ZYMO RESEARCH

THE Epigenetics COMPANY™

EZ-96 DNA Methylation™ Kit (Shallow-Well Format)

For high-throughput bisulfite conversion of DNA

Highlights

- High throughput (96-well), proven procedure for bisulfite conversion of DNA.
- 96-well desulphonation and recovery of bisulfite-treated DNA.
- Recovered DNA is ideal for downstream analyses including PCR, endonuclease digestion, sequencing, microarrays, etc.

Catalog Numbers:
D5003

Scan with your smart-phone camera to view the online protocol/video.

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682

Table of Contents

Product Contents

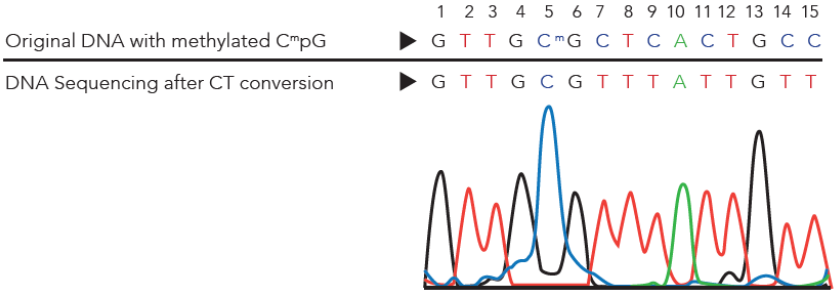
EZ-96 DNA Methylation™ Kit	D5003 (2 x 96 Rxns.)	Storage Temperature
CT Conversion Reagent*	2 Bottles	Room Temp.
M-Dilution Buffer	5.2 ml	Room Temp.
M-Binding Buffer	80 ml	Room Temp.
M-Wash Buffer**	2 x 36 ml	Room Temp.
M-Desulphonation Buffer	40 ml	Room Temp.
M-Elution Buffer	8 ml	Room Temp.
Silicon-A™ Binding Plate	2 Plates	Room Temp.
Conversion Plates w/ Pierceable Cover Film	2 Plates/2 Films	Room Temp.
Collection Plates	2 Plates	Room Temp.
Elution Plates	2 Plates	Room Temp.
Instruction Manual	1	-

* 7.5 ml water and 2.1 ml **M-Dilution Buffer** are added per bottle of **CT Conversion Reagent** and mixed prior to use.
** Add 144 ml of 100% ethanol to the 36 ml **M-Wash Buffer** concentrate before use.

Introduction to DNA Methylation

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 next page).



DNA sequencing results following bisulfite treatment. DNA with methylated CmpG 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.

References:

1. Costello JF, Plass CJ. Med. Genet. 2001; 38(5): 285-303.
2. Stirzaker C. Cancer Res. 1997; 57(11): 2229-2237.
3. Adams RL. Bioessays. 1995; 17(2): 139-145.
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.

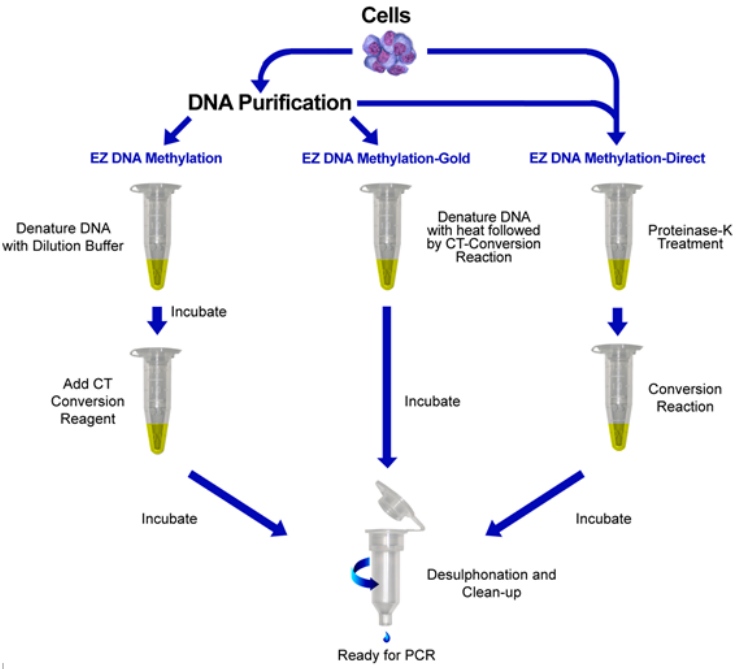
Specifications

- **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 –** > 80%

Product Description

The **EZ-96 DNA Methylation™ Kit** features a high-throughput (96-well), simplified procedure that streamlines bisulfite conversion of DNA. The kit is based on the three-step reaction that takes place between cytosine and sodium bisulfite where cytosine is converted into uracil. The product's innovative desulphonation technology eliminates otherwise cumbersome precipitations. The kit is designed to reduce template degradation, minimize DNA loss during treatment and clean-up, while ensuring complete conversion of the DNA. Purified, converted DNA is ideal for PCR amplification for downstream analyses including endonuclease digestion, sequencing, microarrays, etc.

An outline comparing the **EZ DNA Methylation™ Kit** procedure to Zymo Research's other methylation kits is shown below.



Outline of the **EZ DNA Methylation™**, **EZ DNA Methylation-Gold™** and **EZ DNA Methylation-Direct™ Kit** procedures.

Note: Single spin-column formats are available for processing smaller numbers of samples. Also, MagPrep kits are available (p. 13) for adaptation to liquid handling robots (*e.g.*, Tecan – Freedom EVO®) and automated sample prep.

Selected EZ DNA Methylation™ Kit Citations:

1. Ehrich M, et al. Nuc. Acids Res. 2007; 35 (5): e29
2. Kaneda M, et al. Nature. 2004; 429: 900-903
3. Zhang F, et al. Proc. Natl. Acad. Sci. USA. 2007; 104 (11): 4395-4400.
4. Oda M, et al. Genes & Dev. 2006; 20: 3382-3394.
5. England RPM, et al. Nature Meth. 2005; 2: 1-2.

Protocol

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 7.5 ml water and 2.1 ml of **M-Dilution Buffer** to a bottle 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 bottle of **CT Conversion Reagent** is designed for 96 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 144 ml of 100% ethanol to the 36 ml **M-Wash Buffer** concentrate before use.

Sample Processing

1. Add 5 µl of **M-Dilution Buffer** to each DNA sample in a **Conversion Plate** and adjust the total volume to 50 µl with water. Mix each sample by pipetting up and down.

   Example: For 14 µl of a DNA sample add 5 µl M-Dilution Buffer and 31 µl water.

2. Incubate the **Conversion Plate** containing the samples at 37°C for 15 minutes.

3. After the above incubation, add 100 µl of the prepared **CT Conversion Reagent** to each sample and mix.

4. Incubate the **Conversion Plate** in the dark at 50°C for 12-16 hours (e.g., using a thermal cycler).

   The **CT Conversion Reagent** is light sensitive, so try to minimize the reaction's exposure to light whenever possible.

5. Incubate the sample at 0-4°C (*e.g.*, on ice or using a thermal cycler) for 10 minutes. Samples may be kept at 4°C for up to 20 hours.

6. Add 400 µl of **M-Binding Buffer** to each well of a **Silicon-A™ Binding Plate** on a **Collection Plate**.

   Note: The capacity of each well of the Binding Plate is 600 µl. The capacity of each well of the Collection Plate is 800 µl. Empty the Collection Plate whenever necessary to prevent contamination of the Binding Plate contents by the flow-through.

7. Load the samples (from Step 5) into the wells of the **Silicon-A™ Binding Plate** containing the **M-Binding Buffer**. Mix by pipetting up and down.

8. Centrifuge at ≥ 3,000 x *g* (5,000 x *g* max.) for 5 minutes. Discard the flow-through.

9. Add 400 µl of **M-Wash Buffer** to each well and centrifuge at ≥ 3,000 x *g* for 5 minutes.

10. Add 200 µl of **M-Desulphonation Buffer** to each well of and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at ≥ 3,000 x *g* for 5 minutes.

11. Add 500 µl of **M-Wash Buffer** to each well and centrifuge at ≥ 3,000 x *g* for 5 minutes. Add another 500 µl of **M-Wash Buffer** and centrifuge for 10 minutes.

12. Place the **Silicon-A™ Binding Plate** onto **an Elution Plate. Add 30 µl of M-Elution Buffer** directly to the binding matrix in each well. Centrifuge for 3 minutes at ≥ 3,000 x *g* to elute the DNA.

    Note: 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 30 µl can be used if necessary. The elution volume can be > 30 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

Appendix

Bisulfite Conversion and PCR Optimization

1. Incomplete C to T Conversion.

A. Increase temperature in Step 2 of the **Protocol** to 42°C and extend the incubation time to 30 minutes. If the problem persists, use modified conversion conditions (see **B**, below).

B. In Step 1 of the **Protocol**, add 7.5 µl **M-Dilution Buffer** instead of 5 µl (the total volume should remain 50 µl). If this change is made, the preparation of the **CT Conversion Reagent** must also be modified by reducing the volume of **M-Dilution Buffer** from 210 µl to 185 µl. In Step 3 of the **Protocol**, add 97.5 µl prepared **CT Conversion Reagent** per reaction instead of 100 µl.

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′

Note: Methylated "C" is underlined in the examples.

Note: Following bisulfite conversion, the strands are no longer complementary

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**

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.

Zymo Research provides primer design assistance with its Bisulfite Primer Seeker Program, available at:

www.zymoresearch.com/tools/bisulfite-primer-seeker

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: **Zymo*Taq*™** is a "hot start" DNA polymerase specifically designed for the amplification of bisulfite treated DNA.

Frequently Asked Questions

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

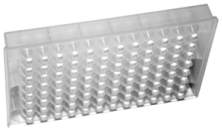
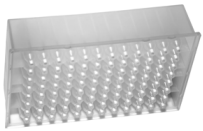
A: *Water, TE or modified TE buffers can be used to dissolve the DNA and do not interfere with the conversion process.*

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

A: *We recommend a "hot start" DNA polymerase (**e.g**., ZymoTaq™ DNA Polymerase).*

Q: Why are there two different catalog numbers for the EZ-96 DNA Methylation™ Kit?

A: *The two different catalog numbers are used to differentiate between the binding plates that are included in the kit. Deep and shallow-well binding plates are available to accommodate most rotors and microplate carriers. Below is a comparison of the two binding plates.*

		
Binding Plate	**Silicon-A™ Plate**	**Zymo-Spin™ I-96 Plate**
Style	Shallow-Well	Deep-Well
Height of Binding Plate	19 mm (0.75 inches)	35 mm (1.38 inches)
Binding Plate/Collection Plate Assembly	43 mm (1.69 inches)	60 mm (2.36 inches)
Binding Cap./Minimum Elution Volume	5 µg/30 µl	5 µg/15 µl
Catalog Numbers	**D5003**	**D5004**

Ordering Information

Product Description	Catalog No.	Size
EZ DNA Methylation™ Kit	D5001 D5002	50 Rxns. 200 Rxns.
EZ-96 DNA Methylation™ Kit (Shallow-Well)	D5003	2 x 96 Rxns.
EZ-96 DNA Methylation™ Kit (Deep-Well)	D5004	2 x 96 Rxns.
EZ-96 DNA Methylation™ MagPrep*	D5040 D5041	4 x 96 Rxns. 8 x 96 Rxns.

* MagPrep kits are adaptable to liquid handling robots *(e.g.*, Tecan – Freedom EVO®) making them ideal for automated sample prep.

Individual Kit Components	Catalog No.	Amount
CT Conversion Reagent	D5001-1 D5003-1	1 Tube 1 Bottle
M-Dilution Buffer	D5001-2 D5002-2	1.3 ml 5.2 ml
M-Binding Buffer	D5005-3 D5006-3 D5040-3	30 ml 125 ml 250 ml
M-Wash Buffer	D5001-4 D5002-4 D5007-4 D5040-4	6 ml 24 ml 36 ml 72 ml
M-Desulphonation Buffer	D5001-5 D5002-5 D5040-5	10 ml 40 ml 80 ml
M-Elution Buffer	D5001-6 D5002-6 D5007-6 D5041-6	1 ml 4 ml 8 ml 40 ml
Zymo-Spin™ IC Columns (capped)	C1004-50 C1004-250	50 Pack 250 Pack
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 Pack 500 Pack 1,000 Pack

MagBinding Beads	D4100-5-3 D4100-5-8 D4100-5-16	6 ml 8 ml 16 ml
Zymo-Spin™ I-96 Binding Plates	C2004	2 Plates
Silicon-A™ Binding Plates	C2001	2 Plates
Conversion Plates w/ Pierceable Cover Film	C2005	2 Plates /Films
Collection Plates	C2002	2 Plates
Elution Plates	C2003	2 Plates

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Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

This product is for research use only and should only be used by trained professionals. It is not for use in diagnostic procedures. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

Use of Methylation Specific PCR (MSP) is protected by US Patents 5,786,146 & 6,017,704 & 6,200,756 & 6,265,171 and International Patent WO 97/46705. No license under these patents to use the MSP process is conveyed expressly or by implication to the purchaser by the purchase of this product.

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