ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ DNA Methylation-Gold™ Kit

Catalog Nos. **D5005 & D5006**

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.

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ZYMO RESEARCH CORP.

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Product Contents:

EZ DNA Methylation-Gold™ Kit	D5005 50 rxns.	D5006 200 rxns.	Storage Temperature
CT Conversion Reagent*	5 Tubes	20 Tubes	Room Temp.
M-Dilution Buffer	1.5 ml	7 ml	Room Temp.
M-Dissolving Buffer	500 µl	1.2 ml	Room Temp.
M-Binding Buffer	30 ml	125 ml	Room Temp.
M-Wash Buffer**	6 ml	24 ml	Room Temp.
M-Desulphonation Buffer	10 ml	40 ml	Room Temp.
M-Elution Buffer	1 ml	4 ml	Room Temp.
Zymo-Spin™ IC Columns	50 ct.	200 ct.	Room Temp.
Collection Tubes	50 ct.	200 ct.	Room Temp.
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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.

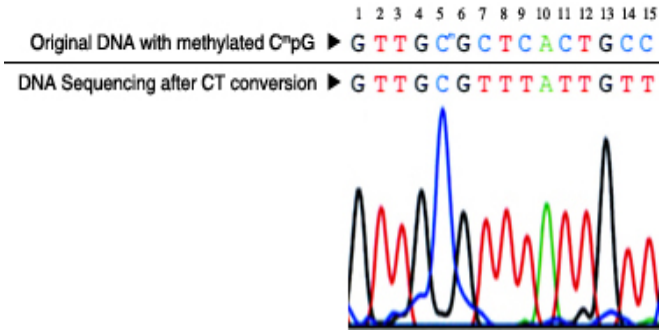
* 900 µl water, 300 µl **M-Dilution Buffer**, and 50 µl **M-Dissolving Buffer** must be added per tube of **CT Conversion Reagent** prior to use.

** Add 24 ml of 100% ethanol to the 6 ml **M-Wash Buffer** concentrate (D5005) or 96 ml of 100% ethanol to the 24 ml **M-Wash Buffer** concentrate (D5006) 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 $C^{m}pG$ 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.

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.

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.

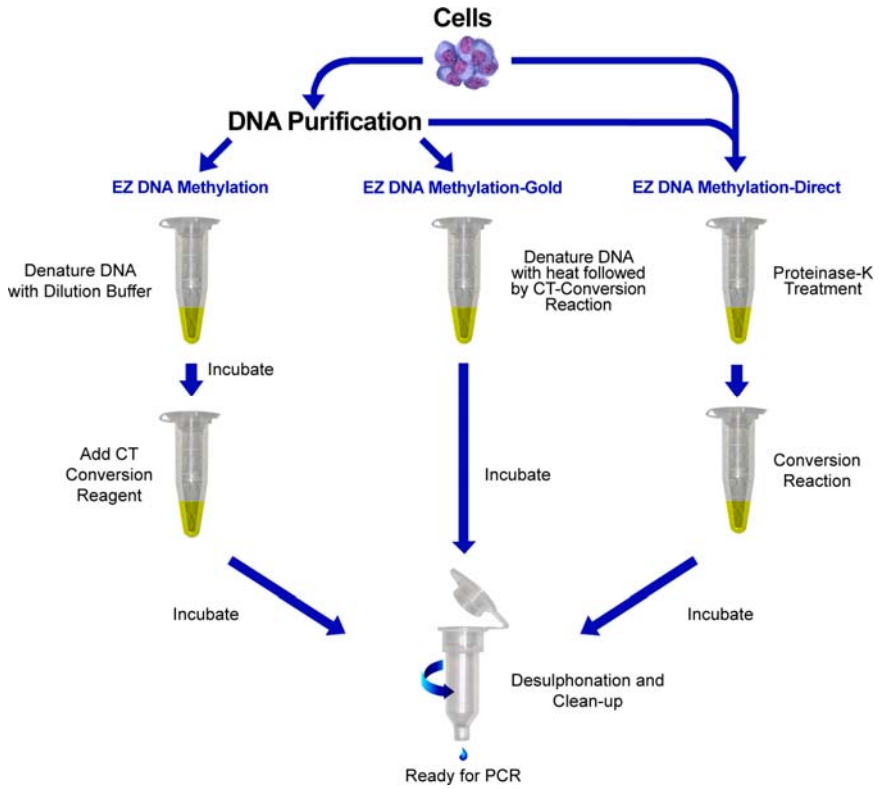
4. Oda M, *et al.* Genes & Dev. 2006; 20: 3382-3394.

5. England RPM, *et al.* Nature Meth. 2005; 2: 1-2.

Product Description:

The **EZ 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.

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 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 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**.

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 **Zymo-Spin™ IC Column** and place the column into a provided **Collection Tube**.

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

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.

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°C – 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.

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

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: 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. **PCR Primer Design.** Generally, primers of 24 to 32 bases are required for amplification of bisulfite converted DNA. For most eukaryotes, all non-methylated cytosine residues will be converted into uracil during the bisulfite treatment. These Cs should be treated as Ts for primer design purposes. For example, for the sequence 5'-AACCTTACAGGCAC-3', the corresponding primer should be 5'-AA**TT**TTA**T**AGG**T**A**T**-3'.

   If the primer contains CpG dinucleotides with uncertain methylation status, then mixed bases with C and T can be used. Usually, there should be no more than three mixed positions per primer and they 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 500 pg. The optimal amount of DNA per bisulfite treatment is 200 to 500 ng. Although, up to 2 µg of DNA can also 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 with optimization of the bisulfite reaction and PCR conditions. We have found that 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. Thus, it may be necessary to reduce the annealing temperature accordingly.

   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, non-methylated cytosine residues are converted into uracil. The recovered DNA is typically A, U, and T-rich. The original base-pairing no longer exists. Instead, it 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.

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: At what temperature and for how long can converted DNA be stored?

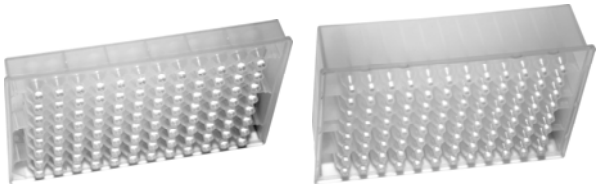
A: *The sample should be stored at ≤ -20ºC whenever possible. The quality of the DNA should remain relatively unchanged for up to 3 months.*

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

A: *We recommend any "Hot-Start" DNA polymerase.*

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.*



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	D5007	D5008

Ordering Information:

Product Description	Catalog No.	Kit Size
EZ DNA Methylation-Gold™ Kit	D5005	50 rxns.
EZ DNA Methylation-Gold™ Kit	D5006	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.

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	30 ml 125 ml
M-Wash Buffer	D5001-4 D5002-4 D5007-4	6 ml 24 ml 36 ml
M-Desulphonation Buffer	D5001-5 D5002-5	10 ml 40 ml
M-Elution Buffer	D5001-6 D5002-6	1 ml 4 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
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

Popular DNA Purification & Analysis Products from Zymo Research

Product	Description	Kit Size (Preps)	Catalog No. (column format)
DNA Clean & Concentrator-5™	Clean & concentrate DNA from any reaction or "crude" preparation in 2 minutes. A 6 µl minimum elution volume allows for highly concentrated DNA. Designed for samples containing up to 5 µg of DNA.	50 200 50 200	**D4003** (uncapped) **D4004** (uncapped) **D4013** (capped) **D4014** (capped)
DNA Clean & Concentrator-25™	Clean & concentrate DNA in minutes. 25 µl minimum elution volume allows for highly concentrated DNA. Designed for purifying up to 25 µg of DNA.	50 200 50 200	**D4005** (uncapped) **D4006** (uncapped) **D4033** (capped) **D4034** (capped)
DNA Clean & Concentrator-100™	Clean & concentrate DNA in minutes. 100 µl minimum elution volume allows for highly concentrated DNA. Designed for purifying up to 100 µg of DNA.	25 50	**D4029** **D4030**
DNA Clean & Concentrator-500™	Clean & concentrate DNA in minutes. 1 ml minimum elution volume allows for highly concentrated DNA. Designed for samples containing up to 500 µg of DNA.	10 20	**D4031** **D4032**
ZR-96 DNA Clean & Concentrator-5™	Quick (15 minute), high-output recovery of pure DNA from PCR, endonuclease digestions, plasmid preparations, etc. 10-15 µl minimum elution volume allows for highly concentrated DNA. Designed for samples containing up to 5 µg of DNA.	2x96 4x96	**D4023** **D4024**
Zymoclean™ Gel DNA Recovery Kit	Purify DNA from high and low-melting agarose gels in minutes	50 200	**D4001** **D4002**
ZR-96 Zymoclean™ Gel DNA Recovery Kit	High-throughput DNA purification from high and low-melting agarose gels.	2x96 4x96	**D4021** **D4022**
Pinpoint Slide DNA Isolation System™	Recover genomic DNA from paraffin-embedded or fresh tissue sections for PCR. Ideal for isolating DNA from clinical tissue samples.	50	**D3001**
Zyppy™ Plasmid Miniprep Kit	Pellet-Free™ plasmid DNA purification in minutes: (alkaline lysis/spin column format for low 30 µl elution volume).	50 100 400	**D4036** **D4019** **D4020**
Zyppy™ Plasmid Midiprep Kit	Pellet-Free™ plasmid DNA purification in minutes: (alkaline lysis/spin column format and 150 µl minimum elution volume).	25 50	**D4025** **D4026**
Zyppy™ Plasmid Maxiprep Kit	High-purity plasmid DNA purification in minutes: (alkaline lysis/spin column format and 2 ml minimum elution volume).	10 20	**D4027** **D4028**
ZR Genomic DNA I Kit™	Genomic DNA isolation from whole blood, tissue culture cells, solid tissue and liquid samples. (Silica bead format is scalable to fit your requirements).	100 400	**D3004** **D3005**
ZR Genomic DNA II Kit™	Genomic DNA purification from whole blood, tissue culture cells, solid tissue and liquid samples. No requirement for beads or phenol chloroform.	50 200 50 200	**D3006** (uncapped) **D3007** (uncapped) **D3024** (capped) **D3025** (capped)
ZR-96 Genomic DNA Kit™	High-output genomic DNA purification from whole blood, tissue culture cells, solid tissue and liquid samples. No requirement for beads or phenol chloroform.	2x96 4x96	**D3010** **D3011**
ZR Soil Microbe DNA Kit™	Simple, rapid isolation of humic-free, PCR-quality genomic DNA from soil microbes.	50	**D6001**
ZR Fungal/Bacterial DNA Kit™	Simple, rapid isolation of PCR-quality genomic DNA from fungi.	50	**D6005**
ZR Fecal DNA Kit™	Simple, rapid isolation of PCR-quality genomic DNA from feces.	50	**D6010**
ZR Viral DNA Kit™	Isolation of viral DNA from cell-free body fluids or sample mixtures containing cells at concentrations less than 10^5 cells per ml.	50 200	**D3015** **D3016**
ZR-96 Viral DNA Kit™	High-output (96-well) isolation of viral DNA from cell-free body fluids or sample mixtures containing cells at concentrations less than 10^5 cells per ml.	2x96 4x96	**D3017** **D3018**
EZ DNA Methylation™ Kit	Streamlined kit for the conversion of unmethylated cytosines in DNA to uracil via the chemical-denaturation of DNA using our specially designed CT Conversion Reagent™. DNA is then desulphonated and subsequently cleaned using *Fast-Spin* column technology. Ultra-pure recovered DNA can be used for PCR and bisulfite sequencing applications.	50 200 2x96 2x96	**D5001** **D5002** **D5003** (Shallow-well) **D5004** (Deep-well)
EZ DNA Methylation-Direct™ Kit	Bisulfite conversion of DNA directly from cells, tissue, and blood samples.	50 200 2x96 2x96	**D5020** **D5021** **D5022** (Shallow-well) **D5023** (Deep-well)

**Bulk quantities are available upon request. Please contact: busdev@zymoresearch.com or call 1-888-882-9682 for assistance.*