

Methylation-specific PCR unraveled

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Abstract. Methylation-specific PCR (MSP) is a simple, quick and cost-effective method to analyze the DNA methylation status of virtually any group of CpG sites within a CpG island. The technique comprises two parts: (1) sodium bisulfite conversion of unmethylated cytosine's to uracil under conditions whereby methylated cytosines remains unchanged and (2) detection of the bisulfite induced sequence differences by PCR using specific primer sets for both unmethylated and methylated DNA. This review discusses the critical parameters of MSP and presents an overview of the available MSP variants and the (clinical) applications.

Keywords: DNA methylation, epigenetics, methylation-specific PCR, tumor suppressor gene silencing, tumor marker

1. DNA methylation

DNA methylation is the post-replicative addition of a methyl group to the 5-carbon position of the cytosine ring, resulting in 5-methylcytosine. The transfer of a methyl group from S-adenosylmethionine (methyl donor) to cytosine (methyl acceptor) is catalyzed by DNA methyltransferases (DNMTs) and predominantly occurs on cytosines located 5' to guanosine, the so-called CpG dinucleotides [33]. CpG dinucleotides are underrepresented in the genome as a result of evolution-induced depletion related to the propensity of methylcytosine to deaminate spontaneously [15, 27,50,51]. Only small regions of DNA named CpG-islands contain CpG dinucleotides at its mathematically predicted frequency [2,21]. A CpG-island is defined as a 0.5–4 kb long stretch of DNA with a G:C content > 55% and an observed over expected frequency > 0.65 [21,33]. These CpG-islands contain approximately 20% of all CpG dinucleotides and are associated with the promoter regions of approximately half of all genes [27]. CpG-island containing promoters are normally protected from methylation, while CpG dinucleotides which are not associated with CpG-islands are heavily methylated [9]. Methylated cytosines are associated with DNMTs, methyl binding

proteins, histone deacetylases and transcription repressor proteins which collectively organize a transcriptional repressive, late-replicating chromatin structure [8,27,34]. DNA methylation is essential for normal mammalian development and is associated with genomic imprinting, transcriptional inactivation of the X-chromosome, ageing [9,27]. Recently it has been shown that aberrant promoter methylation plays a role in cancer by transcriptional silencing of tumor suppressor genes and inactivation of DNA repair genes [27]. Analyzing promoter hypermethylation can contribute to the understanding of cancer and has been proposed as molecular marker for diagnostic purposes [37].

2. Detection of DNA methylation

Various methods have been developed to analyze DNA methylation. Amongst these methods are restriction enzyme- and sodium bisulfite based approaches which directly detect methylation at the level of a single gene or the whole genome (for extensive reviews see [19,37]).

Restriction-enzyme based methods are based on the inability of methylation sensitive restriction enzymes to cleave methylated cytosines in their recognition site. The identification of the methylation status relies on Southern hybridization techniques or PCR and is based on the length of the digested DNA fragment [19,42]. The inability to digest methylated sequences results in longer fragments, indicating a methylated CpG dinu-

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cleotide. Although restriction-enzyme based methods are simple, rapid and highly sensitive, the technique is limited to specific restriction sites and requires a substantial amount of high quality DNA. In addition, incomplete digestion can lead to false positive results. The technique is suitable for genome-wide methylation analyses and marker discovery techniques but is less convenient for analyzing the methylation status of specific CpG-sites [19].

The introduction of sodium bisulfite genomic sequencing by Frommer et al. in 1992 [20] has made methylation studies accessible to a wide range of laboratories. Sodium bisulfite (NaHSO_3) based detection of DNA methylation can circumvent the problems associated with restriction enzyme analysis and is now extensively used. Treatment of single-stranded DNA with sodium bisulfite results in sequence differences due to deamination of unmethylated cytosines to uracil under conditions whereby methylated cytosines remain unchanged. The difference in methylation status marked by bisulfite reactivity can accurately be determined and quantified by PCR-based technology. Bisulfite sequencing techniques provide qualitative data on the methylation status of 5-methylcytosines in the amplicon between the sequence primers and thus requires primers specific for bisulfite converted, but not specific for unmethylated or methylated DNA. Although this approach provides detailed information on the methylation status of all CpG-sites, the method is laborious and time-consuming [19,37].

3. Methylation-specific PCR

In 1996, Herman and colleagues [28] introduced methylation specific PCR (MSP). MSP is based on the use of two distinct methylation specific primer sets for the sequence of interest (see Fig. 1). The unmethylated (U) primer will only amplify sodium bisulfite converted DNA in unmethylated condition, while the methylated (M) primer is specific for sodium bisulfite converted methylated DNA. MSP provides a positive, sensitive (detection of 1 methylated allele in a background of 1000 unmethylated alleles), quick and cost-effective test to analyze the methylation status of CpG dinucleotides in a CpG-islands making the technique suitable for high-throughput analysis of clinical samples [19,28]. Here we describe the most critical parameters determining the success and specificity of MSP, i.e. bisulfite conversion, primer design and PCR and discuss several post PCR validation approaches. For a detailed MSP-protocol, see [26].

3.1. Sodium bisulfite treatment

- Chemical reaction conditions to obtain optimal conversion of cytosine to uracil and limited degradation and fragmentation of the DNA have to be achieved. This can be ensured by treating (up to 1 μg) single stranded DNA (protein- and RNA free) with sodium bisulfite (pH 5.0, final concentration 2.5–3 M) for 16 hours at 50°C. Increasing the amount of input DNA carries the risk of incomplete conversion and leads to false-positive results. Shorter incubation times in combination with higher incubation temperatures are also possible.
- When using low quantities or paraffin-derived DNA, it is recommended to add a carrier (e.g. glycogen) to facilitate DNA precipitation after bisulfite treatment.
- Commercial kits, containing all reagents necessary for sodium bisulfite modification of DNA, are available.
- After sodium bisulfite conversion, DNA is single stranded, thus making the DNA sensitive for degradation. Therefore, sodium bisulfite treated DNA should be treated as RNA and stored at -20°C to prevent further degradation. To avoid repeated freezing and thawing, aliquoting is recommended.

3.2. Primer design

- The primers should contain one to three CpG dinucleotides in the 3' region of the primers to accomplish optimal discriminative power between methylated and unmethylated DNA and to increase the specificity of the primer annealing.
- Since amplification of unconverted unmethylated CpG dinucleotide will lead to an overrepresentation of methylation levels, the primers must be specific for sodium bisulfite converted DNA and therefore have to contain a substantial number of non-CpG cytosines in the original template. To assure sodium bisulfite conversion-specific primer annealing, the MSP primers should be tested on non-converted genomic DNA.
- The primer should be at least 23–24 bp in length to achieve gene specific primer annealing.
- It is preferable to design both U and M primer pairs with similar melting temperatures. Since the sodium bisulfite conversion decreases the GC content of the U primer, the sequence of the

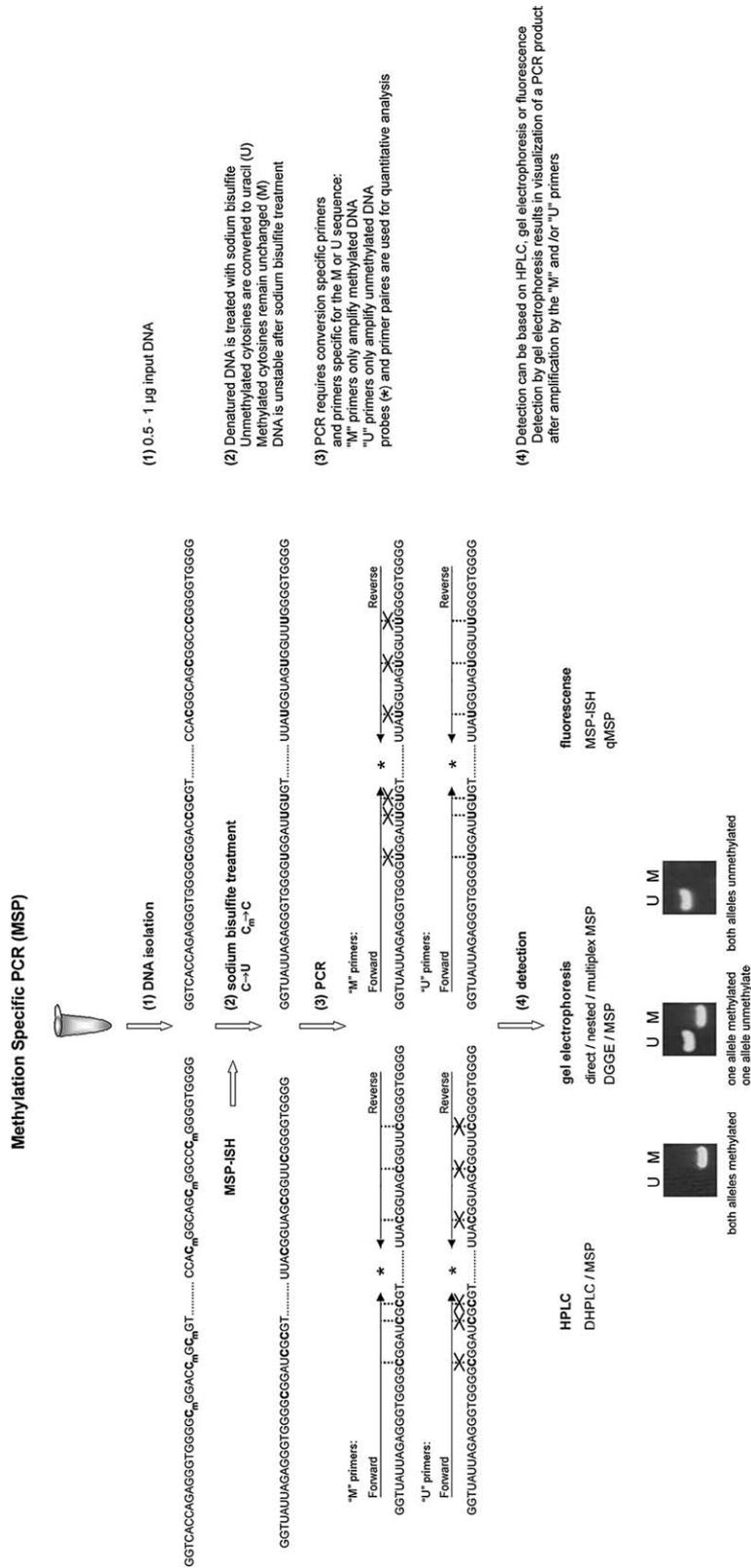


Fig. 1.

(1) 0.5 - 1 µg input DNA

(2) Denatured DNA is treated with sodium bisulfite
 Unmethylated cytosines are converted to uracil (U)
 Methylated cytosines remain unchanged (M)
 DNA is unstable after sodium bisulfite treatment

(3) PCR requires conversion specific primers
 and primers specific for the M or U sequence:
 "M" primers only amplify methylated DNA
 "U" primers only amplify unmethylated DNA
 probes (*) and primer pairs are used for quantitative analysis

(4) Detection can be based on HPLC, gel electrophoresis or fluorescence
 Detection by gel electrophoresis results in visualization of a PCR product
 after amplification by the "M" and/or "U" primers

U-primer needs to be extended into the 5' region. This constraint will inevitably lead to a variation in start position and length but allows paired analyses in a single thermocycler and provides a convenient way to recognize each amplicon after gel electrophoresis.

- To achieve good sensitivity and specificity, the U and M primer should be comparable in their sequence and amplify the same region.
- To facilitate MSP on fragmented DNA obtained from formalin fixed, paraffin-embedded archival clinical tissue, the MSP product to be amplified should not exceed 150 bp [59].
- Software tools are available to support MSP primer design [39,53].

3.3. PCR conditions

- To facilitate amplification of GC-rich DNA sequences it is recommended to use β -mercaptoethanol in the PCR buffer.
- The annealing temperature for specific determination of methylation patterns is critical. MSP relies on the specific annealing of primers based on matches or mismatches of the primer sequence to bisulfite treated DNA. Predicted annealing temperatures should be used to examine amplification of unmethylated or methylated sequences, but ideally the most specific amplification will be determined empirically through examination of multiple annealing temperatures. This is most efficiently accomplished by using a thermocycler with a gradient block. Published annealing temperatures should be confirmed with the thermocycler in use in an investigators laboratory prior to embarking on additional methylation analysis.
- Cycles of amplification must be determined for each MSP reaction condition. If high numbers of amplification cycles are used, even specific M-primers can rarely accept mismatches when annealing to DNA, which will result in the appearance of amplification in the methylated reaction. Typical amplification should not exceed 35 cycles, but must be determined empirically.
- To control PCR efficiency, a dilution series of methylated DNA in a background of unmethylated DNA can be included.

To interpret the MSP results properly, it is important to include positive, negative and H₂O controls. Positive controls are specific for U and M sequences and give an impression of the specificity of the respective reactions. DNA known to be U for the gene of interest can be used as positive control for the U condition. Methylated PCR product in the M reaction of the U control implicates lack of specificity of the M reaction and disturbs proper interpretation of the results. As positive control for the M reaction, *in vitro* methylated DNA can be used.

After amplification, the products of the U and M reactions can be visualized by 6–8% nondenaturing polyacrylamide gel electrophoresis, allowing clear separation of the small products. High percentage horizontal agarose gels can be used as alternative. Fully unmethylated samples will reveal a PCR product only when U-primers are used, as fully methylated samples will give a distinct band in the M reaction. Since clinical tissue samples should be considered heterogeneous, both U and M amplicons can be expected.

3.3.1. Post-PCR validation

- Following PCR amplification, specificity of amplification can be confirmed by product analysis. MSP amplification results in loss of information of the CpG-sites contained within the primer, but all CpG-sites between primers in the PCR product remain informative. False priming, for reasons described above, can be examined by post-PCR analysis of the PCR product. Three principle ways can be employed. The use of fluorescent dyes (Syber Green) can facilitate the examination of differences in melting temperature based on CG content, much the same way as DGGE (described below). This however, does not provide much additional information on location of the methylation changes in the amplified product. Post-PCR restriction analysis can provide information for specific sequences present in restriction sites [28]. In addition, sequencing of the PCR product can be performed and provides complete CpG methylation information on all CpG-sites within the PCR product.

MSP has been widely used for promoter methylation profiling of cancer [14], diagnosing Prader–Willi and Angelman Syndrome by making use of abnormal methylation at the small nuclear ribonucleoprotein-associated polypeptide N (SNRPN) gene [36] and to evaluate X chromosome inactivation [57].

4. Variants and applications of MSP

4.1. (Multiplex) nested MSP

Since the publication of the 'original' MSP protocol [28], several variants have been developed. To increase the sensitivity of MSP for detection of methylated DNA in a background of unmethylated DNA, Palmisano et al. (2000) developed a nested, two-stage MSP [46]. The first round PCR is performed using primers which amplify sodium bisulfite-modified DNA, although these primers do not discriminate between methylated and unmethylated alleles. This means that the primers are located in regions flanking the MSP primers and harbouring a substantial number of cytosines but no CpG dinucleotides (analogues to bisulfite sequence primers). The obtained PCR product is diluted 50–100 fold and subjected to second round PCR reactions using U and M specific MSP primers. Nested MSP allows a more sensitive (1 methylated allele in >50,000 unmethylated alleles) detection of methylation in clinical samples harbouring small amounts of poor quality DNA.

False positives, caused by the lack of the single-stranded conformation, renaturation and uncompleted sodium bisulfite conversion caused by the protection of a methylated cytosine of the neighbouring unmethylated outer cytosine, have been reported [19,25,49]. To check the completion of the bisulfite treatment, an internal standard that consists of an *in vitro* methylated oligonucleotide can be included [25]. If DNA obtained from formalin fixed, paraffin-embedded archival tissue is used, it is recommended to design first round primers to obtain amplicons smaller than 150 bp to further enhance the success rate [59]. van Engeland et al. (2003) multiplexed the first round primers of different genes allowing simultaneous analysis of multiple colorectal cancer tumor suppressor gene promoters [59]. This approach offers the additional advantage that the PCR-product obtained with the first round PCR is stable and can be stored for a long time. This strategy has now been used successfully for methylation profiling of gastrointestinal stromal tumours [29] and hepatocellular carcinoma [65].

Classical MSP and its nested variant are very suitable for detection of methylated tumour DNA in a variety of body fluids. Colorectal- [23,43,44,64,66], lung- [16], liver- [61,62], head and neck- [54] and brain [3] tumour derived DNA has been detected in serum and plasma. Methylated gene promoters in urine and semen have been used to diagnose prostate cancer [22]

and methylation markers present in ductal lavage has reported to be a useful adjunct to mammography in the early diagnosis of breast cancer [17]. Sputum [6,46] and bronchial brushes [6,56] have been analyzed for promoter hypermethylation in order to diagnose lung cancer while Rosas and co-workers reported that aberrant promoter methylation in saliva can be used for detecting and monitoring head and neck cancer [52].

4.2. Quantitative MSP

To exactly quantify the number of methylated alleles, several quantitative MSP (Q-MSP) protocols have been developed which take advantage of the development of quantitative real-time PCR. Amongst these assays are approaches using fluorescently labelled MSP primers and those based on Taqman[®] technology (MethyLight) [13,40]. Two primers and a fluorogenic, dual-labelled probe (reporter dye (e.g. FAM) and quencher dye (e.g. TAMRA)) specific for unmethylated or methylated DNA are used in MethyLight [13]. During the extension phase of the PCR, the 5' to 3' exonuclease activity of the TaqDNA Polymerase cleaves the reporter from the probe, releasing it from the quencher, resulting in an increase in fluorescence emission. Data are normalized to an internal reference gene, e.g. myoblast determination protein 1 (MYOD1) or β actin (ACTB) which are designed in a region that does not contain any CpG nucleotides to allow an unbiased amplification. Rand et al. reported false positive results due to the amplification of unconverted DNA and recommend the inclusion of a sodium bisulfite conversion detection step (ConLight-MSP) to avoid overestimation of DNA methylation [47]. Primer design improvements, as described above for MSP primers, can also eliminate this amplification of unmodified DNA. Lo and colleagues include primers as well as probes specific to methylated, unmethylated or unconverted wild-type DNA [40,63]. Most commonly, these studies determine only methylated bisulfite converted DNA for the gene of interest and compare these results to an internal reference gene [10,24,30,31,38,48,55,58]. It should be noted that the quantitation provided is for the relative amounts of methylated DNA with a particular methylation pattern compared to some input DNA (reference control gene or unmethylated sequence). This quantitation may reflect actual changes in methylation density or differences in tumor DNA within a specific sample. Recently, Fackler et al. introduced a quantitative multiplex nested MSP (QM-MSP) assay to further increase the sensitivity of

this technology [18]. In the first PCR reaction external primers lacking CG nucleotides, thereby obtaining DNA amplification independent of methylation status, are used. In the second reaction, quantitative analysis of methylated and unmethylated DNA for each gene was performed separately with the primer pairs and probes.

An additional advantage of Q-MSP is that it does not involve additional PCR steps, gel electrophoresis or hybridisation. These characteristics make Q-MSP applicable for fast screening of DNA methylation in clinical samples [13]. Several studies describe the applicability of Q-MSP for analyzing formalin-fixed, paraffin-embedded tissues. Jeronimo and colleagues successfully compared GSTP1 promoter methylation in formalin-fixed, paraffin-embedded tissue of non-neoplastic prostatic tissue with organ-confined prostate adenocarcinoma [31]. Also, formalin-fixed, paraffin-embedded specimens of the upper gastrointestinal tract [55], prostate biopsies [12], breast tumours [18] and even laser-assisted micro dissected samples of breast tumours [38] have been analyzed for promoter hypermethylation quantitatively. In addition, Q-MSP has been used to detect aberrant methylation profiles of circulating tumor DNA in serum of patients with oesophageal- [35], prostate- [32], lung- [58], liver- [63] and cervical [60] cancer. Furthermore, promoter hypermethylation has been detected by Q-MSP in cervical scrapings [48].

4.3. *In situ* MSP

MSP data represent only a small percentage of the analyzed cells in a population unless the PCR is done semi-quantitative, with the result that the extent of heterogeneity for loss of expression during tumorigenesis cannot be defined. Furthermore, the precise timing of DNA methylation changes in specific cell types during embryonic development and carcinogenesis cannot be analyzed. To overcome this issue, Nuovo and coworkers (1999) combined MSP with *in situ* hybridization (ISH) allowing *in situ* detection of methylated DNA on tissue slides [45]. Three sequential tissue sections are placed on a slide, treated with sodium bisulfite and analyzed by MSP. One slide is analyzed with U-primers, one slide with M-primers, while no primers are applied to the control slide. After amplification, *in situ* hybridization (ISH) is performed using a U- or M-specific internally digoxin-labeled probe, corresponding to the U and M products generated by solution-phase MSP. Visualization can be performed

by nuclear fast red staining which colours the negative cells pink and the positive cells blue. *In situ* MSP offers information at the cellular level since visualization of the methylation of specific alleles in individual cells is possible. The method can be used to examine the role of CpG-island methylation during embryogenesis, developmental timing of -and aberrant imprinting in specific cell types, mammalian X chromosome inactivation and tumour progression in clinical samples with heterogeneity and the precise cellular consequences of the methylation for gene expression [19,45].

In addition to gel- and fluorescence based detection of MSP products, HPLC and DGGE based MSP-variants have been developed.

4.4. HPLC

Recently, denaturing high-performance liquid chromatography (DHPLC) in combination with MSP for the analysis of methylation profiles has been described [4,5,7]. After sodium bisulfite conversion, PCR amplification with primers specific for sodium bisulfite converted DNA, but not for unmethylated and methylated alleles is achieved, followed by denaturation and renaturation of the PCR products, enabling the formation of heteroduplex DNA detectable by HPLC. The principle of the method is based on different denaturing temperatures between bisulfite converted methylated and unmethylated fragments caused by different G/C contents. DHPLC in combination with MSP allows for the detection of minimal CpG methylation as well as complete CpG methylation of a sample. Furthermore, the degree of CpG-site methylation can be estimated from the degree of heteroduplex formation and the presence of additional and distinct heteroduplex signals can indicate the presence of a mutation in the fragment analyzed.

Matin et al. also used HPLC to differentiate between methylated and unmethylated amplicons using ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC), which is based on distinction of polynucleotide chain length after bisulfite treatment and primer extension reactions carried out with deoxy- and dideoxynucleotides [41].

4.5. DGGE

Epigenetically modified genotypes can be characterized by combining MSP with denaturing gradient gel electrophoresis (DGGE) [1]. The principle of this application is that after bisulfite conversion, the am-

plicon of methylated DNA has a higher GC content compared to unmethylated DNA, resulting in different melting temperatures, thus the basis for separation is based on thermal stability. In addition to detection of almost any sequence change in every DNA region, the technique allows also detection of differentially methylated sequences which is not managed by the usage of restriction enzymes. The first studies described the ability to make a distinction between fully, hemi and unmethylated sites [11]. Later on, a more sensitive method was developed by using a GC-clamp and the distinction between methylated or unmethylated CpG-sites could be made on single base sequence changes in a certain fragment [1]. This approach is not technically MSP, but can be used to examine overall methylation differences.

In summary, MSP is a rapid method to detect aberrant promoter methylation in tissue and body fluids and provides a non-invasive method for molecular diagnosis of diseases associated with aberrant DNA methylation.

Acknowledgements

Conflict of interest statement: "J.G.H. is a paid consultant to OncoMethylome Sciences. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies."

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