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Simplified and Versatile Method for Bisulfite-Based DNA Methylation Analysis of Small Amounts of DNA

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Epigenetic alterations of gene function play a central role in the pathogenesis of many tumors and in the process of aging. Abnormal methylation at transcriptional sites of genes results in epigenetic silencing of the genes that protect against tumor formation or that repair DNA. To date, several studies have analyzed methylation status by oligonucleotide arrays, restriction analysis (COBRA), methylation-specific amplification, and sequence analysis. This requires high concentration of bisulfite-treated DNA, which mandates use of commercially available expensive kits, and is an often laborious and time-consuming task. In this article, we report a simplified high-throughput method, which can serve as a surrogate for screening methylation profiles of various genes and has high sensitivity compared with the other methods described previously. J. Clin. Lab. Anal. 23:172–174, 2009. © 2009 Wiley-Liss, Inc.

Key words: bisulfite modification; DNA methylation; epigenetic changes; gene silencing

INTRODUCTION

Methylation of regulatory gene elements is a well-known epigenetic change that acts as an important alternative for gene inactivation. Methylation of CpG islands located in the 5′ region of tumor suppressor genes (TSGs) is known to inhibit transcriptional initiation and cause permanent silencing of the genes (1,2). In several clinical conditions such as aging, carcinoma of the stomach, and other diseases many such genes have been demonstrated to be inactivated by DNA methylation mechanism (3–5). Therefore, methods to study DNA methylation play a pivotal role in biological research. Various approaches such as methylation-specific PCR (MSP) and bisulfite-based genomic sequencing are the major experimental innovations available, which allow the detection of these methylated cytosines in the genomic DNA.

Most of the bisulfite modification procedures are not only laborious and extensive to perform but also require large quantities (1–2 μg) of the source DNA (3–7). Besides loss of surplus quantity of DNA, the yield of modified DNA is often very less, which limits amplification procedures to only few reactions. To overcome these practical drawbacks, we modified and developed a versatile and less extensive protocol of bisulfite modifications that can be employed to modify miniscule quantity of DNA obtained from human saliva and gastric biopsy tissue. The present protocol is a modification of the method described by Olek et al. (8) and was designed mainly to:

(1) Reduce the overall cost of DNA isolation and bisulfite modification by using common laboratory chemicals.
(2) Ensure complete conversion of cytosines to uracils and not modify 5-methylcytosine.
(3) Reduce the time and labor.
(4) Ensure high reproducibility with least degradation of modified DNA.

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This protocol also ensured that the quality of the DNA be useful for all methylation-based applications such as MSP and methylation-specific sequencing.

(a) *Isolation of the genomic DNA from human saliva and gastric biopsy*: The method described below is an adaptation of the protocol described elsewhere (9,10) to which major modifications were done by eliminating proteinase K treatment and extending the ethanol washings post precipitation of DNA pellet. Finally, the pellet was finally resuspended in 30 μL of Tris EDTA buffer (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0).

(b) *Preparation of DNA embedded agarose beads*:

- To 30 μL of DNA (approx. 1.7845 mg mL^{-1}), NaOH sol. (SD Fine-Chemicals Ltd, Mumbai, India) was added to a final concentration of 0.3 M. This was incubated at 55°C for 15 min in a thermal cycler (Bio-Rad iCycler, Hercules, CA).
- This mixture was then mixed with approximately 1vol of 2% melted low EEO agarose (Hi Media Laboratories Pvt. Ltd, Mumbai, India).
- This was then pipeted out in 100 μL of chilled mineral oil (Sigma Aldrich Life Sciences, St. Louis, MO) to form beads.

(c) *Sodium bisulfite modification of DNA embedded agarose beads*:

- Sodium bisulfite solution was prepared as follows: 4.05 g of mixture of sodium bisulfite and sodium metabisulfite in the ratio of 1:1 (SD Fine-Chemicals Ltd) was added to 8 mL of autoclaved MilliQ water and pH was adjusted to 5.0.
- A total of 500 μL of hydroquinone solution (0.022 g of hydroquinone [Hi Media Laboratories Pvt. Ltd] dissolved in 1 mL of MilliQ water) was added to the above solution and the volume was made to 10 mL with sterile distilled water. The prepared reagent was stored in dark.
- A total of 100 μL of the bisulfite reagent was added to beads in oil. The tube was inverted to move the beads in the sodium bisulfite solution and was incubated at 55°C in a water bath (New Brunswick Scientific Inc., Edison, NJ) avoiding direct light contact for 4 hr.
- Bisulfite treatment was stopped by equilibrations against 1 mL of 1 × TE buffer (6 times × 15 min), followed by desulfonation in 500 μL of 0.5 M NaOH (2 times × 15 min). The reaction was neutralized with 1/5 vol of 1 M HCl (SD Fine-Chemicals).
- Finally, the beads were washed with 1 × TE buffer followed by equilibrations against 1 mL of ddH2O (2 times × 15 min).

(d) *Elution of DNA from sodium bisulfite modified agarose beads*: The beads were heated in microwave boiled water for 10 min in the presence of 40 μL of LMT elution buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). A total of 0.5 μL of this solution was used directly as the template for subsequent PCR amplification.

(e) *Quantification of DNA*: The DNA sample was quantified by Beckman DU604B spectrophotometer (Fullerton, CA). A total of 1 μL of eluted DNA was diluted to 1,000 μL in TE buffer. The DNA was allowed to diffuse in the TE buffer without vortexing for 20 min. The OD was recorded at 260 and 280 nm.

To compare the efficiency of the present protocol, commercially available EzWay DNA methylation detection kit (Koma Biotech, Seoul, Korea) was procured and bisulfite modification reaction was performed as per the instruction manual using the DNA from the same source material (saliva and gastric biopsy).

AMPLIFICATION OF THE MODIFIED DNA SAMPLE

The sensitivity and efficacy of both the methods was assessed by amplification using methylation specific primers for *p*16 gene. Mastermix was prepared using 10 × PCR buffer, 10 mM dNTPs, forward and reverse primers (separate for methylated and unmethylated reactions), Taq DNA polymerase, and 0.5 μL of template DNA from both the methods were added. PCR conditions were optimized and a touch down PCR reaction was employed. The amplification conditions used are tabulated in Table 1. After amplification the products were electrophoresed on 1.8% agarose gel stained with ethidium bromide (Fig. 1). The products of amplification were recorded in automated gel documentation system (Bio Rad). We could observe bright clear bands in the DNA modified by our method but were unable to observe any band in the DNA modified using the kit. Amplifications were repeated thrice to reconfirm the results obtained. Methylation patterns of six other genes, namely MGMT, DAPK, *p*15, CDH1, hMLH1, and TIMP-3, were also assessed using the modified DNA reported in this communication. All of them

TABLE 1. Amplification Conditions Used for Assessing the Methylation Status of *p*16 Gene

Step	Temperature (°C)	Time	No. of cycles
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	20
Annealing	69 (−0.5°C/cycle)	30 sec	
Extension	72	30 sec	
Denaturation	94	30 sec	25
Annealing	61	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	1
	4	∞	

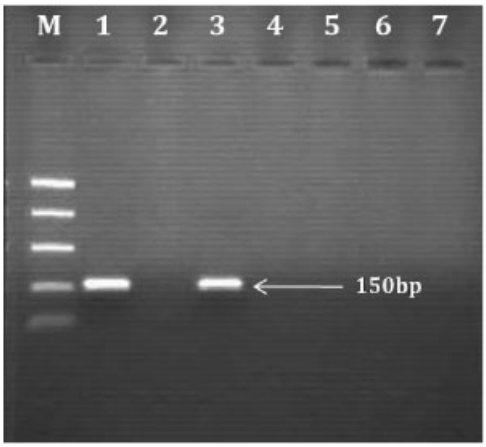

Fig. 1. Gel picture showing methylated and unmethylated amplification status of *p*16 gene after bisulfite modification of saliva and biopsy DNA, **lane "M"** represents PCR marker (New England BioLabs. Inc., Ipswich, MA).

resulted in successful amplification giving expected product sizes, respectively (data not shown).

The results of this study effectively demonstrated bisulfite modification by embedding DNA in low EEO agarose on par with using low melting point (LMP) agarose as reported by Olek et al. (8) thereby reducing the cost of bisulfite modification. This study also focused on lowering the overall cost of DNA isolation with high yield and purity without the use of proteinase K. Further, this method also reduced the time required for sodium bisulfite modification to less than 4 hr with maximum reproducibility compared with other methods, which require at least 12–16 hr with a high concentration of DNA input (3–7). It was also found that agarose embedding and mineral oil overlay helped in reducing the degradation of DNA (data shown above), which otherwise lowered the yield and the subsequent amplification. This technique was also successful in maximum conversion of cytosine to uracil without converting 5-methylcytosines, which is shown by subsequent PCR amplification using respective primers.

Therefore, the major advantages of this assay can be outlined as follows:

It requires low amount of DNA and ensures complete conversion of cytosine to uracil, does not require restriction digestion, and addition of any carrier molecule. Relative loss of DNA after bisulfite modification is less. It further eliminates the need for dialysis, purification of DNA by glass beads, usage of commercial DNA desalting, and purification kits and LMP agarose. The method reported here is not only fast and easy to perform but is also highly sensitive with greater reproducibility.

In summary, we report a simple method for DNA methylation, which can serve as a surrogate for screening methylation profiles of various genes. This method has the advantage of being easier to perform than previous methods and reduces the time required and the overall cost of reaction for methylation analysis giving high productivity of the modified DNA. Further, future study warranting modification of larger chromosomal fragments should be designed to prove its feasibility and efficiency in large scale.

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