Notes:

- Proteinase K digested material can be stored for several months at -20°C.
- If procedure A is used, the CT-Conversion Reagent can be added directly to the samples in a PCR tube.
- 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.
- Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments.
- Methylated "C" is underlined in the examples.
- Following bisulfite conversion, the strands are no longer complementary.
- 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.

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Instruction Manual No. ZD5020

EZ DNA Methylation-Direct Kit

Cat. No. ZD5020

Highlights

• Complete bisulfite conversion of DNA directly* from blood, tissue, or cells.
• Compatible with small sample inputs – as few as 10 cells or 50 pg DNA.
• Well-suited for FFPE and LCM-derived samples.

For In Vitro Research Use Only

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EZ DNA Methylation-Direct Kit Cat. # ZD5020

EZ DNA Methylation-Direc Kit	ZD5020 50 rxns.	Storage Temperature
Proteinase K and Storage Buffer*	5 mg set	–20°C(after mixing)
M-Digestion Buffer (2X)	4 ml	Room Temp.
CT Conversion Reagent**	5 tubes	Room Temp.
M-Dilution Buffer	1.5 ml	Room Temp.
M-Solubilization Buffer	4.5 ml	Room Temp.
M-Reaction Buffer	1 ml	Room Temp.
M-Binding Buffer	30 ml	Room Temp.
M-Wash Buffer***	6 ml	Room Temp.
M-Desulphonation Buffer	10 ml	Room Temp.
M-Elution Buffer	1 ml	Room Temp.
Spin IC Columns	50 columns	Room Temp.
Collection Tubes	50 tubes	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.

* Add 260 µl **Proteinase K Storage Buffer** to the **Proteinase K** tube prior to use. The final concentration of **Proteinase K** after the addition of **Proteinase K Storage Buffer** is 20 mg/ml.

** 790 µl **M-Solubilization Buffer** and 300 µl **M-Dilution Buffer** are added per tube of **CT Conversion Reagent**, mixed, and then 160 µl **M-Reaction Buffer** is added prior to use.

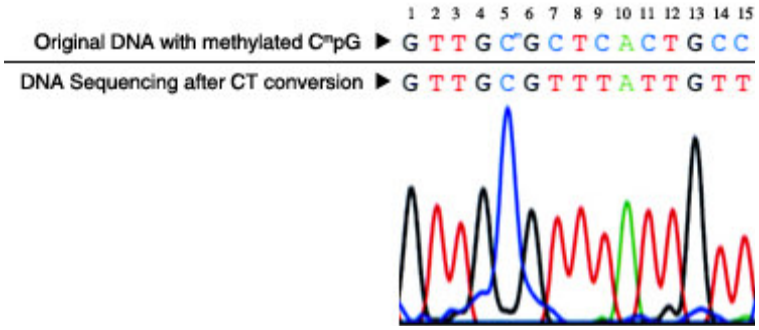
*** **Add 24 ml of 100% ethanol to the 6 ml** M-Wash Buffer **concentrate (ZD5020) before use.**

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

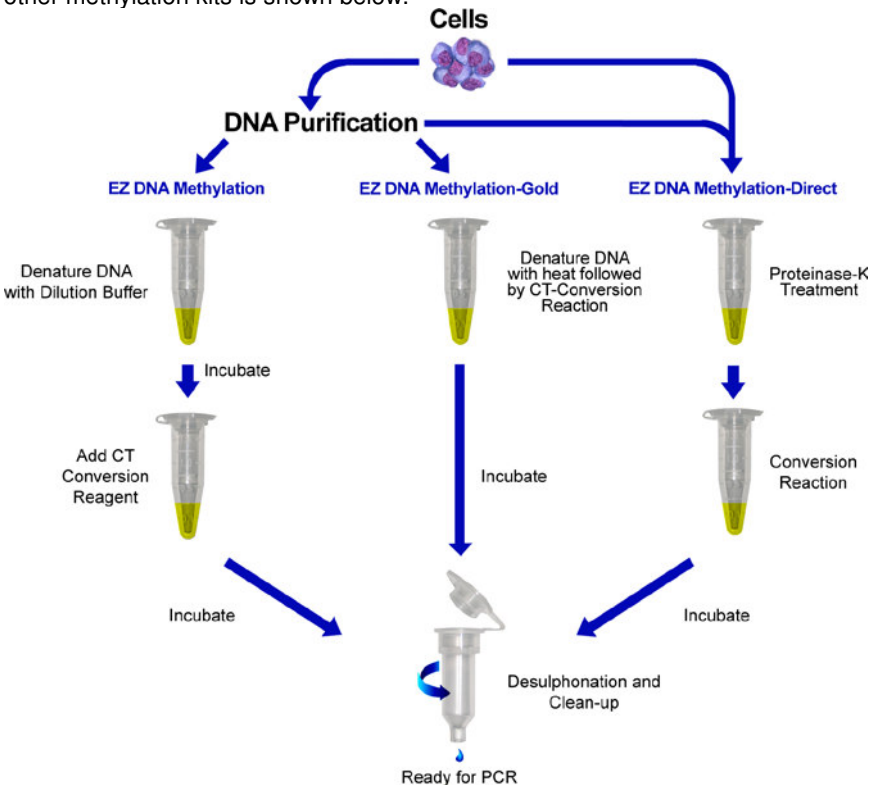
animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (3). The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 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 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.

Product Description:
The EZ DNA Methylation-Direct Kit features simple and reliable DNA bisulfite conversion directly from blood, tissue, and cells without the prerequisite for DNA purification. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. Like the EZ DNA Methylation-Gold Kit, DNA denaturation and bisulfite conversion processes are combined into a single step (see below). All kits streamline the three step process of bisulfite conversion of non-methylated cytosine in DNA into uracil. In addition the methylation kits share innovative in-column desulphonation technology that eliminates otherwise cumbersome DNA precipitation steps while ensuring 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 restriction endonuclease digestion, sequencing, microarrays, etc.
An outline comparing the EZ DNA Methylation-Direct Kit procedure to GSI's other methylation kits is shown below.



Outline of the EZ DNA Methylation kit

Specifications:

• Starting Materials:

Cells: Compatible with cells from solid tissue, tissue culture, whole blood, buffy coat, biopsies, LCM (Laser-Capture Micro-Dissection) and FFPE samples, etc. The number of cells per standard treatment can range from 10-10^5 cells. For optimal results, the cell number should be from 1 x 10^3-8 x 10^4 cells.
Purified DNA: Samples containing 50 pg - 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%

• Sensitivity of Detection (Lower Limit): 10 cells for successful PCR amplification.

Reagent Preparation:

• Preparation of Proteinase K

Add 260 µl (ZD5020) of **Proteinase K Storage Buffer** to the tube containing **Proteinase K**. Dissolve completely and store at -20 ℃.

• 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 790 µl of **M-Solubilization Buffer** and 300 µl of **M-Dilution Buffer** to a tube of **CT Conversion Reagent**.

2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

3. Add 160 µl of **M-Reaction Buffer** and mix an additional 1 minute.

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℃, or up to one month at -20℃. Stored **CT Conversion Reagent** solution must be warmed to 37℃, then vortexed prior to use.

• **Preparation of M-Wash Buffer**

Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate (ZD5020) before use.

Protocol:

Either blood, tissue, cells, or purified DNA can be used as the starting material for the **EZ DNA Methylation-Direct Kit.** If purified DNA is used, then proceed directly to **Section II**.
If blood, tissue, or cells are used, see **Appendix I** for sample-specific recommendations (e.g., FFPE and LCM samples). For optimal results, the cell number should be between 1 x 10_3-8 x 10_4 per treatment, although the cell number can range from 10-10_5 cells. *Using more cells than the recommended limit may result in incomplete bisulfite conversion of the DNA.*

Section I: Sample Digestion with Proteinase K.

Digestions should be performed in a tube (e.g., PCR tube) using either procedure **A** or **B** (below) based on the number of cells and/or tissue type. Digestions are scalable to facilitate multiple samples or to increase the ease of manipulation. Sufficient volumes of reagents are included with this kit to increase the overall **Proteinase K** digestion volume 5-fold.

1. **A**. Setup the following digestion for samples containing up to 2 x 10_3 cells.
10 µl M-Digestion Buffer (2X)
Up to 9 µl Sample (≤ 2 x 10_3 cells)
1 µl Proteinase K
X µl H_2O
20 µl Total Volume

Important! "Difficult to digest" samples result in the formation of visible debris following digestion. These should be processed according to procedure **B**.

B. Setup the following digestion for samples containing up to 1 x 10_5 cells. This should also include all "difficult to digest" samples that form debris or precipitate following **Proteinase K** digestion– see **Appendix I**.
13 µl M-Digestion Buffer (2X)
Up to 12 µl Sample (≤ 10_5 cells)

1 µl Proteinase K
X µl H_2O
26 µl Total Volume
2. Incubate the sample(s) for 20 minutes at 50℃.

Note: For FFPE, LCM and other "fixed" tissue samples, adjust the incubation time to 4 hours (see **Appendix I**).
3. If following procedure **A**, proceed directly to **Section II**.

If following procedure **B**, mix the contents of the reaction thoroughly then centrifuge for 5 minutes at 10,000 x *g.* Use 20 µl of the supernatant for bisulfite conversion as detailed in **Section II**.

Section II. Bisulfite Conversion of DNA

1. Add 20 µl of sample from Step 3 (**Section I**) to 130 µl of **CT Conversion Reagent** solution in a PCR tube. Mix the sample and then centrifuge briefly to ensure no droplets are in the cap or sides of the tube.

Note: If purified DNA is used, add up to 20 µl of DNA to 130 µl of **CT Conversion Reagent** solution. If the volume of DNA is less than 20 µl, compensate with water.
2. Place the PCR tube(s) in a thermal cycler and perform the following steps:

1. 98℃ for 8 minutes
2. 64℃ for 3.5 hours
3. 4℃ storage for up to 20 hours
3. Add 600 µl of **M-Binding Buffer** into 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.

Appendix I: Recommendations for Specific Cells and Tissues

The following guidelines are provided as recommendations when sampling specific cell and tissue sources. *Most importantly,* the optimal amount of DNA used for bisulfite treatment (**Section II**) should be from 1 x 10^3-8 x 10^4 cells, although DNA from as few as 10 to as many as 10^5 cells may be used. *Caution: using more cells than the recommended maximum may result in incomplete bisulfite conversion of the DNA.*

Important! "Difficult to digest" samples result in the formation of visible debris following digestion and should be processed according to digestion procedure **B** on page 5. This can occur with samples that are large or resistant to **Proteinase K** digestion, including: connective tissue (e.g., cartilage), adipose tissue, some fixed tissue, etc. *If debris is not removed by centrifugation, it may interfere with the bisulfite conversion process resulting in incomplete conversion of the DNA.*

Whole Blood: Use up to 0.5 µl whole blood per **Proteinase K** digestion (procedure **A** or **B**) However, the volume of the **Proteinase K** digestion can be adjusted when processing multiple samples or for convenient sample manipulation. For example, to

increase the sample volume 5-fold for digestion procedure **A**: add 2.5 µl of blood to 50 µl **M-Digestion Buffer**, 42.5 µl H_20, and 5 µl of **Proteinase K**.

Solid Tissue (Fresh or Frozen): Use up to 0.1 mg or 0.1 µl tissue per **Proteinase K** digestion (procedure **A** or **B)**. However, the volume of the **Proteinase K** digestion can be adjusted when processing multiple samples or for convenient sample manipulation. For example, to increase the sample volume 5-fold for digestion procedure **B**: add 0.5 mg or 0.5 µl of tissue to 65 µl **M-Digestion Buffer**, 59.5 µl H_20, and 5 µl of **Proteinase K**.

Cultured Cells and Other Cell-Containing Liquids: Both monolayer and cells in suspension may be processed either directly from the culture container or after harvesting. Small amounts of culture medium do not adversely affect the procedure but should be kept to a minimum. Ideally, cells should be suspended in PBS or Tris-buffered solutions prior to **Proteinase K** digestion.

Other cell-containing liquids (e.g., those derived from FACS or buffy coat) may also be used directly as sample sources. If the composition of the liquid is not "defined", then pellet the cells by centrifugation and remove the supernatant. Cells should be resuspended in PBS or Tris-buffered solutions. Generally, cells in body fluids can be used directly for **Proteinase K** digestion.

FFPE (Formalin-Fixed Paraffin-Embedded) and Other "Fixed" Tissues: Paraffin-embedded tissues must be deparaffinized prior to use. This can be accomplished according to conventional xylene-ethanol protocols. The **Proteinase K** digestion must be extended from 20 minutes to 4 hours for FFPE and any other fixed tissue samples.

LCM (Laser Capture Micro-Dissection): Tissue samples from LCM should be in PBS or Tris-buffered solutions. The **Proteinase K** digestion must be extended from 20 minutes to 4 hours for LCM and any other fixed tissue samples.

Appendix II: 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′–**GACCGTTCCAGGTCCAGCAGTGCGCT**–3′
B: 3′–**CTGGCAAGGTCCAGGTCGTCACGCGA**–5′
Bisulfite Converted: **A:** 5′–**GATCGTTTTAGGTTTAGTAGTGCGTT**–3′
B: 3′–**TTGGCAAGGTTTAGGTTGTTATGCGA**–5′

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′–**GATCGTTTTAGGTTTAGTAGTGCGTT**–3′
Primers: Reverse: 3′–**ATCATCACRCAA**–5′ **R= G/A**
Forward: 5′–**GATYGTTTTAGGT**–3′ **Y= C/T**

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 Ab_{260} = 1.0 when determining the concentration of the recovered bisulfite-treated DNA.