Appendix: Bisulfite Conversion and PCR Optimization

1. Reaction Conditions: The reaction conditions given in Step 2 of the Protocol will generate consistent results for both easy and difficult to convert template DNAs including those that are GC rich. However, the two protocols provided below (alternative 1 & 2) may yield better results in PCR amplification of longer DNA fragments. However, should the DNA template have >80% GC composition, then these conditions may result in incomplete template cytosine to uracil conversion.

Alternative 1:

1. 98° C for 10 minute
2. 53° C for 30 minute
3. 53° C for 6 minutes \ 8 cycles
4. 37° C for 30 minute/
5. 4° C storage

Alternative 2:

1. 98° C for 10 minutes
2. 53° C for 4 hours
3. 4° C storage

2. Bisulfite Conversion of Double Stranded DNA Templates. The following illustrates what occurs to a DNA template during bisulfite conversion.

Template: A: 5'-GACCGTTCCAGGTCCAGCAGTGCGCT-3'
B: 3'-CTGGCAAGGTCCAGGTCGTCACGCGA-5'
Bisulfite Converted: A: 5'-GATCGTTTTAGGTTTAGTAGTGCGTT- 3'
B: 3'-TTGGCAAGGTTTAGGTTGTTATGCGA-5'

3. PCR Primer Design. Generally, primers 26 to 32 bases are required for amplification of bisulfite converted DNA. In general, all Cs should be treated as Ts for primer design purposes, unless they are in a CpG context. See example below.

Bisulfite Converted: A: 5'-GATCGTTTTAGGTTTAGTAGTGCGTT-3'
Primers: Reverse: 3'-ATCATCACRCAA-5' R= G/A
Forward: 5'-GATYGTTTTAGGT-3' Y= C/T

4. Amount of DNA Required for Bisulfite Conversion. The minimal amount of human or mouse genomic DNA required for bisulfite treatment and subsequent PCR amplification is 100 pg. The optimal amount of DNA per bisulfite treatment is 200 to 500 ng. Although, up to 2 µg of DNA can be processed, it should be noted that high input levels of DNA may result in incomplete bisulfite conversion for some GC-rich regions.

5. PCR Conditions. Usually, 35 to 40 cycles are required for successful PCR amplification of bisulfite converted DNA. Optimal amplicon size should be between 150-300 bp; however larger amplicons (up to 1 kb) can be generated by optimizing the PCR conditions. Annealing temperatures between 55-60°C typically work well.

As most non-methylated cytosine residues are converted into uracil, the bisulfite-treated DNA usually is AT-rich and has low GC composition. Non-specific PCR amplification is relatively common with bisulfite treated DNA due to its AT-rich nature. PCR using "hot start" polymerases is strongly recommended for the amplification of bisulfite-treated DNA.

Note: Methylated "C" is underlined in the examples.
Note: Following bisulfite conversion, the strands are no longer complementary.
Note: Only one strand (A) is amplified by a given primer set. Only the reverse primer binds to the converted DNA, the forward primer will bind the strand generated by the reverse primer. If the primer contains CpG dinucleotides with uncertain methylation status, then mixed bases with C and T (or G and A) can be used. Usually, there should be no more than one mixed position per primer and it should be located toward the 5' end of the primer. It is not recommended to have mixed bases located at the 3' end of the primer.

Instruction Manual No. ZD5005

EZ DNA Methylation-Gold Kit

Cat # ZD5005

For In Vitro Research Use Only

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Highlights

- Complete bisulfite conversion of GC-rich DNA in less than 3 hours.
- A coupled heat denaturation/conversion reaction step streamlines the conversion of unmethylated cytosines into uracil.
- DNA precipitations are omitted. Instead, DNA is cleaned and desulphonated in a single step using state-of-the-art spin columns.
- Eluted, ultra-pure DNA is ideal for use in subsequent molecular-based analyses.

EZ DNA Methylation-Gold Kit	**ZD5005** 50 rxns.
CT Conversion Reagent*	5 Tubes
M-Dilution Buffer	1.5 ml
M-Dissolving Buffer	500 µl
M-Binding Buffer	30 ml
M-Wash Buffer**	6 ml
M-Desulphonation Buffer	10 ml
M-Elution Buffer	1 ml
Spin IC Columns	50 ct.
Collection Tubes	50 ct.
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Note - Integrity of kit components is guaranteed for one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

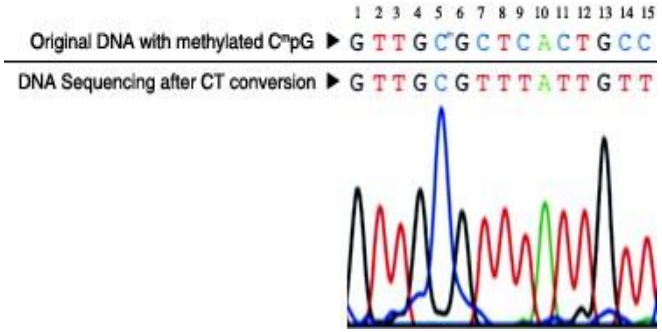
* 900 µl water, 300 µl **M-Dilution Buffer**, and 50 µl **M-Dissolving Buffer** must be added per tube of **CT Conversion Reagent** prior to use.

** Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate (ZD5005) or 96 ml of 100% ethanol to the 24 ml **M- Wash Buffer** concentrate (ZD5006) before use.

Introduction

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression (1). It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (2). DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation. In many plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (3). The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes.

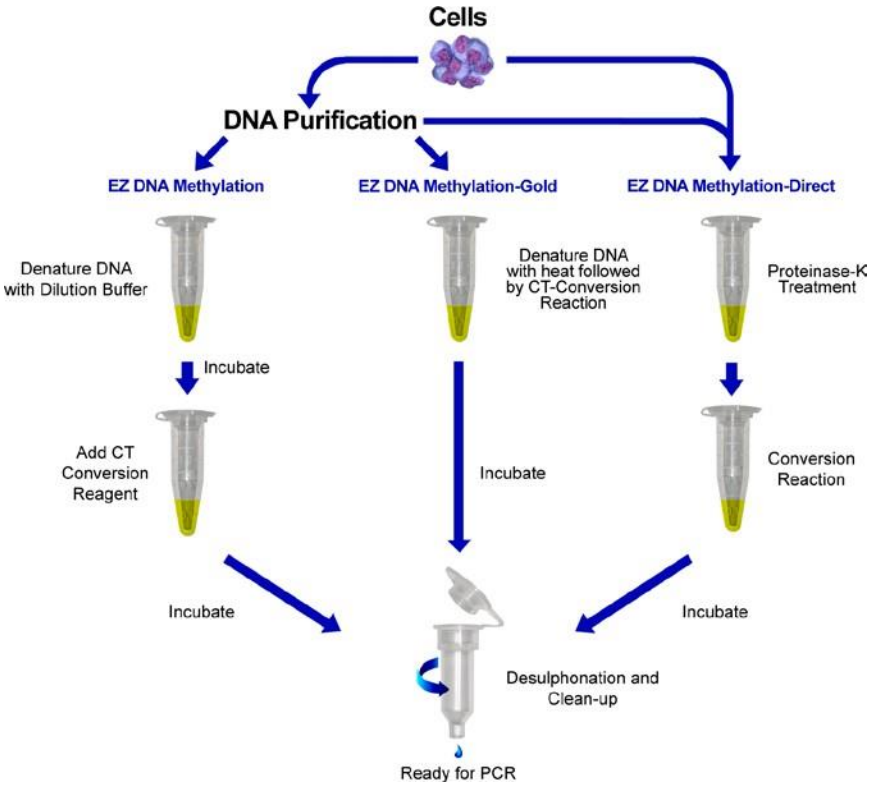
The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis (4) and methylation-sensitive arbitrarily primed PCR (5). However, the most common technique used today remains the bisulfite conversion method (6). This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing (see below).



DNA sequencing results following bisulfite treatment. DNA with methylated C^{m}pG at nucleotide position #5 was processed using the **EZ DNA Methylation™ Kit**. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remained intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 were completely converted into uracil following bisulfite treatment and detected as thymine following PCR.

Product Description

The EZ DNA Methylation-Gold Kit is a refinement of our popular EZ DNA Methylation Kit. The EZ DNA Methylation-Gold Kit integrates DNA denaturation and bisulfite conversion processes into one-step. This is accomplished using temperature denaturation to replace chemical denaturation with sodium hydroxide in the previous protocol. Also, the kit has been streamlined for high yield recovery of DNA following DNA bisulfite conversion. Both kits are based on a three-step reaction process between cytosine and sodium bisulfite resulting in cytosine being converted into uracil. The EZ DNA Methylation-Gold and EZ DNA Methylation™ Kits share innovative in-column desulphonation technology that eliminates cumbersome DNA precipitation steps while providing researchers consistent results every time. The kits have been designed to minimize template degradation, loss of DNA during treatment and clean-up, and to provide complete conversion of unmethylated cytosines. Recovered DNA is ideal for PCR amplification for downstream analyses including endonuclease digestion, sequencing, microarrays, etc.



Specifications

- DNA Input: Samples containing 500 pg - 2 µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500 ng.
- Conversion Efficiency: > 99% of non-methylated C residues are converted to U; >99% protection of methylated cytosines.
- DNA Recovery: > 75%

Reagent Preparation:

- **Preparation of CT Conversion Reagent**

The CT Conversion Reagent supplied within this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:

1. Add 900 µl water, 300 µl of M-Dilution Buffer, and 50 µl M-Dissolving Buffer to a tube of CT Conversion Reagent.
2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

Note: It is normal to see trace amounts of undissolved reagent in the CT Conversion Reagent. Each tube of CT Conversion Reagent is designed for 10 separate DNA treatments.

Storage: The CT Conversion Reagent is light sensitive, so minimize its exposure to light. For best results, the CT Conversion Reagent should be used immediately following preparation. If not used immediately, the CT Conversion Reagent solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored CT Conversion Reagent solution must be warmed to 37°C, then vortexed prior to use.

- **Preparation of M-Wash Buffer**

Add 24 ml of 100% ethanol to the 6 ml M-Wash Buffer concentrate (D5005) or 96 ml of 100% ethanol to the 24 ml M-Wash Buffer concentrate (D5006) before use.

Protocol:

Note: For DNA volumes >20 µl, an adjustment needs to be made during the preparation of the CT Conversion Reagent. The amount of water is decreased 100 µl for each 10 µl increase in DNA sample volume. For example, for a 40 µl DNA sample, 700 µl of water is added to make the CT Conversion Reagent. The volume of CT Conversion Reagent added to the sample must also be decreased by the same volume as the sample is increased, total reaction volume remains 150 µl. The maximum DNA sample volume to be used for each conversion reaction is 50 µl. Do not adjust the volumes of either the M-Dissolving Buffer or M-Dilution Buffer. Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments

1. Add 130 µl of the CT Conversion Reagent to 20 µl of your DNA sample in a PCR tube. If the volume of the DNA sample is less than 20 µl, make up the difference with water. Mix the sample by flicking the tube or pipetting the sample up and down, then centrifuge the liquid to the bottom of the tube.

2. Place the sample tube in a thermal cycler and perform the following steps*:
1. 98° C for 10 minutes
2. 64° C for 2.5 hours
3. 4° C storage up to 20 hours.

*For some samples, alternative parameters may yield improved results (see Appendix). If you have been using this kit with good results using different reaction conditions than described above, you can continue using those same conditions.

3. Add 600 µl of M-Binding Buffer to a Spin™ IC Column and place the column into a provided Collection Tube.

4. Load the sample (from Step 2) into the Spin™ IC Column containing the M- Binding Buffer. Close the cap and mix by inverting the column several times.

5. Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.

6. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.

7. Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.

8. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of M-Wash Buffer and centrifuge for an additional 30 seconds.

9. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C. We recommend using 1-4 µl of eluted DNA for each PCR, however, up to 10 µl can be used if necessary. The elution volume can be > 10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.