Methylation Specific PCR (MSP): Nested PCR vs Unnested PCR

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ABSTRACT
Methylation-specific PCR (MSP) is a valuable technique for studying DNA methylation patterns due to its straightforward design and implementation, high sensitivity in detecting methylated DNA, and ability to analyze large sample sizes cost-effectively rapidly. However, researchers need to be cautious when working with new samples or samples stored in the freezer for an extended period. Freezing does not prevent the action of DNAase enzymes, which can lead to reduced DNA extraction yields. During MSP, DNA undergoes changes, and PCR is performed using two sets of primers that specifically target methylated and unmethylated DNA regions. The use of bisulfite conversion treatment, an essential step in methylation analysis, presents significant challenges. One challenge is that bisulfite treatment can cause DNA fragmentation, particularly when the initial DNA concentration is low. In nested MSP, where an additional round of PCR is performed, smearing may occur due to the high DNA concentrations used. Therefore, for new samples with a DNA concentration of 1-10 µg/µL, it is recommended to use the unnested MSP technique prior to bisulfite conversion treatment.

Keywords: *Bisulfite, Methylation, Nested MSP, and Unnested MSP*

1. INTRODUCTION

Methylation markers are commonly used to diagnose and predict the prognosis of diverse cancers[1], metabolic diseases such as type 2 diabetes[2], organ development disorders[3], and skin disorders[4]. The methylation test is very flexible and can be used in all samples, such as blood, organ, saliva, and cerebral fluid samples—thus, methylation markers are more attractive to use as a diagnostic tool.

Methylation-specific PCR (MSP) is a cost-effective method commonly employed to examine methylation markers, offering an advantageous alternative to more expensive techniques such as bisulfite sequencing. MSP is a robust approach for qualitative DNA methylation analysis, primarily due to its simplicity in design and implementation. It enables the detection of methylated DNA at various sensitivity levels, allowing for the identification of even minute amounts of methylation. Furthermore, MSP facilitates the rapid and cost-efficient screening of a large number of samples, making it particularly suitable for studies that require high-throughput analysis of DNA methylation patterns[5].

The MSP method is a technique used to detect DNA methylation patterns by amplifying specific regions of DNA using PCR. In MSP, primers are designed to target either methylated or unmethylated CpG sites. After the PCR amplification, the resulting amplicons or bands can be visualized on gels made of agarose, boric acid, or nondenaturing polyacrylamide, depending on whether the targeted CpG sites are methylated or unmethylated.

One challenge in analyzing DNA methylation patterns is the fragmentation of genomic DNA caused by bisulfite conversion treatment, which is a process used to convert unmethylated cytosines to uracils while preserving methylated cytosines[6]. This DNA fragmentation can affect the interpretability of the MSP results.

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In light of this, the aim of this study is to investigate the distinctions between unnested MSP and nested MSP methods. The focus is on comparing the performance of these two approaches in detecting and analyzing DNA methylation patterns after bisulfite conversion treatment. The hypothesis suggests that nested MSP, which involves an additional round of PCR amplification using nested primers, may overcome the interpretability issues caused by DNA fragmentation and provide more accurate and sensitive results compared to unnested MSP.

2. MATERIALS AND METHODS

On 1 September 2020, the Research Ethics Committee of the University of Surabaya, Indonesia (No. 138/KE/VIII/2020) approved this research proposal compliant with ethical standards. Using the Favorgen DNA isolation kit, DNA was extracted from Swiss Webster mice muscle samples. After following the kit's instructions for DNA isolation, the bisulfite conversion treatment was applied. Before bisulfite conversion treatment, DNA samples must be between 1-10 µg/µL g. The MSP approach is based on Dwi Putra et al., 2019[7].

Following bisulfite conversion treatment, the DNA samples were subjected to both nested MSP and unnested MSP techniques, followed by Methylation-Specific PCR (MSP). The resulting products from these amplification methods were then diluted and loaded onto a 2% agarose gel for visualization. The gel electrophoresis was conducted to observe and analyze the amplified DNA fragments, allowing for the detection and differentiation of methylated and unmethylated CpG sites based on their migration patterns within the gel.

3. RESULTS AND DISCUSSION

The utilization of nested or unnested Methylation-Specific PCR (MSP) serves the objective of enhancing the sensitivity of the assay for the analysis of DNA methylation. This aim is achieved by using two different methods: the first method involves a single round of PCR (unnested MSP), while the second method includes an additional round of PCR (nested MSP).

Upon analyzing the results obtained from electrophoresis, it becomes evident that the nested MSP approach yields distinct characteristics compared to the unnested MSP approach. In Figure 1, the images of the electrophoresis gel showcase a smeared pattern and intense bands when using the nested MSP method. These distinctive features suggest that the nested MSP technique enhances the sensitivity of the assay, potentially enabling the detection of lower levels of methylated DNA compared to the unnested MSP method.

The MSP primers preferentially amplify only a fully methylated (M) template, the primer binding region should include as many CpG sites as possible. To confirm the locus's non-methylated (U) form, a second set of primers was designed to target its non-methylated (U) form exclusively [8]. There are many significant difficulties concerning the conversion of genomic DNA to bisulfite conversion treatment. The DNA is fragmented after incubation of genomic DNA with bisulfite conversion treatment. Depending on the intended use, this fragmentation may hinder subsequent experiments. This can be mitigated by changing the incubation time of bisulfite conversion treatment or using additional PCR (nested PCR) [6].

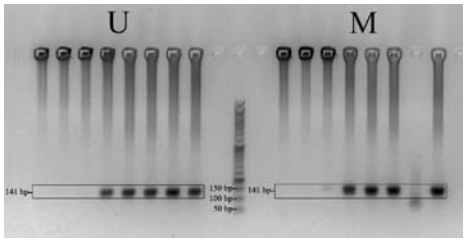
Nested MSP was developed to address the shortcomings of conventional MSP. If an experiment cannot amplify a product enough for direct MSP analysis, nested MSP may be performed. Nested MSP requires an extra primer set consisting of two primers covering the amplified product's sequence. Utilizing the amplified products from the first PCR with nested MSP primers, a second PCR is done using two pairs of primers (each pair targeting a distinct state of methylation) using the first PCR's generated products. The primary advantage of this two-step process is that the first round of amplification salvages fragmented input DNA. The nested MSP technique is strongly recommended when not utilizing contemporary bisulfite kits. (Figure 2) [9,10].

Following MSP, gel electrophoresis is performed to assess the presence of bands by UV transillumination following ethidium bromide staining. Completely unmethylated samples will only provide a PCR product when U-primers are employed since completely methylated samples yield a distinct band in the M-primers [6,11].

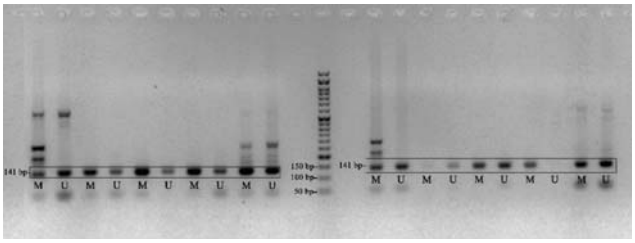
The intensity of the bands observed on the gel was utilized to evaluate the quality of the electrophoresis. Several factors can influence the band brightness, including the amount of DNA loaded, the presence of a molecular weight marker, and the specific gel staining method employed. It is important to note that the thickness of the gel can significantly impact the resolution of the DNA fragments. For optimal resolution, horizontal gels should ideally be cast no thicker than 3-4 mm. Thicker gels, such as those with a thickness of 10 mm, may result in reduced clarity and resolution, leading to a haziness in the visualization of smaller DNA fragments when compared to a 3 mm thick gel, which provides more consistent resolution throughout the gel [12].

The width (thickness) of the comb used to create wells in agarose gels may also influence DNA fragment resolution. With a larger comb, more volume may be inserted into the well, but the DNA bands may be broader. A comb with a width of 1 mm will produce more distinct DNA bands [12], [13]. The amount of DNA that can be loaded onto a gel is influenced by two main factors: the distribution of DNA fragments (including the number and size of target fragments) and the capacity of

the well (referring to its width or depth). When DNA



MSP with Nested MSP



MSP with Unnested MSP

Figure 1 Comparison between MSP Optimization with Nested MSP (2x PCR) and MSP Without Nested MSP (1 time PCR). Note: U: unmethylated sample; M: methylated sample; BP: base pairs; 141 bp: Target Length of the Product

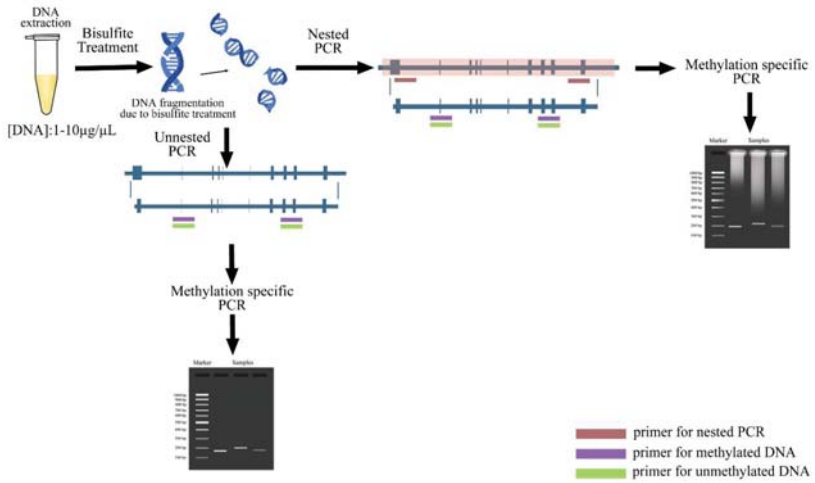


Figure 2. The MSP procedure. DNA was extracted from muscle or other tissues. The bisulfite conversion process may cause DNA fragmentation. Both nested and unnested MSP techniques were utilized to generate bisulfite results. Nested MSP, which involved a higher DNA concentration, resulted in smearing patterns on the electrophoresis gel. In contrast, unnested MSP provided an adequate number of samples for analysis within the recommended DNA concentration range of 1-10 µg/µL.

fragments are larger in size (>10 kb), there is a risk of overloading the gel, which can result in undesirable effects such as lagging and smearing of the DNA bands. This is because larger DNA fragments require more space to migrate properly through the gel matrix, and when the capacity of the well is exceeded, the DNA can have trouble in moving at the desired pace and may exhibit irregular migration patterns [12]. Therefore, it is important to consider the size of the DNA fragments and the well capacity to prevent overloading issues and ensure accurate and clear separation of the DNA bands during gel electrophoresis.

The concentration of DNA in the specific bands of interest is crucial. Careful consideration should be given to the impact of DNAase activity when deciding whether to utilize fresh or frozen samples and how long to store them. It's important to note that freezing the samples does not halt the action of DNAase, leading to a reduction in DNA concentration. When frozen samples were held at -20 °C for a duration of 10 days, approximately 65% loss of DNA concentration and a 90% decrease in the amount of extracted DNA were observed [12,14].

Before performing MSP, bisulfite conversion treatment must be completed. The bisulfite conversion treatment has been instrumental in DNA methylation research. The differing effects of bisulfite conversion treatment on unmethylated and methylated cytosines under acidic circumstances result in deamination and conversion of unmethylated cytosines to uracils. Unaffected methylation of cytosines permits differentiation between methylated and unmethylated cytosines in the transformed DNA [6].

The primary objective of utilizing nested MSP is to enhance the sensitivity of MSP by amplifying a greater amount of DNA following the bisulfite conversion treatment. However, it is important to note that the bisulfite conversion process itself can lead to a reduction in the overall DNA quantity, making the MSP results undetectable. In contrast, unnested MSP samples often yield visible bands that can be analyzed. The electrophoresis results of nested MSP may appear smeared due to the higher concentration of DNA templates used in the process.

These results suggest that researchers can opt for unnested MSP as a substitute when the DNA concentration before bisulfite conversion treatment is between 1-10 µg/µL and fresh samples are accessible. It is important to consider that samples stored for a long duration may undergo DNA degradation caused by DNAase activation, leading to reduced DNA content during analysis. Additionally, careful attention should be given to the size of DNA fragments and the capacity of wells during gel electrophoresis to avoid overloading and ensure precise and distinct separation of DNA bands.

To ensure the success of qualitative DNA methylation analysis, future researchers are advised to thoroughly evaluate the sensitivity requirements of their study. It is crucial to carefully assess the advantages and disadvantages of both nested and unnested MSP methods, taking into account the particular context and the availability of DNA samples. Furthermore, optimizing the DNA concentration and storage conditions should be prioritized to guarantee reliable and reproducible results.

Considering the specific experimental needs, researchers should weigh the benefits and drawbacks of nested and unnested MSP. Although nested MSP provides improved sensitivity and specificity, it comes with increased complexity and higher costs. On the other hand, unnested MSP is simpler and more cost-effective but may have limitations in terms of sensitivity and specificity. Making a well-informed decision between these two techniques is vital for the effective execution of qualitative DNA methylation analysis.

Methylation-Specific PCR is a valuable technique for qualitative DNA methylation analysis. Nested MSP can enhance sensitivity but may exhibit smeared results, while unnested MSP provides visible bands for analysis. Researchers should consider DNA concentration, storage conditions, and the impact of bisulfite conversion treatment when choosing between these methods. The unnested MSP technique is recommended for new samples with a DNA concentration of 1-10 µg/µL prior to bisulfite conversion treatment.

4. CONCLUSION

In conclusion, Methylation-specific PCR (MSP) stands out as a valuable technique for studying DNA methylation patterns due to its simplicity, high sensitivity, and cost-effective analysis of large sample sizes. For samples with a DNA concentration of 1-10 µg/µL, it is advisable to opt for unnested MSP before bisulfite conversion treatment to mitigate issues associated with DNA fragmentation. This precaution ensures the preservation of DNA integrity and enhances the reliability of methylation-specific PCR analyses.

AUTHORS' CONTRIBUTIONS

SEDP and FMH performed a major part of the research work, performed statistical analysis, interpreted results, and wrote the initial draft. LTM revised the figures, improved the initial draft, and helped in lab work. LTAT also helped in lab work. HW, RI, DK, HWS conceived the idea, supervised the work, revised, modified, and approved the final draft.

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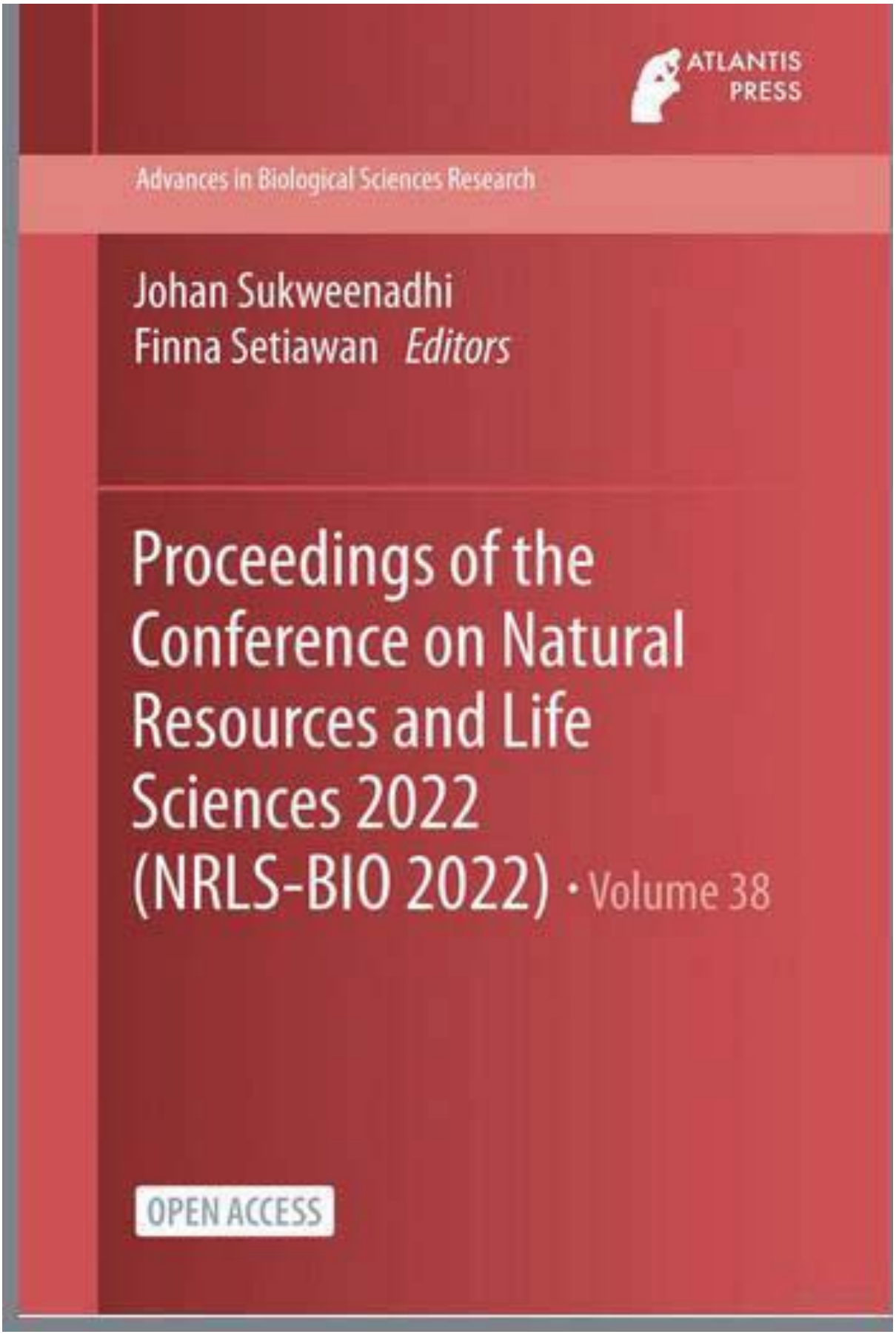
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