12

DNA-Methylation Analysis by the Bisulfite-Assisted Genomic Sequencing Method

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1. Introduction

The postreplicative methylation of DNA at the C5 position of cytosines is found in a broad spectrum of organisms ranging from prokaryotes to human ***(1)***. In prokaryotes the major role of cytosine C5 methylation (like adenine N6 and cytosine N4 methylation) is to protect the genome against DNA degrading nucleases (restriction/modification), whereas in many eukaryotes cytosine C5 methylation (found within CpG dinucleotides) plays a pivotal role in the control of gene expression, inactivation of repetitive sequences, stability of chromosomes, and in cell transformation leading to development of cancer. The growing evidence that the cytosine methylation is also crucial in embryonic development of mammals regulating genomic imprinting, X inactivation and cell differentiation ***(2)*** has caused a demand for effective methods that would detect this modification with high sensitivity and reliability.

Original methods to detect sequence specific genomic methylation were based on the digestion of DNA by methylation-sensitive restriction enzymes and subsequent Southern-blot hybridization ***(3)***. Despite rather high amounts of DNA needed for such experiments and the possibility to investigate just the limited numbers of CpGs situated within suitable restriction sites, the method is still useful as the first indication of methylation in a specific region. To improve the sensitivity, the method was combined with polymerase chain reaction (PCR) amplification ***(4)*** and subsequent quantification of PCR products ***(5,6)***. Although the use of PCR decreased the amount of template

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DNA necessary for the analysis, the whole procedure is highly demanding in terms of strictly standardized conditions of DNA preparation and PCR, since quantification is only possible within the exponential phase of amplification. Additionally, incomplete digestion of chromosomal DNA might be a frequent source of artefacts. Another disadvantage of such methods is that they provide data only about the average level of methylation; it is neither possible to discriminate between mosaic or even methylation patterns nor to address hemi-methylation, which remains in general undetected.

The first information about the methylation of cytosine residues irrespective of their sequence context was obtained using a genomic sequencing protocol ***(7)***. This method identifies a position of 5-methylcytosine (5-MeC) in the genomic DNA as a site that is not cleaved by any of the Maxam and Gilbert sequencing reactions ***(8)*** and thus appears as a gap in a sequencing ladder. Although a detailed distribution of methylation in a given sequence can be analyzed by this method, it still requires relatively large amounts of genomic DNA and a certain level of experience in interpreting the sequencing results as bands of varying intensity and shadow bands may occur. An elegant combination of the chemical cleavage method with ligation mediated PCR ***(9)*** increases the sensitivity, but this modification makes the whole procedure rather laborious and technically challenging.

With a bisulphite genomic-sequencing method ***(10,11)***, a qualitatively and quantitatively new approach to methylation analysis has appeared. The bisulphite reaction leads to the conversion of cytosines into uracil residues, which are recognized as thymines in subsequent PCR amplification and sequencing, whereas the modified cytosines do not react and are therefore detected as cytosines. Thus the method allows direct and positive determination of methylation sites in the genomic DNA, as only methylated cytosines are detected as cytosines. Products of PCR-amplified bisulphite-treated DNA can be used directly for sequencing (detection of average methylation status) or cloned and sequenced individually, when the information about the methylation pattern of single molecules is desired. Not only the methylation status of each single molecule but also the pattern of each DNA strand can be investigated, as the strands are no longer complementary following the bisulphite treatment and are amplified and sequenced separately.

Several modifications of the original bisulphite sequencing protocol improving the sensitivity and quality of the results have been published ***(11–15)***. In some cases a direct sequence analysis of the PCR products obtained may be desirable to estimate the average methylation at specific sites. For such direct quantitation Gonzalgo and Jones ***(16)***, proposed an elegant and simple procedure (Ms-SNuPE). A more sophisticated protocol for direct quantitation of sequencing results is described by Paul et al. ***(17)***.

The attributes of high sensitivity, the ability to detect single-molecule methylation patterns as well as the possibility of addressing nonsymmetrical methylation make bisulphite-based genomic sequencing the method of choice for a variety of applications. The following protocol used routinely in our laboratory is based on the previously published procedure ***(14)***; several modifications are included leading to easier handling and less time-consuming experimental procedure.

2. Materials

2.1. Embedding of Material into Agarose and Bisulphite Reaction

1. Trypsin: 0.25% [w/v] in PBS (Biochrom).
2. Mineral oil: heavy white mineral oil (Sigma).
3. LMP agarose (SeaPlaque Agarose, FMC).
4. Proteinase K (Boehringer Mannheim).
5. Hydroquinone (Sigma).
6. Phenylmethylsulphonyl fluoride (PMSF) (Sigma).
7. Sodium disulphite: (Sodium metabisulphite) (Merck).

Note: Batches of commercially available sodium bisulphite are mixtures of sodium bisulphite and sodium metabisulphite. The ratio between the substances may vary among different batches. We recommend working with pure sodium metabisulphite, which facilitates accurate preparation of solutions with the desired molarity.

Common laboratory solutions and buffers like sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), Tris-HCl, pH8.0, NaOH, TE were prepared according to **ref.** ***17a***.

2.2. PCR, Purification, and Cloning of PCR Product

1. Taq polymerase (Boehringer Mannheim).
2. Geneclean II (Bio 101), or equivalent method, for purification of PCR fragments from agarose gels.
3. TA cloning kit (Invitrogene) with INVα F` ultra-competent *Escherichia coli* cells.

For some bisulphite–PCR fragments we observed a clonal selection against fully converted templates. In those cases we were able to overcome the problem using a different cloning vector system (pGEM-T, Promega) in combination with competent Sure *E.coli* cells.

3. Method

3.1. Chemistry of the Bisulphite Reaction

The reaction of cytosine residues with sodium bisulphite leading to selective conversion to uracils was first published in early 1970s ***(18,19)*** for conformational studies of single- and double-stranded regions in DNA and

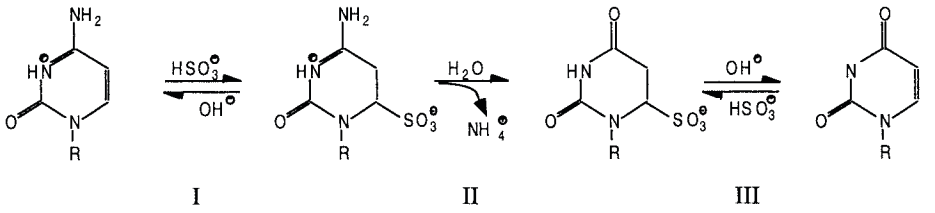

Fig. 1. Chemistry of the reaction steps: I) sulphonation at the position C6 of cytosine, II) irreversible hydrolytic deamination at the position C4 generating 6-sulphonate-uracil, and III) subsequent desulphonation under alkaline conditions. Note that methylation at the position C5 impedes sulphonation at the C6 position (step 1).

RNA *(20–22)*. The reaction generally consists of three major steps (**Fig. 1**): 1) sulphonation, 2) deamination, and 3) desulphonation *(18)*.

1. Reversible sulphonation of cytosine residues to cytosine-6-sulphonate. The sulphonation is favored at low pH and low temperature; at 0°C the equilibrium state is reached within 20 min.
2. Irreversible hydrolytic deamination of cytosin-6-sulphonate to uracil-6-sulphonate. This reaction is favored at higher concentrations of sodium bisulphite and at higher temperatures; the pH optimum is between pH 5.0 and 6.0.
3. Reversible desulphonation of uracil-6-sulphonate to uracil. The elimination reaction is favored at high pH.

Only cytosines in single-stranded DNA (or its components) can be efficiently modified by sodium bisulphite; cytosines in nondenatured, double-stranded DNA are almost refractory to react. Furthermore, under the conditions described, the reaction is highly selective for nonmethylated cytosine residues, which are quantitatively modified (converted to uracils), whereas only 2–3% of 5-methylcytosine residues do react and are converted to thymines *(23)*.

3.2. Principles of the Method

The following protocol is a modified version of the original bisulphite-based methylation-analysis technique described by Frommer et al. *(10)*. As a main difference we routinely embed the material under investigation (i.e., either isolated DNA or intact cells) into low melting point (LMP) agarose. All the following modification steps are performed in the agarose in which the DNA is physically captured. The described modification greatly facilitates the handling of the probes and reduces the loss of DNA in the procedure, thus allowing to work with minute DNA or cell/tissue quantities. The embedding also reduces the reannealing of denatured DNA strands therefore ensuring highest quality and reproducibility in the bisulphite reaction. The principles described in this

paragraph refer to a procedure when working with intact cells (*see* **Subheadings 3.3.** and **3.4.**). A modified version of this protocol (*see* **Subheading 3.4.**) should be applied when using purified DNA.

When intact cells are used as a starting material they are embedded into an agarose, lysed, and treated with Proteinase K to make the genomic DNA accessible for subsequent bisulphite treatment. We recommend including an endonuclease restriction step (with a methylation-insensitive enzyme) to obtain smaller DNA fragments (of about 3–6 kb), which enhances the spatial separation of complementary DNA strands after the following denaturation. Agarose-embedded (and -digested) DNA is denatured by alkaline treatment and boiling. Nonmethylated cytosine residues in single strands are subsequently modified in the presence of 2.5 *M* sodium bisulphite (see experimental procedures concerning preparation of solutions), converted to uracil residues by following alkaline treatment, washed extensively and stored in minimal volumes of TE (**Fig. 1**). The sequence of interest is finally amplified by (nested) PCR. Due to the bisulphite treatment, DNA strands are no longer complementary, and therefore are amplified and analyzed separately (**Fig. 2**). The PCR products can be used directly for sequencing, which allows the quantitation of average values of cytosine methylation at individual positions. Alternatively, the PCR product may be cloned and individual clones sequenced, the latter revealing information about individual chromosomes.

3.3. Preparation of Cells for Bisulphite Treatment

The following procedure should be used when working with limited amount of tissue (containing less than 1 ng of DNA in total) or only a few cells, in which cases the DNA isolation is difficult. When larger quantities of cellular material are available like biopsies, paraffin-embedded tissues, sperm samples, and so on; we recommend first isolating DNA and then proceeding with procedures described in **Subheading 3.4.**

1. When starting with tissue samples, this material should be trypsinized to obtain a single-cell suspension. In cases of individually collected cells (e.g., oocytes, zygotes, etc.) proceed directly to **step 2**.
2. Wash and recover the cells in 1X PBS solution at a maximum density of 60 cells/µL. (In case of oocytes/zygotes, 30–50 cells should be used per agarose bead.)
3. Mix 3 µL of cell suspension with 6 µL of hot (80°C) 2% (w/v) LMP agarose (SeaPlaque Agarose, FMC) (prepared in 1X PBS).
4. Add 500 µL of heavy mineral oil, incubate in boiling water bath for 30 min, and transfer to ice (additional 30 min) to solidify the agarose/cell mixture.
5. Incubate the agarose bead in 500 µL of the lysis solution (10 m*M* Tris-HCl, 10 m*M* EDTA, 1% SDS, 20 µg/mL proteinase K) under the mineral oil at 37°C overnight.

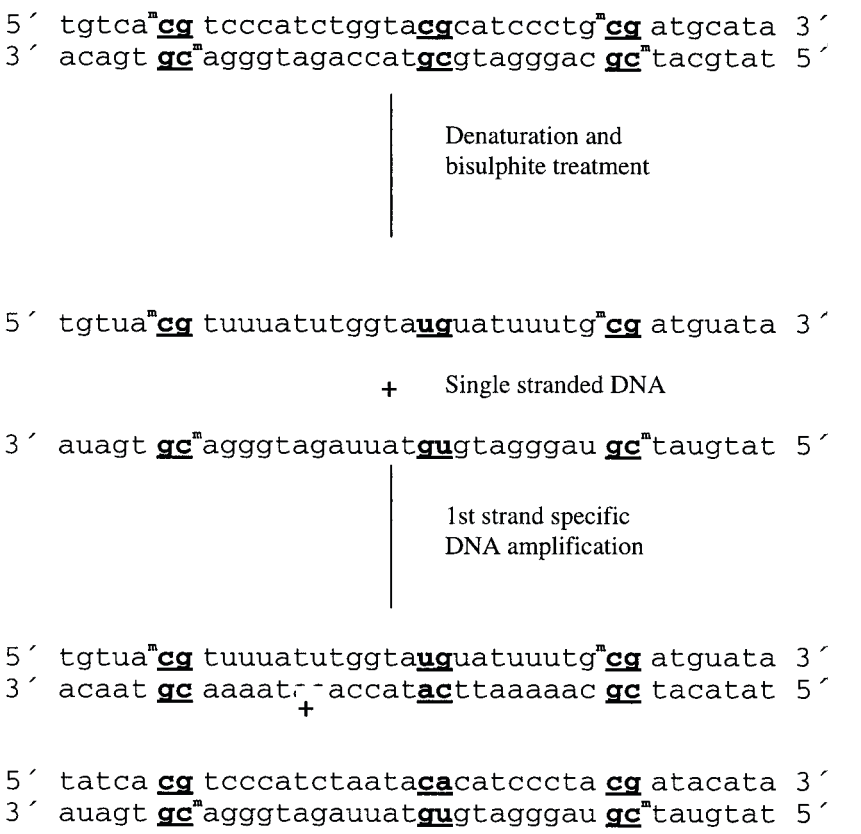

Fig. 2. Schematic diagram of the bisulphite conversion of a DNA sequence; note that the upper and lower strands are no longer complementary after the bisulphite treatment (a, adenine; c, cytosine; ^{m}c, 5-methyl-cytosine; g, guanine; t, thymine; u, uracil).

6. Remove the lysis solution and the oil and inactivate proteinase K in 500 µL of TE, pH 7.0, containing 40 µg/mL PMSF 2 × 45 min at room temperature (RT). (This step is optional.)
7. Remove the solution and wash with 1 mL of 1X TE, pH 9.0, 2 × 15 min (i.e. 2 washes, 15 min each).
8. Equilibrate against 100 µL of restriction buffer 2 × 15 min.
9. Remove the solution and add 100 µL of 1X restriction buffer containing 20 units of restriction endonuclease and incubate overnight. (Alternatively add 50 units for 1 h digestion.)
10. Remove the restriction buffer and incubate with 500 µL of 0.4 *M* NaOH 2 × 15 min.
11. Wash with 1 mL of 0.1 *M* NaOH for 5 min.

12. Remove all the solution and overlay with 500 µL of mineral oil.
13. Boil the beads in a water bath for 20–30 min to separate the individual DNA strands.
14. Chill on ice for 25 min to re-solidify the agarose bead.
15. Prepare the bisulphite/hydroquinone solution according to **Subheading 3.4., step 6**.
16. Add 500 µL of the (ice-cold) bisulphite/hydroquinone solution. The agarose bead should be in the (lower) aqueous phase.
17. Proceed according to **Subheading 3.4.**, **step 9**.

3.4. Bisulphite Treatment of Isolated DNA

Bisulphite and hydroquinone solutions are light-sensitive, thus should be protected from light in all steps.

1. Digest genomic DNA with a suitable restriction enzyme (which does not cut within the region to be amplified) in a volume of 21 µL. In order to achieve a complete bisulphite conversion, we recommend using not more than 700 ng DNA for the restriction, so that the DNA content of each (later on) formed agarose-DNA bead does not exceed 100 ng, *see* **step 8**.
2. Boil for 5 min in a water bath.
3. Chill on ice and quickly spin down.
4. Add 4 µL of 2 *M* NaOH (final concentration 0.3 *M* NaOH) and incubate 15 min at 50°C.
5. Mix with 2 vol (50 µL) of melted (50–65°C) 2% (w/v) LMP agarose (SeaPlaque Agarose, FMC; prepared in water).
6. Prepare 2.5 *M* bisulphite solution, pH 5.0, as follows: dissolve 1.9 g of sodium bisulphite in a mix of 2.5 mL H_2O and 750 µL of 2*M* NaOH (freshly prepared), dissolve 55 mg of hydroquinone in 500 µL of H_2O at 50°C, and mix both solutions.
7. Pipet 1 mL of the bisulphite/hydroquinone solution into a 2-mL Eppendorf tube and overlay with 750 µL of heavy mineral oil (tubes should be kept for 30 min on ice before proceeding).
8. Pipet up to seven 10 µL-aliquotes of the DNA-agarose mixture into ice-cold mineral oil to form beads. (Each bead should contain up to 100 ng of DNA.) Make sure that all beads have entered the aqueous phase; beads can be pushed into the bisulphite solution using a pipet tip.
9. Leave on ice for 30 min.
10. Incubate at 50°C for 3.5 h.
11. Remove all solutions; wash with 1 mL of 1X TE, pH 8.0 for 4 × 15 min.
12. Add 500 µL of 0.2 *M* NaOH 2 × 15 min.
13. Remove NaOH solution and wash with 1 ml of 1X TE, pH 8.0, 3 × 10 min. Store in a small volume of TE, pH 8.0, at 4°C (beads are stable for at least several weeks).
14. Prior to amplification wash the beads with H_2O for 2 × 15 min.

3.5. General Recommendations

3.5.1. Primer Design

The guidelines for primer design for the amplification of bisulphite-treated DNA presented here concern methylation patterns found in mammalian genomes (i.e., methylation mainly at CpG sites). Different requirements for primer design have to be considered when analyzing methylation patterns in organisms with a broader methylation spectrum (as CpNpG or nonsymmetrical cytosine methylation in plants or fungi, and so on; *see* **Subheading 4.**).

1. A bisulphite-treated DNA sequence should be generated using any word processor to replace all Cs for Ts except at CpG sites (e.g., for DNA methylation patterns in mammalian genomes). Any primer designing software that will help to avoid any hairpin structures and possible primer dimers can use this modified sequence.
2. The length of the oligos should be at least 20 nucleotides and up to 25–30 nucleotides.
3. The primers should be located in an originally cytosine-rich region so that they selectively amplify converted DNA.
4. Overlapping of the primers with CpG dinucleotides should be strictly avoided especially at the 3′ end of the oligos.
5. Extensive T and A stretches in both primers, which are typical for bisulphite-treated DNA, should be avoided to minimize the formation of primer dimers.

3.5.2. Optimizing PCR Conditions

1. The PCR conditions for amplifying bisulphite-treated material should be carefully optimized. The bisulphite treatment reduces the sequence specificity (by changing all cytosines to uracils) and thus the selectivity for primer annealing.
2. It is recommended that the length of the product does not exceed 600–700 bp as longer fragments may be more difficult to amplify from a bisulphite-treated DNA (due to depurination of DNA as a result of low pH during the bisulphite treatment).
3. A nested or at least a seminested approach for amplifying the target region is recommended to increase the sensitivity when working with limited numbers of cells and to ensure the specificity of the product.
4. To avoid any contamination with previous PCR products, the bisulphite treatment and handling of the DNA or cells should be carried in a separate room and using separate pipets.
5. A gradient PCR cycler is recommended to optimize the annealing temperature.

3.5.3. Cloning and Sequencing

1. To increase the efficiency of cloning, the specific PCR product should be purified from any unspecific band(s) or primer dimers by agarose-gel elution.

Table 1
Frequently Encountered Problems

Problem	Recommended solution
1. The hydroquinone solution turns red.	a. Protect the solution from light and do not heat over 50°C to dissolve.
2. During incubation of the bisulphite-hydroquinone solution on ice crystals appear.	a. This will not affect the results: proceed normally.
3. The beads disappear after entering the bisulphite solution.	a. The mineral oil layer is not cold enough; the tubes containing the mineral oil should be pre-incubated on ice for a longer period (at least 20 min) or alternatively they can be kept at –20°C for 10 min, then the bisulphite solution added after the formation of the beads. b. Use only heavy mineral oil. c. Increase the concentration of LMP agarose.
4. No PCR product is obtained.	a. Inefficient bisulphite conversion (*see* **Subheading 3.4.**). b. The amount of template is not sufficient. c. The PCR product is too long for bisulphite-treated DNA; design primers that amplify smaller fragments. d. Use nested primers to increase the sensitivity and the yield of the amplification. e. Try different set of primers.
5. The PCR product is difficult to clone.	a. If a T/A cloning vectors are used: prior to ligation, incubate the PCR product with additional amount of Taq polymerase and dATP (this would help to add a flanking A to the 3′ end of the product). b. Try different cloning vectors and *E. coli* strains.
6. Unconverted sequences are frequently observed.	a. Primers are not selective enough for converted DNA. The primers should be located in a C-rich region to increase the specificity of amplification for converted sequences. b. Incomplete bisulphite conversion may be caused by excess of DNA in reaction; the maximum recommended amount of DNA per bead is 100 ng. c. DNA was not properly denatured, make sure that denaturation steps and desulphonation steps are properly made using fresh NaOH solution. d. The bisulphite and hydroquinone solutions should be prepared fresh and stored no longer than 24 h before use.

2. Cloning of the PCR product can be improved by additional incubation of the purified product in presence of dATP and Taq polymerase for 5 min at 95°C followed by 60 min at 72°C. This will increase the percentage of DNA molecules with flanking As at the 3′ end.
3. To verify the presence of the correct insert in plasmids, we routinely use a colony PCR protocol. Products of the correct size can be directly sequenced using internal primers.
4. According to our experience, blue/white screening of colonies is not always reliable (especially when short fragments are cloned). In such situation, we recommend analyzing all colonies, as the blue ones may contain an insert.

3.6. Drawbacks of the Bisulphite-Based Methylation Analysis

Although the bisulphite-based methylation analysis is a powerful tool to obtain detailed genomic-methylation data, it is connected with specific experimental or technical problems, which are briefly discussed here.

1. In order to perform a bisulphite-based methylation analysis, a detailed sequence information of the genomic region of interest is required.
2. The upper and lower strands of the bisulphite-treated DNA samples are analyzed separately. Therefore it is impossible (except for a single-cell approach) to obtain data about the original double-stranded DNA.
3. Amplifications (or cloning of PCR products) from the upper and lower strand may not work equally well in each case.
4. In case of analyzing methylation patterns of unknown distribution as, e.g., in plants and fungi, it may be difficult to design primers for the PCR amplification of the bisulphite-treated DNA. In this case primers can be designed that contain either C or T at the respective positions. However, the use of such primers with "wobble" positions greatly reduces the sensitivity of the PCR reaction and may cause a bias in the amplification towards specific (mostly not fully converted) products.
5. A systematic analysis ***(24)*** nicely demonstrated that the choice of primers might cause a bias in the PCR reaction, such that either the low or highly methylated template DNA is predominantly amplified. Another selection against a specific subset of PCR products may occur during the cloning procedure. The problem of biased amplification or cloning has to be tested individually and several control experiments should be carried out. First, different templates with a known content of methylated cytosine residues should be mixed in different ratios and the bisulphite treatment and amplification steps should be carried out as usual. The distribution of nonconverted and converted cytosine residues in the analyzed products will then allow determination of whether and in which magnitude a bias occurs. Furthermore, independent experiments (including different techniques) should be used to analyze the methylation state of a given template, e.g., conventional Southern-blot hybridization or the Ms-SNuPE assay ***(16)***. Both experiments may be very helpful to obtain an independent impression of the real ratio of modified and unmodified cytosines within the sequence of interest.

Acknowledgments

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