Chapter 7

Methylation-Specific PCR

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Abstract

Defining DNA methylation patterns in the genome has become essential for understanding diverse biological processes including the regulation of gene expression, imprinted genes, and X chromosome inactivation and how these patterns are deregulated in human diseases. Methylation-specific (MS)-PCR is a useful tool for qualitative DNA methylation analysis with multiple advantages, including ease of design and execution, sensitivity in the ability to detect small quantities of methylated DNA, and the ability to rapidly screen a large number of samples without the need for purchase of expensive laboratory equipment. This assay requires modification of the genomic DNA by sodium bisulfite and two independent primer sets for PCR amplification, one pair designed to recognize the methylated and the other pair the unmethylated versions of the bisulfite-modified sequence. The amplicons are visualized using ethidium bromide staining following agarose gel electrophoresis. Amplicons of the expected size produced from either primer pair are indicative of the presence of DNA in the original sample with the respective methylation status.

Key words DNA methylation, Polymerase chain reaction, Primer design, Annealing temperature, Gel electrophoresis

1 Introduction

Methylation-specific PCR (MS-PCR) as described herein is a qualitative technique used for detecting the presence of methylation in bisulfite-converted DNA [1]. The procedure relies on a standard PCR protocol modified to include two sets of methylation-specific primer pairs, which are designed to anneal to sequences containing CpG dinucleotides in the region to be analyzed (Fig. 1). Primers designed to detect methylated DNA (M primers) are designed under the assumption that the region will be fully methylated, and thus will contain cytosines in the sequence at CG dinucleotides. Conversely, the primers designed to detect unmethylated DNA (U primers) are designed assuming the region is unmethylated, and therefore will contain thymines rather than cytosines at CG dinucleotides. The annealing temperature for M primers will be higher than that for U primers, given their correspondence to the same genomic DNA sequence. This is due to the presence of

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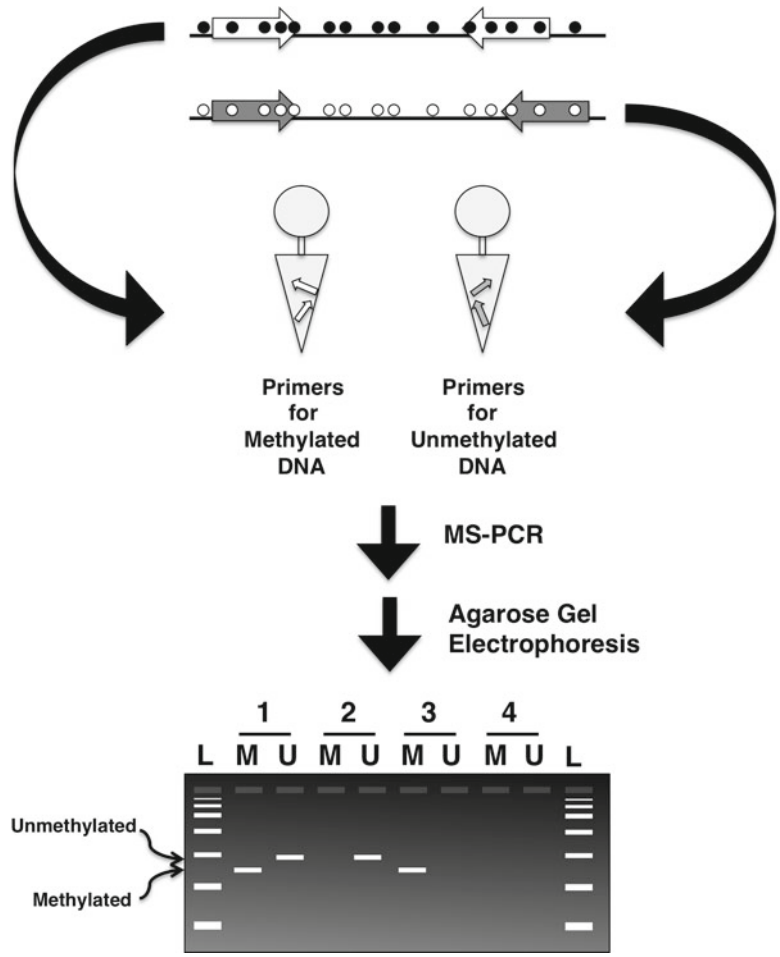

Fig. 1 Methylation-specific PCR. Methylation-specific PCR involves use of two sets of primer pairs that are designed to the methylated (methylated cytosines are represented by the *filled circles*) and unmethylated (*unfilled circles*) versions of the bisulfite-modified genomic DNA. It is helpful if the primers are designed such that there is a distinguishable size difference in the amplicons produced. Following PCR amplification, the amplicons are resolved by agarose gel electrophoresis. Hypothetical results are presented in the figure. Sample 1 shows amplicons for both the methylated (M) and unmethylated (U) primers sets; sample 2 contains only unmethylated DNA; sample 3 contains only methylated DNA; and sample 4 is a negative control. *L* DNA ladder

cytosines in the M primer sequence versus thymines in the U primer sequence for the same CG positions and the higher temperature required to melt C-G hydrogen bonds versus T-A hydrogen bonds in the DNA. The annealing temperature can usually be increased for the U primers to a level similar to that of the M primers by simply extending the number of bases at the 5′ end of the U primers.

The two primer sets are generally used to amplify template DNA in separate reactions, although with careful design and optimization, multiplexing the two primer sets in MS-PCR reactions is possible [2]. In this case, design the M and U primer pairs such that the amplicons produced are sufficiently divergent in size to enable clear separation upon agarose gel electrophoresis. This has the added benefit of enabling amplification from the same template and reaction mix. There are limitations to this approach, including that the sequences to which the primers anneal will be in different locations, which may not be homogeneous in methylation status, and there may be bias in amplification efficiency for one or the other amplification product (e.g., the shorter amplification product may be produced faster and thus will be at higher molarity than the longer product, all else being equal). PCR is followed by gel electrophoresis in which the presence of bands under UV transillumination following ethidium bromide staining qualitatively suggests either the presence or absence of methylation in the template DNA. Because MS-PCR as described here is nonquantitative, these results provide a "present-or-absent" insight into methylation.

Primer design for methylation analysis can be challenging because the sequence to be analyzed is often CG-dense and has reduced complexity after bisulfite modification due to conversion of non-CG cytosines to uracils. MethPrimer is a free online primer design tool specifically for methylation studies (available at http://www.urogene.org/methprimer) [3]. This software provides suggestions for design of primers for both MS-PCR and bisulfite sequencing. The selected genomic sequence can be input using a simple copy and paste function; the software then performs an in silico bisulfite conversion and provides suggestions for primer sequences and positioning. Parameter settings include the target size, regions to exclude, number of primer pairs to be output, the product size, primer Tm, primer length, number of CGs to include in the primers, and number of non-CG cytosines to include in the primers as well as the maximum Tm difference for MS-PCR primers.

MS-PCR is a simple, sensitive, and specific method for detecting the methylation status of virtually any genomic region. Although numerous techniques for study of DNA methylation have been developed, MS-PCR remains a useful approach widely used for DNA methylation analysis. In addition to the ability of MS-PCR to detect aberrant methylation of genes in cancer, MS-PCR is also used to assess DNA methylation relevant to other biological processes, such as genomic imprinting and X chromosome inactivation. However, the nonquantitative "methylation-present-or-absent" results obtained from MS-PCR reactions can be a disadvantage, as can the potential for false positive results. Appropriately designed primers, optimization of PCR reactions, and inclusion of appropriate control reactions can improve the quality of the analysis.

This chapter describing the MS-PCR technique is divided into subsections that explain preparing and running the PCR reactions, agarose gel electrophoresis and interpreting results.

2 Materials

2.1 Kits

1. EpiTect Unmethylated and Methylated Bisulfite-Converted Controls (Qiagen, Valencia, USA).
2. HotStarTaq Polymerase Kit (Qiagen, Valencia, USA): DNA polymerase, 10× PCR buffer, 25 mM $MgCl_2$.

2.2 Reagents and Supplies

Prepare all solutions using nuclease-free water. Unless otherwise indicated, store reagents at room temperature.

1. Bisulfite-converted DNA (20 ng/μl). The bisulfite-converted DNA is stable for up to 6 months at –80°C, up to 3 months at –20 °C, and for 1–2 weeks at 4°C. Avoid freeze-thaws.
2. Primers specific to bisulfite-modified, methylated DNA.
3. Primers specific to bisulfite-modified, unmethylated DNA.
4. 100 mM dNTP set, PCR grade (Invitrogen, Carlsbad, USA). dNTPs should be stored at –20 °C in 10–20 μl aliquots. Dilute to 10 mM with nuclease-free water before use.
5. Tris–Borate–EDTA buffer (TBE): 0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA, pH 8.3.
6. Parafilm® M Barrier Film (SPI Supplies, West Chester, USA).
7. Ethidium bromide in dropper bottle (0.625 mg/ml) (Genesee Scientific, Research Triangle Park, USA).
8. 10× Orange G gel loading buffer. Dissolve 15 ml of glycerol and 100 mg of Orange G powder in water up to final volume 50 ml, vortex to mix. Store at 4 °C.
9. 50 bp DNA Ladder, 1,000 μg/ml (New England Biolabs, Ipswich, USA). Prior to use, dilute 20 μl DNA Ladder into 160 μl nuclease-free water. Add 20 μl Orange G Gel Loading Buffer. Store at 4 °C.
10. GenePure LE Quick Dissolve Agarose (ISC BioExpress, Kaysville, USA).

2.3 Equipment

1. Thermocycler.
2. Gel casting tray and running box.
3. Power supply.
4. UV Transilluminator.

3 Methods

3.1 Polymerase Chain Reaction

1. Prepare the PCR Master Mix with the primer sets specific to the methylated and unmethylated bisulfite-modified DNAs. An example for a 25 μl PCR reaction using the Qiagen HotStarTaq PCR kit is shown below (*see* **Notes 1** and **2**):

Stock	Reaction mix volumes (μl)	Final concentration
10× PCR buffer	2.5	1×
25 mM $MgCl_2$	1.5	1.5 mM
dNTP mix (10 mM each)	0.5	0.2 mM each
M forward primer	1	100–500 nM
M reverse primer	1	100–500 nM
U forward primer	1	100–500 nM
U reverse primer	1	100–500 nM
HotStarTaq DNA polymerase	0.2	2.0–2.5 U
DNA (to be added last)	1	10–50 ng
Nuclease-free water	17.3	
Total volume	25	

2. Thoroughly mix the PCR Master Mix by vortexing for several seconds. Spin down briefly to insure liquid is collected at the bottom of the tube.
3. Transfer the appropriate amount of PCR Master Mix (25 μl minus the volume of DNA to be added) to reaction wells (*see* **Note 3**).
4. Add template DNA to Master Mix in reaction wells.
5. Seal the reaction wells. Mix the PCR mixture by vortexing for several seconds. Spin down briefly.
6. Transfer reaction wells containing PCR mixture to a thermocycler. Perform PCR as follows: initialization step 95 °C/15′, then cycling by denaturation step 94 °C/30″, annealing step *X*°C/30″ (*see* **Note 1**), and extension step 72 °C/30″, for 25–35 cycles followed by extension at 72 °C for 10′. The samples can be analyzed immediately or can be stored at –20 °C prior to use.

3.2 Gel Electrophoresis

1. For an 8 × 10 cm, 2 % agarose gel, add 50 ml of 1× TBE Buffer to 1.0 g of agarose in a glass beaker (*see* **Note 4**). Swirl gently to mix.

2. Microwave the buffer/gel mixture until the agarose is fully dissolved (usually 1–2 min). Watch closely, as it may be necessary to pause the microwave occasionally for a few seconds to avoid having the mixture boil over.
3. In a fume hood, add one drop of ethidium bromide solution (*see* **Note 5**) to the molten agarose mixture (final concentration 0.5 μg/ml) and swirl gently until fully mixed.
4. Still in the fume hood, carefully pour molten agarose into a gel casting tray with barrier ends and comb(s) in place. Let solidify for 15–20 min.
5. Remove the end barriers from the gel. Fill the gel box with 1× TBE buffer so that the surface of the gel is just submerged in the buffer solution. Carefully remove the comb(s) using a very gentle rocking motion while constantly applying slight upward pressure.
6. Add 10 μl of prepared 50 bp DNA ladder to the desired marker lane(s) for each row of samples to be run.
7. For each of the samples that will be run on the gel, pipet 1 μl of Orange G Gel Loading Buffer onto a short strip of Parafilm® M Barrier Film. The liquid will bead up on the film, making it easy to pipet. Carefully pipet 10 μl of the PCR product onto one of the Orange G drops, pipet up and down to mix, and then slowly load the sample into the corresponding well of the gel, making sure to keep the pipet tip within the well while slowly dispensing the sample so that any runover of sample into adjacent wells is prevented. Repeat for the remaining samples.
8. Run gel at an appropriate voltage for the desired time (*see* **Note 6**).

3.3 Interpreting Results

1. After the gel has finished its run, and using gloved hands, pour the 1× TBE gel running buffer into the sink.
2. Carefully remove the gel from the gel box and place onto the UV transilluminator (*see* **Note 7**).
3. Turn on the UV light. PCR amplicons are visible on the gel due to intercalation of the ethidium bromide into the double-stranded DNA, which causes fluorescence. There should not be any visible bands in the no template controls. If there are bands present in these lanes of the anticipated size, then there is contamination and the reactions must be redone to determine the source of the contamination. The positive control methylated DNA should show an appropriately sized amplicon for the M primer set only, while the positive control unmethylated DNA should show an appropriately sized amplicon for only the U primer set (*see* **Notes 8** and **9**).
4. Dispose of the gel, which contains ethidium bromide, according to relevant guidelines.

4 Notes

1. Optimal reaction conditions (incubation times and temperatures, concentrations of primers, $MgCl_2$, and template DNA) vary and need to be optimized. The primer annealing temperatures are a good point at which to start when optimizing the PCR reactions, using the lowest annealing temperature for the primer sets. Every effort should be made to try and choose primers with similar annealing temperatures to avoid bias in performance during the PCR reactions.
2. High-performance liquid chromatography (HPLC) purification of the primers helps to assure that the primers are full length, thus improving the specificity of the primers for the intended target sequence.
3. The template DNA required varies and should be optimized. We typically elute 800 ng of bisulfite-converted DNA with 40 μl of elution buffer (with the concentration of bisulfite-converted DNA at about 20 ng/μl, assuming that all DNA was recovered after the bisulfite modification process) and use 1–3 μl (20–60 ng of input DNA) in each 25 μl PCR reaction.
4. The concentration of TBE used to prepare the gel must match that used as running buffer. If the gel is prepared with 1× TBE, it must be run in 1× TBE running buffer.
5. Ethidium bromide intercalates double-stranded DNA and is therefore a suspected mutagen. However, it is not considered hazardous waste at low concentrations. Guidelines for disposal vary. Those relevant to your institution or place of employment should be followed.
6. The timing of the run and power used will vary depending on the size of the gel, the percentage of agarose used to prepare the gel, and the distance required for the amplicons to be resolved by migration through the gel.
7. Protect your eyes and skin from UV exposure by wearing an appropriate UV protective face shield, long-sleeved lab coat, and gloves.
8. We usually include 3–4 CG sites in primer sequences. CG sites in primers work best to increase specificity of primer binding to the appropriate template when they are located as close as possible to the 3′ end for both M (CG) and U (TG) primers.
9. We recommend inclusion of as many non-CG cytosines as possible within primer binding sequences to increase specificity for the completely modified DNA.

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