CpG MethylQuest™
DNA Isolation Kit

Cat. No. 17-10035
30 Reactions

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

The CpG MethylQuest DNA Isolation Kit is used to isolate methylated double stranded DNA from genomic samples. Specific binding of methylated double stranded DNA is facilitated by the CpG MethylQuest GST-MBD fusion protein. In this kit the CpG MethylQuest protein is pre-coupled to glutathione paramagnetic beads. To capture methylated sequences using this kit, simply incubate fragmented genomic DNA with CpG MethylQuest beads. The high affinity of the CpG MethylQuest protein for methylated CpG regions results in highly specific and selective binding of methylated DNA fragments while non-methylated DNA remains in solution. After binding, the CpG MethylQuest beads and the bound methylated DNA are collected by drawing the beads to the side of a reaction vessel using a permanent magnet. The supernatant containing non-methylated DNA is removed leaving magnetic beads containing methylated DNA behind. This isolated methylated DNA is then eluted from the beads using a heat treatment. The resulting DNA is ready for downstream analysis without the need for additional clean-up steps.

This kit contains reagents sufficient for processing 30 DNA samples. Control DNA sufficient for 5 reactions as well as control primers for analysis by PCR amplification is provided. Assuming all controls are used, up to 5 controls and 25 samples can be processed. The CpG MethylQuest kit accommodates a range of input DNA allowing the processing of as little as 1 ng to as much as 1 µg of input genomic DNA per sample. Should larger amounts of material need to be processed, it is possible to scale up the protocol to isolate larger quantities of methylated double stranded DNA.

CpG MethylQuest Protein –High Specificity and Affinity for Methylated DNA

The CpG MethylQuest DNA isolation kit utilizes a high-affinity GST-MBD protein (CpG MethylQuest Protein, Cat. #14-921). This protein is an effective and versatile tool for the study of CpG methylation in DNA. This purified recombinant protein contains the methyl binding domain (MBD) of the mouse MBD2b protein fused to a glutathione-S-transferase protein (GST) from *S japonicum*. These two protein domains are separated by a linker containing a thrombin cleavage site (see figure 1).

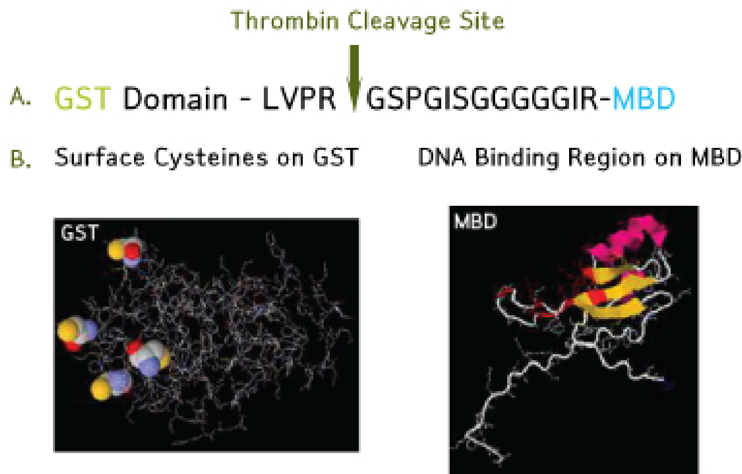


Figure 1A. GST and MBD domains separated by thrombin cleavage site. Arrow indicates site of thrombin specific cleavage.
Figure 1B. Surface cysteine groups available for chemical modification.

The MBD from the MBD2b protein has the highest affinity among the known methyl CpG binding proteins for Me-CpG sites and the lowest cross reactivity with unmethylated CpG sequences (Fraga M.F., et al. (2003). *Nuc. Acids Res*, 31, 1765–1774). In addition to its high affinity for methylated DNA, the CpG MethylQuest protein binds to methylated CpG regardless of sequence context. This is in contrast to isolation of methylated DNA using proteins such as MeCP2, which requires a run of A-Ts near a CpG site. This combination of sequence independent, high affinity binding to methylated CpG sites and extremely low affinity for non-methylated sites results in a greater number of methylated CpG sites being recognized allowing for comprehensive analysis of methylation patterns across the genome.

As shown in figure 2, CpG MethylQuest protein is highly specific for DNA methylated on both strands. In contrast, unmethylated and hemi-methylated DNA are not effectively bound by this protein.

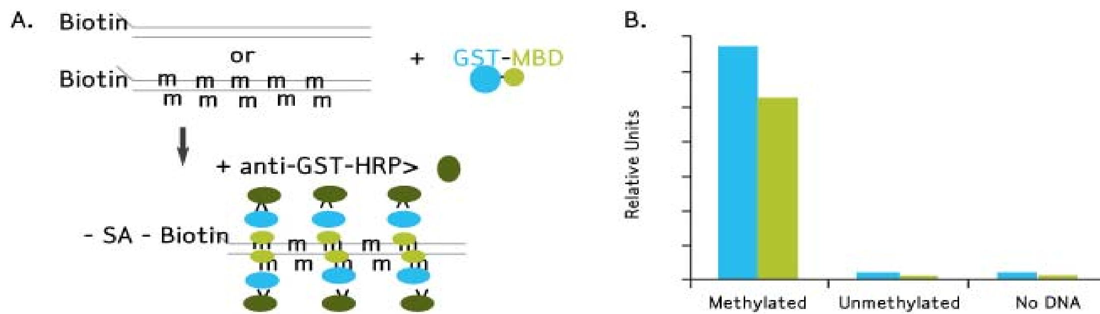


Figure 2. Purified MethylQuest protein (Cat# 14-921) incubated with a 550 bp biotinylated p16 amplicon (fully methylated, unmethylated, or no DNA) immobilized to magnetic beads. CpG MethylQuest protein was incubated with immobilized DNA for 1 hour. After washing, bound CpG MethylQuest protein was detected with an anti-GST antibody-horseradish peroxidase conjugate). Different colored bars represent duplicate experiments.

To simplify the protocol and remove the potential for experimental variability, the GST-MBD protein provided in this kit has been pre-bound to magnetic beads. For applications requiring CpG MethylQuest Protein not bound to any substrate, CpG MethylQuest protein (Cat 14-921) is available. This unbound protein has several unique properties that can be incorporated into your own customized assays for the study of DNA methylation.

- Competitive elution of the GST-MBD protein or GST-MBD-methylated DNA complexes with glutathione.
- Elution of intact complexes by thrombin digestion
- Immobilization of protein DNA complexes or GST-MBD protein to other solid surfaces such as microtiter plates coated with GST antibodies.
- Visualization of complexes using labeled GST antibodies or GST antibodies and labeled secondary antibodies.
- Surface cysteine groups on the GST protein can be chemically modified to allow the addition of other reporters, affinity tags, or functional groups.

Analysis of Isolated DNA by PCR

The CpG MethylQuest kit includes pre-cut control DNA as well as positive and negative control primers. These primers can be used for either endpoint PCR followed by electrophoretic analysis or for qPCR using SYBR® Green or similar fluorescent dyes.

As a positive control, primers that amplify a 230 bp fragment of the imprinted SNRPN locus are provided. These primers are used to assay for the presence of methylated DNA, Figures presented on page 13 in the appendix of this manual provide examples of the results of a control amplification using this positive control primer set.

A second primer pair for the COX2 CpG Island is included as a negative control. This primer pair is designed for use with the provided HeLa genomic control DNA. When genomic DNA derived from HeLa cells is used, amplification of CpG MethylQuest fractions produce a 442 bp amplicon in the input DNA and supernatant fraction. For further details on the proper use of control primers please refer to page 11 "Analysis of Eluted DNA and Use of Provided Control Primers".

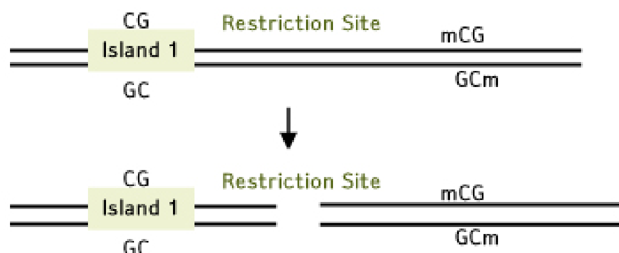
Overview of CpG MethylQuest Procedure

The CpG MethylQuest procedure is simple and can be broken down into three key steps.

- DNA isolation and fragmentation
- Methylated DNA binding and enrichment
- Elution of methylated DNA fragments

DNA Isolation and Fragmentation

To begin the procedure, simply isolate genomic DNA using any established genomic DNA purification procedure or a commercial kit. The input DNA should be in either TE or water. After purification prepare the genomic DNA for binding to the CpG MethylQuest beads by fragmentation using a restriction digest in order to physically separate methylated CpG islands from other regions that also may be methylated. Typically four cutter type II restriction enzymes, such as *Mse I*, that theoretically cut DNA into 256 bp fragments are used. *Mse I* cuts at the sequence TTAA and is not affected by CpG methylation.



Fragmentation is a key step and care should be taken to ensure complete digestion/fragmentation of your sample. Failure to adequately fragment DNA could lead to co-purification of unmethylated fragments with neighboring methylated regions which can confound analysis. Consequently it is recommended fragmented samples be analyzed to ensure complete digestion before proceeding.

Alternatively, sonication can be used to fragment the genomic DNA. Optimization of the sonication condition is recommended to ensure the average size distribution of fragments is ~500 bp.

Methylated DNA Binding and Enrichment

To capture the DNA fragments containing methylated CpG sites, digested DNA is incubated with CpG MethylQuest beads. The MBD protein is already attached to the magnetic beads eliminating the need to bind the capture protein before starting your experiment.

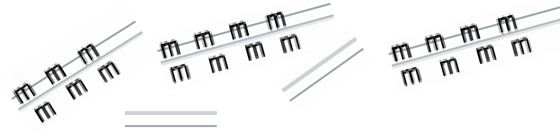
Typically the DNA added to the beads is a mixture of both methylated and unmethylated CpG sites. During the incubation, the high affinity CpG MethylQuest protein binds DNA fragments containing methylated CpG islands while unmethylated CpG fragments remain unbound and are removed in subsequent washes.

Elution of Methylated DNA Fragments

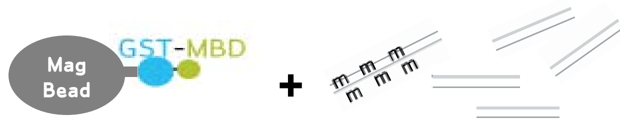
Because the methylated DNA is bound by a protein, a heat treatment is all that is required to release the bound DNA. Simply add TE or sterile water to the beads, mix, and heat in a water bath to disrupt the structure of the CpG MethylQuest protein and release methylated DNA. At this point the DNA is ready to use and no additional clean-up of the eluted material is required for most downstream applications.

CpG MethylQuest Work Flow

1. Fragment genomic DNA by restriction digestion



2. Add Fragmented DNA to CpG MethylQuest Beads



3. Incubate to allow binding of methylated DNA.

4. Remove or collect supernatant containing unmethylated DNA

5. Wash beads and heat to elute methylated DNA

CpG MethylQuest Kit Components and Storage

Part #	Component Description	Supplied As	Volume	Storage
CS204326	CpG MethylQuest Binding Buffer	1 bottle	6 mL	4˚C
CS204329	CpG MethylQuest Magnetic Beads*	1 vial	165 µL	-20˚C
CS204325	CpG MethylQuest Wash Buffer 1	3 vials	3.3 mL	4˚C
CS204327	CpG MethylQuest Wash Buffer 2	2 bottles	26.4 mL	4˚C
CS204328	TE Buffer	1 bottle	13.2 mL	4˚C
CS204319	Control HeLa DNA 110 ng, MseI cut, 2ng/µl	1 vial	55 µL	-20˚C
CS204331	Positive Control Primers 10 µM each	1 vial	16.5 µL	-20˚C
CS204318	Negative Control Primers 20 µM each	1 vial	16.5 µL	-20˚C
	User Manual	Print Copy	1	RT

CpG MethylQuest Kits are shipped on 'blue ice'. All components should be stored at indicated temperatures upon receipt. Performance is guaranteed for 3 months from date of receipt when stored and used according to recommendations.

*****CpG MethylQuest Beads: IMPORTANT STORAGE INFORMATION*****

Do not expose CpG MethylQuest beads to temperatures below -20˚C. The beads should not be stored in a frost-free freezer. Store beads in an insulated cooler if your freezer has a defrost cycle. Avoid extreme temperature shifts and prolonged exposure of beads to temperature above -20˚C.

Additional Materials and Equipment Required

- Microcentrifuge tubes (~1.5 mL polypropylene) and microcentrifuge
- Pipettes and pipette tips (aerosol barrier tips are suggested to minimize potential cross contamination)
- Sample mixing equipment (e.g. Eppendorf Thermomixer, shaker or tube rotator)
- Thermocycler or real-time PCR instrument
- PCR reagents for control reactions (e.g. Novagen NovaTaq™ Hot Start DNA Polymerase cat# 71091-3 and 10 mM dNTP mix cat# 71004-3 or NovaTaq™ Hot Start Master Mix Kit cat# 71676-3)
- DNAse-free ultrapure water (e.g. Millipore cat# 3098)
- Rare-earth magnet or magnetic rack (e.g. Millipore Magna GrIP™ Rack cat# 20-400 or Millipore PureProteome™ Magnetic Stand cat# LSKMAGS08)

CpG MethylQuest DNA Isolation Kit Protocol

IMPORTANT NOTES BEFORE STARTING

Starting with fragmented DNA, this protocol takes approximately 2 hours to complete. It is recommended that you review the entire protocol before starting and plan out your work for best results.

For the binding of the DNA to the beads (step 3 on page 9) either 1.5 mL or 1.7 mL microcentrifuge tubes can be used. Which tube to use depends upon the equipment used to mix the binding and wash reactions. Use a tube that allows for mixing and appropriately fits your equipment. Performing binding with a mixing device that has a horizontal rotary motion is ideal. An end-over-end mixer can be used as well. If an end-over-end device is used, some of liquid may adhere to the tube wall and cap. To settle the tube contents, spin the samples in a microcentrifuge for a few seconds. Do not exceed 500 x g (~2700 rpm).

Rare Earth Magnet Advisory:
This product requires the use of magnetic stands containing rare earth magnets (e.g. Millipore Magna GrIP™ Rack cat# 20-400 or Millipore PureProteome™ Magnetic Stand cat# LSKMAGS08). These magnets can be extremely powerful. Exercise care when handling them. Improper use can result in potential damage to instruments and magnetic media. Strong magnets can attract tools and metal objects, causing pinch, cut, or entrapment hazards. People with Pacemakers or Automatic Internal Cardiac Defibrillators should exercise caution.

Prepare Genomic DNA (Materials not provided)

1. Isolate and purify DNA from cells or tissues of interest. Most commercially available genomic DNA isolation kit or established genomic DNA isolation procedures result in DNA of sufficient purity for the CpG MethylQuest isolation procedure.

2. Fragment DNA sample using 4-base-recognition restriction endonucleases biased to A-T rich sequences (e.g. *Mse* I, TTAA, and *Tsp* 509I, AATT). Alternatively sonication can be used.

3. Evaluate the completeness of digestion by running the DNA on the agarose gel or performing PCR with a primer pair flanking a known restriction site verses a primer pair that does not have an intervening site. A positive control with undigested DNA should also be performed.

4. Heat inactivate the enzyme using a time and temperature appropriate for the enzyme used. It is important to conduct this step prior to adding the DNA to the binding buffer. Phenol extraction and ethanol precipitation of the DNA is optional unless quantitative comparisons are to be made between the CpG MethylQuest eluted fraction and the supernatant fraction or input DNA sample.

Isolate Methylated DNA Fragments Using CpG MethylQuest Kit

Before starting:

Thaw the reagents and keep them at room temperature. Remove an aliquot of CpG MethlyQuest beads required for your experiment then immediately return the beads to the freezer.

Invert buffer tubes and mix bottles upon thawing to ensure homogeneous mixtures. Separate mixing instructions for beads are given in step 1.

1. Prepare CpG MethylQuest Beads

a. Gently resuspend the CpG MethylQuest beads by gently flicking the vial to create a uniform suspension of beads.

***IMPORTANT! DO NOT VORTEX THE BEADS.**

Note: To prevent loss of beads, avoid pipetting up and down to resuspend the stock vial of CpG MethylQuest beads provided with this kit.

b. For each sample to be processed, transfer 5 µL of resuspended beads per reaction to separate 1.5 mL microcentrifuge tubes. This 5 µL volume of beads is suitable for 1 ng to 1 µg of DNA. Optimal performance is typically achieved with 300 ng of DNA.

c. Add 100 µL of Wash Buffer 1 to each tube.

d. Transfer the tubes to a magnetic stand. Allow at least 1 minute for beads to separate. Remove and discard the wash buffer without disturbing the beads.

2. Add Binding Buffer and DNA samples to prepared CpG MethylQuest Beads.

To the prepared beads, add the following components in order:

80 µL Binding Buffer
20 µL fragmented DNA (1 ng-1ug)*

*For controls add 10 µl of 2 ng/µL HeLa DNA plus 10 µl of Ultrapure DNase free water

Note: If less than 20 µL of DNA is used, add the volume difference in Ultrapure DNAse free water to the Binding Buffer before adding the DNA. If a DNA volume greater than 20 µL is used, add 4 volumes of Binding Buffer per volume of DNA.

3. Bind methylated DNA fragments to CpG MethylQuest Beads

a. Pipette gently to resuspend the beads and mix the DNA solution. If desired, a portion of this input sample can be saved for comparative analysis.

b. Incubate the beads at room temperature (18-23°C) for 1 hour with gentle mixing.

Note: The beads can be mixed during the incubation with equipment that allows for a horizontal rotary motion (e.g. Eppendorf Thermomixer® set at 1,000 rpm). Alternatively, a rotating end over-end mixer set at 8 rpm can be used. If end-over-end mixing is used, liquid may adhere to the tube wall and the lid. To settle the contents of the tube and remove residual liquid, spin the samples in a microcentrifuge for a few seconds at no more than 500 x g (~2700 rpm).

4. Wash CpG MethylQuest Bead to remove unbound DNA fragments

a. Spin the samples in a microcentrifuge for a few seconds if droplets have collected on the walls of the tubes. Do not exceed 500 x g.

b. Place the tubes in the magnetic separation rack and allow beads to separate for 1 minute. Carefully remove the supernatant fraction without disturbing the beads. This fraction contains the unbound, unmethylated DNA. If desired, this fraction can be saved for analysis.

c. Wash the beads with 0.4 mL of Wash Buffer 2. Apply buffer to the side of the tube and ensure all beads are recovered from tube wall.

d. Incubate the beads for 5 min at room temperature with gentle mixing as done for the incubation step. If needed, spin the samples in a microcentrifuge for a few seconds at no more than 500 x g (~2700 rpm) to remove residual liquid from the cap and sides of tube.

e. Place the tubes in the magnetic separation rack allow beads to separate for 1 minute. Carefully remove the supernatant fraction without disturbing the beads. Perform one additional wash and 5 minute incubation with Wash Buffer 2 as described in steps c and d above.

f. Place the tubes in the magnetic separation rack allow beads to separate for 1 minute. Carefully remove the supernatant fraction without disturbing the beads

g. Add 0.4 mL of TE buffer to vial and gently resuspend beads. Place the tubes in the magnetic separation rack allow beads to separate for 1 minute. Carefully remove the supernatant fraction without disturbing the beads. No incubation is needed for this step

5. Elute Methylated DNA

Note: TE is used in the standard procedure to elute DNA from the magnetic bead: MBD2 complex. If quantitative comparison to the supernatant fraction is desired, it is recommended to elute the DNA from the beads using an 80% Binding Buffer (vol/vol) solution to match the ionic content of the unbound material collected following step 4b. Although heating the sample is the recommended approach for elution, alternative approaches can be used.
For other elution options please refer to the appendix.

a. Resuspend the beads in TE or 80% (v/v) Binding Buffer. The suggested volume of elution buffer will vary depending on the amount of input DNA used. Use the table on page 11 to determine the appropriate elution volume to add to the MBD2-DNA bead complex.

b. After adding the appropriate volume to the sample for elution, incubate 10 minutes at 80˚C with mixing.

Input DNA	Elution Volume
1 ng to < 20 ng	20 µl
20 ng to < 50 ng	50 µl
50 ng to 1 µg*	100 µl

c. Place the tubes in the magnetic separation rack allow beads to separate for 1 minute. Immediately and carefully remove and save the supernatant fraction containing the eluted DNA. Avoid disturbing the beads. This eluted DNA is ready to use for most downstream applications and can be stored at -20˚C.

Analysis of Eluted DNA and Use of Provided Control Primers

Once the methylated DNA fraction has been eluted, analysis can be carried out using several methods. For PCR or qPCR, follow the manufacturer's recommendations for amplification and detection. To reduce the risk of non-specific amplification, hot start PCR is recommended.

Control Reactions

Control DNA as well as positive and negative control primers are provided with the CpG MethylQuest DNA Isolation Kit. Primers that amplify the imprinted SNRPN locus serve as a positive control to confirm methylated DNA Isolation using PCR. SNRPN is an imprinted gene and is an excellent positive control for methylation in normal human DNA samples. Amplification of HeLa DNA isolated with the CpG MethylQuest Kit using the provided primer pair will produce a 230 bp amplicon in both the supernatant fraction of the binding reaction (the unbound, unmethylated copy) and the DNA fraction eluted from the GST-MBD beads (the methylated copy). The figures in the appendix on page 11 provide an example of the results of a control amplification using this primer set.

As a negative control, a second primer pair for the COX2 CpG Island is included for use with HeLa DNA. This region is hypomethylated in HeLa DNA. Consequently this locus is not enriched by the CpG MethylQuest procedure. Amplification using fractions from the provided HeLa produce a 442 bp amplicon only in the input and supernatant fractions.

IMPORTANT NOTES ON CONTROL PRIMERS:

The positive and negative control primers included in this kit are designed for use with the fragmented HeLa genomic DNA control. The positive control primers will detect enriched DNA from cells imprinted at the SNRPN locus. Imprinting may be affected by cellular transformation, so a 1:1 ratio of paternal and maternal alleles may not be observed in all samples. Similarly, the methylation of the CpG Island at the COX2 promoter may differ depending upon the sample type. Alternatively, sets of control primers can be developed for samples being analyzed in your lab based upon published methylation patterns or in house results.

PCR Amplification Protocol

A total of 110 ng (2 ng/µl) of fragmented HeLa DNA is included in the kit, together with primers for 5 control samples plus 1 no-DNA control for each sample. Control PCR reactions should be done after processing 20 ng of fragmented HeLa DNA with the CpG MethylQuest DNA Isolation Kit using the standard protocol followed by elution using 50 µl of TE buffer and heat. PCR reactions should be done on both the supernatant fraction and the elution fraction. What follows is a general end-point PCR amplification protocol using a hot start Taq DNA polymerase. Similar conditions can be used if qPCR using SYBR Green or similar fluorescent dyes that bind double stranded DNA.

1. Set up reactions according to the table below.

Table 1. Taq Hot Start PCR (Volumes for a single reaction)

Component	Volume per 20 µl	Final Concentration
10X PCR buffer	2.0 µl	1X
Ultrapure water	10.4 µl	-
dNTPs (10 mM, 2.5 mM each)	1.6 µl	800 µM (200 µM each)
Control Primer mix	1.0 µl	0.5 µM, or 1µM each[2]
DMSO	1.0 µl	5% v/v
$MgCl_2$ (25 mM)	1.6 µl	2 mM
CpG MethylQuest DNA fraction[1] or no DNA control	2.0 µl	
Hot start Taq DNA polymerase (5 units/µl)	0.4 µl	2 units

[1] Unbound supernatant fraction (unmethylated) or eluted fraction (methylated). Replace with 2 µl of ultrapure water for the no DNA control.

[2] The positive control primer final concentrations are 0.5 µM each and the negative control primer final concentrations are 1 µM each.

2. Place reaction tubes in a thermal cycler set to run the following program:

1. 95°C, 1 min
2. 95°C, 30 sec
3. 60°C 30 sec
4. 72°C 1 min
5. Repeat steps 2-4 35 times
6. 72°C 5 min
7. HOLD 20°C

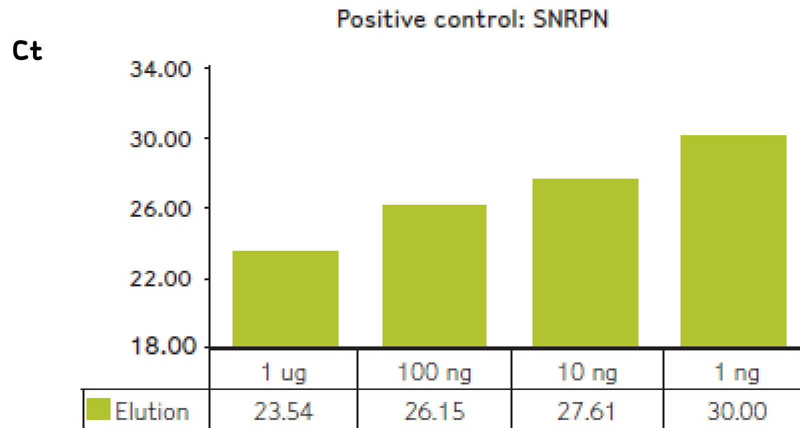
3. Analyze amplification products by electrophoresis on 1% agarose gel.

Expected size fragments are 230 bp for the positive SNRPN primers and 442 bp for the COX2 negative control primers.

Appendix

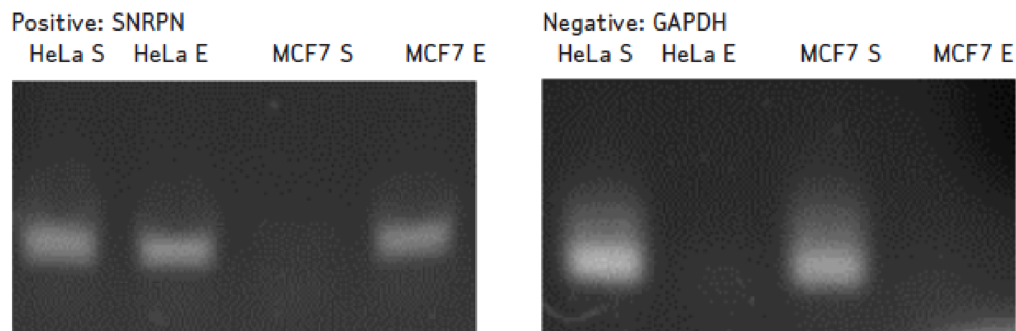
Real-time PCR with Limiting Amounts of DNA: High Sensitivity and Specificity

The CpG MethylQuest DNA Isolation Kits can be used with as little as 1 ng of input DNA or as much as 1 µg of input sample DNA. Shown in the figure below, increasing amounts of MCF7 sonicated genomic DNA were purified with the CpG MethylQuest DNA Isolation Kit and eluted in TE (100 µl elution for 1ug and 100 ng samples and 20 µl of TE for 10 ng and 1 ng samples) by heating the samples at 80°C for 10 minutes. Real-time PCR analysis was conducted on 2 µl of eluted DNA using SNRPN primer.



Endpoint PCR Analysis Showing High Specificity for Methylated DNA

To demonstrate the specificity for methylated DNA, 20 ng of HeLa genomic DNA (MseI digested) and 100 ng MCF7 genomic DNA (sonicated) was purified with the CpG MethylQuest DNA Isolation Kit. The supernatants were retained for analysis of unbound material and bound DNA was eluted in TE. Using conventional PCR, 2 µl samples were amplified using primer for either SNRPN or GAPDH. After 30 cycles of PCR the samples were analyzed on an agarose gel. Because the GAPDH locus is not methylated, DNA fragments are not captured and are not detected in either the eluted fraction from MCF7 or HeLa after 30 cycles of PCR. Conversely, for the methylated SNRPN locus in HeLa DNA, product is detected in both supernatant fraction (unbound, unmethylated copy of gene) and the fraction eluted from the GST-MBD beads (bound methylated copy). For the MCF7 samples, SNRPN product is detected only in the elution fraction from the GST-MBD beads (the methylated copy). Note that while the methylation pattern of the imprinted gene SNRPN is expected to be consistent over normal human DNA samples, deviations from a 1:1 ratio of methylated to unmethylated copies are possible for tumor DNA samples.



Alternative Methods to Recover Methylated DNA

Although heat treatment is the preferred approach to recover methylated DNA from the beads, the CpG MethylQuest kit allows for alternative elution methods. Materials for these alternative elution methods are not provided with the kit.

Alternative Elution Approach 1: Competitive Elution Using Glutathione

Important: This approach is not recommended if downstream analysis will be bisulfite modification or qPCR with SYBR® Green.

Note: Glutathione elution buffer can lose potency with extended storage due to oxidation of the glutathione. The recommended formulation for glutathione elution buffer is 60 mM reduced glutathione, 10 mM Tris, pH 8.0, 0.05% (v/v) Tween 20. If glutathione elution buffer is made fresh or will be used within a time frame of 1 month or less, then a concentration of 20 mM glutathione is sufficient. Glutathione elution buffer should be stored at -20°C.

a. Resuspend the beads in elution buffer as following the guidelines in step 5 of the protocol and incubate 10 min with mixing
b. Place the tubes in the magnetic separation rack allow beads to separate for 1 minute. Carefully remove and save the supernatant fraction containing the eluted DNA without disturbing the beads.

Alternative Elution Approach 2: Elution by Thrombin Cleavage

The CpG MethylQuest protein has been engineered to include a Thrombin cleavage site that links the GST and MBD domains. Thrombin digestion releases DNA that may contain a bound MBD domain. Bovine Thrombin does not interfere with PCR, and targets can be successfully amplified if samples are used immediately without inactivation of the protease. However, contaminating nucleases may be present in thrombin preparations. Consequently, one should inactivate nucleases by heating samples to 65 °C for 20 minutes or adding EDTA to a final concentration of 10 mM.

Following the suggested volumes given in step 5 of the protocol, resuspend the beads in a Thrombin cleavage buffer (10 mM TrisCl pH 8, 150 mM NaCl). Calcium chloride can be omitted from the cleavage buffer to avoid possible interference with downstream analysis.

a. Add 2 units/50 μl of Bovine Thrombin to each sample and incubate at room temperature (22 to 25 °C) for 20 minutes with gentle mixing.
b. Place the tubes in the magnetic separation rack allow beads to separate for 1 minute. Carefully remove and save the supernatant fraction containing the eluted DNA without disturbing the beads.

Troubleshooting

Problem	Potential Cause	Possible Solution
No PCR product detected in any of the fractions	Primers flank a restriction site	Check sequence to make certain the target amplicon does not contain restriction sites for the enzyme used to fragment genomic DNA.
	GST-MBD2 protein is non-functional/degraded	Ensure magnetic beads complexed with GST-MBD2 are stored properly in a non-frost free -20° C freezer.
	PCR failure	Utilize positive and negative controls to ensure PCR amplification reagents are working properly.
	Input genomic DNA was degraded	Check an aliquot of your input material to verify integrity of the sample.
Unmethylated DNA in the elution fraction	Beads not washed thoroughly	Rigorously follow wash instructions to ensure unmethylated DNA is removed before elution
	Incomplete digestion of genomic DNA	Make sure the DNA is completely digested by analyzing on an agarose gel or PCR.
Methylated DNA in the supernatant	Binding capacity of the beads has been exceeded	Check concentration of input DNA. Always use less than or equal to 1 µg of DNA per 5 µL of beads. If processing more DNA, increase the volume of beads used.
	Binding capacity of beads is reduced due improper storage or handling	Use in vitro methylated control DNA (i.e. Millipore cat# S7821) to confirm decreased binding capacity. Increase volume of beads.
	Binding capacity of the beads is reduced because of non-optimal buffer conditions	Use only the reagents supplied with the CpG MethylQuest kit.
	DNA sample contains methylated and unmethylated DNA	Check source of DNA, tissue samples may contain mixed populations of cells with different methylation patterns.
Signal appears only in the supernatant fractions of all samples	Failure of the beads to bind methylated DNA	Check kit receipt date. The CpG MethylQuest kit must be used within 3 months of receipt. Use control DNA to verify performance. Ensure that the beads are thoroughly mixed during the binding reaction. Incubate the beads at 80°C for 10 min in TE buffer and amplify the eluted fraction.
	Failure to release methylated DNA from the beads with glutathione buffer	Check kit receipt date. The CpG MethylQuest kit must be used within 3 months of receipt. Ensure that the beads are thoroughly mixed during the binding reaction. Incubate the beads at 80°C for 10 min in TE buffer and amplify the eluted fraction.
High background in the eluted fraction of the negative control	Incomplete washing	Ensure that the walls of the tubes are rinsed when applying Wash Buffer 2 Briefly spin the binding reactions at 500 x g before washing to collect droplets from the tube walls.

NOTICE TO PURCHASER: This product is for Research Use Only and may not be resold. Not for use in diagnostic or therapeutic procedures.

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