Product Manual

BioArray™ Methylated DNA IP Kit

ENZ-45012-0010

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Enzo Life Sciences

Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

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). 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 (2). 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 (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 (3) and methylation-sensitive arbitrarily primed PCR (4), 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 (5). Immunoprecipitation (IP) of methylated DNA with an antibody is another powerful tool in the developing study of genome-wide methylation.

ASSAY PRINCIPLE

The **BioArray™ Methylated DNA IP Kit** features 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 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 (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.

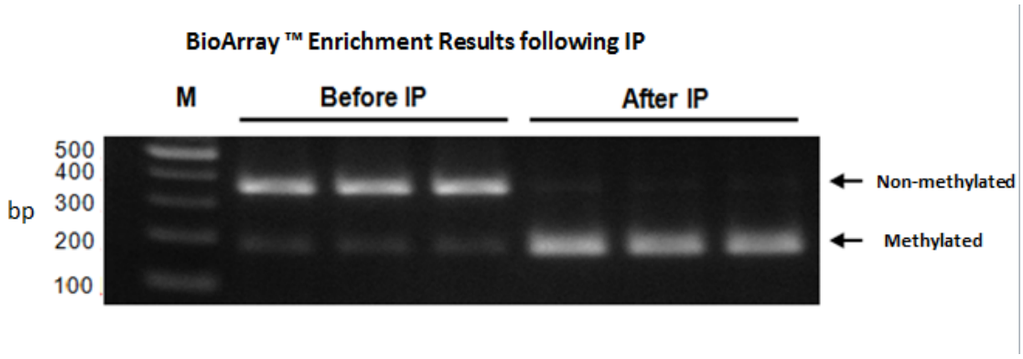


Figure 1 In this example, Salmon sperm genomic DNA "spiked" with non-methylated/methylated control DNA was processed with the kit. The eluted DNA was amplified by PCR using the supplied control primers. Digestion of the amplicons with *Nco* I produced two 175bp fragments for the methylated DNA control or one 350bp fragment for the non-methylated DNA control. 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.

- **Enrichment Factor for Methylated vs. Non-methylated DNA:** > 100 fold.
- **Control DNA:** Supplied at a 1:4 (methylated : non-methylated) DNA ratio at 1ng/µL in 20µL.
- **Control Primer I and 2**: Each supplied at a 20µM concentration in 20µL.

MATERIALS SUPPLIED

Reagent	Storage Conditions	Format	Comments
Protein A Beads	4°C	200µL	Upon arrival store at 4°C
Anti-5 Methylcytosine (Mouse)	4°C	50µL	Upon arrival, store antibody at -80°C for long term storage. Store at 4°C for frequent usage.
Control DNA (Methylated/Non-methylated)	-20°C	20µL	Upon arrival store at -20°C
Control Primer 1	-20°C	20µL	Upon arrival store at -20°C
Control Primer 2	-20°C	20µL	Upon arrival store at -20°C
IP Buffer	0°C to RT	20ml	
DNA Denaturing Buffer	0°C to RT	1ml	
DNA Elution Buffer	0°C to RT	10ml	
Rods (Magnetic)	-	4	

STORAGE

Store reagents at suggested temperatures indicated in the Materials Supplied Section.

STABILITY

12 months upon receipt

OTHER MATERIALS NEEDED

1. Calibrated pipette
2. Microtube centrifuge (Counter)
3. PCR tube
4. Magnetic tube rack (optional)
5. Tube rocker or rotator
6. -20°C or -70°C freezer
6. DNA input (50-500ng optimal)
7. Agarose Gel (TAE/EtBr) and Gel illuminator

REAGENT PREPARATION

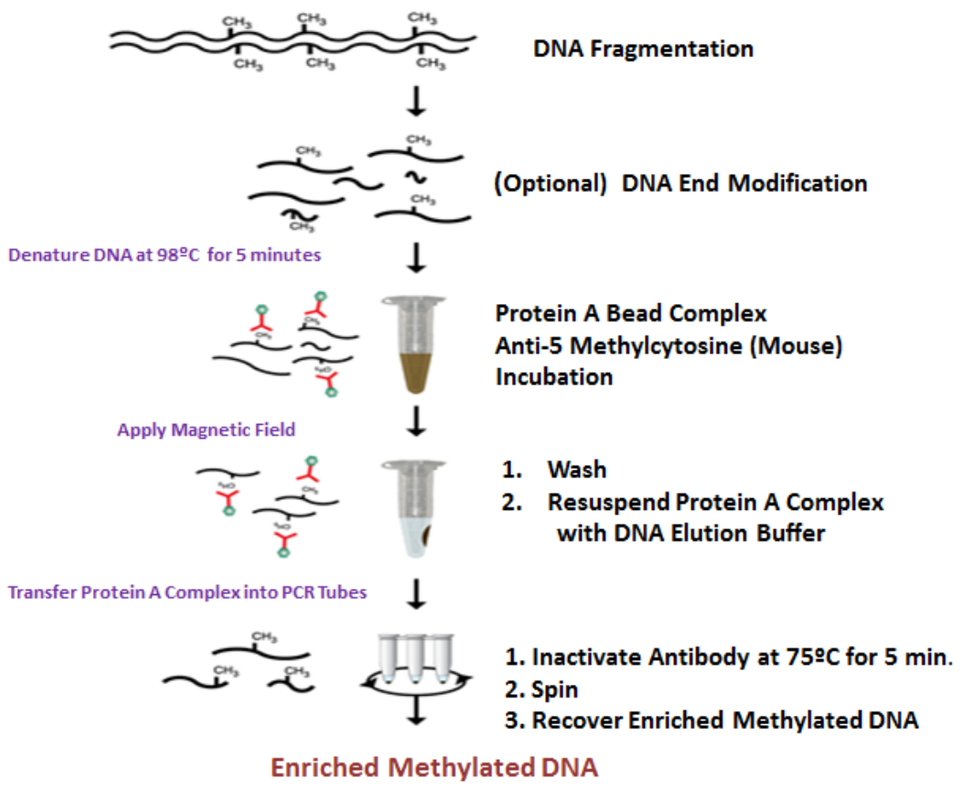
NOTE: Prior to use, thaw indicated -20°C reagents to 4°C. *Reagents should be briefly centrifuged to ensure contents are fully collected. Ensure reagents are homogenous.*

Test DNA Input Control Solution

We recommend “spiking” input DNA samples with the control DNA for monitoring the methylated DNA IP process.

METHODS

The procedure described in this manual assumes that the user is familiar with the basic principles of IP and using proper sterile technique.



OVERALL PROCEDURE

Important: *PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Prepare and use appropriate desired controls for each experiment. Prior to beginning experiment, turn on the PCR equipment and set to desired parameters.*

Considerations and Precautions

1. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately.

   Interpretation of the results is the sole responsibility of the user.

2. **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

3. **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.

4. **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 (in terms of µg) is recommended. For example, use 160 ng DNA : 1.6 µg (1.6 µl) McAb. Ratios higher than 1:10 (i.e., more DNA) may bias richly-methylated sequences in the recovered DNA. Conversely, ratios lower than 1:15 (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.

5. **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 2** 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 3). See Appendix for detailed information regarding the **Control DNA** and **Control Primers I and 2**.

PROTOCOL

Note: The entire procedure takes approximately 3 hours to complete and requires a magnetic tube rack (small magnetic rods provided can be used alternatively). Input DNA needs to be fragmented using an established procedure before beginning. This is essential! It is also recommended that a 1:10 ratio of input DNA to antibody (by mass) 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. Optimization might be required.

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 **IP Buffer**

   b. Add 15 µl of **Protein A Bead Complex** (Pipet up-and-down to expel beads from pipette tip)

      Note*:* ***Protein A Bead Complex*** *must be resuspended completely by gently flicking and inverting the tube prior to use*

   c. Add 1.6 µl **Anti-5-Methylcytosine (Mouse)**

      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-inutes during the incubation.

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

6. Add 500 µl of **IP 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 **IP 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.

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

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

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.-
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG
GGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT
ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA***ACATGT***
GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCAC
AAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACT
ATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTC-978 nt.

Primer position on pUC19m sequence:

636 nt.-
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG
GGCGCTCTTCCGCT
TCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCG
AGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGA
ATCAGGGGATAACGCAGGAAAGA***CCATGG***GAGCAAAAGGCCAGC
AAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTC
CATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTC
AAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGG
CGTTTCCCCCTGGAAGCTCCCTC-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 I2:

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.

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