qPCR Supermix Universal	200 rxns 1000 rxns	11784-200 11784-01K
EXPRESS qPCR Supermix Universal	200 rxns 1000 rxns	11785-200 11785-01K
Platinum® PCR SuperMix High Fidelity	100 rxns 500 rxns	12532-016 12532-024
UltraPure™ DEPC-treated Water	4 × 100 ml 1 L	750024 750023
UltraPure™ DNase/RNase-Free Distilled Water	10 × 500 ml	10977-023

Applied Biosystems Products for Real-Time PCR

Applied Biosystems has a wide range of industry-standard instruments, reagents, and other products for real-time PCR. Visit their website at www.appliedbiosystems.com.

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

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