Video Article

Methylated DNA Immunoprecipitation

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URL: http://www.jove.com/video/935
DOI: doi:10.3791/935

Keywords: Cell Biology, Issue 23, DNA methylation, immunoprecipitation, epigenomics, epigenetics, methylcytosine, MeDIP protocol, 5-methylcytosine antibody, anti-5-methylcytosine, microarray

Date Published: 1/2/2009

Citation: Thu, K.L., Vucic, E.A., Kennett, J.Y., Heryet, C., Brown, C.J., Lam, W.L., Wilson, I.M. Methylated DNA Immunoprecipitation. *J. Vis. Exp.* (23), e935, doi:10.3791/935 (2009).

Abstract

The identification of DNA methylation patterns is a common procedure in the study of epigenetics, as methylation is known to have significant effects on gene expression, and is involved with normal development as well as disease [1-4]. Thus, the ability to discriminate between methylated DNA and non-methylated DNA is essential for generating methylation profiles for such studies. Methylated DNA immunoprecipitation (MeDIP) is an efficient technique for the extraction of methylated DNA from a sample of interest [5-7]. A sample of as little as 200 ng of DNA is sufficient for the antibody, or immunoprecipitation (IP), reaction. DNA is sonicated into fragments ranging in size from 300-1000 bp, and is divided into immunoprecipitated (IP) and input (IN) portions. IP DNA is subsequently heat denatured and then incubated with anti-5'mC, allowing the monoclonal antibody to bind methylated DNA. After this, magnetic beads containing a secondary antibody with affinity for the primary antibody are added, and incubated. These bead-linked antibodies will bind the monoclonal antibody used in the first step. DNA bound to the antibody complex (methylated DNA) is separated from the rest of the DNA by using a magnet to pull the complexes out of solution. Several washes using IP buffer are then performed to remove the unbound, non-methylated DNA. The methylated DNA/antibody complexes are then digested with Proteinase K to digest the antibodies leaving only the methylated DNA intact. The enriched DNA is purified by phenol:chloroform extraction to remove the protein matter and then precipitated and resuspended in water for later use. PCR techniques can be used to validate the efficiency of the MeDIP procedure by analyzing the amplification products of IP and IN DNA for regions known to lack and known to contain methylated sequences. The purified methylated DNA can then be used for locus-specific (PCR) or genome-wide (microarray and sequencing) methylation studies, and is particularly useful when applied in conjunction with other research tools such as gene expression profiling and array comparative genome hybridization (CGH) [8]. Further investigation into DNA methylation will lead to the discovery of new epigenetic targets, which in turn, may be useful in developing new therapeutic or prognostic research tools for diseases such as cancer that are characterized by aberrantly methylated DNA [2, 4, 9-11].

Video Link

The video component of this article can be found at http://www.jove.com/video/935/

Protocol

DNA EXTRACTION AND SAMPLE PREPARATION

DNA from a variety of different samples (cultured cells, fresh frozen as well as formalin-fixed paraffin embedded tissues) can be used for MeDIP. It is important to use highly purified DNA without associated proteins such as histones. It is also important to remove as much RNA as possible from the sample, as it can interfere with both DNA quantitation and antibody binding. The quantity of DNA used for MeDIP can range from 200 ng to 1 µg depending on the amount of DNA available. To demonstrate this protocol, 1 µg of DNA will be used. The following protocol will provide high quality double stranded DNA from cultured cells. Other protocols should be employed for extraction of DNA from other sample types.

1. Add 400 µl of digestion buffer to a cell pellet in a 1.7 ml Eppendorf tube.
2. Add 100 µg of proteinase K to the tube, and incubate overnight at 50°C.
3. Add 500 µl phenol pH 7 and mix gently but thoroughly by inverting.
4. Spin at 13 000 g for 10 mins at room temperature.
5. Remove aqueous (top) fraction to a new tube.
6. Repeat Steps 3 through 5 once.
7. Add 500 µl 1:1 phenol/chloroform pH 7 and mix gently but thoroughly by inverting.
8. Spin at 13 000 g for 10 mins at room temperature.

9. Remove aqueous (top) fraction to a new tube.
10. Add 40 µg of RNase A and incubate 1 hr at 37°C.
11. Add 500 µl phenol pH 7 and mix gently but thoroughly by inverting.
12. Spin at 13 000 g for 10 mins at room temperature.
13. Remove aqueous (top) fraction to a new tube.
14. Repeat Steps 11 through 13 once.
15. Add 500 µl 1:1 phenol/chloroform pH 7 and mix gently but thoroughly by inverting.
16. Spin at 13 000 g for 10 mins at room temperature.
17. Remove aqueous (top) fraction to a new tube.
18. Add 1/10th volume 3 M sodium acetate (40 µl) and mix well.
19. Add 2 volumes (900 µl) of 100% ethanol, mix well, and place at -20°C for 20 mins.
20. Spin at 13 000 g for 20 mins at 4°C.
21. Remove ethanol, pulse spin, and remove residual ethanol.
22. Add 500 µl cold 70% ethanol to wash. Spin at 13 000 g for 20 mins at 4°C.
23. Remove 70% ethanol, pulse spin, and remove residual ethanol by pipetting.
24. Air dry the pellet with the cap open for 10 mins at room temperature to remove all traces of residual ethanol.
25. Resuspend DNA in 50 µl of sterilized dH2O overnight at 4°C.
26. Quantify the DNA using a NanoDrop Spectrophotometer. An A_{260}:A_{280} ratio of 1.8 is ideal.
27. Determine DNA quality and size range on an agarose gel with 100 bp ladder. As little as 10 ng of genomic DNA can be run on a 1.7% agarose gel followed by staining using a dye that is highly sensitive to small amounts of DNA such as SYBR Gold.
28. In one siliconized tube per sample, prepare 1 µg of DNA in a total volume of 50 µl with the remainder of the volume made up with sterilized dH_2O.

DNA SONICATION

DNA sonication and the MeDIP protocol must be performed in siliconzied tubes to prevent non specific binding of proteins to tube walls. Optimal sonication times for the DNA samples are based on the degree of DNA sample fragmentation as determined from gel electrophoresis (Step 27). For example, DNA extracted from cultured cells should be of very high molecular weight, and will subsequently require more sonication than DNA extracted from archival samples which are often partially degraded. If samples are of uniformly high molecular weight, it is reasonable at this point to proceed with the sonication as described in this protocol without checking each sample individually. If samples are fragmented as with archival samples, it will be necessary to adapt the sonication procedure likely by decreasing sonication times to obtain 300-100bp fragments. If you expect to process samples that are partially degraded, optimization of sonication parameters can be performed on a representative sample from those of interest. Based on the degree of DNA fragmentation observed from gel electrophoresis (Step 27), the experimenter can predetermine the optimal sonication time for the sample. Here we describe a method to obtain 300-1000 bp DNA fragments by sonication with an automated sonicating device (Bioruptor from Diagenode, UCD-200 TM) using high molecular weight DNA.

1. Water in Biorupter must be at 4°C.
2. Sonicate for 7 mins on automatic settings (30 sec on 30 sec off at maximum power).
3. Remove 800 ng (40 µl) of sonicated product and place in siliconized 1.7 ml centrifuge tube for the immunoprecipitation (IP) reaction.
4. Set aside remaining 200 ng (10 µl) to serve as input (IN) reference DNA (store at 4°C).

IMMUNOPRECIPITATON OF METHYLATED DNA

1. Denature the DNA that will be used for IP reaction (800 ng) at 95°C for 10 mins in a water bath.
2. Cool immediately on ice. Let DNA cool completely (approximately 5 mins on ice) before proceeding with next step.
3. Add 5 µg monoclonal antibody.
4. Add IP buffer (used at room temperature throughout this protocol) to a final volume of 500 µl.
5. Incubate for 2 hrs at 4°C in rotating tube holder.
6. Just before step 5 is complete, prepare Dynabeads by washing (Steps 6-11). First, resuspend the beads thoroughly in the vial by vortexing.
7. Transfer 30 µl (~ 2 x 10^7) of resuspended Dynabeads per reaction plus 1, into a new siliconized tube (for example, if doing 8 reactions, remove enough beads for 9, i.e. 270 µl).
8. Place the tube on the magnetic rack for 2 mins at room temperature.
9. Pipette off the supernatant. When removing supernatant, avoid touching the beads against inside wall (where the beads attract to the magnet) with the pipette tip.
10. Remove the tube from the magnet, and resuspend the beads in an excess volume of IP buffer (750 µl-1000 µl). Place the tube back on the magnetic rack for 2 mins at room temperature.
11. Repeat the wash once more, and then resuspend the washed beads in IP buffer in the original volume removed in Step 7.
12. Add 30 µl of washed Dynabeads to each IP reaction
13. Incubate in a rotating tube holder for 2 hrs at 4°C.
14. After incubation is complete, place the tube on the magnetic rack for 2 mins at room temperature.
15. Pipette off the supernatant. Avoid touching the inside wall of the tube (where the beads attract to the magnet) with the pipette tip. Add 500 µl of IP buffer. Mix the tube contents and put it back on the magnetic rack for 2 mins. Repeat wash with 500 ul IP buffer one time.
16. After removing the supernatant from the last wash, resuspend the beads in 400 µl of digestion buffer.
17. Treat the reaction with 100 µg of Proteinase K and incubate overnight at 50°C.

PURIFICATION OF IMMUNOPRECIPITATED DNA

1. Add 500 µl 1:1 phenol/chloroform pH 7 and vortex thoroughly.

2. Spin at 13 000 g for 10 mins at room temperature.
3. Remove aqueous (top) fraction to a new tube.
4. Repeat Steps 1 through 3 if the interphase between the aqueous and organic layers appears cloudy.
5. Add 1/10th volume 3 M sodium acetate (40 µl) and vortex.
6. Add 1 µl of glycogen (20 µg/µl) and vortex.
7. Add 2 volumes (1000 µl) of 100% ethanol, vortex, and place at -20°C for 20 mins.
8. Spin at 13 000 g for 20 mins at 4°C.
9. Remove ethanol, pulse spin, and remove residual ethanol.
10. Add 500 µl cold 70% ethanol to wash. Vortex briefly, and spin at 13 000 g for 20 mins at 4°C.
11. Remove 70% ethanol, pulse spin, and remove residual ethanol by pipetting.
12. Air dry the pellet with the cap open for 10 mins at room temperature to remove all traces of residual ethanol.
13. Resuspend DNA pellet in 10 µl sterilized dH_20.

VALIDATION BY PCR

1. You may test to ensure that your MeDIP procedure is working by performing the MeDIP protocol using normal human DNA (male or female), and subsequently assaying a region known to be enriched for methylation.
2. Remove 30% of MeDIP product to PCR tube, and another 30% of MeDIP product to another PCR tube.
3. Put 10 ng of IN DNA into a PCR tube, and 10 ng of IN DNA into another PCR tube. You should now have four separate PCR reactions to set up.
4. Perform PCR using H19 and CTRL primers as indicated in Table 1.
5. Prepare two mastermixes (one for each primer set) for PCR reactions with 12.5 µl total volume as outlined in Table 2.
6. Thermocycle the PCR using the conditions outlined in Table 3.
7. Run 5 µl of PCR products on a 2% agarose gel for visualization.
8. The expected results for successful MeDIP are shown in Table 4.

Tables

Table 1: H19 and CTRL primers for PCR validation.

Primer Set	Forward Primer	Reverse Primer	Anticipated Product Size
H19	H19_F 5'-cgagtgtgcgtgagtgtgag	H19_R 5'-ggcgtaatggaatgcttgaa	174 bp
CTRL (control)	CTRL_F5'-gagagcattagggcagacaaa	CTRL_R 5'-gttcctcagacagccacattt	139 bp

Table 2. Mastermixes for PCR reactions.

	H19 Mix (per Rxn)	CTRL Mix (per Rxn)
ddH_2O	6.875	6.875
10X Buffer	1.25	1.25
dNTP mix (10 mM each)	0.25	0.25
$MgCl_2$ (50 mM)	0.25	0.25
Primers (H19_F/R or CTRL_F/R) (10 µM each F/R)	0.625	0.625
Platinum Taq	0.25	0.25

Table 3. PCR thermocyling conditions.

	95°C	5:00
40X	95°C	0:30
	56°C	0:30
	72°C	0:15

Table 4. Expected PCR results for successful MeDIP.

	Template DNA	
Primer used	IN	IP
H19	Positive	Positive
CTRL	Positive	Negative

Discussion

There is a growing awareness of the significant role DNA methylation plays in disease, therefore the development of assays to measure this modification are becoming increasingly important [3, 12, 13]. The MeDIP technique is an amenable tool for screening at both the whole-genome and locus-specific level [6, 7]. This technique provides a rapid view of DNA methylation levels using limited amounts of starting DNA and allows for easy comparisons between different sources. Downstream applications using the MeDIP product include a variety of microarrays such as whole genome and CpG island oligonucleotide arrays, direct PCR assays for loci of interest, as well as sequencing.

MeDIP provides a distinct approach to DNA methylation detection that relies upon antibodies to distinguish methylated and unmethylated DNA [6]. While MeDIP is faster than traditional bisulfite sequencing approaches and it is not limited to the analysis of specific sequences like restriction enzyme analysis, the immunoprecipitation will be dependent on DNA sequence including CpG density, repetitive element presence and composition. Thus, appropriate controls, as with every experiment, are important to the analysis and interpretation of MeDIP results.

There are several steps throughout the MeDIP technique where extra care must be taken. These include: the use of siliconized tubes to prevent non-specific binding of DNA to tube walls; ensuring adequate fragmentation of DNA after sonication; and ensuring that DNA is completely denatured. Additionally, note that quantification of single-stranded MeDIP product may be difficult because there is a limited amount of material and many common techniques for estimating DNA quantity, such as spectrophotometry, work best with double-stranded DNA. Additionally, it is important to observe proper laboratory safety practices at all times, especially when caustic substances such as phenol are used.

The MeDIP technique can also be modified at several steps. Importantly the protocol can be scaled up or down to allow increased yields, or work with limited sample sizes. An alternative fragmentation approach, such as the use of restriction enzymes (e.g. *Alu*I digestion), reduces equipment requirements, but can also introduce biases potentially limiting DNA pull down in some regions. As with any approach, results are best validated using an alternative approach to DNA methylation detection [1, 14].

Acknowledgements

We wish to thank members of the Brown and Lam labs for their participation in critiquing this video and article. This work was supported by funds from the Canadian Institutes for Health Research and the Michael Smith Foundation for Health Research.

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