Standard DNA methylation analysis in mouse epidermis: bisulfite sequencing, methylation-specific PCR and 5-methyl-cytosine (5mC) immunological detection

Jesús Espada $^{1,2,*}$, Elisa Carrasco $^{1,2}$ and María I. Calvo $^{1,2}$

$^1$ Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain

$^2$ Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC-UAM, Madrid, Spain

* Corresponding author: Jesús Espada, Instituto de Investigaciones Biomédicas “Alberto Sols” CSIC-UAM, Arturo Duperier 4, 28029 Madrid, Spain. Fax.: +34 91 585 4400. Email: jespada@iib.uam.es

Short title: Standard DNA methylation analysis in mouse epidermis

Key words: DNA methylation, 5-methyl-cytosine (5mC9, mouse epidermis, hair follicle, bisulfite sequencing, immunological detection

Number of figures: 2.

Fig. 2 must be printed in color (paper edition).
Chapter …

Standard DNA methylation analysis in mouse epidermis: bisulfite sequencing, methylation-specific PCR and 5-methyl-cytosine (5mC) immunological detection

Jesús Espada, Elisa Carrasco and María I. Calvo

Summary

In mammals, methylation of cytosine C-5 position is a major heritable epigenetic mark on the DNA molecule. Maintenance of proper DNA methylation patterns is a key process during embryo development and in the maintenance of adult tissue homeostasis. The use of experimental procedures based on the chemical modification of cytosine by sodium bisulfite and the development of antibodies recognizing 5mC have essentially contributed to our knowledge on DNA methylation dynamics in normal and disease states. Here we describe standard procedures for bisulfite sequencing, methylation-specific PCR and 5mC immunodetection using mouse skin and the hair follicle stem cell niche as model tissues.

1. Introduction

Methylation of the C-5 position of cytosine in CpG nucleotides is a major epigenetic mark in mammalian genomes implicated in key transcriptional regulation events during embryo development and in the maintenance of adult tissue homeostasis [1, 2].
Consequently, deregulation of DNA methylation patterns severely impairs cell and tissue viability and is associated to carcinogenesis [1-3]. At present, DNA methylation is considered the most stable and the only demonstrated as mitotically heritable epigenetic mark in mammals. However, the recent discovery that the methylated C-5 position of cytosine can undergo further dynamical changes in the form of hydroxy-methylation [4], that can act as an intermediate in CpG demethylation processes, adds additional complexity levels to the regulatory roles of DNA methylation in the cell.

In a typical, non-transformed mammalian somatic cell, 5-methyl-cytosine (5mC) comprises a roughly 0.75-1% of all the nucleotides in the DNA molecule. Most of this 5mC content is found in CpG dinucleotides and 60-90% of CpG sites are methylated [1]. 5mC is largely concentrated in the repetitive fraction of the mammalian genome, expanding satellites α (alphoid DNA) and β (Sau3A family) in centromeric heterochromatin, LINES (LINE1 and 2 families), SINES (Alu and Mir families) and parasitic transposon and retroviral sequences [1]. An unsuspected high density of CpG dinucleotides, the so-called CpG islands, is present in the 5´ promoter region of about 40% mammalian genes [5, 6]. A significantly elevated cytosine methylation ratio at these CpG islands has been causally associated to strong transcriptional repression [5, 6]. Although a consistent methylation of CpG islands is consistently found only on imprinted genes and on a small proportion of non-imprinted genes, the maintenance of proper global CpG methylation patterns is thought to be essential in every step of development and in adult somatic tissues [5, 6].

The implementation of robust methodologies based on the chemical modification of non-methylated cytosines in the DNA molecule by sodium bisulfite treatment and subsequent conversion to uracil, including bisulfite sequencing [7] and methylation-specific PCR [8] (Fig. 1), has enormously contributed to the improvement of biochemical DNA methylation analysis at a single-nucleotide resolution scale. These methodologies have also paved the way for the development of power techniques for
large-scale, genome wide analysis of global changes in DNA methylation patterns during normal development or in disease states using Next Generation Sequencing (NGS) approaches [3, 9].

Similarly, the generation of antibodies specifically recognizing 5mC [10] is a key landmark in the field allowing a precise intranuclear compartment localization and quantification of this modified nucleotide in cell culture and tissue samples [11, 12]. In the same way, these type of antibodies have foster the development of large-scale immunological methods for the immunoprecipitation of DNA methylated regions in the chromatin (methylated DNA immunoprecipitation; MeDIP) [13]. Immunoprecipitated DNA-methylated chromatin can further constitute the start material for high-throughput, genome wide scale analysis using high-resolution DNA microarrays (MeDIP-chip) or NGS (MeDIP-seq) [14].

As a straight consequence of the successful completion of several whole-genome sequencing projects in the last decade, we are witnessing to an unprecedented emergence of vast amounts of data related to chromatin dynamics and gene transcriptional regulation. In the context of DNA methylation, data obtained from different high-throughput experimental approaches at genome wide scales are of unquestionable analytical value, but require correct experimental validation to be interpreted in an adequate biological background. In this sense, standard methods for DNA methylation analysis not only are highly informative in different experimental situations but also are the methods of choice to validate high-throughput data. Here we describe standard procedures for bisulfite sequencing and 5mC immunolocalization using mouse skin and the hair follicle as biological models.

2. Materials

2.1. Isolation of epidermis from mouse tail skin.
1. C57BL/6j mice.

2. Sterile water.

3. PBS, 5mM EDTA in PBS, 0.02 % sodium azide in PBS.

4. Insulin type syringes, needles, sterile surgical scalpel blades, scissors and tweezers.

**2.2. DNA extraction, sodium bisulfite sequencing and methylation-specific PCR**

1. 3M NaOH (Merck).

2. 3M NaC₂H₃O₂ (sodium acetate, Merck).

3. 10M AcNH₄ (ammonium acetate, Merck).

4. 10 mg/ml glycogen (Roche).

5. Ethanol (Merck).


7. 16mM hydroquinone (Sigma).

8. 4M NaHSO₃ pH 5.0 (sodium bisulfite; Sigma; see **Note 1**).

9. 10% SDS.

10. Proteinase K Buffer: 50mM Tris-HCl pH 8, 100mM EDTA, 100mM NaCl.

11. Proteinase K (Roche).

12. RNase A (Roche).

13. Phase Lock Gel 2ml tubes (Eppendorf).

14. 2mM dNTPs.
15. Bisulfite-sequencing or methylation-specific oligonucleotides (see Note 2).

16. Taq DNA Polymerase of choice (in our case, FastStart Taq DNA Polymerase set, Roche, including 10x Taq polymerase buffer and 50 mM MgCl₂)

17. Wizard DNA clean-up kit (Promega).

18. QIAquick Gel Extraction kit (Qiagen).

19. QIAGEN Plasmid Mini kit (Qiagen).

20. pGEM-TEasy Vector System kit (Promega).

21. JM109 competent cells.

22. LB plates with ampicillin/IPTG/X-gal.

23. DNA 100bp ladder (Invitrogen).

24. Standard 1xTBE DNA electrophoresis buffer.

25. For DNA electrophoresis, use 0.8% agarose (Pronadisa) in 1xTBE buffer containing 1x SYBR® Safe DNA gel stain (Invitrogen).

26. DNA electrophoresis equipment

27. Heating blocks.

28. Polytron.

29. Rotator wheel.

30. Refrigerated tabletop minifuge.

2.3. Immunodetection of 5mC in epidermal whole-mounts.

1. 1N HCl (Merck).
2. 1x Tris-borate-EDTA (TBE) buffer.

3. Fixation solution: 3.7 % formaldehyde (Merck) in PBS (see Note 3).

4. Permeabilization and blocking (PTG) buffer solution: 0.5 % Triton X-100 (Sigma), 0.2% gelatin (Merck) in PBS (see Note 4).

5. Specific antibodies raised against 5-methyl-cytidine or 5-methyl-cytosine (see Note 5)

6. Mounting medium: Vectashield (Vector Labs)-DAPI (Sigma) (see Note 6).

7. Leica TCS SP2 or SP5 AOBS Laser Scanning Spectral Confocal Microscopes.


   http://www.csc.mrc.ac.uk/microscopy/links.html


3. Methods

3.1. Isolation of epidermis from mouse tail skin

This procedure essentially follows the guidelines stated by Braun et al. [15].

1. Sacrifice the mice following the guidelines of the corresponding housing animal facility, cut the tail and peel the skin in a whole piece from the tail backbone.

2. Incubate the peeled tail skin in PBS with 5 mM EDTA at 37 °C for 4 h.

3. Separate the epidermis from the dermis using tweezers as a whole sheet of tissue.

3.2. DNA extraction, sodium bisulfite sequencing and methylation-specific PCR
1. Add 500 µl of Proteinase K Buffer to a 0.5 cm x 0.5 cm piece of fresh or frozen (-80°C) epidermal sheet obtained in step 3.1.3 and homogenize the tissue using a Polytron.

2. Add 50 µl of 10% SDS, 50 µl of a 200 mg/ml Proteinase K stock and 20 µg/ml of RNase A to the homogenate and incubate overnight at 37°C on a rotator wheel.

3. Liquate the jellified homogenate by gently pipetting and transfer the volume to a Phase Lock Gel tube.


5. Transfer to Phase Lock Gel tubes, centrifuge at maximum speed for 5 min in a tabletop minifuge and transfer the aqueous phase supernatant to a 2ml tube.

6. Add 50 µl of 3M sodium acetate and 1.5 ml of 100% ethanol, mix well by inversion and centrifuge at maximum speed in a tabletop minifuge for 10 min at 4°C.

7. Discard the supernatant and wash the DNA pellet two times with 1 ml of 70% ethanol, resuspend the pellet in 50-100 µl of sterile water, evaporate the residual ethanol content by heating the open tube at 50°C for 5 min and quantify the DNA content.

8. Add 1 µg of genomic DNA to a final volume of 50 µl of sterile water.

9. Add 5.7 µl of 3M NaOH to the DNA volume and incubate 10 min at 37°C.

10. Add 33 µl of 16mM hydroquinone and 530 µl of 4M NaHSO₃ and incubate 6-8 h at 50 °C (see Note 7).

11. Purify the DNA using the Wizard DNA-Clean up kit following the instructions of the manufacturer.
12. Elute the DNA in 50 µl of sterile water, add 5.7 µl of 3M NaOH and incubate 15 min at 37°C.

13. Precipitate the DNA by adding 1 µl of 10 mg/ml glycogen, 17 µl of 10M AcNH₄ and 450 µl of 100% ethanol. Mix well by inversion and incubate overnight at -80°C.

14. Centrifuge at maximum speed in a tabletop minifuge, discard the supernatant and wash the DNA pellet two times with 1 ml of 70% ethanol. Resuspend the pellet in 50-100 µl of sterile water, evaporate the residual ethanol content by heating the open tube at 50°C for 5 min and quantify the DNA content.

15. Design methylation-specific nucleotides (see Note 2) and amplify the genomic region of interest from bisulfite modified DNA by PCR (see Note 8).

16. Resolve and separate PCR amplicons by DNA electrophoresis in 0.8% agarose gels. Extract and purify PCR amplicons using QIAquick Gel Extraction kit following the instructions of the manufacturer.

17. Clone each PCR amplicon in JM109 competent cells using the pGEM-TEasy Vector System as indicated in the instructions of the manufacturer. A typical ligation reaction for cell transformation combines 3 µl of purified PCR amplicon, 1 µl of pGEM-T vector, 1 µl of T4 ligase and 2.5 µl of reaction buffer.

18. Plate each transformed clone on LB plates containing ampicillin/IPTG/X-gal exactly as described in the manufacturer’s instructions and growth overnight at 37°C.

19. After the growth of clearly distinguishable white and blue colonies, pick up at least 10 white positive colonies from each individual plate. Inoculate each picked white colony into 1,250 ml of ampicillin containing LB medium and growth overnight at 37°C.

20. Purify plasmids from each grow colony using QIAGEN Plasmid Mini kit and sequence cloned inserts using the method of choice.
3.3. Immunodetection of 5mC in epidermal whole-mounts.

1. Fix the whole sheet of epidermal tissue obtained in step 3.1.3 with 3.7 % formaldehyde in PBS at 4 °C for at least 24 h.

2. Thoroughly wash the tissue with PBS and store at 4 °C in PBS containing 0.02% sodium azide.

3. Cut fixed epidermal sheets into 0.5 cm x 0.5 cm pieces and incubate the epidermal pieces with 1 N HCl for 45 min at 37 °C to hydrolyse chromatin (see Note 9). Wash before and after the incubation with distilled water.

4. Incubate with 1xTBE for 5 min at room temperature (RT) to neutralize acid hydrolysis and thoroughly wash with distilled water.

5. Permeabilize and block tissue pieces by incubating with PTG buffer for 30 min at RT.

6. Incubate tissue samples with specific antibodies recognizing 5mC (see Note 5) diluted 1:50 in PBS overnight at 37 °C.

7. Thoroughly wash samples at least three times in PBS and mount in Vectashield-DAPI on slides facing upwards the inner side of the epidermis. Keep samples in the dark at 4 °C until confocal microscopy analysis.

4. Notes

1. Combine 7.5 gr of sodium bisulfite and 20 ml of sterile H₂O. Adjust solution to pH 5.0 by adding 500 µl of 10M NaOH.

2. Two types of oligonucleotides can be used to amplify by PCR a particular genomic region in a bisulfite-modified DNA pool: bisulfite-sequencing or methylation specific primers.
Methylation-specific primers are designed to produce a PCR amplicon only if a set of CpG nucleotides in the target sequence that are contained in each forward and/or reverse oligonucleotide sequence are transformed (CpG-UpG transition; unmethylated) or not (CpG-CpG transition; methylated) after sodium bisulfite DNA treatment. This Methylation Specific PCR (MSP) can provide a preliminary hint on the CpG methylation status of the genomic region of interest. The sequence of these oligonucleotides should contain a significant number of informative CpG nucleotides.

Bisulfite-sequencing primers are designed to amplify the target sequence regardless of the CpG-UpG transformation degree after bisulfite treatment inside the amplicon. The resulting PCR amplicon is intended for further DNA sequencing and the resulting data provide information on the methylation status of all CpGs in the target genomic region. The sequence of these oligonucleotides must not contain CpGs.

Ideally for both types of oligonucleotides, the annealing temperature (Tm) of each primer pair should be similar (+/-5ºC) on a 55-65ºC range and the size of the amplicon should not exceed 350bp. Different overlapping amplicons can be used to map a particular genomic region of interest that is larger than 400bp. There are different software packages that help to design both types of oligonucleotides. We can recommend Methyl Primer Express software (Applied Biosystems; https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=602121).

3. The fixation solution should be kept in a neutral pH range (6.8-7.5). Acidification of the solution, which progressively takes place at RT, results in a drastic reduction of formaldehyde fixative properties.

4. For PTG buffer preparation, weight 100 mg of gelatin powder, heat 20 ml of PBS at 60 ºC in a glass beaker, slowly pour the gelatin powder into the PBS volume and
thoroughly stir until the solution reaches RT. At this point, complete the PBS volume up to 50 ml, add 250 µl of Triton X-100 and stir for additional 20 min.

5. There are several commercially available antibodies raised against 5-methyl-cytidine or 5-methyl-cytosine that specifically recognize 5mC on the chromatin fiber or on the isolated DNA molecule. Here we have used a non-commercial original batch of a mouse monoclonal antibody raised against 5-methyl-cytidine, kindly supplied by A. Niveleau [10, 11]

6. Prepare a DAPI stock solution, 10 mg/ml in distilled water and store at -20 ºC. Combine 5 µl of concentrated DAPI stock with distilled water to a final volume of 1 ml, obtaining a 50 ng/ml intermediate stock. Add 100 µl of DAPI intermediate stock solution to 900 µl of Vectashield, to obtain 1 ml of mounting medium containing DAPI at 5 ng/ml. Store at 4 ºC in the dark. Samples should be kept in mounting medium a minimum of 30 min before microscopy analysis to ensure that minor groove insertion of DAPI in the DNA molecule reaches a suitable chemical equilibrium for optimal detection. Mounted samples can be stored at -20 ºC in the dark 1-2 weeks showing good emission and resolution efficiencies.

7. Sodium bisulfite induces severe DNA degradation. Longer incubation times (16-18 h, or overnight) proposed in other protocols can enhance this process. In the same, a 4M sodium bisulfite concentration can cause excessive DNA degradation in some tissue and cell types.

8. For amplicons up to 400bp size, guideline standard PCR amplification conditions can be as follows:

Initial denaturation step, 1 cycle, 5 min, 95ºC;

Amplification step, 35 cycles:

Denaturing, 30 sec, 95ºC
Annealing, 30 sec at the lower Tm of the primer pair

Extension, 30 sec, 72ºC

Final extension step, 1 cycle, 5 min, 72ºC

9. Mild acid hydrolysis of chromatin is critical. A short hydrolysis time results in a defective nucleotide epitope unmasking, while excessively longer hydrolysis results in extensive chromatin degradation.

Acknowledgements

This work was supported by grants of the Spanish Ministerio de Economía y Competitividad (SAF 11-23493) and the Comunidad Autónoma de Madrid (SkinModel, CAM S10/BMD-2359) to J.E.; E.C and M.I.C. are supported by PhD fellowship grants of the Spanish Ministerio de Educación and Universidad Autónoma de Madrid, respectively.

References


Figure legends

Figure 1. DNA methylation status of the rDNA gene start promoter region and of the Major Satellite repetitive consensus sequence in normal adult mouse epidermis.

Figure 2. Distribution of 5mC in normal adult mouse telogen hair follicles.
Methylated DNA

Non-Methylated DNA

Sodium bisulfite treatment

MSP-F  MSP-F

MSP-R  MIP-R

MSP-F  MIP-F

MSP-R  MIP-R

PCR amplicon sequencing

A

B

C

Figure 1. Espada et al. 2013
Figure 2. Espada et al. 2013