



ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ-96 DNA Methylation™ Kit

Catalog No. **D5004 (Deep-Well Format)**

Highlights

- High throughput (96-well), proven procedure for bisulfite conversion of DNA.
- 96-well desulphonation and recovery of bisulfite-treated DNA.
- Recovered DNA is ideal for downstream analyses including PCR, endonuclease digestion, sequencing, microarrays, etc.

Contents

Product Contents	1
Introduction to DNA Methylation.....	2
Product Description.....	3
Product Specifications.....	4
Reagent Preparation	4
Protocol.....	5
Appendix.....	6
Frequently Asked Questions	7
Ordering Information	8
List of Related Products	9

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.

	D5004	Storage Temperature
EZ-96 DNA Methylation™ Kit	2 x 96 rxns.	
CT Conversion Reagent*	2 bottles	Room Temp.
M-Dilution Buffer	5.2 ml	Room Temp.
M-Binding Buffer	80 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 Plate	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.

* 7.5 ml water and 2.1 ml **M-Dilution Buffer** are added per bottle of **CT Conversion Reagent** and mixed prior to use.

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

The Polymerase Chain Reaction (PCR) process is covered by U.S. Pat. Nos. 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche. Patents pending in other countries. No license under these patents to use the PCR process is conveyed expressly or by implication to the purchaser by the purchase of Zymo Research's EZ DNA Methylation kits. Further information on purchasing licenses to practice the PCR process can be obtained from the director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404 or at Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501.

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.

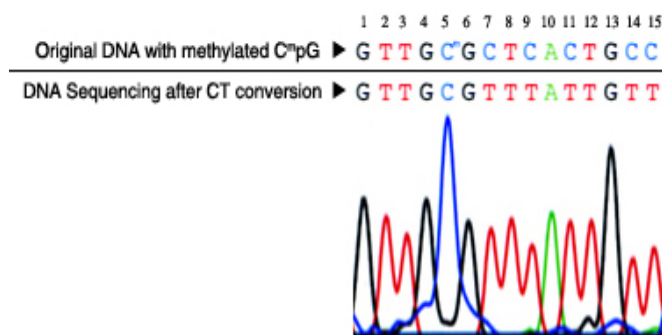
Note - ™ Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by trained professionals. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

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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.

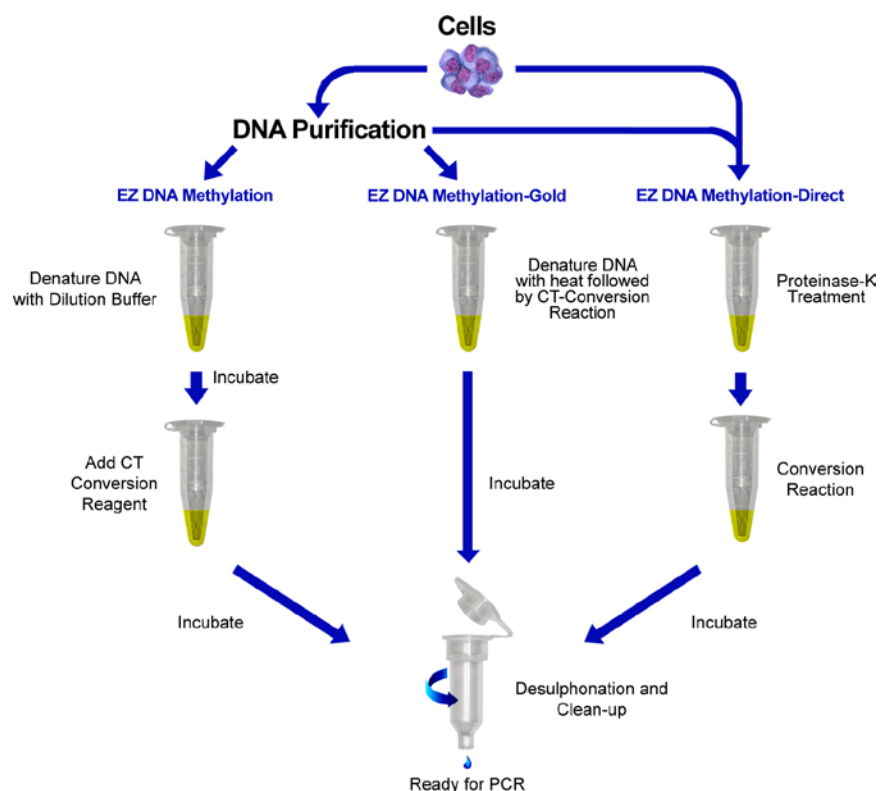
Product Description:

Selected EZ DNA Methylation™ Kit Citations:

1. Ehrlich 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.

The **EZ-96 DNA Methylation™ Kit** features a high-throughput (96-well), simplified procedure that streamlines bisulfite conversion of DNA. The kit is based on the three-step reaction that takes place between cytosine and sodium bisulfite where cytosine is converted into uracil. The product's innovative desulphonation technology eliminates otherwise cumbersome precipitations. The kit is designed to reduce template degradation, minimize DNA loss during treatment and clean-up, while ensuring complete conversion of the DNA. Purified, converted DNA is ideal for PCR amplification for downstream analyses including endonuclease digestion, sequencing, microarrays, etc.

An outline comparing the **EZ DNA Methylation™ 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:** > 80%

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 7.5 ml water and 2.1 ml of **M-Dilution Buffer** to a bottle 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 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.

Protocol:

1. Add 5 µl of **M-Dilution Buffer** to each DNA sample in a **Conversion Plate** and adjust the total volume to 50 µl with water. Mix each sample by pipetting up and down.

Example: For 14 µl of a DNA sample add 5 µl M-Dilution Buffer and 31 µl water.

2. Incubate the **Conversion Plate** containing the samples at 37°C for 15 minutes.
3. After the above incubation, add 100 µl of the prepared **CT Conversion Reagent** to each sample and mix.
4. Incubate the **Conversion Plate** in the dark at 50°C for 12 – 16 hours (e.g., using a thermal cycler).

Please see **Appendix** (page 6) for alternative incubation conditions (e.g., when using the Illumina Infinium® Methylation Assay)

5. Incubate the sample at 0 - 4°C (e.g., on ice or using a thermal cycler) for 10 minutes.
6. Add 400 µl of **M-Binding Buffer** to each well of a **Zymo-Spin™ I-96 Binding Plate** on a **Collection Plate**.
7. Load the samples (from Step 5) into the wells of the **Zymo-Spin™ I-96 Binding Plate** containing the **M-Binding Buffer**. Mix by pipetting up and down.
8. Centrifuge at $\geq 3,000 \times g$ (5,000 $\times g$ max.) for 5 minutes. Discard the flow-through.
9. Add 500 µl of **M-Wash Buffer** to each well and centrifuge at $\geq 3,000 \times g$ for 5 minutes.
10. Add 200 µl of **M-Desulphonation Buffer** to each well of and let stand at room temperature (20°C – 30°C) for 15 - 20 minutes. After the incubation, centrifuge at $\geq 3,000 \times g$ for 5 minutes.
11. Add 500 µl of **M-Wash Buffer** to each well and centrifuge at $\geq 3,000 \times g$ for 5 minutes. Add another 500 µl of **M-Wash Buffer** and centrifuge for 10 minutes.
12. Place the **Zymo-Spin™ I-96 Binding Plate** onto an **Elution Plate**. Add 15 µl of **M-Elution Buffer** directly to the binding matrix in each well. Centrifuge for 3 minutes at $\geq 3,000 \times g$ 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 15 µl can be used if necessary. The elution volume can be $> 15 \mu\text{l}$ depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

The **CT Conversion reagent** is light sensitive, so try to minimize the reaction's exposure to light whenever possible.

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 ≥ 6.0) can be used for elution if required for your experiments.

Appendix: Bisulfite Conversion and PCR Optimization

1. Incomplete C to T Conversion.

A. Increase temperature in step 2 of the **Protocol** to 42°C and extend the incubation time to 30 minutes. If the problem persists, use modified conversion conditions (see **B**, below).

B. In Step 1 of the **Protocol**, add 7.5 µl **M-Dilution Buffer** instead of 5 µl (the total volume should remain 50 µl). If this change is made, the preparation of the **CT-Conversion Reagent** must also be modified by reducing the volume of **M-Dilution Buffer** from 2.1 ml to 1.85 ml. In Step 3 of the **Protocol**, add 97.5 µl prepared **CT-Conversion Reagent** per reaction instead of 100 µl.

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'-AATTTTATAGGTAT-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 $A_{260} = 1.0$ when determining the concentration of the recovered bisulfite-treated DNA.

6. Alternative Incubation Conditions When Using the Illumina Infinium® Methylation Assay. For Steps 4 & 5 of the protocol, incubate the sample(s) in a thermocycler at...

(95 °C for 30 sec., 50 °C for 60 min.) x 16 cycles, then "hold" at 4 °C

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

Infinium® is a registered trademark of Illumina, Inc.

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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 $\leq -20^{\circ}\text{C}$ 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 a “hot start” DNA polymerase (e.g., ZymoTaq™, page 9).*

Q: Why are there two different catalog numbers for the EZ-96 DNA Methylation™ 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	D5003	D5004

Ordering Information:

Product Description	Catalog No.	Kit Size
EZ DNA Methylation™ Kit	D5001	50 rxns.
EZ DNA Methylation™ Kit	D5002	200 rxns.
EZ-96 DNA Methylation™ Kit (Shallow-Well)	D5003	2 x 96 rxns.
EZ-96 DNA Methylation™ Kit (Deep-Well)	D5004	2 x 96 rxns.

For Individual Sale	Catalog No.	Amount(s)
CT Conversion Reagent	D5001-1	1 tube
	D5003-1	1 bottle
M-Dilution Buffer	D5001-2	1.3 ml
	D5002-2	5.2 ml
M-Binding Buffer	D5001-3	20 ml
	D5002-3	80 ml
M-Wash Buffer	D5001-4	6 ml
	D5002-4	24 ml
	D5007-4	36 ml
M-Desulphonation Buffer	D5001-5	10 ml
	D5002-5	40 ml
M-Elution Buffer	D5001-6	1 ml
	D5002-6	4 ml
	D5007-6	8 ml
Zymo-Spin™ IC Columns (capped)	C1004-50	50 columns
	C1004-250	250 columns
Collection Tubes	C1001-50	50 tubes
	C1001-500	500 tubes
	C1001-1000	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

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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 <u>chemical-denaturation</u> of DNA and a specially designed CT Conversion Reagent. <i>Fast-Spin</i> 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 <u>heat/chemical-denaturation</u> of DNA and a specially designed CT Conversion Reagent. <i>Fast-Spin</i> 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™	Desulphonation 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)
ZymoTaq™ 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)
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