Notes & Tips

A mobility-shift detection method for DNA methylation analysis using phosphate-affinity polyacrylamide gel electrophoresis

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Short title: Mobility-shift detection of methylated DNA

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[1] *Abbreviations used:* ^{5m}C, 5-methylcytosine; MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Zn^{2+}–Phos-tag, 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex; dcm, DNA cytosine C5 methylase

Abstract

Herein, we describe a procedure for DNA methylation analysis using the bisulfite-mediated C-to-U conversion of a target DNA followed by methylation-specific polymerase chain reaction (MSP) and phosphate-affinity polyacrylamide gel electrophoresis (PAGE). The MSP was performed using a 1:1 mixture of 5'-phosphorylated methylation-specific and 5'-OH non-methylation-specific primers. The PAGE using an immobilized phosphate-binding tag molecule (*i.e.*, a polyacrylamide-bound dizinc(II) complex, Zn^{2+}–Phos-tag), which selectively captures the 5'-phosphorylated DNA fragment, enabled the mobility-shift detection of the methylation-specific product as a slower migration band. Using this novel procedure, we demonstrated the detection of a methylated cytosine base in a pUC19 plasmid.

Key words: DNA methylation; Epigenetics; Methylation-specific PCR; Phosphate-affinity PAGE; Phos-tag

DNA methylation is well-characterized epigenetic DNA modification to play a role in the regulation of gene expression during cell and cancer development [1]. Many methods for the analysis of genomic DNA methylation status have been developed. Currently, almost all such methods employ the bisulfite-mediated deamination of denatured DNA, which effectively converts cytosine (C) to uracil (U), leaving 5-methylcytocine (^{5m}C)[1] intact [2]. The determination of U or ^{5m}C in the resultant DNA sample can then be achieved by methylation-specific polymerase chain reaction (MSP), sequencing, or other techniques [2–4]. MSP is a widely accepted technique to obtain the methylation status of the genomic CpG sites of interest. The specificity of the polymerase chain reaction (PCR) products can be specified by an appropriate method, such as denaturing high-performance liquid chromatography, denaturing gradient gel electrophoresis, or real-time quantitative PCR, using a TaqMan probe [3, 5]. However, these methods used after the MSP technique require a special apparatus or expensive fluorescent primers.

Recently, we developed a novel type of phosphate-affinity polyacrylamide gel electrophoresis (PAGE) using a phosphate-binding tag molecule, Zn^{2+}–Phos-tag (1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex) [6], for genotyping of single nucleotide polymorphisms [7]. This method is based on the principle that the binding of Zn^{2+}–Phos-tag immobilized in the gel to the phosphate group at the 5'-end of DNA fragment results in slower electrophoresis migration of the 5'-OPO_3^{2-}-labeled DNA fragment compared with the corresponding non-labeled one. The combination of the allele-specific PCR technique and phosphate-affinity PAGE (Zn^{2+}–Phos-tag PAGE) enables the separation of the 5'-OPO_3^{2-}-labeled allele-specific product as a slower migration band from the non-labeled counterpart. The DNA bands can be visualized by staining with ethidium bromide.

In this report, we introduce a simple post-MSP validation method using the Zn^{2+}–Phos-tag PAGE. As the first model samples of the methylated and non-methylated DNA sequences for the novel method, we used pUC19 DNA plasmids prepared from *Escherichia coli* strains of DH5α and ET12567. The DH5α strain has a DNA cytosine C5 methylase (dcm, encoded by the *dcm* gene), which methylates the internal cytosine residue in the sequences CCAGG and CCTGG at the C5 position, and the 14-cytosine bases in the pUC19 sequence

prepared from the strain are methylated. On the other hand, the corresponding bases were not methylated in the plasmid from the ET12567, which is the *dcm*-deficient strain. We designed the following MSP primers set for the analysis of the methylation at cytosine 137 in the plasmid sequence (referring to the sequence site number of GenBank Accession No. M77789): 5'-GTTTATTTATTAGGTATTTCA (T_m = 50 ˚C) for the methylation-specific primer, 5'-GTTTATTTATTAGGTATTTTA (T_m = 48 ˚C) for the non-methylation-specific primer (underlined at the methylation position), and 5'-CTTAACATAATCATAATCATA for the reverse primer to produce a 115-bp fragment. The methylation-specific primer was phosphorylated with T4 polynucleotide kinase before the MSP reaction. The non-methylation-specific primer and the reverse primer were used without the kinase treatment.

The bisulfite treatment of each pUC19 (1.0 μg) from the DH5α and ET12567 was first performed using the MethylEasy DNA Bisulphite Modification Kit (Human Genetics Signatures, North Ryde, Australia). Next, we amplified a 1191-bp DNA fragment including the target site (cytosine 137) in a primary PCR using each bisulfite-modified plasmid (10 ng) as a template DNA and a pair of primers, 5'-TGTAGAAAAGATTAAAGGAT (0.20 μM) and 5'-CCTCTTCACTATTACACCAACT (0.20 μM), and then each PCR product was cloned using the pGEM-T Easy Vector System (Promega, Madison, WI) for the accurate and reliable assay without non-specific products derived from the primary PCR. The cloning procedure is a precaution for the first demonstration of our method. Ten colonies from each of the methylated and non-methylated samples were subjected to colony-direct PCR using the MSP primers set described above (a 1:1 mixture of the MSP primers (each 0.10 μM) and a 0.20 μM reverse primer) and 0.10 U KOD-plus-DNA polymerase (Toyobo, Osaka, Japan). Each resultant reaction mixture (a total volume of 5.0 μL) was added to 2.5 μL of a loading buffer (50 mM EDTA (pH 8.0), 30% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol). Then, 1.0 μL of each sample solution was applied to Zn^{2+}–Phos-tag PAGE. The electrophoresis was carried out using a 1-mm-thick, 9-cm-wide, and 9-cm-long gel prepared with 20% (w/v) polyacrylamide (99:1 ratio of acrylamide to *N,N'*-methylenebisacrylamide), an acrylamide-pendant Phos-tag ligand (20 μM), which was

obtained from the Phos-tag consortium (http://www.phos-tag.com/english/index.html), 2 equivalents of $Zn(NO_3)_2$, and 375 mM Tris-HCl (pH 8.8) on a standard mini-slab PAGE apparatus (model AE-6500; ATTO, Tokyo, Japan) at 15 mA/gel for 70 min at room temperature. The electrophoresis running buffer (pH 8.4) was 25 mM Tris and 192 mM glycine. The DNA bands were stained with an aqueous solution (100 mL/gel) of ethidium bromide (10 μg/mL) after electrophoresis. The scheme of the novel procedure for DNA methylation analysis using the combination of the MSP technique and Zn^{2+}–Phos-tag PAGE is summarized in Fig. 1.

To optimize the condition for colony-direct MSP in this experiment, three annealing temperatures (44, 47, and 50 ˚C) and three $MgSO_4$ concentrations (1.2, 1.0, and 0.8 mM) were examined. The temperatures were predicted from the calculated T_m values of primers used. As a template, we used each colony harboring the non-converted cytosine 137 (methylated sample) and the converted thymine 137 (non-methylated sample), which were confirmed by DNA sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) (Supplemental Fig. S1). The MSP products were then analyzed using Zn^{2+}–Phos-tag PAGE (Fig. 2A). The amplified 115-bp products gave two migration bands for each lane in the following conditions: 1.2 – 0.8 mM $MgSO_4$ at 44 ˚C of annealing temperature and 1.2 – 1.0 mM at 47 ˚C. The slower band detected at an R_f value of 0.52 was a 5'-OPO_3^{2-}-labeled product from the methylation-specific primer, and the faster band at an R_f value of 0.54 was a non-labeled product from the non-methylation-specific primer (see Fig. 1). Under the condition of 0.8 mM $MgSO_4$ at 47 ˚C of annealing temperature, each MSP product was specifically amplified. Thus, we adopted this condition of MSP for the first demonstration of a mobility-shift detection method using Zn^{2+}–Phos-tag PAGE. We examined randomly 10 colonies from the methylated and non-methylated samples, respectively. As a result, all MSP products from the methylated and non-methylated samples were clearly specified in the phosphate-affinity PAGE, as shown in Fig. 2B.

In summary, the combination of the MSP technique and Zn^{2+}–Phos-tag PAGE enabled simple and accurate analysis of DNA methylation without using a special apparatus or expensive fluorescent primers. Since this procedure requires a general PCR apparatus, a

mini-slab PAGE system, and a gel-bound Zn^{2+}–Phos-tag molecule, it would be a very convenient tool to obtain the data of the genomic DNA methylation status from a small number of samples. It is worthwhile to consider using Zn^{2+}–Phos-tag PAGE in the medical field for the analysis of DNA methylation.

Acknowledgments

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Figure legends

Fig. 1. Scheme of the novel procedure for DNA methylation analysis using the combination of MSP technique and Zn^{2+}–Phos-tag PAGE.

Fig. 2. Analysis of DNA methylation by the combination of the MSP technique and Zn^{2+}–Phos-tag PAGE. (A) Optimization of the MSP condition. Three annealing temperatures (44, 47, and 50 ˚C) and three $MgSO_4$ concentrations (1.2, 1.0, and 0.8 mM) were examined. The colony-direct MSP products were analyzed using Zn^{2+}–Phos-tag PAGE (20 μM polyacrylamide-bound Zn^{2+}–Phos-tag and 20% (w/v) polyacrylamide). (B) Mobility-shift detection of methylated DNA. The colony-direct MSP products from the colonies for 10 non-methylated and 10 methylated samples were analyzed using Zn^{2+}–Phos-tag PAGE (20 μM polyacrylamide-bound Zn^{2+}–Phos-tag and 20% (w/v) polyacrylamide).

Figure 1

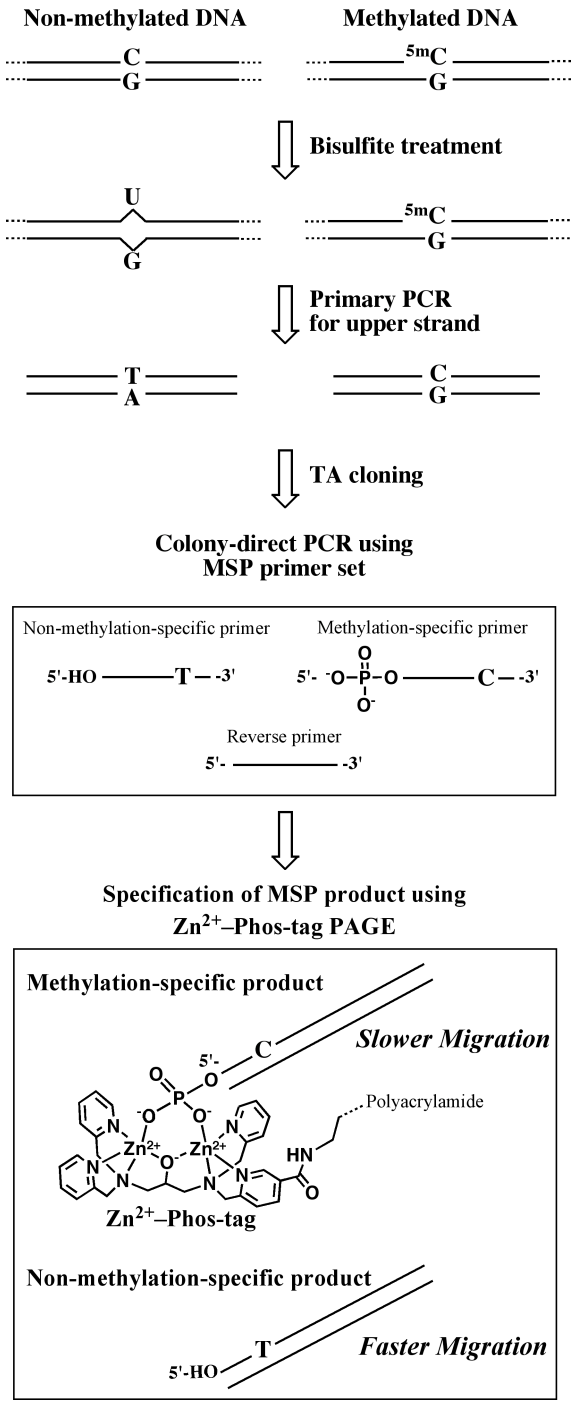
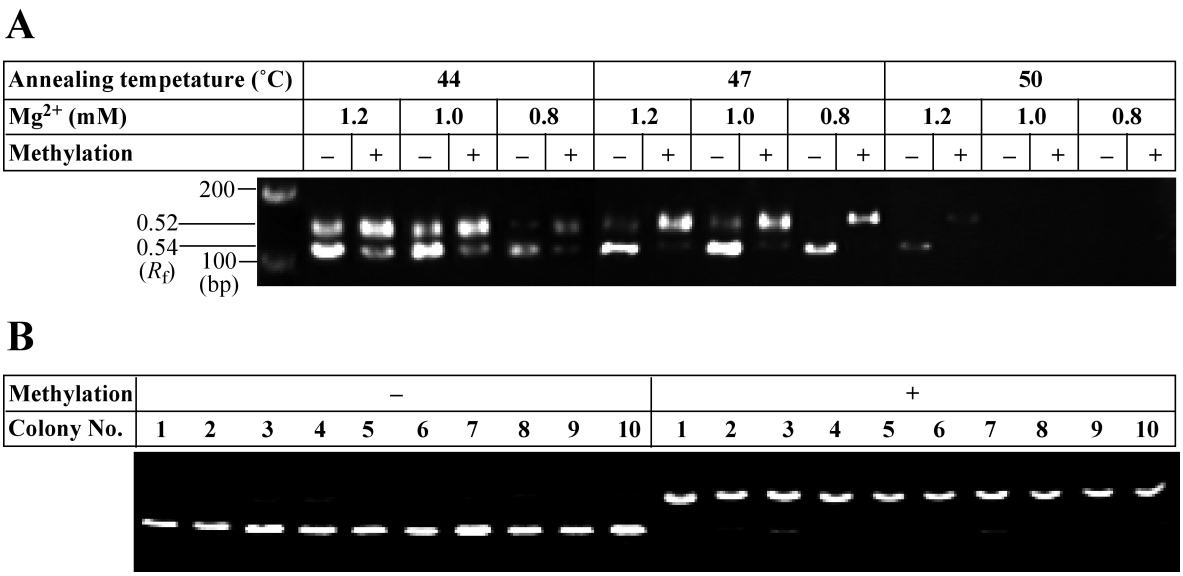

Non-methylated DNA
Methylated DNA
Bisulfite treatment
Primary PCR
for upper strand
TA cloning
Colony-direct PCR using
MSP primer set
Non-methylation-specific primer
Methylation-specific primer
Reverse primer
Specification of MSP product using
Zn2+–Phos-tag PAGE
Methylation-specific product
Slower Migration
Polyacrylamide
Zn2+–Phos-tag
Non-methylation-specific product
Faster Migration

Figure 2


A
Annealing tempetature (˚C)
44
47
50
Mg2+ (mM)
1.2
1.0
0.8
1.2
1.0
0.8
1.2
1.0
0.8
Methylation
– + – + – + – + – + – + – + – + – + – +
200
0.52
0.54
(Rf)
100
(bp)
B
Methylation
–
+
Colony No.
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10