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CpG WIZ™ p16 Amplification Kit
S7800

CpG WIZ™ p15 Amplification Kit
S7802

CpG WIZ™ E-cadherin Amplification Kit
S7804

CpG WIZ™ Prader-Willi/Angelman Amplification Kit
S7806

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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I. INTRODUCTION

Using This Manual

Please read the entire instruction manual prior to using CpG WIZ™ Amplification Kits. Note that in the Procedures and Troubleshooting sections, instructions are separate for the CpG WIZ™ Prader-Willi/ Angelman Amplification Kit (S7806). 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. The exception is extensive methylation of CpG islands associated with transcriptional inactivation of regulatory regions of imprinted genes (2, 3) such as those associated with Prader-Willi/Angelman Syndrome (4) and genes on the inactive X-chromosome of females (5, 6). Aberrant methylation of normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells (7) and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers (8, 9). E-cadherin, p16, and p15 are examples of genes that exhibit 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 (10). 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 (S7820) contains the reagents necessary to perform the initial bisulfite reactions, while CpG WIZ™ Amplification Kits contain the reagents required for the PCR amplification reactions.

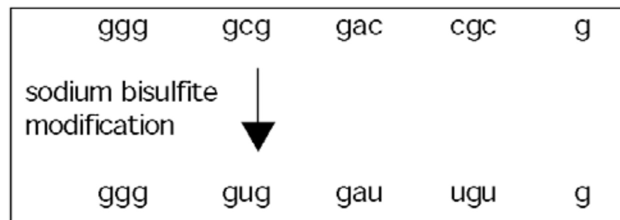
Principles of the Technique

MSP, performed using the CpGenome™ DNA Modification Kit and CpG WIZ™ Amplification Kits, permits sensitive detection of altered DNA. Due to the fact that it is a PCR-based assay, it is extremely sensitive, facilitating the detection of low numbers of methylated alleles and the study of samples containing low 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.

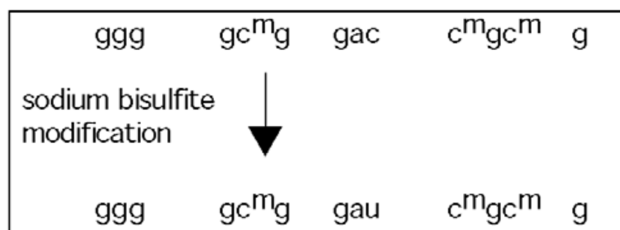
Methylation-specific PCR employs an initial bisulfite reaction to modify the DNA, followed by a "hot start" PCR amplification with specific primers designed to distinguish methylated DNA from unmethylated DNA. As shown in Figure 1, in the bisulfite reaction, all unmethylated cytosines are converted to 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 CpG WIZ™ Amplification Kits 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



II. KIT COMPONENTS

The components of CpG WIZ™ Amplification Kits 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: CpG WIZ™ p16 (S7800), p15 (S7802) and E-cadherin (S7804) Amplification Kit Components (color-coded microcentrifuge tube caps)

Description	Amount	Storage Conditions
U Primer Set 5 µM each primer (25X)	35 µL (white cap)	-15°C to -25°C
M Primer Set 5 µM each primer (25X)	35 µL (red cap)	-15°C to -25°C
W Primer Set 5 µM each primer (25X)	35 µL (green cap)	-15°C to -25°C
U control DNA 0.1 µgµL	50 µL (white cap)	-15°C to -25°C
M control DNA 0.1 µg/µL	50 µL (red cap)	-15°C to -25°C
W control DNA 0.05 µg/µL	50 µL (green cap)	-15°C to -25°C
Universal 10X PCR Buffer	265 µL (blue cap)	-15°C to -25°C

Table 2: CpG WIZ™ Prader-Willi/Angelman (S7806) Amplification Kit Components (color-coded microcentrifuge tubes)

Description	Amount	Storage Conditions
P (paternal) primer set 5 µM each primer (25X)	70 µL (white cap)	-15°C to -25°C
M (maternal) primer set 5 µM each primer (25X)	70 µL (red cap)	-15°C to -25°C
Normal control DNA 0.1 µg/µL	20 µL (green cap)	-15°C to -25°C
10X PCR buffer	175 µL (blue cap)	-15°C to -25°C

Materials Required But Not Supplied

Equipment and Supplies

a. Thermocycler

b. Gel electrophoresis apparatus (vertical or horizontal)

c. Power Supply

d. Screw-cap tubes for PCR amplification

e. Aerosol-resistant pipette tips

f. Microcentrifuge (to 12,000 X g)

g. 302 nm UV transilluminator, camera and film

Reagents

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

b. Taq polymerase

Note: *For S7806 (CpG WIZTM Prader-Willi/Angelman Amplification Kit), the use of a "hot start" enzyme is strongly recommended. See Sec. III.* Protocols.

c. "Hot start" PCR reagents for **S7800**, **S7802**, and **S7804** (see Sec. III. *Protocols*).

d. Reagents for gel electrophoresis (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
h. Bisulfite Modified DNA (CpGenome™ DNA Modification Kit, S7820)

III. Protocols

CpG WIZ™ p16 (S7800), p15 (S7802) and E-cadherin (S7804) Amplification Kits

Experimental Design

Primer Sets

CpG WIZ™ Amplification Kits contain primers that can be used for analysis of DNA samples by MSP. However, the samples must first undergo bisulfite modification prior to PCR amplification. CpGenome™ DNA Modification Kit, 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 by the following accession numbers: **p16**, X94154; **p15**, S75756; **E-cadherin**, L34545.

“Hot Start” PCR

The three sets of primers used in the CpG WIZ™ Methylation Assay are 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

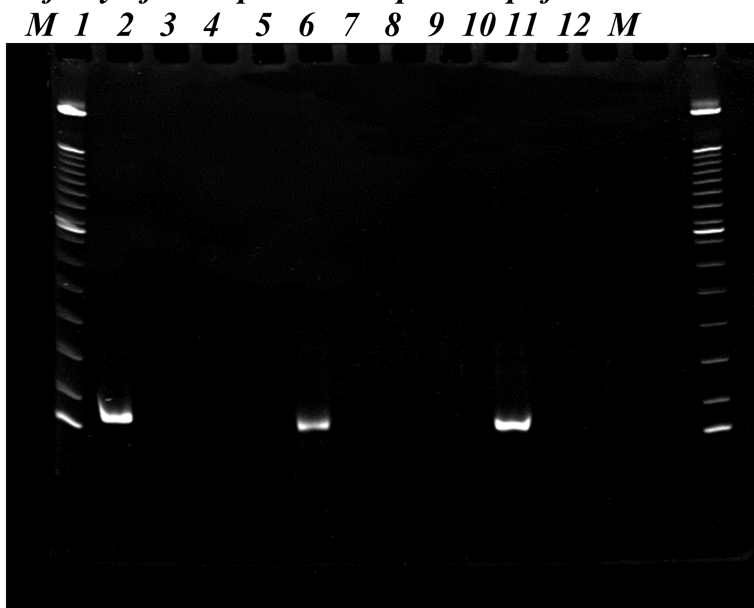
The methylated (red cap) and unmethylated (white cap) control DNAs must undergo bisulfite modification prior to PCR amplification using the CpGenome™ DNA Modification Kit (S7820). When used with their respective U and M primer sets, a single PCR product of an expected size is obtained in each case.

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.

Specificity of the Assay

The specificity of the CpG WIZ™ p16 Amplification Kit is shown in Figure 2. With a complete chemical modification reaction, U primers amplify only unmethylated DNA (154 bp, lane 1) and M primers amplify only methylated DNA (145 bp, lane 5). W primers amplify only DNA which is not chemically modified, or "wild type W" (142 bp, lane 9).

Figure 2: Specificity of the CpG WIZ™ p16 Amplification Kit.



Polyacrylamide gel analysis using the primers and the control DNA samples included in the kit is shown. (Lanes 1-3: U control DNA; lanes 4-6: M control DNA; lanes 7-9: W control DNA; lanes 10-12: Minus DNA control. Lanes 1, 4, 7, 10: U primers; lanes 2, 5, 8, 11: M primers; lanes 3, 6, 9, 12: W primers. M lane: 100 base-pair marker.)

Experiment Setup

CpG WIZ™ Amplification Kits (S7800, S7802, and S7804) include 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 U, M, and W primers

Amplification Protocol

To prevent PCR contamination, read Sec. VI. *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 (S7820).

STEP 2. Amplification

"Hot start" PCR is recommended for this assay (refer to Sec. III. *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. III. *Protocols, Experimental Design*).

b. Prepare three (3) "master mixes" which correspond to the 3 possible primer sets U, M and W (primer cap colors-white, red and green) 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 color: 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" (white, red and green). 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 (white, red and green)	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" (white, red and green) into corresponding PCR tubes.

d. Add:

   2 µL of water to the no DNA control 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 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 5 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.*

CpG WIZ™ Prader-Willi/Angelman (S7806) Amplification Kit

Experimental Design

Primer Sets

The CpG WIZ™ Prader-Willi/Angelman Amplification Kit contains primers that can be used for analysis of DNA samples by MSP. However, the first step of MSP is the bisulfite modification of the DNA samples. CpGenome™ DNA Modification Kit (S7820), contains the reagents necessary to perform the modification. This 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.

P (Paternal) Primer Set will anneal to unmethylated DNA that has undergone a chemical modification.

M (Maternal) Primer Set will anneal to methylated DNA that has undergone a chemical modification.

Amplified Regions

The P and M primer sets are designed to amplify a differentially methylated site present at the CpG island of the small nuclear ribonucleoprotein-associated polypeptide N (SNRPN), a candidate gene for Prader-Willi Syndrome. 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 primer pair. The nucleotide numbering system is that used in the GenBank submission identified by the accession number L32702.

"Hot Start" PCR

The two sets of primers used in the CpG WIZ™ Prader-Willi/Angelman Amplification Kit are derived from sequences closely related to each other, which introduces the possibility of mispriming. In order to avoid this and other PCR-related artifacts, it is recommended that a "hot start" enzyme be used.

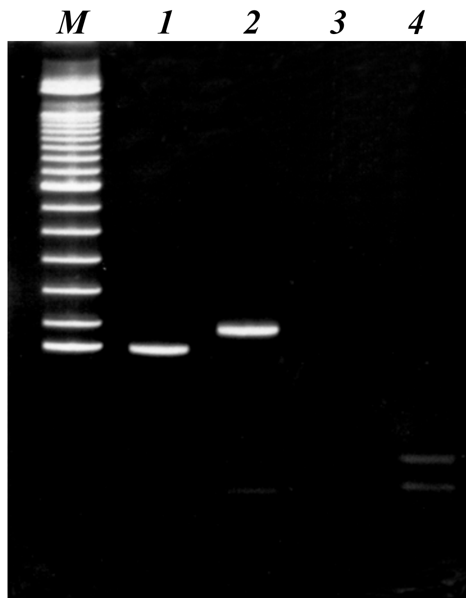
Normal Control DNA

The normal control contains DNA from both maternal and paternal homologs which means that both methylated and unmethylated SNRPN sequences are represented. Therefore, PCR products will be obtained after the bisulfite modification of the normal control DNA when using either the M or the P primer sets. The M primer set will generate a 174 bp product while the P primer set produces a 100 bp fragment. Multiplex amplification is feasible since the fragment sizes are sufficiently dissimilar.

Specificity of the Assay

The specificity of the CpG WIZ™ Prader-Willi/Angelman Amplification Kit is shown in Figure 3. With a complete chemical modification reaction, P primers amplify only unmethylated DNA (100 bp, lane 1) and M primers amplify only methylated DNA (174 bp, lane 2). The smaller bands present in the lanes where the M primer set was used in the PCR reaction represent excess primers.

Figure 3: Specificity of the CpG WIZ™ Prader-Willi/Angleman Amplification Kit.



Polyacrylamide gel analysis using the primers and the control DNA samples included in the kit is shown. (Lane 1: P primer set with modified control DNA; Lane 2: M primer set with modified control DNA; Lane 3: P primer set with unmodified control DNA; Lane 4: M primer set with unmodified control DNA. Marker Lane: 100 bp ladder)

Experiment Setup

The CpG WIZ™ Prader-Willi/Angelman Amplification Kit includes sufficient reagents to analyze 25 samples with appropriate controls (70 PCR reactions). For each batch of experimental DNA samples to be analyzed, the experimental samples and the normal control DNA must first undergo bisulfite modification. In the subsequent PCR reactions, the chemically modified DNA samples, both experimental and control, are amplified using both the maternal (M) and paternal (P) primer sets. In addition, 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 and proper controls includes a total of 14 lanes:

Lanes 1-2	Experimental sample 1 with P and M primers
Lanes 3-4	Experimental sample 2 with P and M primers
Lanes 5-6	Experimental sample 3 with P and M primers

Lanes 7-8	Experimental sample 4 with P and M primers
Lanes 9-10	Experimental sample 5 with P and M primers
Lane 11	Chemically modified normal control DNA with P primers
Lane 12	Chemically modified normal control DNA with M primers
Lanes 13-14	No DNA Control with P and M primers

Amplification Protocol

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

STEP 1. Modification

Prior to performing PCR with the primer sets in the CpG WIZ™ Prader-Willi/Angelman Amplification Kit, the DNA samples must undergo bisulfite modification. CpGenome™ DNA Modification Kit (S7820), contains the reagents necessary to perform the modification.

STEP 2. Amplification

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

b. Prepare two (2) "master mixes" which correspond to the 2 possible primer sets P and M (primer cap colors-white and red) 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 color: 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" (white and red). 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**	2.0 μL	
P or M primers (white, red)	2.0 μL	
"Hot start" enzyme (5 U/μL)	0.1 μL	(0.5 U)
dH_2O	16.4 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" (white and red) into corresponding PCR tubes.

d. Add:
   2 μL of water to the no DNA control tube.

   2 μL of modified sample DNA to each of the sample tubes.

   2 μL of modified normal DNA control to each control tube.

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

Denature:
   for "hot start" enzyme check manufacturer's specifications

Then, perform 35 cycles of the following conditions:

denature	95°C / 30 seconds
anneal	62°C / 30 seconds
extend	72°C / 30 seconds

Final Extension: 72°C /10 minutes

f. Remove the tubes from the thermocycler block. From this point on, it is important to designate separate pipettors 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 5 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.*

IV. DATA ANALYSIS

CpG WIZ™ p16 (S7800), p15 (S7802) and E-cadherin (S7804) Amplification Kits

Table 3: Sizes of Expected Products from Controls

Controls	p16	p15	E-cadherin
U primer/U control DNA	154	162	212
M primer set/M control DNA	145	154	206
W primer set/W control DNA	142	137	194

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

Table 4: Sizes of Expected Products from Samples

Experimental Samples	p16	p15	E-cadherin
U primer set/ unmethylated DNA	154	162	212
M primer set/methylated DNA	145	154	206

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.

CpG WIZ™ Prader-Willi/Angelman (S7806) Amplification Kit

Table 5: Sizes of Expected Products

Template	Primer Set	
	P	M
Normal Control DNA	100	174
Experimental Methylated DNA	—	174
Experimental Unmethylated DNA	100	—

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

In a clinical research sample obtained from a normal individual, a 100 bp and a 174 bp fragment will be obtained when using the P primer set and M primer set, respectively, in PCR reactions. MSP performed on a research sample from an individual with Prader-Willi Syndrome will produce the 174 bp fragment with the M primer set but will generate no product with the P primer set. Conversely, a research sample from an individual with Angelman Syndrome will produce the 100 bp fragment with the P primer set, but will generate no product with the M primer set.

V. TROUBLESHOOTING

CpG WIZ™ p16 (S7800), p15 (S7802) and E-cadherin (S7804) Amplification Kits

? 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. VI. *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 PCR product produced with the W primer set is always smaller than that produced with either U or M.

? 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.

CpG WIZ™ Prader-Willi/Angelman (S7806) Amplification Kit

? 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. Confirm that the PCR polymerase is still active.
e. Confirm that no additional Mg^{2+} was added to the PCR reaction mix.
f. The optimal annealing temperature is 62°C. If items #a-e have not remedied the problem, re-optimize annealing conditions to suit your amplification instrument.

? No amplification product is generated in the experimental samples using the P and M 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.

? P 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. VI. *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.

VI. 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

VII. REFERENCES

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