

INSTRUCTION MANUAL

EZ-96 DNA Methylation-Gold™ Kit

Catalog No. D5008 (Deep-Well Format)

Highlights

- Complete, high-throughput (96-well) 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.
- Eluted, ultra-pure DNA is ideal for use in subsequent molecular-based analyses.

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Product Contents:

Note: Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888-882-9682.

EZ-96 DNA Methylation-Gold™ Kit	D5008 2 x 96 rxns.	Storage Temperature
CT Conversion Reagent*	2 bottles	Room Temp.
M-Dilution Buffer	7 ml	Room Temp.
M-Dissolving Buffer	1.2 ml	Room Temp.
M-Binding Buffer	125 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.
Zymo-Spin™ I-96 Binding Plates	2 plates	Room Temp.
Conversion Plates w/ Pierceable Cover Film	2 plates/films	Room Temp.
Collection Plates	2 plates	Room Temp.
Elution Plates	2 plates	Room Temp.
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.

EZ DNA Methylation-Gold™ Kit technologies are patent pending.

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.

Note - The Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by trained professionals. 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.

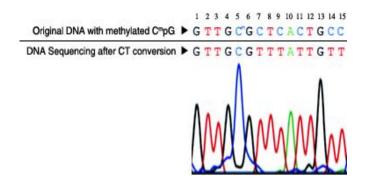
^{* 9} ml water, 500 µl **M-Dissolving Buffer**, and 3 ml **M-Dilution Buffer** must be added per bottle of **CT Conversion Reagent** prior to use.

^{**} 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 below).



DNA sequencing results following bisulfite treatment. DNA with methylated C^mpG 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.

Note: Single spin-column formats are available for processing smaller numbers of samples. Also, MagPrep kits are available (p. 8) for adaptation to liquid handling robots (e.g., Tecan – Freedom EVO®) and automated sample prep.

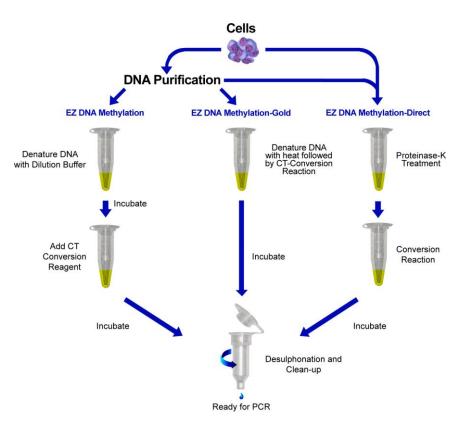
Selected EZ DNA Methylation™ Kit Citations:

- 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.

Product Description:

The EZ-96 DNA Methylation-Gold™ Kit is a refinement of our popular EZ-96 DNA Methylation™ Kit. The EZ-96 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.

An outline comparing the **EZ DNA Methylation-Gold™ 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.

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 9 ml water, 500 μl **M-Dissolving Buffer**, and 3 ml of **M-Dilution Buffer** to a bottle of **CT Conversion Reagent**.
- 2. Mix at room temperature with frequent vortexing or shaking for 15 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.

*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 1 ml for each 10 µl increase in DNA sample volume. For example, for 40 µl DNA samples, 7 ml 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 45 µl. Do not adjust the volumes of either the M-Dissolving **Buffer** or M-Dilution Buffer

The capacity of each well of the Binding Plate is 1.1 ml. 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.

Alternatively, water or TE $(pH \ge 6.0)$ can be used for elution if required for your experiments.

Protocol:

- 1. Add 130 μl of the **CT Conversion Reagent** to 20 μl* of each DNA sample in a **Conversion Plate**. If the volume of the DNA sample is less than 20 μl, make up the difference with water. Mix the samples by pipetting up and down.
- 2. Seal the plate with the provided film. Transfer the **Conversion Plate** to 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 for up to 20 hours

Note: The 4°C storage step is *optional*. 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 the wells of a **Zymo-Spin™ I-96 Binding Plate** mounted on a **Collection Plate**.
- Transfer the samples from the Conversion Plate (Step 2) to the wells of the Zymo-Spin™ I-96 Binding Plate. Mix by pipetting up and down.
- 5. Centrifuge at \geq 3,000 x g (5,000 x g max.) for 5 minutes. Discard the flow-through.
- 6. Add 400 μ I of **M-Wash Buffer** to each well of the plate. Centrifuge at \geq 3,000 x g for 5 minutes.
- 7. Add 200 μ l of **M-Desulphonation Buffer** to each well and allow the plate to stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at \geq 3,000 x q for 5 minutes. Discard the flow-through.
- 8. Add 400 μl of **M-Wash Buffer** to each well of the plate. Centrifuge at ≥ 3,000 x g for 5 minutes. Discard the flow-through. Add another 400 μl of **M-Wash Buffer** and centrifuge for 10 minutes.
- 9. Place the **Zymo-SpinTM I-96 Binding Plate** onto an **Elution Plate**. Add 15 μ I of **M-Elution Buffer** directly to each well. After 5 minutes, centrifuge at \geq 3,000 x g for 3 minutes 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 μ l of eluted DNA for each PCR, however, up to 15 μ l can be used if necessary. The elution volume can be > 15 μ l depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

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 minutes
- 2. 53°C for 30 minutes
- 3. 53°C for 6 minutes
- 4. 37°C for 30 minutes 8 cycles
- 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

Zymo Research provides primer design assistance with its <u>Bisulfite Primer Seeker Program</u>, 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: Methylated "C" is underlined in the examples.

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

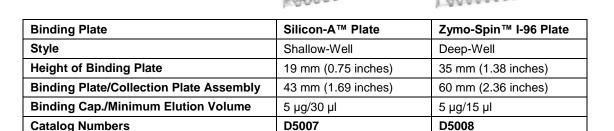
Note: Only one strand (A) is amplified by a given primer set. Only the reverse primer binds to the converted DNA, the forward primer will bind the strand generated by the reverse primer.

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.

Zymo Taq™ is a "hot start" DNA polymerase <u>specifically designed</u> for the amplification of bisulfite treated DNA. (see page 9 for details)

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™, page 9).
- Q: Why are there two different catalog numbers for the EZ-96 DNA Methylation-Gold™ 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.



Ordering Information:

Product Description	Catalog No.	Kit Size
EZ DNA Methylation-Gold™ Kit	D5005 D5006	50 rxns. 200 rxns.
EZ-96 DNA Methylation-Gold™ Kit (Shallow-Well)	D5007	2 x 96 rxns.
EZ-96 DNA Methylation-Gold™ Kit (Deep-Well)	D5008	2 x 96 rxns.
EZ-96 DNA Methylation-Gold™ MagPrep*	D5046 D5047	4 x 96 rxns. 8 x 96 rxns.

For Individual Sale	Catalog No.	Amount(s)
CT Conversion Reagent	D5001-1 D5003-1	1 tube 1 bottle
M-Dilution Buffer	D5005-2 D5006-2	1.5 ml 7 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
M-Dissolving Buffer	D5005-6 D5006-6	500 μl 1.2 ml
Zymo-Spin™ IC Columns (capped)	C1004-50 C1004-250	50 columns 250 columns
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 tubes 500 tubes 1,000 tubes
MagBinding Beads	D4100-2-6 D4100-2-8 D4100-2-12 D4100-2-16 D4100-2-24	6ml 8 ml 12 ml 16 ml 24 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

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



Epigenetics Products From Zymo Research

Product	Description	Kit Size	Cat No. (Format)
	Bisulfite Kits for DNA Methylation Detection	on	
EZ DNA Methylation™ Kit	For the conversion of unmethylated cytosines in DNA to uracil via the chemical-denaturation of DNA and a specially designed CT Conversion Reagent. Fast-Spin technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5001 (spin column) D5002 (spin column) D5003 (shallow-well plate) D5004 (deep-well plate) D5040 (magnetic bead) D5041 (magnetic bead)
EZ DNA Methylation- Gold™ Kit	For the fast (3 hr.) conversion of unmethylated cytosines in DNA to uracil via heat/chemical-denaturation of DNA and a specially designed CT Conversion Reagent. Fast-Spin technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5005 (spin column) D5006 (spin column) D5007 (shallow-well plate) D5008 (deep-well plate) D5042 (magnetic bead) D5043 (magnetic bead)
EZ DNA Methylation- Direct™ Kit	Features simple and reliable DNA bisulfite conversion directly from blood, tissue (FFPE/LCM), and cells without the prerequisite for DNA purification in as little as 4-6 hrs. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5020 (spin column) D5021 (spin column) D5022 (shallow-well plate) D5023 (deep-well plate) D5044 (magnetic bead) D5045 (magnetic bead)
EZ DNA Methylation- Lightning™ Kit	Complete bisulfite conversion in about an hour using a unique liquid format conversion reagent that requires no preparation. Fast-Spin technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5030 (spin column) D5031 (spin column) D5032 (shallow-well plate) D5033 (deep-well plate) D5046 (magnetic bead) D5047 (magnetic bead)
EZ DNA Methylation- Startup™ Kit	Designed for the first time user requiring a consolidated product to perform DNA methylation analysis. Includes technologies for sample processing, bisulfite treatment of DNA, and PCR amplification of "converted" DNA for methylation analysis.	1 Kit	D5024
	Methylated DNA Standards		
Jniversal Methylated Human DNA Standard	Human (male) genomic DNA having all CpG sites methylated. To be used for the evaluation of bisulfite-mediated conversion of DNA. Supplied with a control primer set.	1 set	D5011
Jniversal Methylated Mouse DNA Standard	Mouse (male) DNA having all CpG sites methylated. To be used for the evaluation of bisulfite-mediated conversion of DNA. Supplied with a control primer set.	1 set	D5012
	Other		
ChIP DNA Clean & Concentrator™	Clean and concentrate DNA from any reaction or "crude" preparation in 2 min. A 6 µl minimum elution volume allows for highly concentrated DNA. Designed for samples containing up to 5 µg of DNA.	50 Preps. 50 Preps.	D5201 (uncapped column D5205 (capped column)
Genomic DNA Clean & Concentrator™	Genomic DNA clean-up in minutes. Unique spin column technology for recovery of ultra-pure large-sized DNA (100 bp to ≥200 kb) DNA from any impure preparation (e.g., Proteinase K digestion).	25 Preps. 100 Preps.	D4010 D4011
Zymo <i>Taq</i> ™ DNA Polymerase	ZymoTaq™ "hot start" DNA Polymerase is specifically designed for the amplification of "difficult" DNA templates including: bisulfite-treated DNA for methylation detection. The product generates specific amplicons with little or no by-product formation. Available either as a single buffer premix or as a polymerase system with components provided separately.	50 Rxns. 200 Rxns. 50 Rxns. 200 Rxns.	E2001 (system) E2002 (system) E2003 (premix) E2004 (premix)
Methylated-DNA IP Kit	IP with a highly specific anti-5-methylcytosine monoclonal antibody. Designed for the enrichment of 5-methylcytosine-containing DNA from any pool of fragmented genomic DNA for use in genome-wide methylation analysis.	10 Rxns.	D5101
	Services		