

# Promoter Methylation PCR Kit

Cat. # MP1100

Product User Manual  
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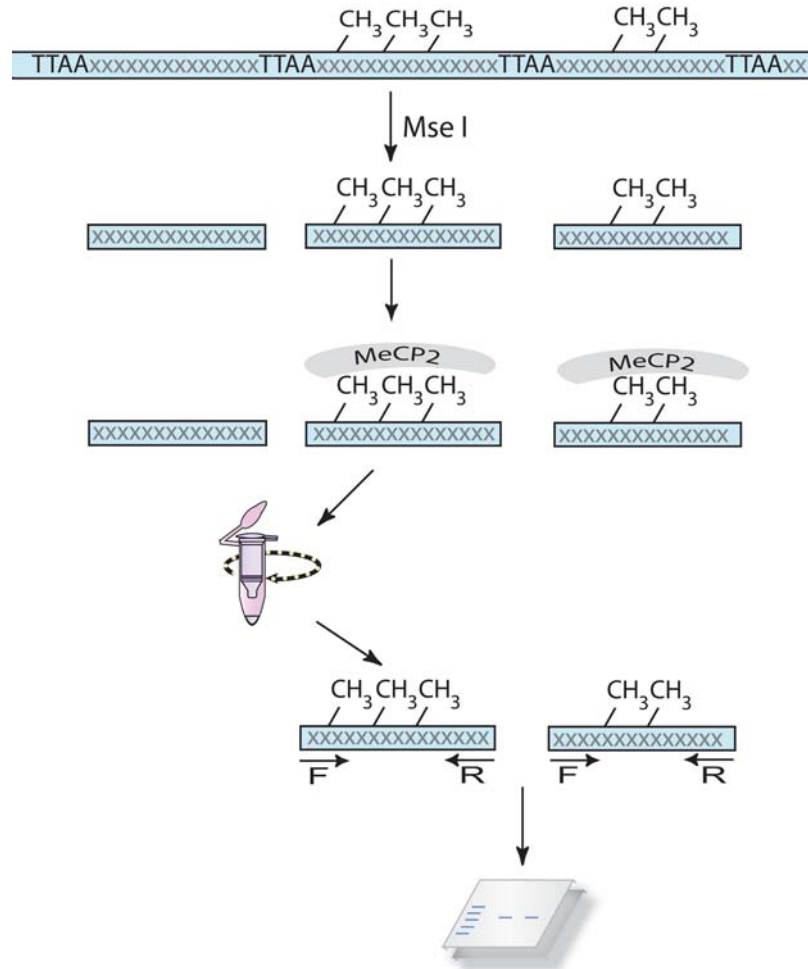
## 1. INTRODUCTION

DNA methylation is a commonly occurring modification of human DNA. It involves the addition of a methyl group to cytosine residues at CpG dinucleotides, a reaction that is catalyzed by DNA methyltransferase (DNMT) enzymes. CpG dinucleotides are gathered in clusters called CpG islands, which are unequally distributed across the human genome. There are approximately 30,000 CpG islands in the genome and 50-60% of these are found within the promoter region of genes. CpG islands are primarily unmethylated in normal tissues, and the aberrant methylation of CpG islands is clearly related with diseases, such as cancer.

MeCP2 is a member of a family of proteins that selectively recognizes methylated CpGs. The binding of these proteins to DNA leads to an altered chromatin structure, which subsequently prevents the binding of transcription machinery and thus precludes gene expression. The abnormal methylation causes transcriptional repression of numerous genes, leading to tumor growth and development. Studies of DNA methylation in cancer have uncovered new potential targets for the diagnosis, prognosis and ultimately the treatment of human cancer.

There has been a delay in the appreciation of methylation as an important epigenetic event in cancer progression. This has been due to the difficulties associated with the analysis of DNA methylation, as standard molecular biology techniques do not preserve methylation of the genomic DNA. Panomics has developed a new PCR method for analyzing methylation status of a specific promoter of interest. The method is based on MeCP2 binding, which differentiates methylated from unmethylated promoters. Compared to sodium bisulphate-based conversion of methylated bases in conjunction with PCR amplification, this method is very simple and straight forward.

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**Figure 1: Flow chart of the Promoter Methylation PCR procedure.** Three basic steps are involved: (1) Genomic DNA is digested with a restriction enzyme. (2) The DNA fragments are incubated with MeCP2 (a methylation binding protein or MBP), resulting in formation of protein/DNA complexes. (3) Methylated fragments are subsequently isolated and amplified by PCR. The PCR products are visualized by agarose gel electrophoresis.

NOTE: PCR primers for Promoter Methylation PCR Kit are sold separately. Please see [www.panomics.com](http://www.panomics.com) for a current listing of primers.

## 2. MATERIALS PROVIDED

### Box 1: Reagents (4 °C)

- DNA Purification Columns (6 each)
- Separation Columns (6 each)
- 1X Column Incubation Buffer (2 x 2 ml)
- 1X Column Wash Buffer (2 x 10 ml)
- 1X Column Elution Buffer (200 µl)
- PE Buffer (5 ml)
- PB Buffer (650 µl)

### Box 2: Reaction Kit (-20 °C)

- Control Primer Pair<sup>a</sup> (5 µl)
- MBP (2 x 6 µl)
- Distilled H<sub>2</sub>O (dH<sub>2</sub>O; RNase, DNase free; 500 µl)
- Control Genomic DNA<sup>a</sup> (10 µl)
- 5X Binding Buffer (2 x 25 µl)

## 3. ADDITIONAL MATERIALS REQUIRED

### 3.1 Reagents and Solutions

- Forward and Reverse PCR Primers<sup>b</sup>
- Genomic DNA Extraction kit (e.g., Panomics Genomic DNA Extraction Kit, Cat. # AY2005)
- MseI Restriction Enzyme (10,000 U/ml)
- Agarose gel electrophoresis reagents (see reference 2)
- Panomics PCR Reagent Kit (Cat. # MP0000)
- OR-
- PCR reagents from any commercially available PCR kit

### 3.2 Materials and Equipment

- 0.5-ml and 1.5-ml microfuge tubes
- Pipetman and tips
- Microcentrifuge
- Agarose-gel electrophoresis apparatus with 8-mm wide combs
- Thermocycler for PCR

<sup>a</sup>Provided as positive control.

<sup>b</sup>Panomics offers PCR primers for 40 different gene promoter regions. Please see [www.panomics.com](http://www.panomics.com) for a current listing of primers. If using your own primers, see section 7 for primer design tips.

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### 4. PREPARING GENOMIC DNA FROM CELLS OR TISSUES

Genomic DNA can be prepared using a commercially available kit, such as Panomics' Genomic DNA Extraction Kit (Cat. # AY2005). For best results, your DNA should have a concentration of 100 - 500 ng/ $\mu$ l.

### 5. FRAGMENTATION OF GENOMIC DNA

In this section, you will digest the genomic DNA with MseI restriction enzyme, to produce small fragments of DNA that retain the CpG islands. Control Genomic DNA is provided as a positive control.

5.1 Set up the following restriction digest:

Genomic DNA (400 ng/ $\mu$ l)	10 $\mu$ l
10X NE Buffer 2 with BSA	2 $\mu$ l
MseI (10,000 U/ml)	1 $\mu$ l
dH <sub>2</sub> O	7 $\mu$ l

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Total Volume	20 $\mu$ l
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5.2 Mix well by pipetting and incubate at 37°C for 2 hours.

5.3 Add 100  $\mu$ l PB Buffer to the digest reaction and transfer all solution to the **DNA Purification Column**.

5.4 Bind the DNA to the column, centrifuge at 10,000g, for 30 - 60 s.

5.5 Discard flow-through.

5.6 Add 750  $\mu$ l PE Buffer and centrifuge at 10,000g, for 30 - 60 s.

5.7 Discard the flow-through and centrifuge the column at maximum speed, for 1 min.

5.8 Elute the DNA by adding 10  $\mu$ l dH<sub>2</sub>O to the center of the column membrane and let the column stand for 5 min. Then centrifuge the column at maximum speed, for 1 min.

## 6. ISOLATION OF METHYLATED DNA FRAGMENTS

In this section you will isolate the methylated DNA fragments, from the non methylated fragments.

### 6.1 Prepare MBP/DNA complexes:

Add the following components to a 0.5 ml microfuge tube:

MBP	2 $\mu$ l
Purified DNA (from step 5.8)	6 $\mu$ l
5X Binding Buffer	4 $\mu$ l
dH <sub>2</sub> O	8 $\mu$ l
<hr/>	
Total Volume	20 $\mu$ l

- 6.2 Mix components by pipetting and incubate at 15°C, for 30 min.
- 6.3 Meanwhile, wash the **Separation Column** by adding 500 $\mu$ l chilled 1X Column Incubation Buffer and centrifuging at 7,000 rpm for 30 sec at room temperature.
- 6.4 Add 20 $\mu$ l 1X Column Incubation Buffer to the MBP/DNA complexes, from step 6.1. Transfer the entire mixture on to the membrane of the Separation Column.
- 6.5 Incubate the Separation Column **on ice** for 30 min.
- 6.6 Centrifuge column at 7,000 rpm for 30 sec at 4°C and discard the flow-through.
- 6.7 Add 600 $\mu$ l 1X Column Wash Buffer to the Separation Column and incubate for 10 min, **on ice**.
- 6.8 Centrifuge column at 7,000 rpm for 30 sec at 4°C and discard the flow-through.
- 6.9 Wash the column by adding 600 $\mu$ l 1X Column Wash Buffer to the Separation Column and centrifuging at 7,000rpm for 30 sec at 4°C.
- 6.10 Repeat 6.9 an additional 3 times.
- 6.11 Remove residual 1X Column Wash Buffer with an additional centrifugation at 7,000 rpm for 30 sec at 4 °C.

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- 6.12 Add 10  $\mu$ l 1X Column Elution Buffer to the center of the Separation Column and incubate at room temperature for 5 min.
- 6.13 Place the Separation Column in a clean 1.5ml microcentrifuge tube and centrifuge for 1 minute at 10,000 rpm at room temperature.
- 6.14 Place the microfuge tube, containing the collected flow through on ice for use in the following steps. This tube contains your isolated methylated DNA fragments. To amplify your promoter region of interest using PCR, proceed to step 7.

This tube can be stored at -20 °C for future use.

## 7. PCR AMPLIFICATION OF METHYLATED DNA FRAGMENTS

In this step the purified methylated DNA fragments will be amplified by PCR, for subsequent visualization by agarose gel electrophoresis. If designing your own primers, please see the following guidelines for optimal primer design:

- Locate sequential MseI restriction sites (TTAA) in your promoter region(s).
- Locate CpG islands between these MseI sequential restriction sites.
- Your forward and reverse primers should correspond to the DNA sequences after and before sequential MseI sequences, respectively. Be sure that this region between these sequential MseI sites contains a CpG island.
- The predicted melting temperature ( $T_m$ ) of your primers should optimally be ~55 °C. Your primers should have be at least 22 nucleotides in length.

For greater accuracy, we strongly recommend that the customer amplify **more than one region** within the promoter region of interest. By designing primers for multiple regions of the promoter area, the customer is more likely to detect methylation within the promoter region.

7.1 If you are using the **Panomics PCR Reaction Kit** (Cat # MP0000), mix the following components in a PCR tube:

Methylated DNA (from step 6.14)	1 $\mu$ l
Panomics PCR Buffer	40 $\mu$ l
Forward Primer	1 $\mu$ l
Reverse Primer	1 $\mu$ l
Panomics PCR Polymerase	1 $\mu$ l

*NOTE: For positive control, use 2  $\mu$ l of **Control Primer Pair** in place of Forward and Reverse Primers.*

If you are using another commercially available PCR kit, please follow the directions supplied by the manufacturer. A typical PCR reaction mixture is listed below:

Methylated DNA (from step 6.14)	1 $\mu$ l
10x PCR Buffer	4 $\mu$ l
Forward Primer	1 $\mu$ l
Reverse Primer	1 $\mu$ l
10 mM dNTP Mix	4 $\mu$ l
ddH <sub>2</sub> O	29 $\mu$ l
TOTAL	40 $\mu$ l

7.2 Mix well by pipetting and carry out the following PCR steps:

82°C	2 min
35 cycles of the following steps:	
94°C	1 min
55°C	1 min
72°C	2 min
4°C	Forever

If you plan to run your agarose gel electrophoresis at a later time, store your PCR reaction tubes at -20 °C.

## **8. AGAROSE GEL ELECTROPHORESIS**

To detect the amplified PCR product, perform agarose gel electrophoresis using any commercially available agarose gel electrophoresis apparatus.

- 8.1 Pour molten (55 °C) 1.5 - 2.0 % agarose gel in 1X TAE and 0.5 µg/ml ethidium bromide into gel tray. Add comb to tray.
- 8.2 After gel solidifies, place in electrophoresis apparatus and add sufficient 1X TAE to cover gel. Remove comb.
- 8.3 In a clean microcentrifuge tube, prepare the following:  

18 µl PCR reaction mixture (from step 6.2)
2 µl 10X gel-loading buffer (commercially available)
20 µl Total
- 8.4 Load entire 20 µl of each PCR reaction into separate wells. Be sure to reserve one well for the 1kB Ladder.
- 8.5 Run gel at 1-5 V/cm (distance between positive and negative electrodes). Observe gel under UV while running, to ensure sufficient migration of 1kB Ladder fragments.

## 9. TROUBLESHOOTING GUIDE

Problem	Cause	Recommendation
Positive spot is visible on Panomics Promoter Methylation Array Kit, but no PCR product for corresponding promoter region is visible.	PCR reaction is not optimized.	Use Panomics PCR Reaction Kit for your PCR reaction.  Perform PCR reaction for 45 cycles.
	PCR product is present, but in a quantity not detectable with ethidium bromide.	Repeat PCR using Panomics Biotin Labeling Kit, for detection by chemiluminescence.
No PCR product was detected using your own PCR primers, for a promoter region known to undergo methylation.	Primers do not flank methylated CpG island within promoter region.	Repeat PCR reaction using PCR primers for a different area of the promoter region.

## 10. REFERENCES

1. Sambrook, J. and Russell, D.W. Molecular Cloning. 3rd Ed. CSHL Press, New York, 2001.

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### RELATED PRODUCTS

Please visit our website at [www.panomics.com](http://www.panomics.com) for the most up-to-date information about our products.

Products	Size	Catalog#
Promoter Methylation PCR Primers	15 $\mu$ l	MPxxxx
PCR Reaction Kit	1 kit	MP0000
TranSignal™ Promoter Methylation Array	1 kit	MA7010
TranSignal™ Promoter Methylation Array Refill	1 kit	MA7011