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INSTRUCTION MANUAL

Methylated-DNA IP Kit

Catalog No. **D5101**

Highlights

- The use of a highly specific anti-5-methylcytosine monoclonal antibody allows for precise and accurate 5-mC DNA pulldown.
- Includes control DNA and primers for easy monitoring immunoprecipitation efficiency.
- Eluted, ultra-pure DNA is ideal for use downstream applications (e.g., qPCR, MeDIP-Seq, etc.).

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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 (10 reactions)	**D5101**	**Storage Temperature**
ZymoMag Protein A[1]	200 µl	4°C.
Anti-5-Methylcytosine (0.5 µg/µl)	50 µl	-20°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
DNA Elution Buffer	10 ml	0°C to RT
Magnetic Rods	4	-
Instruction Manual	1	-

Note - Integrity of kit components are 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.

[1] Upon arrival, store the ZymoMag Protein A at 4°C.

[2] Upon arrival, store the Mouse Anti-5-Methylcytosine monoclonal antibody at -80°C for long-term storage or at -20˚C for frequent usage.

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

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.

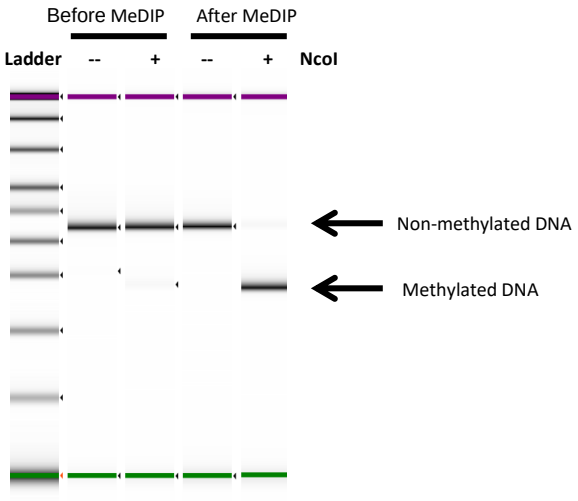
Use of Methylation Specific PCR (MSP) is protected by US Patents 5,786,146 & 6,017,704 & 6,200,756 & 6,265,171 and International Patent WO 97/46705. No license under these patents to use the MSP process is conveyed expressly or by implication to the purchaser by the purchase of this product.

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.

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. The most common technique used in the study of DNA methylation remains the treatment of DNA with bisulfite prior to analysis. Immunoprecipitation (IP) of methylated DNA with an antibody is another quick and simple method used to study genome-wide methylation.

The **Methylated-DNA IP Kit** enriches 5-methylcytosine (5-mC) containing DNA from any pool of fragmented genomic DNA for use in genome-wide methylation analysis. The kit includes 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. 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, qPCR, whole-genome amplification, MeDIP-Seq, etc.



Efficient enrichment of methylated DNA using Methylated-DNA IP Kit. Salmon sperm genomic DNA was "spiked" with 1 ng of control DNA comprised of a mixture of methylated/non-methylated DNA (1:4 ratio) and immunoprecipitated following the protocol. Digestion of amplicons with *NcoI* produced two 175 bp fragments for methylated DNA control or one 350 bp fragment for non-methylated control. The results show an efficient enrichment of methylated DNA vs. non-methylated DNA in immunoprecipitated DNA (After MeDIP) compared to non-precipitated (Before MeDIP) samples. The products were visualized using D1000 Tape on TapeStation 2200 (Agilent, Santa Clara, CA).

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

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** (below).
- **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.

Considerations for Experimental Design:

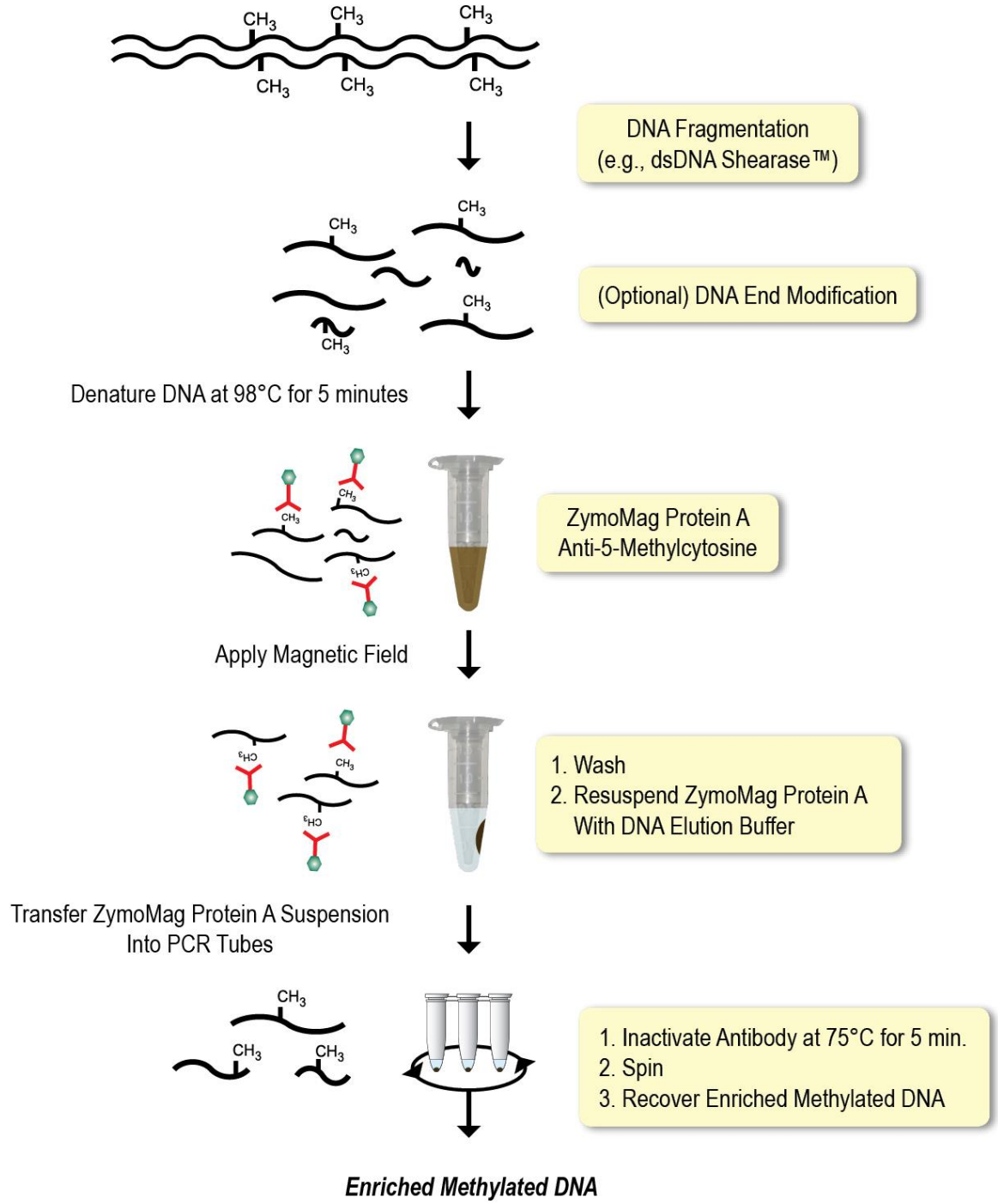
A. **DNA Fragmentation -** The input DNA for use with the **Methylated-DNA IP Kit** should be fragmented according to the specific requirements of your experiment. Typical sizes average from 200-500 bp. For genomic DNA fragmentation, we strongly recommend our **dsDNA Shearase™ Plus** (Cat No. E2018) as it is perfect for random fragmentation and compatible with DNA end modifications. Other methods for fragmentation including nebulization, sonication, restriction endonuclease and nuclease digestion can be employed.

B. **DNA End Modification -** Different adaptors can be added to the ends of fragmented DNA using any established procedure 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 important factor for methylated DNA IP procedure. A DNA:McAb ratio of 1:2.5 (in terms of ng) is recommended. For example, use 160 ng DNA:400 ng (0.8 µl) McAb. Ratios higher than 1:2.5 (i.e., more DNA) may bias richly-methylated sequences in the recovered DNA. Conversely, ratios lower than 1:2.5 (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.

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**.

Outline of the Methylated-DNA IP Kit Procedure



Protocol:

The entire procedure takes about 3 hours to complete and requires a magnetic tube rack (small magnetic rods provided can be used alternatively). It is very important that input DNA be fragmented using an established procedure before beginning. It is also recommended that a 1:2.5 ratio of input DNA to antibody (µg/µg) is used. The following protocol is designed for 160 ng of input DNA, though it can be adjusted for DNA samples ranging from 50-500 ng (see **Considerations for Experiment Design**, page 3).

1. Dilute and denature input DNA samples as follows:

   Dilute 1-40 µl of sample containing 160 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 diluted input DNA at 98˚C for 5 minutes.

2. *Complete this step while the DNA is being denatured, or set up tubes before Step 1:*
   In order, add the following reagents to a 1.5 ml microcentrifuge tube:
   a. Add 250 µl **MIP Buffer**
   b. Add 15 µl of **ZymoMag Protein A** (Pipet up-and-down to expel beads from pipette tip)
      *Note: **ZymoMag Protein A** must be resuspended completely by gently flicking and inverting the tube prior to use*
   c. Add 0.8 µl **Anti-5-Methylcytosine** antibody

   Invert the tube 2-4 times to mix the antibody/Protein A mixture.

3. Add the denatured DNA *immediately* to the antibody/Protein A mixture after Step 1 above is complete.

4. Incubate the antibody/Protein A/DNA mixture at 37˚C for 0.5-1 hour on a rotator or rocker. Alternatively, invert tubes every 10-15 minutes during the incubation.

5. Place tubes on a magnetic tube rack, allow time for the beads to cluster, then remove and discard the supernatant.

   It is beneficial to try and remove the beads from the microcentrifuge tube cap using a quick flick of the wrist prior to using the magnetic tube rack in order to maximize yield.

6. Add 500 µl of **MIP Buffer** to each 1.5 ml microcentrifuge tube and secure all the caps. Invert tubes several times and vortex briefly to resuspend the beads. Remove and discard supernatant using the magnetic tube rack.

7. Repeat wash step (Step 6.) twice more – first with 500 µl **MIP Buffer** and then with 500 µl of **DNA Elution Buffer**.

8. Once the supernatant from the final wash step has been removed and discarded, add 15 µl of **DNA Elution Buffer** to each tube and resuspend the beads by gently flicking the tube or pipetting up and down. Transfer each bead suspension to a clean 0.2 ml PCR tube.

   Alternatively, water can be used for elution if required for your experiments.

9. Incubate the PCR tubes at 75˚C for 5 minutes and follow with a 2-minute spin in a mini-centrifuge.

   Instead of the 2-minute spin, a magnetic PCR-tube rack or individual magnets can be used to cluster the beads in order to recover the enriched DNA.

10. Transfer the supernatant to new 1.5 ml microcentrifuge tubes without disturbing the beads (if beads are disturbed PCR tubes can be re-spun). This is the recovered DNA.

    The recovered DNA is mostly single stranded and suitable for PCR based amplification and other downstream DNA methylation analyses. It 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.-TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT
TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAG
GCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA***ACATGT***GAGCAAAAGGCCAG
CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGAC
GAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
GCGTTTCCCCCTGGAAGCTCCCTC-978 nt.

Primer position on pUC19m sequence:

636 nt.-TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCT
TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAG
GCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA***CCATGG***GAGCAAAAGGCCAG
CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGAC
GAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
GCGTTTCCCCCTGGAAGCTCCCTC-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:

Control Primer I: 5′-GGTTAATGAATCGGCCAACGCGCG-3′
Control Primer II: 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 sec. We recommend using Zymo*Taq*™ Premix with the following conditions…

1. 95.0˚C – 10 min.
2. 94.5˚C – 30 sec.
3. 60.0˚C – 30 sec.
4. 72.0˚C – 1 min 20 sec.
5. Go to Step 2, for 27 - 30 Cycles.
6. 72.0˚C – 7 min.
7. 4.0˚C – 4 min.

Frequently Asked Questions:

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

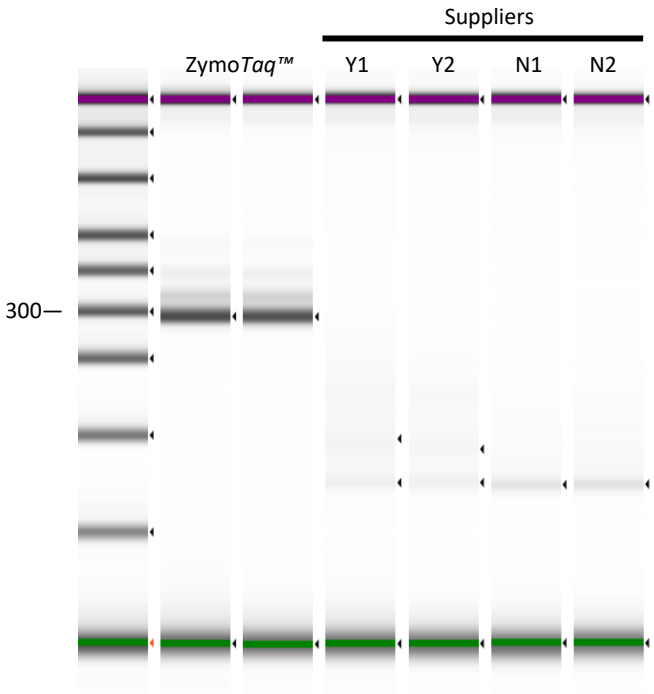
A: *Water, TE, or modified TE buffers can be used to dissolve the DNA and do not interfere with the denaturation or the enrichment process.*

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.*



Efficient PCR amplification of bisulfite treated DNA for methylation detection. The figure shows a 274 bp product amplified from bisulfite-treated DNA using Zymo*Taq*™ DNA Polymerase vs. polymerases from Supplier Y and N. In each case, equal amounts of bisulfite-treated DNA (EZ DNA Methylation-Lightning Kit (Cat # D5030) from Zymo Research) were used for each duplicate PCR reaction and the products visualized using the TapeStation 2200 (Agilent, Santa Clara, CA).

Ordering Information:

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

For Individual Sale	Catalog No.	Amount(s)
ZymoMag Protein A	M2001	200 µl
Anti-5-Methylcytosine (0.5 µg/µl)	A3002-50	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
DNA Elution Buffer	D3004-4-10	10 ml

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