Instruction Manual

# Premium Bisulfite kit

Cat. No. C02030030

Version 1 / 18.09.13

diagenode

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# Content

# Introduction

- Fastest method for complete bisulfite conversion of DNA for methylation analysis.
- Ready-to-use conversion reagent is added directly to DNA.
- High-yield, converted DNA is ideal for PCR, MSP, array, bisulfite and Next-Gen Sequencing.

# Product Contents

| Description | Quantity | Storage |
|---|---|---|
| **Conversion Reagent*** | 5 tubes | Room Temperature |
| **Binding buffer** | 30 ml | Room Temperature |
| **Wash buffer**** | 6 ml | Room Temperature |
| **Desulphonation buffer** | 10 ml | Room Temperature |
| **Elution buffer** | 1 ml | Room Temperature |
| **Spin columns** | 50 columns | Room Temperature |
| **Collection tubes** | 50 tubes | Room Temperature |
| **Instruction Manual** | 1 | - |

**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.

* The **Conversion reagent** is in a ready-to-use liquid format. The reagent should be stored tightly capped at room temperature with minimum exposure to light.

** Add 24 ml of 100% ethanol to the 6 ml **Wash buffer** concentrate (K07291001) before use.

# Introduction to DNA Methylation

Cytosine methylation is a naturally occurring base modification, in both prokaryotic and eukaryotic organisms, consisting of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (1). In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA. DNA methylation in higher eukaryotes functions in the regulation/control of gene expression (2).

The majority of DNA methylation in mammals occurs in 5Đ-CpG-3Đ dinucleotides, although other patterns do exist. About 80 percent of all 5Đ-CpG-3Đ dinucleotides in mammalian genomes are found to be methylated, and the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes. 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 (3). DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, and 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 techniques used today still rely on bisulfite conversion (6).

Treating DNA with bisulfite chemically modifies non-methylated cytosines into uracil, methylated cytosines remain unchanged. Once converted, the methylation profile of the DNA can be determined using the desired downstream application. For single locus analysis, the region of interest is generally amplified following bisulfite conversion (i.e., bisulfite PCR) and then sequenced or processed for Pyrosequencing®. Recent advances in methylation detection also allow the investigation of genome-wide methylation patterns using technologies including array-based methods, reduced representation bisulfite sequencing (RRBS), and whole genome bisulfite sequencing (7).

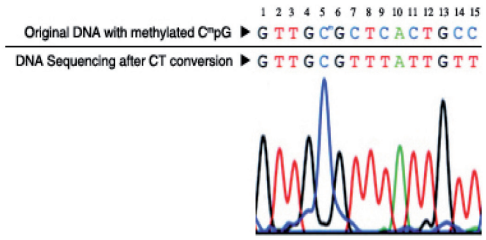



**Figure 2: DNA sequencing results following bisulfite treatment.**

DNA with methylated C at nucleotide position #5 was processed using the Premium Bisulfite kit. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remains intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 are completely converted into uracil following bisulfite treatment (detected as thymine following PCR).

## References

1. Adams RL. Bioessays. 1995; 17(2): 139-145.
2. Costello JF, Plass CJ. Med. Genet. 2001; 38(5): 285-303.
3. Stirzaker C. Cancer Res. 1997; 57(11): 2229-2237.
4. Fraga MF, et al. Electrophoresis. 2000; 21(14): 2990-2994.
5. Gonzalgo ML. Cancer Res. 1997; 57(4): 594-599.
6. Frommer M. Proc. Natl. Acad. Sci. USA. 1992; 89(5): 1827-1831.
7. Rakyan VK, et al. Nat. Rev. 2011, 12(8): 529-541.

MANUAL

# Product Description

The Premium Bisulfite kit features rapid and reliable bisulfite treatment and conversion of DNA for methylation analysis. Key to the fast workflow is the ready-to-use Conversion reagent. No preparation is necessary, simply add this unique reagent to a DNA sample, wait about an hour, and let the reaction proceed to completion. DNA denaturation and bisulfite conversion processes are combined with added heat to facilitate rapid denaturation. Desulphonation and clean-up of the converted DNA is performed using a unique low-elution spin column. High yield, converted DNA is ideal for PCR, array, bisulfite and Next-Generation Sequencing, etc.

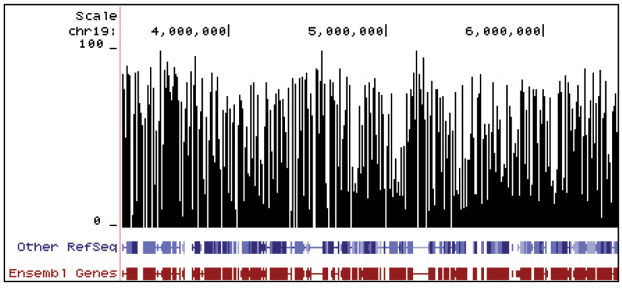
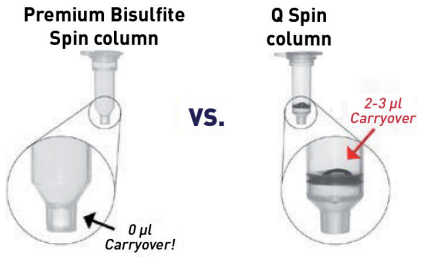



**Figure 2: Methylation Plot From Reduced Representation Bisulfite Sequencing (RRBS).**

Data shows the relative percentage of methylation at individual CpG sites in mouse DNA. Methylation percentage is shown across a ~3 Mb region of mouse chromosome 19. Bisulfite sequencing libraries were prepared using mouse genomic DNA prepped with the Genomic Clean & Concentrator™ (D4010, D4011 – Zymo Research) and bisulfite converted using Premium Bisulfite kit technology prior to Next-Gen Sequencing.




Premium Bisulfite Spin columns Ensure No Buffer Retention

**Figure 3: Spin column Design Characteristics.).**

The image above shows the unique design of the column that facilitates extremely small elution volumes (≥10 µl) without buffer carryover. This is unlike other columns that can retain liquid (binding/wash buffer residue) leading to carryover into the eluate.

# Specifications

- DNA Input: Samples containing between 100 pg to 2 µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500 ng.
- Conversion Efficiency: > 99.5% of non-methylated C residues are converted to U; > 99.5% protection of methylated cytosines.
- DNA Recovery: >80%

# Reagent Preparation

- **Preparation of Wash buffer**

  Add 24 ml of 100% ethanol to the 6 ml Wash buffer concentrate (K07291001) before use.

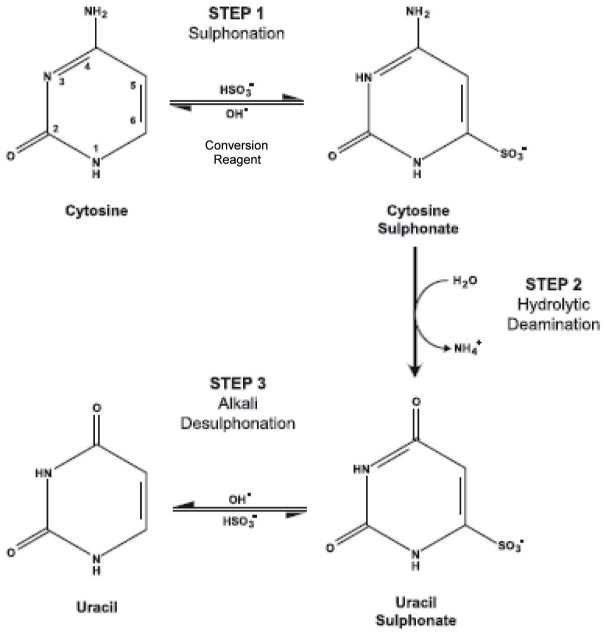



**Figure 4: Overview of Bisulfite Conversion.**

Steps 1 and 2 occur during bisulfite conversion, while Step 3 is performed as the DNA is bound to the column matrix. For the reaction to proceed to completion, it is essential the DNA be fully denatured.

# Protocol

1. Add 130 µl of **Conversion reagent** to 20 µl of a DNA sample in a PCR tube. Mix, then centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.
   **Note**: If the volume of DNA is less than 20 µl, compensate with water.
   ⚠ Samples >20 µl must be processed using multiple conversion reactions. Replicate reactions can be cleaned using the same column for each by repeating steps 3-5.
2. Place the PCR tube in a thermal cycler and perform the following steps:
   **a**. 98°C for 8 minutes
   **b**. 54°C for 60 minutes
   **c**. 4°C storage for up to 20 hours
   **Note**: The 4°C storage step is optional.
3. Add 600 µl of **Binding buffer** to a **Spin column** and place the column into a provided **Collection tube**.
4. Load the sample (from Step 2) into the **Spin column** containing the **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.
   ⚠ The capacity of the collection tube with the column inserted is 800 µl. Empty the collection tube whenever necessary to prevent contamination of the column contents by the flow-through.
6. Add 100 µl of **Wash buffer** to the column. Centrifuge at full speed for 30 seconds.
7. Add 200 µl of **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 **Wash buffer** to the column. Centrifuge at full speed for 30 seconds. Repeat this wash step.
9. Place the column into a 1.5 ml microcentrifuge tube and add 10 µl of **Elution buffer** directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.
   ⚠ Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments.
   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 higher DNA concentrations.

# Appendix: Bisulfite Conversion and PCR Optimization

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

   Template: **A**: 5'-**G**AC<u>C</u>**G**TTCCA**GG**TCCA**G**CA**G**T**G**<u>C</u>**G**CT-3'

   **B**: 3'-CT**GG**<u>C</u>AA**GG**TCCA**GG**TC**G**TCAC**G**<u>C</u>**G**A-5'

   Bisulfite Converted: **A**: 5'-**G**AT<u>C</u>**G**TTTTA**GG**TTTA**G**TA**G**TG<u>C</u>**G**TT-3'

   **B**: 3'-TT**GG**<u>C</u>AA**GG**TTTA**GG**TT**G**TTAT**G**<u>C</u>**G**A-5'

   **Note**: Methylated "C" is underlined in the examples.
   **Note**: Following bisulfite conversion, the strands are no longer complementary.

2. **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'-**G**AT<u>C</u>**G**TTTTA**GG**TTTA**G**TA**G**T**G**<u>C</u>**G**TT-3'

   Primers: Reverse: 3'-ATCATCAC**R**CAA-5' R= G/A

   Forward: 5'-**G**AT**YG**TTTTA**GG**T-3' Y= C/T

   **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.

3. **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.
4. **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.
5. **Quantifying Bisulfite Treated DNA.** Following bisulfite treatment of genomic DNA, the original base-pairing no longer exists since non-methylated cytosine residues are converted into uracil. Recovered DNA is typically A, U, and T-rich and is single stranded with limited non-specific base-pairing at room temperature. The absorption coefficient at 260 nm resembles that of RNA. Use a value of 40 µg/ml for Ab260 = 1.0 when determining the concentration of the recovered bisulfite-treated DNA.

# Frequently Asked Questions

**Q: Should the input DNA be dissolved in TE, water, or some other buffer prior to its conversion?**

**A:** Water, TE or modified TE buffers can be used to dissolve the DNA and do not interfere with the conversion process.

**Q: Which Taq polymerase(s) do you recommend for PCR amplification of converted DNA?**

**A:** We recommend Diagenode's MethylTaq DNA polymerase (Cat. No. C09010010).

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