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ZYMO RESEARCH
The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ-96 DNA Methylation-Lightning™ Kit

Catalog No. **D5032 (Shallow-Well Format)**

Highlights

- Fastest method for *complete,* high-throughput (96-well) 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.

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

Product Contents:

EZ-96 DNA Methylation-Lightning™ Kit	D5032 2 x 96 rxns.	Storage Temperature
Lightning Conversion Reagent*	2 bottles	Room Temp.
M-Binding Buffer	125 ml	Room Temp.
M-Wash Buffer**	2 x 36 ml	Room Temp.
L-Desulphonation Buffer	40 ml	Room Temp.
M-Elution Buffer	8 ml	Room Temp.
Silicon-A™ 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.

* The **Lightning 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 144 ml of 100% ethanol to the 36 ml **M-Wash Buffer** concentrate before use.

EZ DNA Methylation-Lightning™ 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 - ™ 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. Freedom EVO® is a registered trademark of Tecan Group Ltd. Pyrosequencing® is a registered trademark of Biotage.

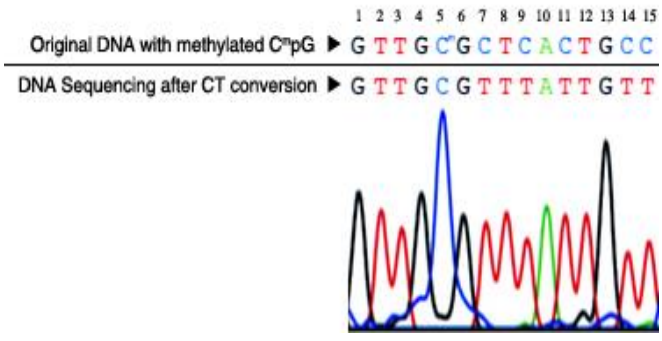
Introduction to DNA Methylation:

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 5′-CpG-3′ dinucleotides, although other patterns do exist. About 80 percent of all 5′-CpG-3′ 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).



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

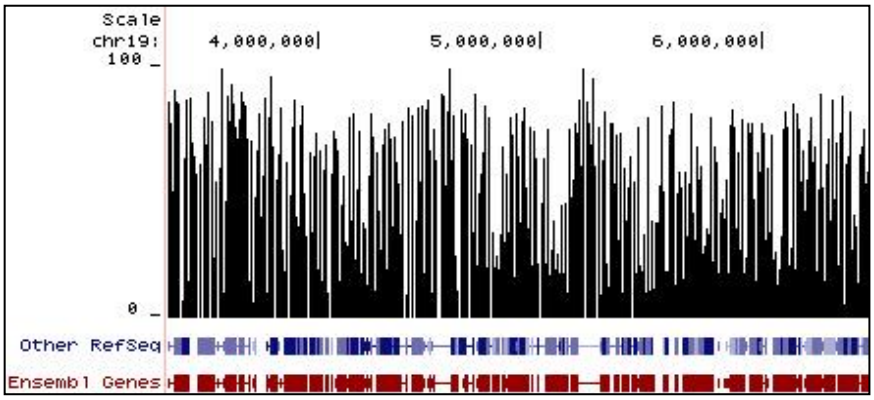
6. Frommer M. Proc. Natl. Acad. Sci. USA. 1992; 89(5): 1827-1831.

7. Rakyan VK, *et al*. Nat. Rev. 2011, 12(8): 529-541.

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.

Product Description:

The **EZ-96 DNA Methylation-Lightning™ Kit** features high-throughput (96-well) bisulfite treatment and conversion of DNA for methylation analysis. Key to the fast workflow is the ready-to-use **Lightning 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 96-well spin-plate. High yield, converted DNA is ideal for PCR, array, bisulfite and next generation sequencing, etc.



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 **EZ DNA Methylation™** technology prior to Next-Gen sequencing.

Select 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.

6. Berman BP, et al. Nature Gen. 2012; 44: 40-46.

7. Leung DC, et al. Proc. Natl. Acad. Sci. USA. 2011; 108 (14): 5718-5723.

8. Hesselink AT, et al. Clin. Cancer Res. 2011; 17: 2459-2465.

9. Campan M, et al. PLoS ONE. 2011, 6 (12): e28141.

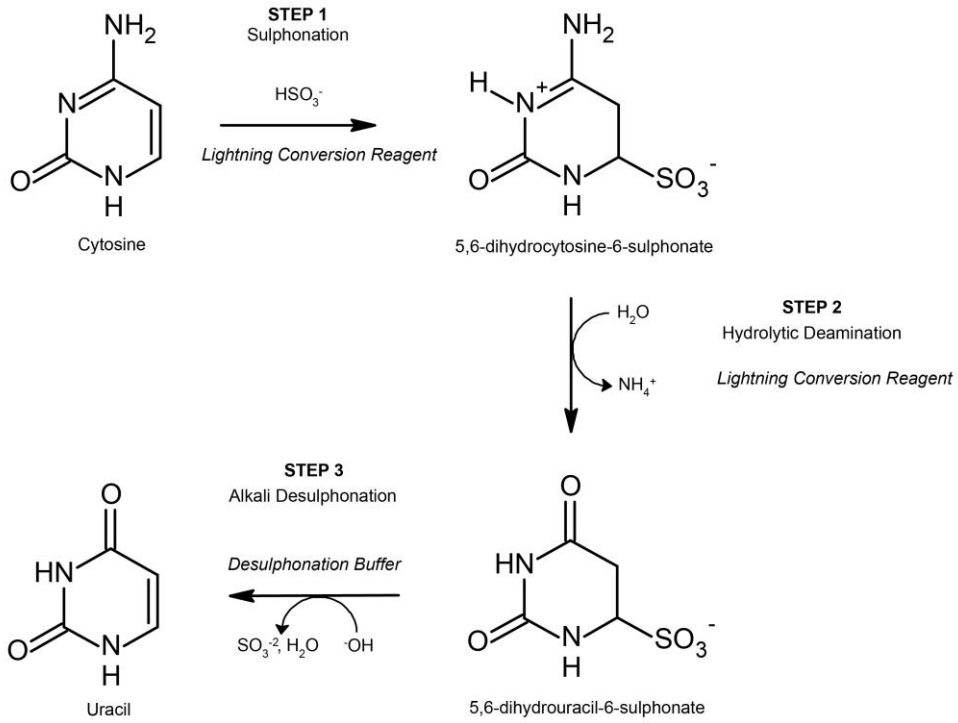
Specifications:

- **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 M-Wash Buffer**

  Add 144 ml of 100% ethanol to the 36 ml **M-Wash Buffer** concentrate before use.



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 **Lightning Conversion Reagent** to 20 µl of a DNA sample in a **Conversion Plate**. Mix the samples by pipetting up and down.

   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 well by repeating steps 3-5.

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 8 minutes
   2. 54°C for 60 minutes
   3. 4°C storage for up to 20 hours

   Note: The 4°C storage step is *optional*.

3. Add 400 µl of **M-Binding Buffer** to the wells of a **Silicon-A™ Binding Plate** mounted on a **Collection Plate**.

4. Transfer the samples from the **Conversion Plate** (Step 2) to the wells of the **Silicon-A™ Binding Plate**. Mix by pipetting up and down.

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.

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

6. Add 400 µl of **M-Wash Buffer** to each well of the plate. Centrifuge at ≥ 3,000 x *g* for 5 minutes.

7. Add 200 µl of **L-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 ≥ 3,000 x *g* 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.

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

9. Place the **Silicon-A™ Binding Plate** onto an **Elution Plate**. Add 30 µl of **M-Elution Buffer** directly to each well. Wait 5 minutes, then centrifuge at ≥ 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 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 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′-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.

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

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

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.

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

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.

Frequently Asked Questions:

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

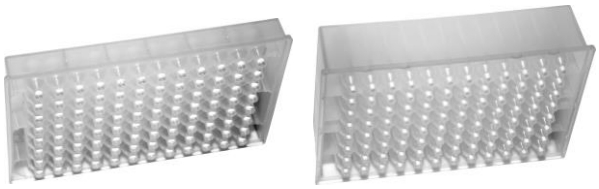
A: *Water, TE or modified TE buffers can be used to dissolve the DNA and do not interfere with the conversion process.*

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-Lightning™ 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	**D5032**	**D5033**

Ordering Information:

Product Description	Catalog No.	Kit Size
EZ DNA Methylation-Lightning™ Kit	D5030 D5031	50 rxns. 200 rxns.
EZ-96 DNA Methylation-Lightning™ Kit (Shallow-Well)	D5032	2 x 96 rxns.
EZ-96 DNA Methylation-Lightning™ Kit (Deep-Well)	D5033	2 x 96 rxns.
EZ-96 DNA Methylation-Lightning™ MagPrep*	D5046 D5047	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.

For Individual Sale	Catalog No.	Amount(s)
Lightning Conversion Reagent	D5030-1 D5032-1	1 tube 1 bottle
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
L-Desulphonation Buffer	D5030-5 D5031-5 D5046-5	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 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

THE Epigenetics COMPANY™

Epigenetics Products From Zymo Research

Product	Description	Kit Size	Cat No. (Format)
Bisulfite Kits for DNA Methylation Detection			
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			
Universal 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**
Universal 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*Taq*™ DNA Polymerase	Zymo*Taq*™ "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			
Available for **DNA Methylation** and **Hydroxymethylation** at http://www.zymoresearch.com/services or inquire at services@zymoresearch.com *…powered by the latest Next-Gen Sequencing technologies!*			