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Original Publication:

Carlos Guerrero-Bosagna and Per Jensen, Optimized method for methylated DNA immuno-precipitation, 2015, MethodsX, (2), e432-e439.
http://dx.doi.org/10.1016/j.mex.2015.10.006

MethodsX 2 (2015) 432–439

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Optimized method for methylated DNA immuno-precipitation

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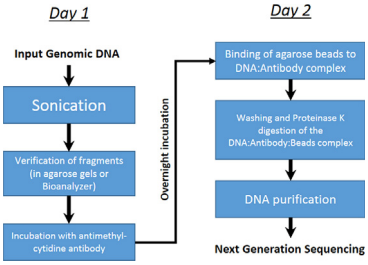
GRAPHICAL ABSTRACT



ABSTRACT

Methylated DNA immunoprecipitation (MeDIP) is one of the most widely used methods to evaluate DNA methylation on a whole genome scale, and involves the capture of the methylated fraction of the DNA by an antibody specific to methyl-cytosine. MeDIP was initially coupled with microarray hybridization to detect local DNA methylation enrichments along the genome. More recently, MeDIP has been coupled with next generation sequencing, which highlights its current and future applicability. In previous studies in which MeDIP was applied, the protocol took around 3 days to be performed. Given the importance of MeDIP for studies involving DNA methylation, it was important to optimize the method in order to deliver faster turnouts. The present article describes optimization steps of the MeDIP method. The length of the procedure was reduced in half without compromising the quality of the results. This was achieved by:

- Reduction of the number of washes in different stages of the protocol, after a careful evaluation of the number of indispensable washes.
- Reduction of reaction times for detaching methylated DNA fragments from the complex agarose beads:antibody.
- Modification of the methods to purify methylated DNA, which incorporates new devices and procedures, and eliminates a lengthy phenol and chloroform:isoamyl alcohol extraction.

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http://dx.doi.org/10.1016/j.mex.2015.10.006

ARTICLE INFO
Method name: Optimized methylated DNA immuno-precipitation
Keywords: MeDIP, DNA methylation, Immunoprecipitation, Epigenetic, Whole genome
Article history: Received 17 August 2015; Accepted 14 October 2015; Available online 19 October 2015

Method details

Background

Methylated DNA immunoprecipitation (MeDIP) is one of the most widely used methods to evaluate DNA methylation on a whole genome scale. MeDIP involves the capture of the methylated fraction of the DNA by an antibody specific to methyl-cytosine [1]. The method was initially coupled with microarray hybridization of the captured methylated DNA in order to detect local DNA methylation enrichments along the genome. When compared against the hybridization of the input DNA, the method has been used to determine absolute DNA methylation levels [2–4]. In other scenarios, competitive hybridization has been performed. In those cases, the hybridization of MeDIP samples from one experimental condition is compared to MeDIP samples from other experimental conditions, in order to determine relative changes of DNA methylation [5–7]. More recently the method has been coupled with next generation sequencing [8], which highlights its current and future applicability in several organism models and experimental designs. In previous articles in which MeDIP was applied [5–7,9] the protocol took around 3 days to be performed. Given the importance of the technique for studies involving DNA methylation, it was important to optimize the method in order to deliver faster turnouts. The present article describes steps of optimization of the MeDIP method. The length of the procedure was reduced to half of the time without compromising the quality of the results. In addition, different incubation times with the antibody were tested in order to decide the most efficient conditions.

Startup solutions, reagents and materials

- 100 mM Na-Phosphate pH 7 buffer
- 5 M NaCl
- Triton X-100
- 1 M Tris–HCl
- 0.5 M EDTA
- 10% SDS
- 0.1 M dithiothreitol (DTT)
- Proteinase K (20 mg/mL)
- TE buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA)
- 700 W sonic dismembrator from Fisher, with probe attached to a cooling cup horn (Fig. 1a and b) and capacity for 8 ultracentrifuge tubes (Fig. 1c)
- Agarose gels and chambers for electrophoresis
- Monoclonal mouse anti 5-methylcytosine from Diagenode (Mab-006-500)
- Protein A/G Plus agarose beads from Santa Cruz (SC-2003)
- Spin-filtering columns from Pierce (with paper filter) (PI69700)
- Glycogen (5 mg/mL) from Ambion (AM9510)
- Ethanol

Buffer preparation

100 mL of 5× IP buffer: Mix 50 mL of 100 mM Na-Phosphate (pH 7.0), 14 mL of 5 M NaCl, 250 μL of Triton X-100 and 35.75 mL of dnase free water. Stir the solution until no solids are visible. Filter-sterilize with filter of 0.2 μm pore size.

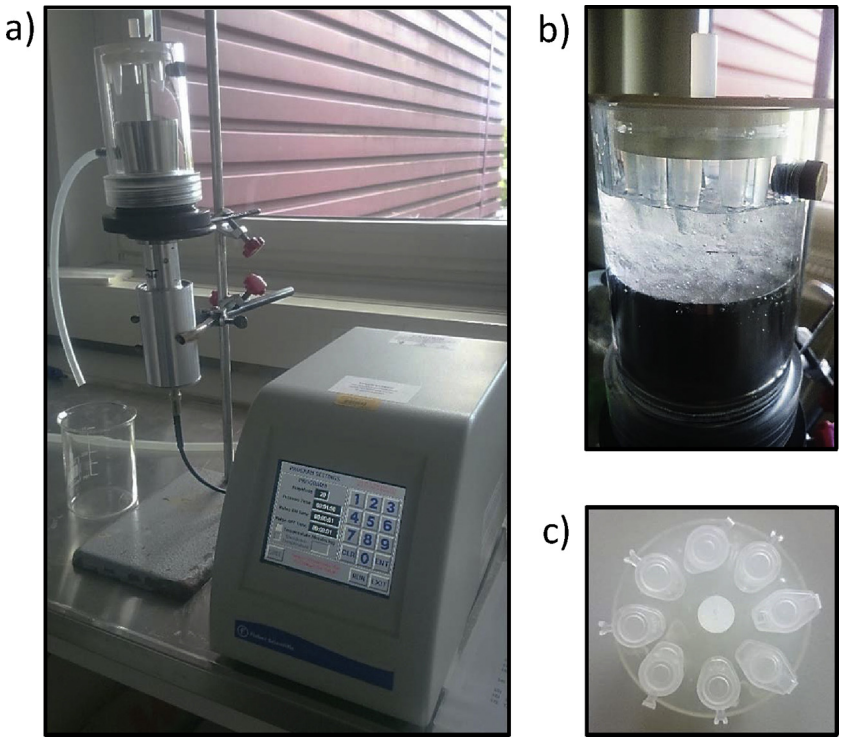

Fig. 1. Set up for sonication: Fisher ultra-sonicator (a) attached to a cooling chamber (cup horn) (b) with capacity for 8 microfuge tubes (c).

100 mL of digestion buffer: Mix 5 mL of 1 M Tris–HCl (pH 8.0), 2 mL of 0.5 M EDTA, 5 mL of 10% SDS and dnase free water.

Procedure

Step 1 (day 1). DNA sonication

1. Sonicate purified genomic DNA using the Fisher Sonicator (Fig. 1a–c):
   a. Dilute 0.5–8 μg genomic DNA in 80 μL deionized water in a 1.5 mL microcentrifuge tube.
   b. Set Sonifier to 20% amplitude.
   c. Sonicate with pulses of 1 s on/off for a total of 2 series 110s in presence of ice in the chamber (Fig. 1b), replenishing it in between series. Rack has to be hanging from the chamber and not in contact with the probe (Fig. 1b).
2. Run 4 μL (300 ng) sonicated DNA and 100 ng unsonicated DNA (ladder) on 1.5% agarose gels, or 1 μL of sonicated DNA in the bioanalyzer, to verify fragment size of 200–800 bp. Verifying fragmentation with bioanalyzer saves considerable amount of time and DNA material in comparison with agarose gels.

Step 2 (day 1). Incubation of DNA with anti-methylcytidine antibody

3. Add 330 μL TE buffer to 70 μL (5.25 μg) of the sonicated DNA.
4. Heat-denature for 10 min at 95 °C, and immediately cool on ice for 5 min.
5. To the denatured sonicated DNA:

a. Add 100 μL of 5× IP buffer.
b. Add 5 μg (2.5 μL) of anti-methylcytosine antibody.
c. Incubate the DNA–antibody mixture overnight on a rotating platform at 4 °C. Rotate at a low enough speed to prevent significant foaming.

Step 3 (day 2). Binding of agarose beads to DNA:antibody complex

6. Shake the bottle of agarose beads to resuspend them.
7. Transfer 80 μL to a 1.5 mL centrifuge tube and centrifuge at 6000 rpm for 2 min at 4 °C.
8. Remove the supernatant and discard.
9. Add 500 μL of DNA–antibody mixture from step 2 to the beads.
10. Incubate 2 h on a rotating platform at 4 °C.

Step 4 (day 2). Washing and proteinase K digestion of the DNA:antibody:beads complex

11. Wash beads two times with 1× IP buffer as follows:
    a. Centrifuge the reaction from step 3.10 at 6000 rpm for 2 min at 4 °C and discard supernatant.
    b. Add 1 mL 1X IP buffer.
    c. Incubate 5 min on a rotating platform at 4 °C.
    d. Centrifuge at 6000 rpm for 2 min at 4 °C. Carefully remove the supernatant while not disturbing the pellet.
    e. Repeat the above steps twice more.
12. Resuspend the beads in 210 μL of digestion buffer.
13. Add 20 μL proteinase K (20 mg/mL) to the re-suspended beads. If the cell type used is sperm cells add 23 μL of DTT 0.1 M, which will disrupt the sulfur bonds in the coating of these cells.
14. Incubate for 2 h on a rotating platform at 55 °C (make sure to seal lids to avoid leaking).

Step 5 (day 2). DNA purification

15. Filter all the content of the previous reaction with the Pierce spin-filtering columns at max speed for 30 s, at RT. Discard the column and preserve the flow through.
16. To the flow through add 3 μL glycogen (5 mg/mL) and mix well.
17. Add 20 μL 5 M NaCl and then 750 μL ethanol and mix well. It is important to perform this step using ice cold reagents.
18. Precipitate the DNA for 30 min. This can be performed on ice or at room temperature (see discussion about these options on the section "Step 5. DNA purification")
19. Centrifuge at 14,000 rpm for 30 min at 4 °C. Carefully remove the supernatant, while not disturbing the pellet.
20. Completely dry samples in the heating block at 50 °C for 5 min.
21. Resuspend in 30 μL H_2O. Incubate in the heating block at 50 °C for 5 min and measure the DNA concentration. The expected yield is 300–500 ng (10–15 ng/μL).

Observations and detailed optimization measures taken in each step of the protocol

Input DNA sample amounts

We constructed a curve based on data testing the use of different amounts of DNA as input ranging from to 0.5 to 8 μg. All the different amounts of input DNA used yielded measurable amounts of output MeDIP DNA (Fig. 2). Therefore, the amount of input DNA should be chosen within the range of 0.5–8 μg, based on the availability of input DNA and the MeDIP DNA amounts needed for further sequencing. The DNA used in the present optimization was from chicken (*Gallus gallus*) blood.

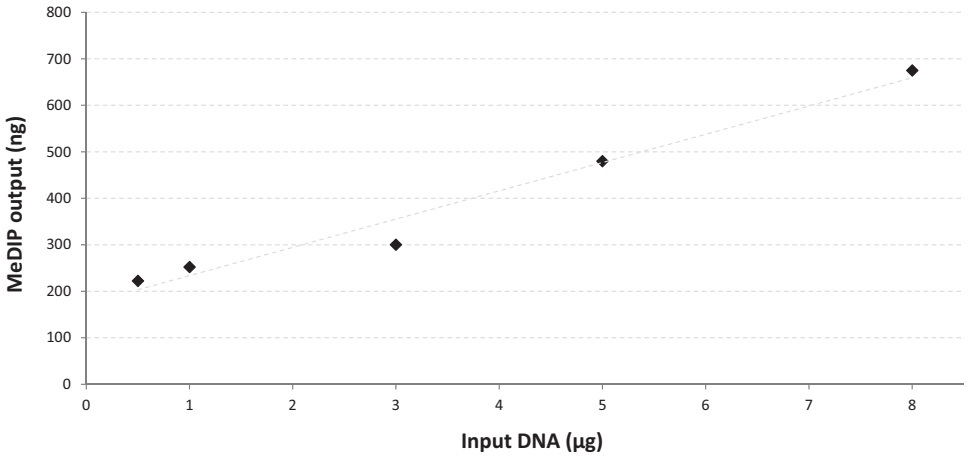

Fig. 2. Correlation between the amount of DNA used as input for the MeDIP procedure and the DNA enriched for DNA methylation obtained as output.

Step 1. DNA sonication

We currently use the ultra-sonicator from Fisher (700 W sonic dismembrator) attached to a cooling chamber (cup horn) with capacity for 8 microfuge tubes (Fig. 1a–c). This set up was chosen due to the fact that this machine does not need insertion of the probe inside the sample for the sonication, thereby, avoiding cross-contamination and reducing processing times. Eight samples can be sonicated simultaneously with this device. Also the cost of this machine is considerably lower compared to similar options from other companies (e.g. Bioruptor from Diagenode, M220 Focused-ultrasonicator from Covaris).

Step 2. Incubation of DNA with anti-methylcytosine antibody

The antibody used (monoclonal mouse anti 5-methylcytosine from Diagenode) is for mouse but seems to bind methylated DNA irrespective of the species. Datasheet from the company shows binding to human and mouse DNA. We have tested it positively in DNA from rats [6,7], Darwin finches [9] and chickens (current set of samples).

Our usual protocol uses overnight incubation of the DNA with the antibody. Because some protocols have described reduced incubation times with the anti-methylcytosine antibody [1,4,10], we tested 2.5 h versus overnight incubations. We performed MeDIP on two blood samples from chickens, each tested with both incubation times. The coverage of samples sequenced was evaluated with the R script MEDIPS. We found that the percent of CpGs not covered in the genome was considerably increased with the short incubation time. Moreover, the fraction of highly covered CpGs (>5X) was considerably increased with the overnight incubation (Fig. 3).

Step 3. Binding of agarose beads to DNA:antibody complex

This was one of the major optimization steps performed in the protocol. Previously [5–7,9], we performed series of four washes of the agarose beads with 2% bovine serum albumin (BSA) in the 1× IP buffer in order to avoid unspecific binding of the beads. Since Santa Cruz agarose beads are pre-coated with BSA, we performed tests of non-specific binding in order to determine the need of performing these washes. In these tests, fragmented DNA was incubated with the agarose beads in absence of the antibody to determine the degree of non-specific binding. It was observed that agarose beads bind non-specifically to approximately 3–6% of the input DNA, in spite of the number of washes (0, 1 or 4) (Fig. 4).

Step 4. Washing and proteinase K digestion of the DNA:antibody:beads complex

In this step it was tested how many washes with 1× IP buffer were truly needed for a complete elimination of DNA unbound to the DNA:antibody:beads complex. It was found that the number of

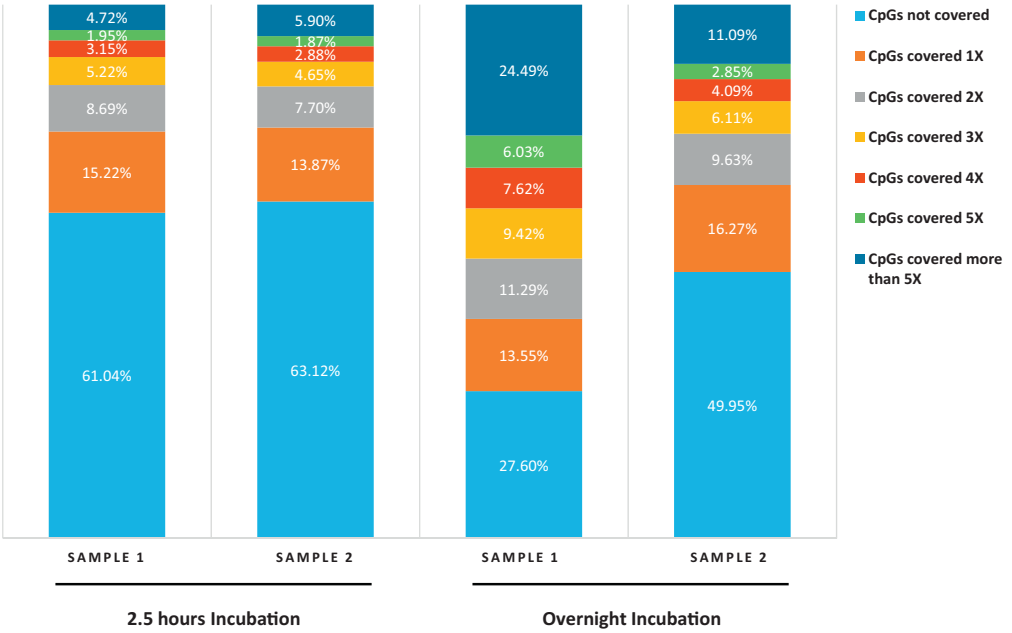

Fig. 3. Bioinformatic analysis to identify differences in the coverage of CpGs after sequencing MeDIP material using two incubation times with the antibody (2.5 h and overnight).

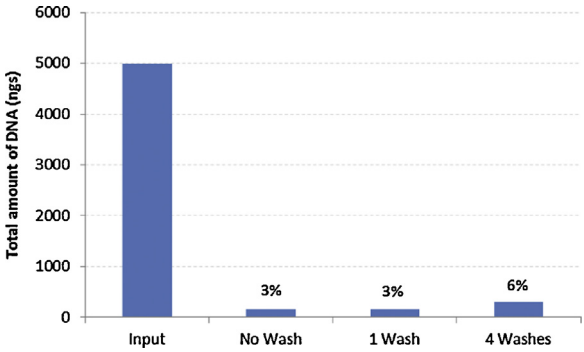

Fig. 4. Test of non-specific binding of fragmented DNA to the agarose beads. Plot shows the DNA obtained from the agarose beads in absence of the antibody, after pre-treating the beads with different conditions (no wash, or 1 or 4 washes with PBS-BSA).

washes used previously, i.e. three, was appropriate in order to eliminate all DNA unbound to the complex DNA:antibody:beads (Fig. 5). The proteinase K digestion was also optimized. Initially we performed the digestion reaction overnight at 55 °C. However, we found that the same efficiency is obtained with a 2 h reaction, incubating at the same temperature.

Step 5. DNA purification

The final step for the optimization of the MeDIP protocol was improving the DNA precipitation procedure, which initially was lengthy, performed with toxic reagents such as phenol and chloroform:isoamyl alcohol, and included two events of ethanol precipitation. The optimization eliminated these steps and introduced the filtering out of the agarose beads immediately after the digestion reaction with protein K. After a wide range of columns and other methods were tested, the

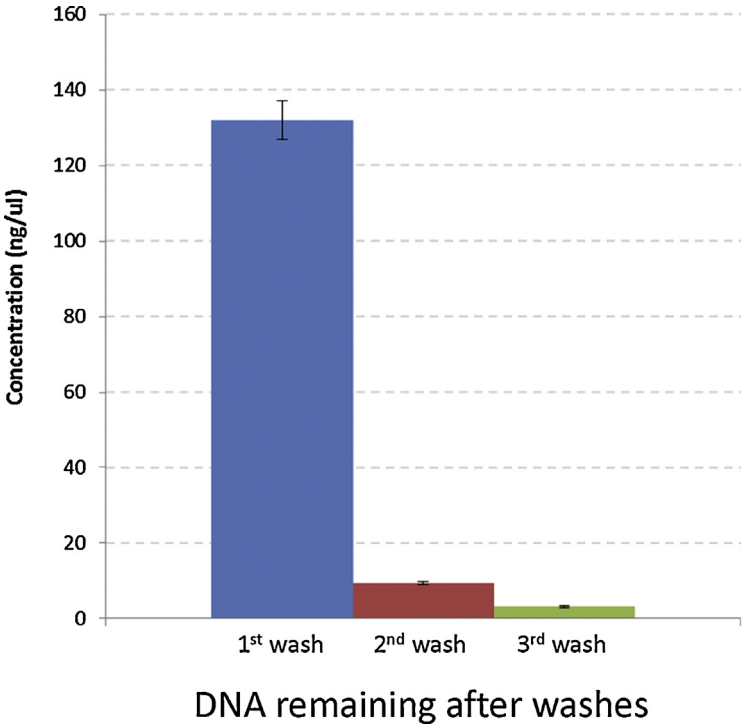

Fig. 5. Test of washing conditions of the DNA:antibody:beads complex. Plot shows unbound DNA remaining after each washing step with 1× IP buffer.

spin-filter Pierce columns produced optimal results for separating the agarose beads from the solution. At this stage, it is very important to precipitate the DNA with methods that do not overestimate the absorbance reading, due to the small amount of DNA being measured. The Pierce columns achieved this because did not introduce any other reagents to the mixture. Finally, because the mixture was cleaner from the start, only one step of ethanol precipitation was needed in order to obtain pure sample of DNA enriched for methylation, thereby reducing inherent losses that occur with each event of precipitation.

Also, because of the low amount of DNA to be precipitated, it is very important that the parameters for ethanol precipitation are carefully chosen. Precipitation was performed on ice or at room temperature instead of negative temperatures, based on previous studies that evaluated ethanol precipitation of the DNA [11,12]. These studies reported that incubation with higher temperatures (0–4 °C) generates better yield than incubations with extremely low temperatures (−20 or −80 °C). Concordantly, our best results were obtained with incubations on ice or even room temperature. However, precipitation at room temperature seems to give a slightly cleaner absorbance curve, therefore, it is the condition we actually employ in our experiments. It is also important to mention that when solutions previously maintained at room temperature were added, the yield was significantly reduced. Therefore, even though the incubation was performed at room temperature, it seems important that the solutions used for the ethanol precipitation are kept cold in advance.

Acknowledgements

The authors are grateful of inputs provided by Dr. Dominic Wright and Andrey Höglund. The authors greatly appreciate funding support by the European Research Council Advances Research Grant Genewell 322206, held by Dr. Per Jensen.

MethodsX thanks the reviewers of this article for taking the time to provide valuable feedback.

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