ZYMO RESEARCH

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INSTRUCTION MANUAL

Methylated-DNA IP Kit

Catalog No. **D5101**

Highlights

- Methylated DNA enrichment for large-scale DNA methylation analysis.
- A highly specific anti-5-methylcytosine monoclonal antibody for defined, reproducible results.
- Includes control DNA and primers for easy monitoring of the entire procedure.
- Eluted, ultra-pure DNA is ideal for use in subsequent molecular-based analyses (e.g., assembling genomic libraries and determining genome-wide methylation status).

Contents

ZYMO RESEARCH CORP.

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

Product Contents

Methylated-DNA IP Kit (Kit Size)	D5101 10 rxns.	Storage Temperature
Protein A-Sepharose Slurry[1]	500 µl	4˚C.
Mouse Anti-5-Methylcytosine[2]	50 µl	4˚C.
Methylated/Non-methylated Control DNA[3]	20 µl	-20˚C.
Control Primers I and II (20 µM)[3]	20 µl	-20˚C.
MIP Buffer	20 ml	0˚C to RT
DNA Denaturing Buffer	1 ml	0˚C to RT
IP DNA Binding Buffer	6 ml	0˚C to RT
DNA Wash Buffer[4]	6 ml	0˚C to RT
DNA Elution Buffer	1 ml	0˚C to RT
Zymo-Spin™ IVM Filter Columns w/ Green Caps	10	0˚C to RT
Zymo-Spin™ I Columns	10	0˚C to RT
Collection Tubes	50	0˚C to RT
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Note - Integrity of kit components is guaranteed for up to one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

Note - Cold components are provided in a wet ice box (Box 2 of 2) together with the Kit box (Box 1 of 2).

[1] Upon arrival, store the Protein A-Sepharose Slurry at 4˚C.

[2] Upon arrival, store the Mouse Anti-5-methylcytosine monoclonal antibody at -80˚C for long-term storage or at 4˚C for frequent usage.

[3] Upon arrival store the Control DNA and Primers at -20˚C.

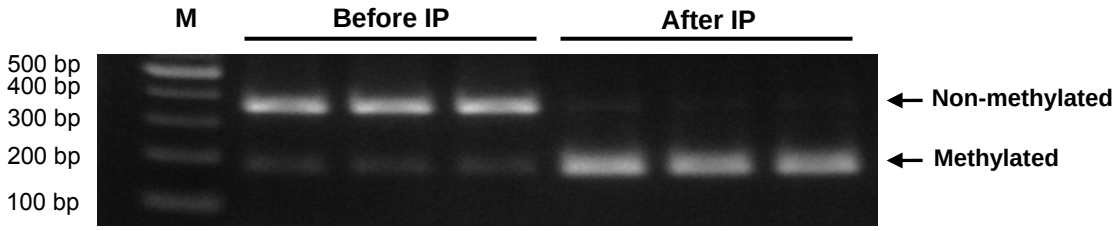
[4] Add 24 ml of 100% ethanol to the 6 ml Wash Buffer concentrate (D4003-2-6).

Product Description:

The ability to detect and quantify DNA methylation (i.e., 5-methylcytosine) efficiently and accurately has become essential for epigenetic-based research into cancer, gene expression, genetic diseases, and other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis (1) and methylation-sensitive arbitrarily primed PCR (2), just to name a few. However, the most common technique used in the study of DNA methylation remains the treatment of DNA with bisulfite prior to analysis (3). Immunoprecipitation (IP) of methylated DNA with an antibody is another powerful tool in the developing study of genome-wide methylation.

The **Methylated-DNA IP Kit** utilizes IP technology for the enrichment of 5-methylcytosine-containing DNA from any pool of fragmented genomic DNA for use in genome-wide methylation analysis. The kit features a highly specific anti-5-methylcytosine monoclonal antibody for the "capture" and separation of methylated DNA from non-methylated DNA in only a few hours (see figure below). Typically, over a hundred-fold enrichment of methylated DNA vs. non-methylated DNA can be achieved with the use of this kit. Recovered DNA is suitable for many downstream applications to analyze genome-wide DNA methylation including: PCR, whole-genome amplification, ultra-deep sequencing and microarray.

Example of Enrichment Results Using Control Primers and DNA



Efficient enrichment of methylated DNA using the Methylated-DNA IP Kit. Salmon sperm genomic DNA "spiked" with non-methylated/methylated control DNA was processed with the kit and the eluted DNA was amplified by PCR using the supplied control primers. Digestion of the amplicons with *Nco* I produced two 175 bp fragments for the methylated DNA control or one 350 bp fragment for the non-methylated DNA control. Thus, differences between the methylated and non-methylated templates can be easily determined. The results show an efficient enrichment of methylated versus non-methylated DNA by the kit. *Nco* I digested products were separated in a 2.0% (w/v) agarose TAE/EtBr gel.

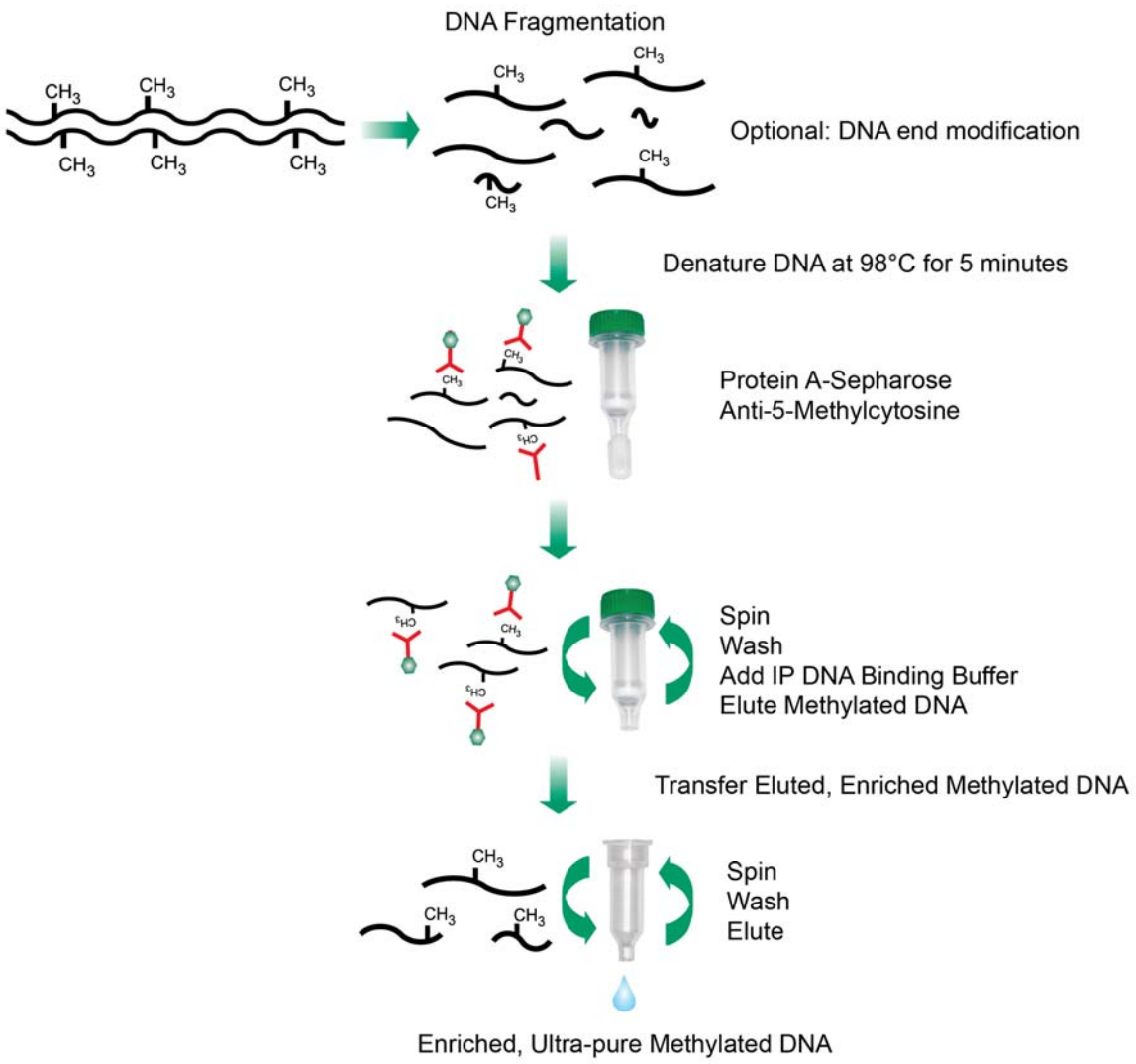
References:

1. Fraga MF, *et al*. Electrophoresis. 2000; 21(14): 2990-2994.

2. Gonzalgo ML. Cancer Res. 1997; 57(4): 594-599.

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

Outline of the Methylated-DNA IP Kit Procedure



Specifications:

- **DNA Input:** Samples containing 50 - 500 ng of DNA yield optimal results. The ratio of input DNA:McAb is an important factor when considering the experimental design (please see **Considerations for Experimental Design** on page 5 for details).
- **Enrichment Factor for Methylated vs. Non-methylated DNA:** > 100 fold.
- **Control DNA:** Supplied at a 1:4 (methylated:non-methylated) DNA ratio at 1 ng/µl in 20 µl. See **Appendix** for more details.
- **Control Primers I and II**: Each supplied at a 20 µM concentration in 20 µl. See **Appendix** for more details.

Reagent Preparation:

- **Preparation of DNA Wash Buffer**

  Add 24 ml of 100% (26 ml of 95%) ethanol to the 6 ml **DNA Wash Buffer** concentrate.

- **Preparation of Protein A-Sepharose Slurry**

  Spin down the Protein A-Sepharose slurry prior to use (beads will stick to the walls of the tube). Pipet bead slurry up-and-down several times to ensure a homogenous aliquot of beads is added to each reaction.

For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

Considerations for Experimental Design:

A. **DNA Fragmentation -** The input DNA for use with the **Methylated-DNA IP Kit** should be fragmented according to the specific requirements of your experiment. Typical sizes average from 200-500 bp. Fragmentation can be accomplished using nebulization, sonication, restriction endonuclease and nuclease digestion, among other established techniques.

B. **DNA End Modification -** Different adaptors can be added to the ends of fragmented DNA using any of a number of established procedures if required for DNA amplification or priming following methylated DNA IP recovery.

C. **Ratio of Input DNA:Monoclonal Antibody -** The ratio of input DNA to monoclonal antibody (McAb) is very important in determining the success of the methylated DNA IP procedure. A DNA:McAb ratio of 1:10-25 (in terms of µg) is recommended. For example, use 160-400 ng DNA:4 µg (4 µl) McAb. Ratios higher than 1:10 (i.e., more DNA) may bias richly-methylated sequences in the recovered DNA. Conversely, ratios lower than 1:25 (less DNA) may bias non-specific, non-methylated sequences in the recovered DNA. Thus, biased recovery of CpG-rich versus low CpG content DNA can be avoided when performing genome-wide methylation analysis simply by adjusting the input DNA:McAb ratio. Bias is a common problem with other methylated-DNA IP (MeDIP, MeIP) methods.

D. **Positive Controls -** We recommend "spiking" input DNA samples with the **Control DNA** included in the kit for easy monitoring of the methylated DNA IP process. This control contains both *in vitro* methylated DNA and non-methylated DNA at a ratio of 1:4, respectively. Methylated DNA IP enrichment efficiency can be determined following PCR with **Control Primers I and II** and then *Nco* I digestion of the PCR products to differentiate methylated from non-methylated DNA template. A *successful* enrichment should invert the ratio from 1:4 to 10:1 or higher (see figure on page 2). See Appendix for detailed information regarding the **Control DNA** and **Control Primers I and II**.

Protocol:

The entire procedure takes about 4 hours to complete. It is very important that input DNA be fragmented using an established procedure before beginning (see **Considerations for Experiment Design**, page 5).

1. ***In order***, add the following reagents to a **Zymo-Spin™ IVM** (**ZS-IVM**) column:
   a. Add 250 µl **MIP Buffer**
   b. Add 50 µl of **Protein A-Sepharose Slurry** (Pipet up-and-down to expel beads from pipette tip)
   c. Add 4 µl **Mouse Anti-5-Methylcytosine**

2. Cap the **ZS-IVM** column tightly with the green cap. Invert the tube 2-4 times to mix the sample. Place the column/sample on a rotator or rocker at room temp. for 30 minutes.

3. During incubation of the antibody/protein A mixture (Step 2), dilute and denature input DNA samples as follows:

   Dilute 1-40 µl of sample containing 160 - 400 ng of DNA in the **DNA Denaturing Buffer** to a final volume of 50 µl.

   Example: For 32 µl genomic DNA, add 17 µl **DNA Denaturing Buffer** and 1 µl **Control DNA** (optional).

   Denature the input DNA at 98˚C for 5 minutes and then *immediately* transfer the denatured DNA to ice.

4. Add the denatured DNA to the antibody/protein A mixture after Step 2 above is complete.

5. Incubate the antibody/protein A/DNA mixture at 37˚C for 0.5-2 hours on a rotator or rocker. Alternatively, invert tubes every 10-15 minutes during the incubation.

6. Break off bottom tip of the **ZS-IVM** sample column, place the column into a **Collection Tube**, and centrifuge samples at 800 x *g* (~3,000 rpm) for one minute. Discard the flow through.

7. Add 500 µl of **MIP Buffer** to the column and centrifuge at 800 x *g* for 30 seconds. Discard the flow through. Repeat wash 2 more times discarding the flow through and saving the pellet in the column after each wash.

   The capacity of the Collection Tube with the ZS-IVM column inserted is 500 µl. Empty the Collection Tube whenever necessary to prevent contamination of the column contents by the flow-through.

8. Transfer the **ZS-IVM** column into a new **Collection Tube**. Add 400 µl of **IP DNA Binding Buffer** to the **ZS-IVM** column and completely re-suspend the pellet. Centrifuge at 10,000 x *g* (~13,000 rpm) for 30 seconds. Save the flow through.

9. Transfer the flow through from Step 8 above into a **Zymo-Spin™ I** (**ZS-I**) column inside a new **Collection Tube** and centrifuge at 10,000 x *g* for 30 seconds. Discard the flow through.

10. Add 200 µl **DNA Wash Buffer** to the **ZS-I** column. Centrifuge at 10,000 x *g* for 1 minute. Repeat wash step and discard the flow through.

11. Add 10 µl **DNA Elution Buffer** directly to the column matrix, place column into a new 1.5 ml microcentrifuge tube, and centrifuge briefly at 10,000 x *g* to elute the DNA.

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

    The recovered DNA is mostly single stranded and suitable for PCR based amplification and other downstream DNA methylation analyses. The eluted DNA can be stored at or below -20°C for later use. For long term storage, it is recommended the DNA be stored at or below -70°C.

Appendix: Methylated/Non-methylated Control DNA and Primers

The kit contains **Control DNA** which is a mixture of fully methylated pUC19 (pUC19m) and non-methylated pUC19 (pUC19) DNA (at a 1:4 ratio) and **Control Primers** for monitoring the different steps of the methylated-DNA IP procedure. The pUC19m DNA contains base-replacement mutations at nucleotide positions 806-811 to create a novel *Nco* I restriction enzyme site. Additionally, the DNA was methylated *in vitro* at all CpG sites using *Sss* I methylase. Since the methylated DNA (pUC19m) contains a *Nco* I restriction site (the non-methylated (pUC19) DNA does not), *Nco* I digestion can be used to differentiate between methylated and non-methylated DNAs. The success of the methylated-DNA IP procedure can be gauged by a significant enrichment of methylated DNA over non-methylated DNA in the PCR amplified end-product. The supplied primers will generate a 350 bp PCR amplicon, that once digested with *Nco* I, will produce two 175 bp fragments for the methylated pUC19m and an intact 350 bp fragment for the non-methylated pUC19. Specifics for the Control DNA and Primers are as follows:

Plasmid Format: Linearized by *Sca* I digestion.

Control DNA Concentration: 1 ng/µl in TE buffer, containing 250 pg/µl methylated pUC19m and 750 pg/µl non-methylated pUC19.

Sequence and Primer Information:

Primer position on pUC19 sequence:

636 nt.-
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTC
GCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGGAAAGA***ACATGT***GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCT
GGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCC
GACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC-978 nt.

Primer position on pUC19m sequence:

636 nt.-
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTC
GCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGGAAAGA***CCATGG***GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCT
GGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCC
GACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC-978 nt.

Note: The annealing positions of Control Primers I and II are underlined. The numbers represent the position of primers relative to the pUC19 sequence. The position of base-replacement mutation for the *Nco* I site is given in ***bold italic***.

Primer Sequences:

Forward: 5′-GGTTAATGAATCGGCCAACGCGCG-3′
Reverse: 5′-GAGGGAGCTTCCAGGGGGAAA-3′
Recommended final concentration of control primers is between 400 nM – 1 µM.

Amplicon Size: 350 bp (2 x 175 bp fragments following pUC19m digestion w/ *Nco* I).

PCR Conditions for Control Primers:

Primer annealing temperature is 60˚C for 30.0 sec. We recommend using Zymo*Taq*™ Premix with the following conditions…

1. 95.0˚C – 10.0 min.
2. 94.5˚C – 30.0 sec.
3. 60.0˚C – 30.0 sec.
4. 72.0˚C – 1.20 min.
5. Go to Step 2, for 27 - 30 Cycles.
6. 72.0˚C – 7.0 min.
7. 4.0˚C – 4.0 min.

Frequently Asked Questions:

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

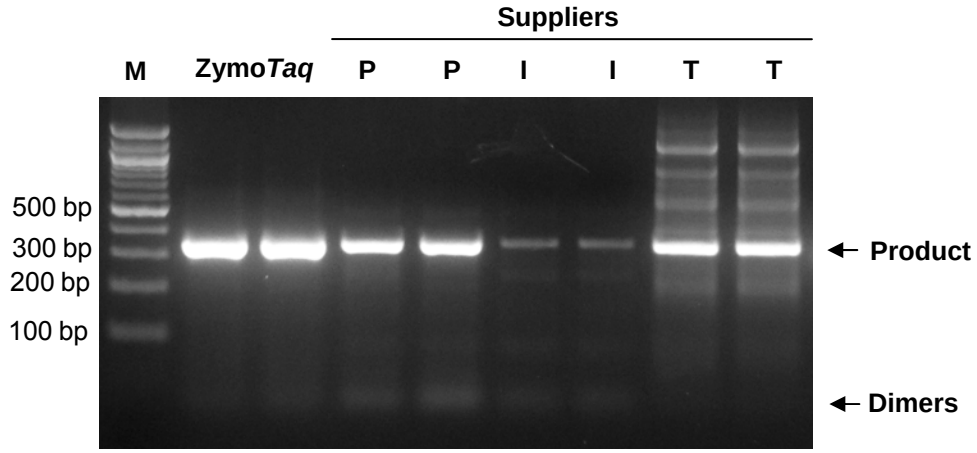
A: *Water, TE, or modified TE buffers can be used to dissolve the DNA and do not interfere with the denaturation or the enrichment process.*

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

A: *The sample should preferably be used immediately or stored at ≤ -20ºC whenever possible.*

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

A: *We recommend "hot start" polymerases like Zymo Research's* **Zymo*Taq*™** ***DNA Polymerase*** *or* ***Premix****. Typically, 27-30 cycles will be enough to yield a robust product. See the figure below for a comparison of hot start polymerases for amplification of immunoprecipitated methylated DNA.*



PCR products of Immunoprecipitated methylated DNA vary depending on the hot start polymerase used. Methylated DNA was immunoprecipitated using the Methylated-DNA IP Kit. The resultant DNA was used in a PCR assay comparing Zymo Research's hot start **Zymo*Taq*™** polymerase vs. that of three other suppliers. Expected amplicon size is 350 bp. PCR products (in duplicate) were separated in a 2.0% (w/v) agarose TAE/EtBr gel. The use of **Zymo*Taq*™** generated specific, robust products with minimal non-specific banding as compared to the others.

Ordering Information:

Product Description	Catalog No.	Kit Size
Methylated-DNA IP Kit	D5101	10 rxns.

For Individual Sale	Catalog No.	Amount(s)
Protein A-Sepharose Slurry	D5101-1-500	500 µl
Mouse Anti-5-Methylcytosine	A3001-50	50 µl
Methylated/Non-methylated Control DNA and Primer Set	D5101-2	1 Set
MIP Buffer	D5101-3-20	20 ml
DNA Denaturing Buffer	D5101-4-1	1 ml
IP DNA Binding Buffer	D5101-5-6	6 ml
DNA Wash Buffer concentrate	D4003-2-6 D4003-2-24	6 ml 24 ml
DNA Elution Buffer	D5101-6-1	1 ml
Zymo-Spin™ IVM Columns w/ Green Caps	C1020-10	10 columns
Zymo-Spin™ I Columns	C1003-50 C1003-250	50 columns 250 columns
Collection Tubes	C1001-50 C1001-500 C1001-1000	50 tubes 500 tubes 1,000 tubes

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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.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns.	**D5001** (spin column) **D5002** (spin column) **D5003** (shallow-well plate) **D5004** (deep-well plate)
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.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns.	**D5005** (spin column) **D5006** (spin column) **D5007** (shallow-well plate) **D5008** (deep-well plate)
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.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns.	**D5020** (spin column) **D5021** (spin column) **D5022** (shallow-well plate) **D5023** (deep-well plate)
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**
EZ Bisulfite DNA Clean-up Kit™	Desulfonation and purification of DNA from any "homebrew" or commercially derived reaction mixture containing bisulfite.	50 Preps. 200 Preps. 2x96 Preps. 2x96 Preps.	**D5025** (spin column) **D5026** (spin column) **D5027** (shallow-well plate) **D5028** (deep-well plate)
	Methylated DNA Standards		
Universal Methylated DNA Standard	pUC19 plasmid 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	**D5010**
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)
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)
Anti-5-Methylcytosine Monoclonal Antibody (clone 10G4)	Mouse monoclonal antibody developed to facilitate the differentiation between methylated and non-methylated cytosines in DNA. Can be used in immunoprecipitation-based procedures including Methylated DNA Immunoprecipitation (MeIP).	50 µg/50 µl 200 µg/200 µl	**A3001-50** **A3001-200**
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**