CpGenome™ Turbo Bisulfite Modification Kit (50 Reactions)

Catalog No. S7847

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

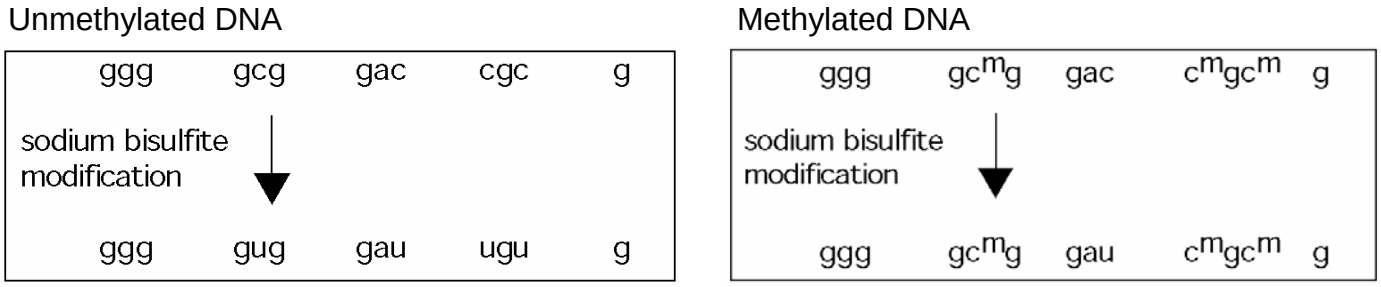
Methylation of cytosines located 5' to guanosine is known to have a profound effect on the expression of many eukaryotic genes (1,2). In normal cells methylation occurs predominantly in CG-poor regions, while CG-rich areas, called CpG-islands remain unmethylated. The exceptions are the extensive methylation of CpG islands associated with transcriptional inactivation of regulatory regions of imprinted genes (3, 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). Hundreds of CpG islands are now known to exhibit the characteristic of hypermethylation in tumors resulting in identification of candidate genes that can be interrogated to determine extent of tumor specific transformation (10).

Several methods have been developed to determine the methylation status of cytosine (11,12). These include the use of antibodies or protein methyl binding domains, digestion with methylation sensitive, insensitive, or dependent restriction enzymes as in restriction landmark genomic scanning, oligonucleotide array hybridization, bisulfite genomic DNA sequencing and Methylation Specific PCR (MSP). Some techniques are more useful for discovery (e.g. RLGS) while others are better used for evaluating sequence specific methylation of known methylated cytosines (e.g. MSP).

Genomic DNA sequencing, although time consuming and labor intensive, offers a more universal detection method (13, 14). MSP is an established technology for the monitoring of abnormal gene methylation in selected gene sequences (15). Utilizing small amounts of DNA, this procedure offers sensitive and specific detection of 5-methylcytosine in promoters. It is being exploited to define tumor suppressor gene function, and to provide a new strategy for early tumor detection by interrogating DNA derived from tissues and bodily fluids.

The initial step of both bisulfite genomic sequencing and MSP is to perform a bisulfite modification of the DNA sample. As shown in Figure 1, in the bisulfite reaction, all unmethylated cytosines are deaminated and sulfonated, converting them to uracils, while 5-methylcytosines remain unaltered. Thus, the sequence of the treated DNA will differ depending on whether the DNA is originally methylated or unmethylated. Also, the initially complementary DNA strands will no longer be complementary after cytosine conversion. Primers for use in MSP can be designed to specifically amplify either a bisulfite-sensitive, unmethylated strand or a bisulfite-resistant, methylated strand, based upon these chemically-induced differences (16). For additional details on MSP please refer to the appendix.

Figure 1. DNA treatment with bisulfite reagent



CpGenome Turbo Bisulfite Modification Kit Overview

The CpGenome Turbo Bisulfite Modification Kit is designed to simplify and streamline the bisulfite modification process. This kit contains all key reagents for bisulfite modification to allow for the recovery of modified DNA in about 90 minutes as compared to 6-12 hours for similar kits. This rapid, but effective approach employs a proprietary mixture of modification reagents to permit shorter conversion times. This unique mixture of reagents allows for complete conversion of unmethylated cytosines while minimizing damage to the input DNA. After modification, and desulfonation, the modifed DNA is purified and eluted using the provided spin columns. This purified, bisulfite-modifed DNA is ready to use in a wide variety of downstream applications.

To complement the CpGenome Turbo Bisulfite Modification Kit, we offer a range of CpG WIZ® Amplification Kits containing the materials required for sensitive detection of bisulfite modified DNA using MSP. To see the collection of targets offered as well as additional technologies for the study of DNA methylation and epigenetics visit us at www.millipore.com/epigenetics.

Kit Components

S7847 CpGenome Turbo DNA Modification Kit Store at Room Temperature (18-25°C)		
Component	**Part #**	**Quantity**
Bisulfite Conversion Reagent 1	CS204366	10.5 g
Bisulfite Conversion Reagent 2	CS204367	3.35 g
Bisulfite Reagent Diluent	CS204355	25 mL
DNA Binding Buffer	CS204332	25 mL
DNA Wash Buffer**	CS204392	4 X 6 mL
DNA Elution Buffer	CS204321	5 mL
Modified DNA Purification Columns	CS204322	50 EA
2 mL Collection Tubes	CS204323	50 EA

** DNA Wash Buffer requires the addition of 100% Ethanol, see the Reagent Preparation section of the protocol for additional details.

Materials Required But Not Supplied

Reagents

- Nuclease-free water
- 96-100% Molecular Biology Grade Ethanol
- 12 N HCl
- 3 N NaOH

Equipment and Supplies

- Thermal cycler, heat block, or water bath
- Microcentrifuge (capable of 12,000 x g)
- Screw-cap microcentrifuge tubes, 1.5-2.0 mL
- Narrow range pH indicator paper (pH 0-6, three different indicators per strip, and/or pH 4-7, one indicator per strip) or a dedicated pH electrode).
- Pipettes and pipette tips (aerosol barrier tips are suggested to minimize potential cross contamination).
- Analytical scale or balance

HAZARDS AND PRECAUTIONS:

This protocol requires the use of strong acids and bases to prepare the bisulfite modification solution. Avoid inhalation, ingestion or contact with skin. Use of a chemical fume hood or particle mask is recommended when weighing the bisulfite reagents.

The DNA binding buffer (CS204332) provided in this kit contains guanidine thiocyanate. This compound is a skin irritant and can form highly reactive compounds when combined with bleach. Do not add bleach or acidic solutions directly to this buffer or solutions containing this buffer.

The use of Personal Protective Equipment and gloves when handling bisulfite modification reagents as well as the DNA purification reagents is strongly recommended.

Bisulfite-conversion Protocol

Please read entire protocol before starting

The CpGenome Turbo DNA Modification Kit provides sufficient reagents to bisulfite convert 50 individual purified DNA samples according to your experimental needs. The purified DNA can be sourced from cells or tissue by standard genomic DNA extraction protocols. Conversion of formalin fixed paraffin embedded samples may require additional steps prior to bisulfite conversion. See Millipore Cat. No. S4530, EX-WAX™ Paraffin-embedded DNA Extraction Kit user guide for details.

Chemical conversion of cytosine residues to uracil using elevated high temperatures and high concentrations of sodium bisulfite are known to cause degradation over time to DNA templates (17). The conditions used in the CpGenome Turbo Bisulfite Modification Kit were developed to minimize damage to the input sample. Please follow the directions in the protocol closely to preserve the integrity of your DNA samples.

Before Starting Protocol

Reagent Preparation

Prepare DNA Wash Buffer

Before use, add 24 mL of 96-100% Ethanol to one bottle of wash buffer for a final volume of 30 mL and mix well. Once ethanol is added this wash buffer can be stored at room temperature (18– 25°C) for up to six months. This kit contains multiple bottles of wash buffer, it is suggested that you mark the bottle as “ethanol added”.

3N NaOH

Dissolve 3 g of NaOH pellets in 25 mL ddH_2O to make 3N NaOH.
Exercise appropriate caution and lab practices when using this caustic base.

Reagent Preparation (continued)

Desulfonation Solution (Prepare fresh prior to each use)

To prepare 1 mL of this solution, combine 900 µl of 100% EtOH, 100 µL of 3N NaOH. For each reaction make 220 µL of this solution.

Dissolve DNA Modification Reagent (Prepare fresh prior to each use)

CAUTION: Powdered reagents can be irritating to the respiratory system and skin. Use appropriate safety procedures to minimize exposure during weighing.

Note: Reagent 2 is slightly hygroscopic. Minimize exposure to air and avoid excessive heat. Clumping may occur after prolonged periods if the product is not stored properly, but this will not usually affect the performance of the product. Use a clean spatula to disintegrate clumps.

For every 10 samples to be modified, weigh 0.42 g of **Bisulfite Conversion Reagent 1** and 0.134 g of **Bisulfite Conversion Reagent 2**. Reconstitute the blended reagent 1 & 2 with 1 mL of **Bisulfite Reagent Diluent**. To prevent the creation of a solid that is difficult to dissolve, pre-blending of the dry reagents for later use is not advised.

Adjust the pH to 5.3–5.4 with approximately 120 µL of 12N HCl. Check the pH with pH indicator paper. To ensure complete solubilization, incubate solution at 70°C with vortexing several times during incubation. Note: Reagent 1 & 2 may not completely solubilize without pH adjustment and heat.

PROTOCOL

Bisulfite Modification

Note: Bisulfite modification can be performed in 0.2 mL PCR tubes (if using a thermal cycler) or 1.5 mL microfuge tubes (if using a heat block or water bath)

1. Add 1.0 µl 3 N NaOH solution to 10 µL DNA in a 0.2 mL or 1.5 mL microfuge tube. The amount of input DNA should be from 1 ng to 1 µg. If the amount of starting material is limited as little as 500 pg can be used.
2. Incubate DNA, NaOH mixture for 10 minutes at 37°C (thermal cycler, heat block or water bath).
3. Add 120 µL of freshly prepared DNA Modification Reagent and vortex.
4. Incubate for 40 minutes at 70°C in thermal cycler or heat block. Proceed immediately to the next step.

Important: Do not extend the incubation period or allow sample to remain in the concentrated bisulfite solultion after the specified incubation period. Immediately proceed with DNA purification to reduce the bisulfite concentration and prevent DNA damage or over conversion of your sample.

Desalting/Desulphonation/Elution

1. Add 500 µL of **DNA Binding Buffer** to the bisulfite modified DNA. Mix briefly ensuring that the sample and binding buffer are completely mixed.

2. For each sample, place a **Modified DNA Purification Column** into a **2 mL Collection Tube** and load the entire sample into the spin column.
3. Centrifuge the column at 11,000 x g for 1 minute to bind the modified DNA to the column membrane. Discard the flow through.
4. Add 700 µL of **DNA Wash Buffer** to each spin column and centrifuge at 11,000 x g for 1 minute.

   Important: Be sure to check that ethanol has been added to the wash buffer. Adding wash buffer that does not contain ethanol will result in the loss of your sample.
5. Add 200 µL of freshly prepared **Desulfonation Solution** to the center of the membrane in the spin column.
6. Incubate at room temperature for 15 minutes. Centrifuge at 11,000 x g for 1 minute and discard the flow through.
7. Add 700 µL of **DNA Wash Buffer** and spin at 11,000 x g for 1 minute. Discard the flow through.
8. Repeat the wash step as described in step 7.
9. Place the column in a new 1.5 mL microcentrifuge tube and centrifuge at 11,000 x g for 1 minute to remove residual Wash Buffer.

   Note: Failure to remove the wash buffer will result in residual ethanol in your final eluted material. Residual ethanol can result in a lower recovery of your modified DNA and inhibition of downstream enzymatic reactions.
10. Place the spin column in a clean 1.5 mL tube. Add 25-50 µL of **DNA Elution Buffer** to the center of the membrane and incubate on the bench top for 1 minute. Spin at full speed for 1 minute.

    Note: For increased DNA recovery, elute the DNA with 25 µL of **DNA Elution Buffer** heated to 65-70°C and then repeat the elution with an additional 25 µL of heated **DNA Elution Buffer**. It is recommended to elute in 25 µL if the starting material is less than 100ng.
11. DNA is now ready for downstream applications. Store at -20°C for up to 2 months.

Analysis of bisulfite converted DNA

Bisulfite-modified DNA can be analyzed by several molecular methods including MSP (methylation specific PCR) using endpoint- PCR or quantitative RT-PCR, cloning and dideoxy-sequencing, microarray analysis or deep sequencing methods.

Useful information on primer design and other considerations for Methylation-Specific PCR (MSP) can be found in the Appendix. Should additional questions arise, assistance is available from Millipore Technical Service.

Performance Evaluation

The CpGenome Turbo DNA Modification Kit is evaluated using control DNAs and primers from the CpG WIZ® BRCA1 Amplification Kit (Cat. No. S7830) and CpG WIZ® MGMT Amplification Kit (Cat. No. S7803) to detect modified DNA. Results shown in figure 2 and 3 demonstrate reliable bisulfite conversion of 1 ng to 1 µg of input DNA.

DNA sequencing of bisulfite treated DNA was used to evaluate conversion and over-conversion of these samples. Results are shown in figure 4 and figure 5. In multiple clones, unmethylated cytosines were completely converted while methylated cytosines were unaffected consistent with complete conversion and no over conversion of the sample.

Millipore offers a selection of CpG WIZ assays to a variety of targets. As appropriate for your sample, these kits can be used as controls to evaluate the success of samples modified with the CpGenome Turbo Bisulfite Modification Kit. Should you wish to design your own unique target specific assays, further details on methylation specific PCR and guidelines for primer design to allow the creation of assays for virtually any gene of interest are presented in the appendix.

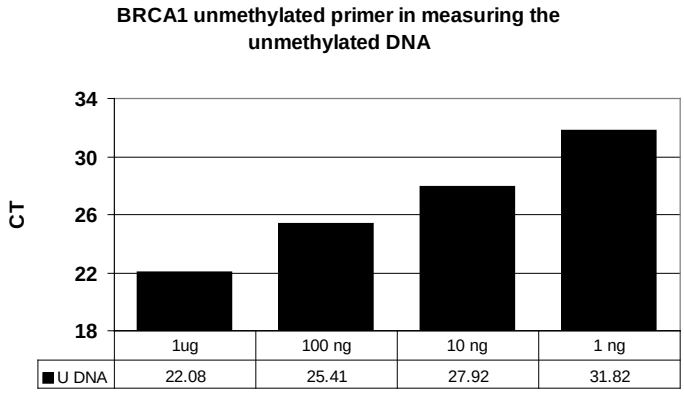


Figure 2. Unmethylated DNA (Cat. No. S7822), diluted from 1 ng to 1 µg, was bisulfite treated as described in the protocol. BRCA1 U primer was used to amplify the converted DNA using quantitative PCR (Note: 1 µg and 100 ng starting material was eluted in 50 µL DNA Elution Buffer while 10 ng and 1 ng was eluted in 25 µL DNA Elution Buffer).

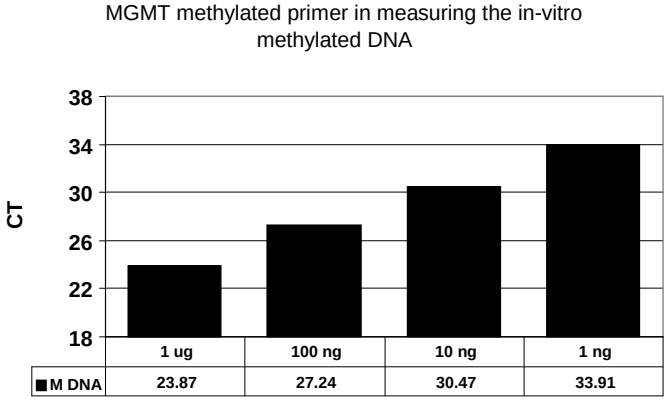


Figure 3. In vitro Methylated DNA (Cat. No. S7821), diluted from 1 ng to 1 µg was bisulfite treated as described in the protocol. MGMT M primer was used to amplify the converted DNA using quantitative PCR (Note: 1 µg and 100 ng starting material was eluted in 50 µL DNA Elution Buffer while 10 ng and 1 ng was eluted in 25 µL DNA Elution Buffer).

GAAAAAGCGCGGGGAATTACAGATAAATTAAAACTG

```
-------T-T---------T-------------T--
-------T-T---------T-------------T--
-------T-T---------T-------------T--
-------T-T---------T-------------T--
-------T-T---------T-------------T--
-------T-T---------T-------------T--
-------T-T---------T-------------T--
```

Figure 4. One microgram of unmethylated DNA (Cat. No. S7822) was bisulfite treated and amplified by PCR using the BRCA1 U primers. The resulting PCR amplicon was sequenced to determine the conversion status of the cytosines within the sequence. Cytosines known to be unmethylated in this DNA sample are underlined. Sequence alignments of seven clones that were analyzed are shown with dashes representing perfect matches and Ts representing cytosines converted to uracil with subsequent conversion to thymidine via PCR amplification.

GGTGCGCACCGTTTGCGACTTGGTGAGTGTCTGGGT

```
----C-T-TC-----C-T------------T-----
----C-T-TC-----C-T------------T-----
----C-T-TC-----C-T------------T-----
----C-T-TC-----C-T------------T-----
----C-T-TC-----C-T------------T-----
```

Figure 5. One microgram of methylated DNA (Cat. No. S7821) was bisulfite treated and amplified by PCR using the MGMT M primers. The resulting PCR amplicon was sequenced to determine the conversion status of cytosines within the sequence. All CpG cytosines in this sequence are *in vitro* methylated and are underlined, as are all non-CpG cytosines. Sequence alignments of five clones are shown to demonstrate conversion of non-methylated cytosines (reflected as thymidine residues following PCR amplification of uracil) and non-conversion events reflected as cytosines (demonstrating lack of over conversion).

Appendix

Methylation Specific PCR (MSP)

For this amplification based analysis method, PCR primers are designed to specifically amplify the promoter regions of the gene of interest. If the sample DNA was originally unmethylated, an MSP reaction product will be detectable when using the primer set (labeled as 'U') designed to be complementary to the unmethylated bisulfite converted DNA sequence. No product will be generated using a primer set (labeled as 'M') designed to be complementary to the derivative methylated DNA sequence. Conversely, an MSP product will be generated only using the M primer set if the sample was originally methylated, and the U primers will not amplify such a template.

Methylation specific PCR permits sensitive detection of altered DNA. Because it is a PCR-based assay, it is extremely sensitive, facilitating the detection of low numbers of methylated alleles and the evaluation of DNA from small samples, including paraffin-embedded, formalin fixed materials. MSP, when coupled with bisulfite sequencing, also allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. Increasing the number of such sites that 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 unmethylated cytosines to uracils, making a region of the genome which is CG rich more easily amplified by PCR.

MSP Primer Design

Methylation Specific PCR consists of chemical modification followed by amplification. Chemical modification creates the sequence differences between the methylated and unmethylated DNA. Ideally, three sets of primers should be designed to anneal to the DNA, based upon these sequence differences. The three primer sets each are designed to detect the different states of a converted DNA sequence.

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

The standard rules for primer design apply toward the creation of MSP primers. The primers should be approximately 20-21 bp in length and should possess similar dissociation temperatures. The amplified product produced with each set of primers should be 100-200 bp in size. Internal secondary structure should be avoided. In order to minimize primer dimer formation, primers should not be complementary, especially at the 3' end. Discrimination between methylated and unmethylated sequences by MSP seems to be greatest when the 3' ends (or anchor region) of the primers are most different from one another.

Alternatively, MethPrimer software package, a functional program that selects oligonucleotide primers from a sequence file for methylation-specific PCR (MSP) analysis can be found at http://www.urogene.org/methprimer/index1.html. This interactive online program will select and generate primer sets that may potentially be used for MSP analysis.

MSP Experimental Design
To perform a thorough analysis of each modified DNA sample, experiments should include amplification reactions using the U, M and W primer sets as described above. Unmodified DNA should be amplified with the W primer set. This reaction serves as a positive control for PCR and verification of the integrity of the genomic DNA sample. In addition, a negative PCR control (i.e. no DNA) should be performed for each set of primers. Depending on the DNA sample, a product may be produced using either the U or M primer sets or both sets may produce a product. Amplification using the W primer set with a modified DNA sample should not produce a product unless the modification was incomplete. However, PCR with the W primer set on unmodified DNA serves as a general positive PCR control product. Appearance of a product in the negative PCR control would indicate possible contamination.

Use of "Hot Start" PCR in MSP
The three sets of primers used in MSP are derived from sequences closely related to each other, which introduces an opportunity for 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. There are multiple approaches to performing a "hot start" PCR reaction. Simple approaches that heat a reaction mix to 95°C in a thermal cycler prior to the addition of Taq can be used or more sophisticated approaches using either Taq-specific antibodies or polymerases modified such they are inactive until brought to 95°C can also be used.

Note: When performing MSP, do not use a polymerase capable of 3-5' mismatch repair (i.e. thermal stable polymerase with proof reading activity). Also avoid use of uracil N-glycosylase containing PCR master mixes when amplifying bisulfite converted DNAs.

Laboratory Setup and Precautions
One of the most important considerations when using the CpGenome Turbo Bisulfite Kit and when performing PCR 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. Some sources of PCR product contamination are pipettes, tips, gel box, buffer, tube racks, notebooks, lab coats and any other items exposed to amplified PCR products. The following precautions should be followed in all steps of the assay protocol.

1. Always wear gloves.
2. Use sterile water for all solutions, aliquot the solutions in small amounts, and use fresh aliquots as working solutions. Discard working solutions after use.
3. Keep the assay solutions (10X PCR buffers, dNTPs, polymerase, etc.) separate from the amplified DNA.
4. Always use aerosol resistant pipette tips.
5. Separate micropipettors and work areas are recommended for the following three steps of the assay:
    - DNA modification and purification
    - Amplification set-up
    - Post-amplification analysis

Troubleshooting

Problem	Potential Cause	Suggestions
DNA degradation or loss of DNA detected	Sample is left in the conversion reagent longer than recommended or temperature exceeded 70°C	Carefully monitor time and temperature of conversion
	Input sample degraded	Confirm starting DNA material was intact using an unconverted template control primer set such as W primers.
	DNA purification procedure failure	Ensure the DNA purification protocol is followed as recommended.
Low bisulfite conversion rate	Chemical modification of the experimental DNA sample is incomplete	Ensure DNA modification buffer is at correct pH. Use pH paper check pH and adjust pH to 5.3-5.4 if required.
	DNA not adequately denatured.	Sodium bisulfite modifies only single-stranded DNA. Double stranded DNA must be denatured in 300mM NaOH prior to modification. Ensure NaOH solution is made fresh from solid pellets.
No PCR product is detected by end-point or real-time PCR	Experimental DNA samples were degraded prior to chemical modification.	Confirm integrity of DNA samples prior to bisulfite conversion using control primer set (i.e. W Primer) in PCR analysis.
		Start with freshly prepared genomic DNA sample and repeat the chemical modification. Methylated and Unmethylated Control DNAs (unmodified) are available from Millipore for use as positive controls.
	DNA samples were degraded after modification.	If chemically modified experimental DNA samples are stored at –20°C for more than two months prior to PCR, repeat the chemical modification on new genomic DNA samples. Do not store DNA in a frost-free freezer.

Troubleshooting -continued

Problem	Potential Cause	Suggestions
No PCR product is detected by end-point or real-time PCR (continued from previous page)	Inhibitor is present following bisulfite conversion and DNA purification	Dilute bisulfite converted DNA in water and use less DNA as template in PCR reactions.
		Ensure ethanolic DNA Wash Buffer is removed during DNA extraction steps by centrifuging columns 5 min in last wash, or heating columns 2-5 min at 70°C prior to elution of bisulfite converted DNA
		Diminish carryover of chaotropic salts by introducing an additional wash step using 700 uL DNA Wash Buffer followed by complete removal of buffer by centrifugation or heating at 70° C
PCR product in No Template Control of PCR reaction	PCR reagents are contaminated with amplification products.	Use fresh aliquots of PCR reagents (i.e. dNTPs, buffer, etc). Use separate set of pipettes for each liquid dispensing. Devote a work area to pre- and post-amplification procedures. Always use aerosol-resistant pipette tips. Always use a clean labcoat and gloves
	PCR primers form primer dimers	Ensure primer design is validated in end point PCR and use melt curve analysis if using a real time capable thermal cycler.

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