

DNA Methylation Detection: Bisulfite Genomic Sequencing Analysis

Yuanyuan Li and Trygve O. Tollefsbol

Abstract

DNA methylation, which most commonly occurs at the C5 position of cytosines within CpG dinucleotides, plays a pivotal role in many biological procedures such as gene expression, embryonic development, cellular proliferation, differentiation, and chromosome stability. Aberrant DNA methylation is often associated with loss of DNA homeostasis and genomic instability leading to the development of human diseases such as cancer. The importance of DNA methylation creates an urgent demand for effective methods with high sensitivity and reliability to explore innovative diagnostic and therapeutic strategies. Bisulfite genomic sequencing developed by Frommer and colleagues was recognized as a revolution in DNA methylation analysis based on conversion of genomic DNA by using sodium bisulfite. Besides various merits of the bisulfite genomic sequencing method such as being highly qualitative and quantitative, it serves as a fundamental principle to many derived methods to better interpret the mystery of DNA methylation. Here, we present a protocol currently frequently used in our laboratory that has proven to yield optimal outcomes. We also discuss the potential technical problems and troubleshooting notes for a variety of applications in this field.

Key words: DNA methylation, Epigenetics, Bisulfite genomic sequencing

1. Introduction

Extensive studies have demonstrated that DNA methylation plays a major role in regulating various physiological and pathological processes in mammals. DNA methylation is an important epigenetic event in modulating embryonic development, genomic imprinting, X inactivation, cellular differentiation, and proliferation (1, 2). However, abnormal patterns of DNA methylation are correlated with DNA instability which may ultimately trigger diseases such as cancer (3). DNA methylation, primarily occurring at C5 of the cytosine ring within cytosine–guanine (CpG) dinucleotides, is frequently

found clustered at gene regulatory sites such as promoter regions (4). Dense methylation of CpGs in the gene promoter region is associated with a compacted chromatin structure resulting in transcriptional silencing of the affiliated gene. If DNA hypermethylation occurs at the promoter regions of certain critical cancer-related genes, it could lead to tumor suppressor gene silencing and ultimately tumorigenesis (5). Therefore, the importance of DNA methylation in a variety of biological processes represents an attractive diagnostic and therapeutic target. A precise and efficient method is required to determine the exact DNA methylation status to further elucidate the essential roles of DNA methylation in biological procedures.

Bisulfite genomic sequencing is regarded as a gold-standard technology for the detection of DNA methylation because it provides a qualitative, quantitative, and efficient approach to identify 5-methylcytosine (5mC) at single base-pair resolution. This method was first introduced by Frommer et al. and it is based on the finding that the amination reactions of cytosine and 5mC proceed with very different consequences after the treatment of sodium bisulfite (6). In this regard, cytosines in single-stranded DNA will be converted into uracil residues and recognized as thymine in subsequent PCR amplification and sequencing; however, 5mCs are immune to this conversion and remain as cytosines allowing 5mCs to be distinguished from unmethylated cytosines. A subsequent PCR process is necessary to determine the methylation status in the loci of interest by using specific methylation primers after the bisulfite treatment. The actual methylation status can be determined either through direct PCR product sequencing (detection of average methylation status) or subcloning sequencing (detection of single molecule distribution of methylation patterns) (Fig. 1). Moreover, bisulfite sequencing analysis can not only identify DNA methylation status along the DNA single strand, but also detect the DNA methylation patterns of DNA double strands since the converted DNA strands are no longer self-complementary and the amplification products can be measured individually.

Over the past few years, several techniques have arisen based on the working basis of bisulfite including methylation-specific PCR (MSP), combined bisulfite restriction analysis (COBRA), methylation-sensitive single nucleotide primer extension (Ms-SNuPE), and several other techniques depending on different applications (7–10). Compared with other DNA methylation approaches based on the sensitivity of restriction enzymes that can specifically recognize methylated cytosine within their cleavage recognition site (11), bisulfite-based DNA methylation analysis has more quantitative accuracy, detection sensitivity, high efficiency, and a wide spectrum for sample analysis.

Bisulfite genomic sequencing, as a fundamental method of DNA methylation analysis, has been widely used in various research and clinical settings. To optimize the final results of the bisulfite

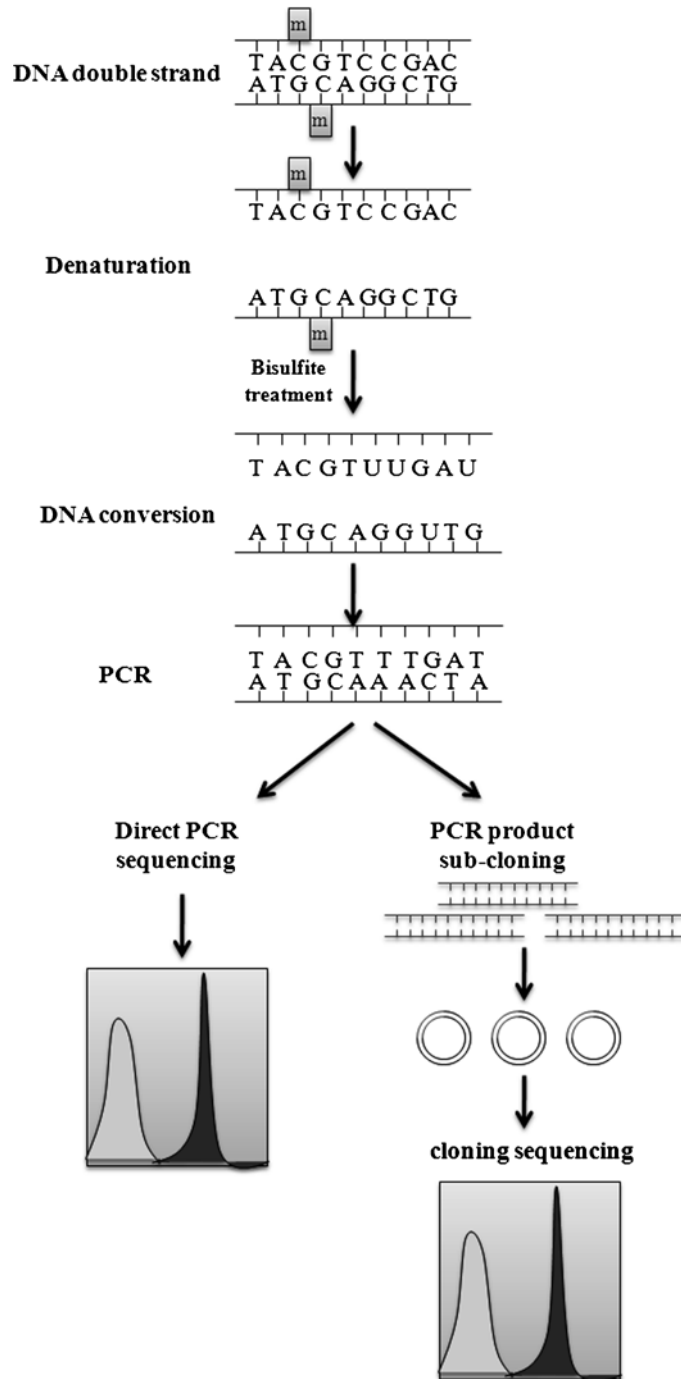


Fig. 1. Principles of methylation analysis using bisulfite genomic sequencing. After treatment with sodium bisulfite, unmethylated cytosine residues are converted to uracil whereas 5-methylcytosine (5mC) remains unaffected. After PCR amplification, uracil residues are converted to thymine. DNA methylation status can be determined by direct PCR sequencing or cloning sequencing.

genomic sequencing protocol, numerous modifications have been explored and have significantly improved the sensitivity and accuracy in this procedure (12–16). In this chapter, we introduce a modified bisulfite sequencing protocol, which is currently used in our laboratory and consistently working well. Detailed discussion of technical problems, solutions, and troubleshooting is also included to enhance this technique.

2. Materials

2.1. Genomic DNA Extraction

1. Wizard Genomic DNA purification kit (Promega).
2. 70% of ethanol.
3. Microcentrifuge.
4. Incubator at 65°C.

2.2. Bisulfite Reaction

1. Commercially available bisulfite reaction kit, EpiTect Bisulfite Kit (Qiagen).
2. For conventional bisulfite genomic treatment, 3 M NaOH solution, 5 M sodium bisulfite solution (with 125 mM hydroquinone, pH 5.0), 5 M ammonium acetate, isopropanol, ethanol, and mineral oil are needed. The mixtures of bisulfite reaction solutions are illuminated in Table 1. Bisulfite and hydroquinone solutions are light-sensitive and should be protected from light in all steps.
3. Wizard DNA clean-up system (Promega) for purification of bisulfite-treated DNA.
4. Disposable 5-ml lure-lock syringes.
5. Deionized water or TE buffer.

2.3. PCR Purification, Cloning, and Sequencing Analysis of Target DNA Fragment

1. Regular 2 × PCR Mastermix.
2. Agarose gel, ethidium bromide, and electrophoresis apparatus.
3. QIAquick PCR Purification Kit (Qiagen) for purification of PCR product.
4. QIAquick Gel Extraction Kit (Qiagen) for purification of target PCR fragment from multiple nonspecific PCR products.
5. pGEM-T Easy vector system II (Promega).
6. For bacterial culturing and positive cloning selection, bacto-tryptone (BD), yeast extract, sodium chloride, ampicillin solution, isopropyl-β-D-thiogalactoside (IPTG), X-Gal (Bio-Rad), and bacterial shaker incubator at 37°C are required.
7. QIAprep Spin Miniprep Kit (Qiagen).
8. ABI 3730 DNA Analyzer.

Table 1
Reagents of 5 M sodium bisulfite solution

Reagents/volumes	1 ml	2 ml	3 ml	4 ml	5 ml
Sodium bisulfite	0.475 g	0.95 g	1.425 g	1.9 g	2.375 g
Deionized water	0.625 ml	1.25 ml	1.875 ml	2.5 ml	3.125 ml
2 M NaOH (80 mg/ml)	175 μ l	350 μ l	525 μ l	700 μ l	875 μ l
1 M hydroquinone (110 mg/ml)	125 μ l	250 μ l	375 μ l	500 μ l	625 μ l

3. Methods

3.1. Genomic DNA Preparation (see Notes 1–5)

Genomic DNA from cultured cells, cultured bacteria, animal tissues, and paraffin-embedded tissue sections can be isolated by using a number of commercially available DNA Extraction Kits followed by the corresponding manufacturer's protocols. Genomic DNA (1–10 μ g) is dissolved in deionized water with 18 μ l final volume. Proceed to Subheading 3.2, step 1.

3.2. Bisulfite Modification

1. Predenature the DNA from Subheading 3.1 by boiling in a water bath for 20 min (see Note 6).
2. Denature the DNA by adding 2 μ l of 3 M freshly made NaOH and 380 μ l 5 M sodium bisulfite solution (Table 1) and mix well (see Note 7).
3. Add 500 μ l of heavy mineral oil on the top of 400 μ l DNA solution from step 2 to diminish evaporation and incubate the solution in the dark at 50°C for 12–16 h (see Note 8).
4. Purification of the bisulfite treated-DNA: We routinely use the Wizard DNA clean-up kit from Promega to purify the bisulfite-treated DNA. After carefully removing the heavy mineral oil from the reaction solution from step 3, the procedure involving this step is followed according to the manufacturer's protocol.
5. The bisulfite-modified DNA is eluted in 50 μ l deionized water and 11 μ l 3 M NaOH is added. Incubate at 37°C for 15 min to desulfonate the DNA.
6. Add 166 μ l 5 M ammonium acetate, 750 μ l of absolute ethanol, and 200 μ l isopropanol to precipitate the DNA at –20°C for 2–4 h.
7. Centrifuge the DNA at maximum speed for 10 min and discharge the supernatant.
8. Wash the DNA with 200 μ l 70% ethanol and centrifuge as in step 7.

9. Carefully remove the ethanol and dry the DNA pellet at room temperature for 10 min. Resuspend the DNA in 10–20 μ l of TE or deionized water (see Note 9).

3.3. Bisulfite PCR Amplification

1. Bisulfite PCR primer design is critical for successful implementation of subsequent bisulfite sequencing analysis. The detailed guidelines for primer design of bisulfite-treated DNA templates are discussed in Note 10.
2. Bisulfite PCR amplification can be performed as a regular PCR reaction. However, the PCR conditions for amplifying bisulfite-treated material should be carefully optimized (see Note 11). The PCR results will be verified by gel-based electrophoresis and a single, bright, and specific band will be considered as a successful PCR amplification.

3.4. Direct PCR Sequencing and Cloning Sequencing (see Note 12)

1. Prior to the direct PCR sequencing, purification of PCR products is necessary to remove the residue of the PCR reaction that might interfere with the outcome of sequencing results. Commercially available kits such as QIAquick PCR Purification Kit (Qiagen) can be used for specific PCR fragments, whereas QIAquick Gel Extraction Kit (Qiagen) can help purify the target PCR product from multiple nonspecific PCR bands. The purified PCR products can be directly sequenced.
2. Cloning sequencing is necessary to observe the distribution of methylation patterns in single molecules. We prefer to use pGEM-T Easy vector system II (Promega) for cloning purposes which provides the T4 DNA ligase system, a pGEM-T Easy vector, and competent JM109 cells as well. By using this kit, purified PCR products can be ligated to the pGEM-T Easy vector and transformed into competent JM109 cells. The JM109 cells that carry the ligated vectors can be selected on agar plates containing ampicillin/X-gal/IPTG by color change where blue colonies represent empty vector, and white colonies represent vectors inserted with target PCR product. The white colonies can then be selected and grown in LB medium. Plasmids containing the target DNA are extracted by using the QIAprep Spin Miniprep Kit (Qiagen) and subjected to standard sequencing analysis. All the procedures follow the manufacturer's protocol.

3.5. Data Interpretation

After successful bisulfite PCR amplification or subcloning procedures, DNA methylation status can be interpreted by subsequent sequencing analysis. Direct sequencing of PCR products may be easily accessible, however, a series of problems limit its application such as failing to read the entire target region and high background interference. Cloning sequencing can provide useful methylation information on a molecular basis. To obtain high confidence in the results, a large number of clones (minimum 5, ideally 10) need to be sequenced, which can be time- and labor-intensive.

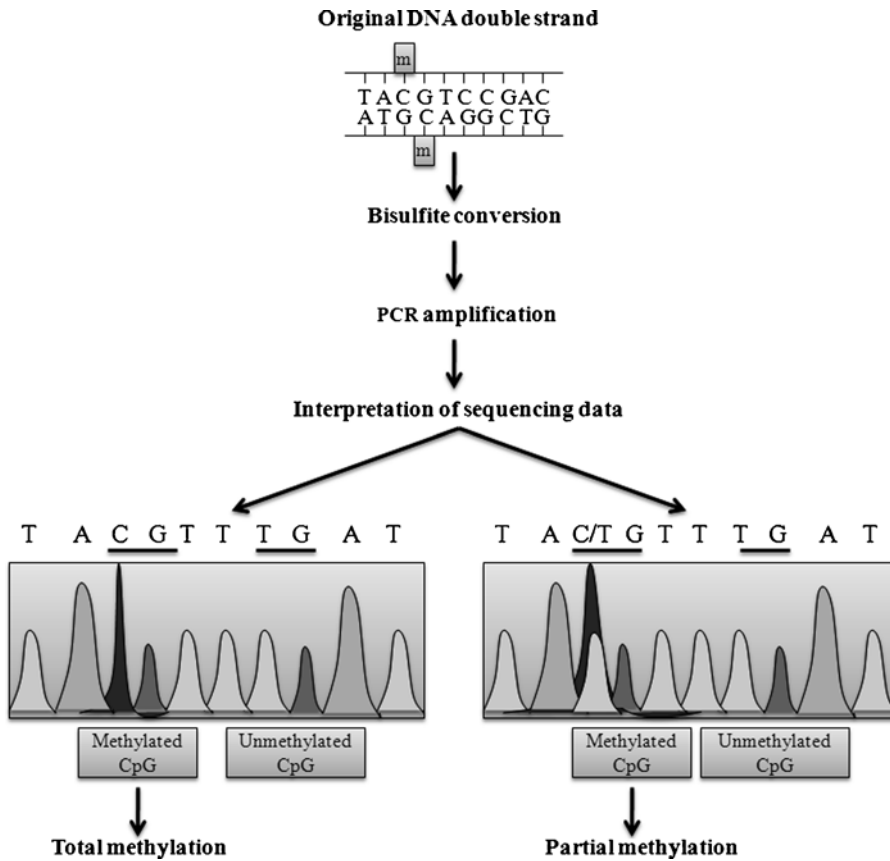


Fig. 2. Interpretation of methylation sequencing results. After bisulfite treatment, all unmethylated cytosines (C) convert to thymine (T) and the presence of a C peak indicates the presence of 5mC in the genome. Total methylation or complete conversion of a single residue shows a single peak. The presence of both C and T peaks indicates partial methylation or potentially incomplete bisulfite conversion.

Both procedures have the potential of artifacts due to incomplete conversion which may be prevented by strictly following Notes 1–9.

DNA methylation status can be interpreted by comparing the sequencing results and the original DNA sequence. Basically, all unmethylated cytosines (C) convert to thymine (T) and the presence of a C peak indicates the presence of 5mC in the genome. If both C and T peaks appear, this indicates partial methylation or potentially incomplete bisulfite conversion has occurred. The proportion of 5mC to C can be interpreted by analyzing the relative square area of these two bands (Fig. 2).

4. Notes

1. The quality and quantity of DNA are important in the bisulfite reaction. Typically, most protocols recommend >1 µg of high-quality DNA extracted from cultured cells or fresh tissue

samples to obtain reliable results. Therefore, samples from formalin-fixed, paraffin-embedded tissue sections may yield unfavorable results due to the limited quantity of initial DNA and subsequent DNA degradation during the bisulfite treatment. Several modifications have integrated into the conventional bisulfite protocol to help optimize analysis of low-quantity DNA such as samples from paraffin-embedded tissue blocks (17).

2. Alternatively, some have recommended an extended proteinase K treatment involving DNA isolation to increase accessibility for subsequent sodium bisulfite to DNA by removing residual protein (18). In this procedure, an overnight proteinase K (2 mg/ml) incubation at 37°C can be employed before proceeding to Subheading 3.2, step 1.
3. An additional DNA digestion by a restriction methylation-insensitive endonuclease can be incorporated prior to bisulfite modification which helps to reduce DNA strand annealing following denaturation (20).
4. A further process that reduces DNA losses during bisulfite modification is to embed the DNA in low-melting-point agarose blocks (14, 16). This modification will allow the subsequent bisulfite reaction to be performed in the agaroses where the DNA is physically captured. This will greatly reduce DNA loss during the procedures, especially when a small amount of DNA sample is applied.
5. Carriers such as salmon sperm DNA and glycogen can be used to increase bisulfite conversion and DNA precipitation, respectively, which also reduce the loss of DNA throughout the whole procedure (19).
6. The most critical step for the bisulfite reaction is DNA denaturation since sodium bisulfite can only react with cytosine in single-stranded DNA. Therefore, complete DNA denaturation is an essential prerequisite for successful DNA conversion by bisulfite treatment. According to the modification protocol originally developed by Frommer et al. (6), genomic DNA is denatured in high sodium bisulfite salt at high temperature and low pH. However, these harsh conditions can cause the DNA double-strand to form an unfavorable conformation leading to partial DNA renaturation, thus increasing the risk of an incomplete conversion reaction (20). Various modifications have been attempted to reduce strand reannealing that are listed below.
 - (a) DNA can be fragmented by the use of proteinase K and appropriate restriction enzymes as mentioned in Notes 2 and 3.
 - (b) DNA can be imbedded in low-melting point agarose block to prevent DNA reannealing as mentioned above in Note 4.

- (c) DNA can be preboiled prior to bisulfite treatment to improve the denaturing step as described in Sub-heading 3.2, step 1.
 - (d) A high concentration of urea (6 M) may be added to the bisulfite solution to destabilize base-pairing in the DNA (21).
7. The bisulfite reaction solution in Table 1 and the NaOH solution must be freshly prepared each time prior to the conversion reaction.
 8. A standard overnight incubation at 50°C ensures a complete reaction. However, the prolonged incubation was found to increase deaminated 5mC in the genome (12, 22) therefore leading to an underrepresentation of 5mC in subsequent PCR analysis. Although several groups have claimed that 4- to 5-h incubations with bisulfite are sufficient for complete conversion (13–15, 20, 23), we prefer to incubate DNA for more than 10 h of bisulfite reaction, which can yield complete conversion without further damage of DNA template. If further PCR reactions give poor results due to extensive DNA damage by a long incubation, a reduced incubation may be employed.
 9. Owing to a noncomplementary DNA conformation after bisulfite treatment, the converted DNA is not stable and repeated freezing–thawing should be avoided. Freshly made bisulfite-modified DNA is recommended to yield optimal results. Using our protocol, the bisulfite-modified DNA can be used for up to a year posttreatment with good quality if stored at –80°C or in liquid nitrogen.
 10. The principles for designing bisulfite PCR primers vary to meet different research purposes and protocols following the conventional bisulfite reaction. The primer guidelines listed below are used for bisulfite genomic sequencing analysis. A more detailed description of methylation primer design is provided in refs. 24, 25.
 - (a) After bisulfite treatment, the unmethylated cytosines convert to thymine and methylated cytosines remain cytosines. Since the methylation status of CpG dinucleotides is unknown, the bisulfite primer sequences should strictly avoid CpG dinucleotides. Therefore, primers should be generated to replace all cytosines to thymines according to the original DNA sequence. Primer designing software can also be used to avoid potential hairpin structures and possible primer dimers based on this modified sequence.
 - (b) The length of the primers should be around 25–30 nucleotides.
 - (c) The length of the PCR product should not exceed 400 bp due to potential DNA degradation during the bisulfite modification that might influence the PCR amplification.

11. The bisulfite PCR conditions should be carefully optimized. Since the bisulfite treatment reduces the specificity of DNA double-strands, the processes for determining the optimal PCR conditions with bisulfite-modified DNA template can be more laborious than regular PCR.
 - (a) Annealing temperature: A gradient PCR thermocycler can help to determine the appropriate annealing temperature. If there is no access to a gradient PCR thermocycler, a touchdown PCR can be applied to increase the annealing sensitivity.
 - (b) PCR reaction system: The commercially available PCR MasterMix which mixes *Taq* DNA polymerase and dNTP with optimal salt concentration can be easily used for bisulfite PCR. If this common PCR reaction system cannot produce a clean band, it is advisable to try a different PCR reaction system. In our laboratory, we normally use JumpStart (Sigma) or SureStart PCR system (Startagene) to improve the bisulfite PCR results.
 - (c) Nested PCR reaction: A nested or a seminested PCR approach is recommended to obtain a sufficient PCR product especially when a limited amount of DNA is used.
12. pGEM-T Easy vector is a T-A cloning system and requires an extra adenosine added to the 5' end of both strands during the PCR reaction. Therefore, it is important to choose the appropriate *Taq* DNA polymerase before cloning. Generally, sequencing requires more than five colonies to determine the accurate methylation patterns on a target region.

Acknowledgments

This work was supported in part by grants from the National Cancer Institute (R01 CA 129415), the Susan G. Komen for the Cure and a Postdoctoral Award (PDA) sponsored by the American Institute for Cancer Research (AICR).

References

1. Bird, A. (2007) Perceptions of epigenetics. *Nature* **447**, 396–398.
2. Li, E. (2002) Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Rev. Genet.* **3**, 662–673.
3. Robertson, K. D. and Wolffe, A. P. (2000) DNA methylation in health and disease. *Nature Rev. Genet.* **1**, 11–19.
4. Bird, A. P. (1986) CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209–213.
5. Jones, P. A. and Baylin, S. B. (2002) The fundamental role of epigenetic events in cancer. *Nature Rev. Genet.* **3**, 415–428.
6. Frommer, M., McDonald, L. E., Millar, D. S., Collis, C.M., Watt, F., Grigg, G. W., Molloy, P. L.,

- and Paul, C. L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, 1827–1831.
7. Rand, K., Qu, W., Ho, T., Clark, S. J. and Molloy, P. (2002) Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. *Methods* **27**, 114–120.
 8. Xiong, Z. and Laird, P. W. (1997) COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* **25**, 2532–2534.
 9. Gonzalgo, M. L. and Jones, P. A. (1997) Rapid quantitation of methylation differences at specific sites using methylationsensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* **25**, 2529–2531.
 10. Suzuki, M. M., and Bird, A. (2008) DNA methylation landscapes: provocative insights from epigenomics. *Nat. Rev. Genet.* **9**, 465–476.
 11. Bird, A. P. (1978) Use of restriction enzymes to study eukaryotic DNA methylation. II: the symmetry of methylated sites supports semi-conservative copying of the methylation pattern. *J. Mol. Biol.* **118**, 48–60.
 12. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22**, 2990–2997.
 13. Raizis, A. M., Schmitt, F., and Jost, J. P. (1994) A bisulphite method of 5-methylcytosine mapping that minimises template degradation. *Anal. Biochem.* **226**, 161–166.
 14. Olek, A., Oswald, J., and Walter, J. (1996) A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res.* **24**, 5064–5066.
 15. Paulin, R., Grigg, G. W., Davey, M. W., and Piper, A. A. (1998) Urea improves efficiency of bisulphite-mediated sequencing of 5-methylcytosine in genomic DNA. *Nucleic Acids Res.* **26**, 5009–5010.
 16. Hajkova, P., El-Maarri, O., Engemann, S., Oswald, J., Olek, A., and Walter, J., (2002) DNA-methylation analysis by the bisulfite-assisted genomic sequencing method. In: Mills, K. I., and Ramsahoye, B. H. (ed) *DNA Methylation Protocols*, Humana Press Inc., New Jersey.
 17. Tan, L. W., and Dobrovic, A. (2001) Methylation analysis of formalin-fixed, paraffin-embedded sections using a nontoxic DNA extraction protocol. *Biotechniques* **31**, 1354, 1356–1357.
 18. Warnecke, P. M., Stirzaker, C., Song, J., Grunau, C., Melki, J. R., and Clark, S. J. (2002) Identification and resolution of artifacts in bisulfite sequencing. *Methods* **27**, 101–107.
 19. Herman, J. G., Graff, J. R., Myohanen S., Nelkin, B. D., and Baylin, S. B. (1996) Methylationspecific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**, 9821–9826.
 20. Rein, T., Zorbas, H., and DePamphilis, M. L. (1997). Active mammalian replication origins are associated with a high-density cluster of mCpG dinucleotides. *Mol. Cell Biol.* **17**, 416–426.
 21. Paulin, R., Grigg, G. W., Davey, M. W., and Piper, A. A. (1998) Urea improves efficiency of bisulphite-mediated sequencing of 59-methylcytosine in genomic DNA. *Nucleic Acids Res.* **26**, 5009–5010.
 22. Wang, R. Y., Gehrke, C. W., and Ehrlich, M. (1980). Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. *Nucleic Acids Res.* **8**, 4777–4790.
 23. Shapiro, R., Servis, R. E., and Welcher, M. (1970b). Reactions of uracil and cytosine derivatives with sodium bisulphite. A specific deamination method. *J. Am. Chem. Soc.* **92**, 422–424.
 24. Li, L. C. (2007) Designing PCR primer for DNA methylation mapping. *Methods Mol. Biol.* **402**, 371–384.
 25. Li, L. C. (2002) Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**, 1427–1431.



<http://www.springer.com/978-1-61779-315-8>

Epigenetics Protocols

Tollefsbol, T. (Ed.)

2011, XI, 332 p. 54 illus., Hardcover

ISBN: 978-1-61779-315-8

A product of Humana Press