CpG WIZ® MGMT Amplification Kit

S7803

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Not for use in diagnostic procedures

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I. INTRODUCTION

Using This Manual

Please read the entire instruction manual prior to using the CpG WIZ® MGMT Amplification Kit. Should additional questions arise, assistance is available from Chemicon Technical Service at techserv@chemicon.com or (800) 437-7500.

Background

Methylation of cytosines located 5' to guanosine is known to have a profound effect on the expression of several eukaryotic genes (1). In normal cells, methylation occurs predominantly in CG-poor regions, while CG-rich areas, called CpG islands, remain unmethylated. Aberrant methylation of normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells (2) and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers (3, 4). O(6)-Methylguanine DNA methyltransferase (MGMT) is an example of a gene that exhibits characteristic hypermethylation.

Previously developed methods to determine the methylation status of cytosine include digestion with methylation sensitive restriction enzymes and genomic DNA sequencing. Both techniques have limitations: restriction enzymes can only detect methylation sites within their recognition sequence and sequencing is time consuming. Increasing the detection sensitivity of CpG island methylation has the potential to define tumor suppressor gene function and provides a new strategy for early tumor detection.

Methylation-specific PCR (MSP) is a new technology for sensitive detection of abnormal gene methylation utilizing small amounts of DNA (5). This process employs an initial bisulfite reaction to modify the DNA, followed by PCR amplification with specific primers designed to distinguish methylated from unmethylated DNA. The CpGenome™ DNA Modification Kit (Cat. No. S7820) contains the reagents necessary to perform the initial bisulfite reactions, while the CpG WIZ® MGMT Amplification Kit contains the reagents required for the PCR amplification reactions.

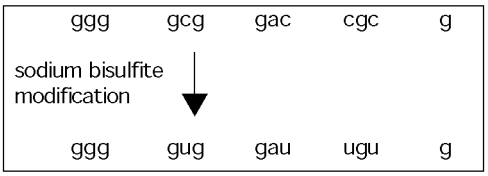
Principles of the Technique

Methylation-specific PCR (MSP), performed using the CpGenome™ DNA Modification Kit and the CpG WIZ® MGMT Amplification Kit, permits sensitive detection of altered DNA. Because this is a PCR-based assay, it is extremely sensitive, facilitating the detection of low numbers of methylated alleles and the study of samples containing small amounts of DNA. MSP also allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. Increasing the number of such sites which can be assessed allows rapid, fine mapping of methylation patterns throughout CpG regions. In addition, the bisulfite modification is ideally suited for analysis of CpG islands since it converts the majority of cytosines to uracils, making a region of the genome which is CG-rich less difficult to amplify by PCR.

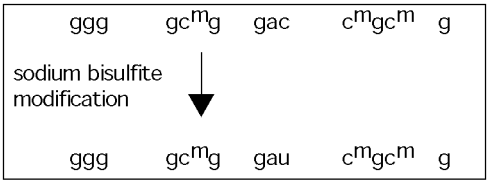
MSP employs an initial bisulfite reaction to modify the DNA, followed by a "hot start" PCR amplification with specific primers designed to distinguish methylated DNA from unmethylated DNA. As shown in Figure 1, in the bisulfite reaction, all unmethylated cytosines are converted to uracils while 5-methylcytosines remain unaltered. Thus, the sequence of the treated DNA will differ if the DNA is originally methylated vs. unmethylated. Primers contained in the CpG WIZ® MGMT Amplification Kit are designed to specifically amplify each of the sequences based upon these chemically-induced differences. If the sample DNA was originally unmethylated, a product will be generated after PCR using the U primer set. Conversely, a product will be generated using the M primer set if the sample was originally methylated.

Figure 1: DNA Treatment with Sodium Bisulfite.

Unmethylated DNA



Methylated DNA



Kit Components

The components of the CpG WIZ® MGMT Amplification Kit include those required for PCR amplification after bisulfite modification of DNA samples. Sufficient reagents are provided to analyze 25 samples with appropriate controls.

Table 1: Kit Components (color-coded microcentrifuge tube caps)

Description	Amount	Part No.	Storage Conditions
U Primer Set 5 µM each primer (25X)	35 µL	90513	-15°C to -25°C
M Primer Set 5 µM each primer (25X)	35 µL	90514	-15°C to -25°C
W Primer Set 5 µM each primer (25X)	35 µL	90515	-15°C to -25°C
U control DNA 0.1 µg/µL	50 µL	90393	-15°C to -25°C
M control DNA 0.1 µg/µL	50 µL	90394	-15°C to -25°C
W control DNA 0.05 µg/µL	50 µL	90395	-15°C to -25°C
Universal 10X PCR Buffer	265 µL	90396	-15°C to -25°C

Materials Required But Not Supplied

Equipment and Supplies

a. Microcentrifuge tubes for PCR amplification

b. Aerosol-resistant pipette tips

c. Thermocycler

d. Gel electrophoresis apparatus (vertical or horizontal)

e. Power Supply

f. 302 nm UV transilluminator, camera and film

Reagents

a 2.5 mM dNTP mix (2.5 mM of each nucleotide)

b. “Hot start” Taq polymerase

c. "Hot start" PCR reagents (see Sec. II. *Protocols*).

d. Reagents for gel electrophoresis (1X TBE and 2% agarose, 10% acrylamide, or suitable high resolution agarose)

e. DNA markers (size range 100-300 bp)

f. Ethidium bromide (10 mg/mL)

g. Gel-loading solution / Loading Dye

h. Bisulfite Modified DNA (CpGenome™ DNA Modification Kit, Cat. No. S7820)

II. PROTOCOLS

Experimental Design

Primer Sets

The CpG WIZ® MGMT Amplification Kit contains primers that can be used for analysis of DNA samples by MSP. However, the samples must first undergo bisulfite modification prior to PCR amplification. The CpGenome™ DNA Modification Kit, Cat. No. S7820, contains the reagents necessary to perform the modification. Chemical modification creates the sequence differences between the methylated and unmethylated DNA. The primer sets in the kit are engineered to anneal to the DNA, based upon the sequence differences.

U Primer Set will anneal to unmethylated DNA that has undergone a chemical modification

M Primer Set will anneal to methylated DNA that has undergone a chemical modification

W Primer Set serves as a control for the efficiency of chemical modification. It will anneal to **any** DNA (unmethylated or methylated) that has NOT undergone chemical modification, hence, the "wild type", or W.

Data interpretation can still proceed in the case of incomplete chemical modification (up to 50%).

Amplification Regions

The amplified region is defined as the sequence between the 3' nucleotide of the sense primer and the complement of the 3' nucleotide of the anti-sense primer for each gene promoter. The nucleotide numbering systems are those used in the GenBank submissions identified as X61657 for MGMT.

“Hot Start” PCR

The three sets of primers used in the CpG WIZ® Amplification Kit is derived from sequences closely related to each other, which introduces the possibility of mispriming. In order to avoid this and other PCR-related artifacts, "hot start" PCR is recommended. "Hot start" PCR permits the Taq polymerase to begin the reaction only after the template and primers are in single-stranded form.

There are several modifications of the standard PCR protocol which allow a "hot start" to occur. In one scenario, the PCR reaction mixture excluding the polymerase can be overlaid with mineral oil prior to heating to 95°C. At the end of the incubation, the enzyme is pipetted directly into the mixture under the mineral oil. A second method involves the physical separation of the polymerase and the rest of the PCR mix with a wax bead. The enzyme combines with the rest of the reaction mixture only after the wax melts. In another variation, an anti-Taq antibody inhibits the polymerase during reaction setup by forming a complex with the Taq enzyme. Taq polymerase becomes active when the complex is abolished due to antibody denaturation during the 95°C incubation. Alternatively, a "hot start" enzyme can be used. Refer to the manufacturer's instructions for enzyme activation protocol.

Note: *Do not use a polymerase with 3'-5' exonuclease activity (i.e. proofreading). Do not use a wax bead that contains* Mg^{2+}*. Any extra* Mg^{2+} *added to the reaction mixture produces suboptimal results.*

Genomic Control DNAs

The methylated and unmethylated control DNAs must undergo bisulfite modification prior to PCR amplification using the CpGenome™ DNA Modification Kit (Cat. No. S7820). When used with their respective U and M primer sets, a PCR product of an expected size (see Table 3) is obtained in each case.

W control genomic DNA is used in the PCR and is NOT to be used in the chemical modification step. When used with the W primer set, it is a positive control for PCR amplification. Failure to generate a PCR product indicates a general failure in the PCR reaction.

Experiment Setup

This CpG WIZ® Amplification Kit includes sufficient reagents to analyze 25 samples with appropriate controls (105 PCR reactions). Each experiment will include chemical modification of seven DNAs: 5 experimental DNA and 2 control DNA samples, (M and U). In the subsequent PCR reactions, each chemically modified experimental DNA sample is amplified with each of three oligonucleotide primer sets U, M and W. The chemically modified genomic control DNAs, U and M, are amplified with their corresponding primer set. Untreated W genomic control DNA is amplified with the W primer set. Lastly, a negative PCR control (i.e. no DNA) is performed for each set of primers.

For example, a typical gel for the analysis of five experimental DNA samples includes a total of 21 lanes:

Lanes 1-3	Experimental sample 1 with U, M and W primers
Lanes 4-6	Experimental sample 2 with U, M and W primers
Lanes 7-9	Experimental sample 3 with U, M and W primers
Lanes 10-12	Experimental sample 4 with U, M and W primers
Lanes 13-15	Experimental sample 5 with U, M and W primers
Lane 16	Chemically modified control U DNA with U primers
Lane 17	Chemically modified control M DNA with M primers
Lane 18	Untreated control W DNA with W primers
Lanes 19-21	No DNA control with (NTC) U, M, and W primers

Amplification Protocol

To prevent PCR contamination, read Sec. IV. *Appendix, Laboratory Setup and Precautions* before beginning.

STEP 1. Modification

Prior to performing PCR with the primer sets provided in CpG WIZ® Amplification Kits, one microgram of purified DNA must undergo bisulfite modification with the reagents contained in CpGenome™ DNA Modification Kit (Cat. No. S7820).

STEP 2. Amplification

"Hot start" PCR is recommended for this assay (refer to Sec. II. *Protocols, Experimental Design*). This is accomplished by several mechanisms, including a wax barrier or anti-Taq antibody. Refer to the instructions specified by the manufacturer of the "hot start" PCR reagents, and modify the amplification "master mix" and reaction conditions accordingly in steps b-f, below.

a. Determine the number of assays to be run in the experiment: run three amplification reactions for each experimental DNA sample plus six control reactions per each set of methylation assays (refer to Sec. II. *Protocols, Experimental Design*).

b. Prepare three (3) "master mixes" which correspond to the 3 possible primer sets U, M and W by mixing all the reagents outlined below **except** for the template DNA.

To analyze five experimental samples with appropriate controls, use the following amount of "master mixes" of each primer set : 7 tubes X 23.0 µL = 161 µL, plus 10% of that volume to adjust for pipetting error. Multiply the volume of each reagent listed below by 7 and add 10% for each "master mix". Thaw all reagents and store on ice while creating the "master mixes". The amount of reagents required in each reaction is:

10X Universal PCR buffer	2.5 µL
2.5 mM dNTP Mix**	2.5 µL
U, M or W primers	1.0 µL
TaKaRa™ *Taq* or "hot start" enzyme (5 U/µL)	0.2 µL (1 Unit)
dH_2O	16.8 µL
	23.0 µL
Template DNA (50 ng/µL)	2.0 µL
TOTAL VOLUME	25.0 µL

** Upon first use, make aliquots of 2.5 mM dNTPs, which should be freeze-thawed no more than 5 times.

c. Aliquot 23 µL of each "master mix" into corresponding PCR tubes.

d. Add:

2 µL of water to the no DNA control (NTC) tube.
2 µL of modified sample DNA to each of the sample tubes.
2 µL of corresponding DNA controls (modified U and M, unmodified W) to each control tube.

e. Place tubes in the thermocycler block, and perform PCR under the following conditions:

Denature:

for Taq Polymerase	95°C / 5 minutes
for "hot start" enzyme	check manufacturer's specifications

Then, perform 35-40 cycles of the following conditions:

denature	95°C / 45 seconds
anneal	60°C / 45 seconds
extend	72°C / 60 seconds

f. Remove the tubes from the thermocycler block. From this point on, it is important to designate separate pipettes and work areas for amplified vs. unamplified samples. This prevents carry-over contamination of future DNA samples with the amplified product.

STEP 3. Gel Electrophoresis

a. After the completion of PCR, add an appropriate amount of loading dye to the sample and analyze 10 μL of the reaction on a 2% agarose, or a 10% native acrylamide or other high resolution agarose gel. Use DNA markers (100-300 bp range) to determine the size of PCR products.

b. After electrophoresis, stain the gel with ethidium bromide. Dilute the 10 mg/mL stock solution 1:10,000 in deionized water. Stain for 10-30 minutes and destain for 10-30 minutes in deionized water at room temperature.

Note: *Ethidium bromide is a known carcinogen. Exercise appropriate caution and good lab practice when using this reagent*

Data Analysis

Table 3: Sizes of Expected Products from Controls

Controls	MGMT
U primer/U control DNA	92
M primer set/M control DNA	80
W primer set/W control DNA	76

In the three no DNA control lanes, no PCR products should be generated.

If the sample is a mixture of unmethylated and methylated DNA, both the U and M primer will produce a PCR product.

If the W primer set produces a PCR product with an experimental sample, it is an indication of incomplete chemical modification.

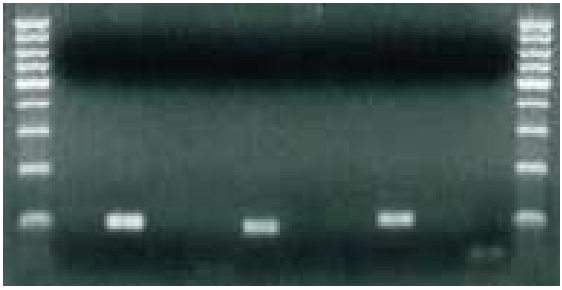
Note: The presence of primer dimers in the amplification reaction may be visible, however can be separated from the specific product band.

Specificity of the Assay

The specificity of the CpG WIZ® MGMT Amplification Kit is shown in Figure 2. With a complete chemical modification reaction, U primers amplify only unmethylated DNA (92 bp, lane 5) and M primers amplify only methylated DNA (80 bp, lane 8). W primers amplify only DNA which is not chemically modified, or "wild type W" (76 bp, lane 2).

Figure 2: Specificity of the CpG WIZ® MGMT Amplification Kit.

M 1 2 3 4 5 6 7 8 9 10 M



2% Agarose gel analysis using the primers and the control DNA samples included in the kit is shown. Lane 1: W primers, No Template Control, Lane 2: W primers, W DNA, Lane 3: U primers, NTC, Lane 4: U primers, M DNA, Lane 5: U primers, U DNA Lane 6: U primers, W DNA Lane 7: M primers, NTC, Lane 8: M primers, M DNA, Lane 9: M primers, U DNA, Lane 10: M primers, W DNA, M lanes: 100 base-pair marker.

III. TROUBLESHOOTING

? There is no visual evidence of products in any lane.

Potential Problem: PCR amplification is not initiated.

Recommendations:

a. Confirm that all PCR components were added to the reaction tube.
b. Confirm that the time and temperature settings on the thermocycler match those described in this manual.
c. If performing "hot start" PCR using a "hot start" enzyme, verify the initial denaturation/activation time of 12 minutes at 95°C.
d. For all other "hot start" methods, confirm the proper use of the reagents.
e. Confirm that the PCR polymerase is still active.
f. Confirm that no additional Mg^{2+} was added to the PCR reaction mix.
g. The optimal annealing temperature is 60°C. If items #a-f have not remedied the problem, re-optimize annealing conditions to suit your amplification instrument.

? No amplification product is generated in the experimental samples using U, M and W primer sets, but products of the correct size are observed with the control samples.

Potential Problem #1: Experimental DNA samples were degraded prior to chemical modification.

Recommendation:
Purify the genomic DNA again and repeat the chemical modification.

Potential Problem #2: Chemically modified experimental DNA samples were stored for more than two months prior to PCR.

Recommendation:
Repeat the chemical modification on new genomic DNA samples.

? U or M primer sets are producing bands in all samples, including the "no DNA" controls.

Potential Problem: PCR reagents are contaminated with amplification products.

Recommendations: see Sec. IV. *Appendix, Laboratory Setup and Precautions.*

a. Use fresh aliquots of every PCR component (i.e. dNTPs, buffer, etc.)

b. Use separate sets of pipettors for pre- vs. post-amplification liquid dispensing.
c. Devote a work area to pre- and post-amplification procedures.
d. Always use aerosol-resistant pipette tips.
e. Always use a clean labcoat and gloves.

? W primer set produces an amplification product in some or all experimental samples, in addition to an amplification product from the U or M primer set.

Potential Problem: Chemical modification of the experimental DNA sample(s) is incomplete.

Recommendation:
This will not jeopardize the validity of the assay as long as a product is also produced using the U or M primer set.

? The only lanes containing a PCR product are from those DNA samples (experimental and control) amplified with the W primer set.

Potential Problem: Chemical modification of the experimental DNA samples did not work.

Recommendation:
If using the CpGenome™ DNA Modification Kit (S7820), check the troubleshooting section of the manual.

IV. APPENDIX

Laboratory Setup and Precautions

One of the most important considerations when performing MSP using CpG WIZ® Amplification Kits is the environment where the initial reaction mixtures are set up. The ideal environment is free of amplified DNA products, which can cause false-positive results. Potential sources of PCR product contamination are: contaminated pipettors and tips, gel box and buffer, tube racks, notebooks, lab coats and any other item exposed to amplified PCR products.

The following precautions should be followed in all steps of the assay protocol:

a. Always wear gloves.

b. Use sterile water for all solutions, aliquot the solutions in small amounts, and use fresh aliquots as working solutions. Discard working solutions after use.

c. Keep the assay solutions (10X PCR buffers, dNTPs, polymerase, etc.) separate from the amplified DNA.

d. Always use aerosol resistant pipette tips.

e. Separate micropipettors and work areas are recommended for the following three steps of the assay:
    1. DNA modification and purification
    2. Amplification setup
    3. Post-amplification analysis

Related Products

Product	Catalog Number
CpGenome™ DNA Modification Kit	S7820
CpGenome™ Universal Methylated DNA (human male genomic DNA)	S7821
DNA Extraction Kit, Non-Organic	S4520
EX-WAX™ DNA Extraction Kit for paraffin-embedded tissue	S4530
TaKaRa Taq Polymerase, 250 U	R001A
TaKaRa Taq Polymerase, 1000 U	R001B
TaKaRa Taq Polymerase, 3000 U	R001C
NucleoClean™ Decontamination Wipes	3097

V. REFERENCES

References Cited in this Manual

1. Bird, A. (1992) The essentials of DNA methylation. *Cell* **70:** 5-8.

2. Antequera, F., J. Boyes and A.Bird. (1990) High levels of De Novo Methylation and Altered Chromatin Structure at CpG Islands in Cell Lines. *Cell* **62:** 503-514.

3. Herman, J.G., F. Latif, Y. Weng, M.I. Lerman, B. Zbar, S. Liu, D. Samid, D.S. Duan, J.R. Gnarra, W.M. Linehan and S.B. Baylin. (1994) Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc. Natl. Acad. Sci.* USA **91:** 9700-9704.

4. Merlo, A., J.G. Herman, L. Mao, D.J. Lee, E. Gabrielson, P.C. Burger, S.B. Baylin and D. Sidransky. (1995) 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nature Medicine* **1:** 686-692.

5. Herman, J.G., J.R. Graff, S. Myohanen, B.D. Nelkin and S.B. Baylin. (1996) Methylation-specific PCR: A novel assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci.* USA **93:** 9821-9826.

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