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A convenient method to generate methylated and un-methylated control DNA in methylation studies

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A B S T R A C T

Methylated and un-methylated control DNA is an important part of DNA methylation studies. Although human and mouse DNA methylation control sets are commercially available, in case of methylation studies on other species such as animals, plants, and bacteria, control sets need to be prepared. In this paper a simple method of generating methylated and un-methylated control DNA is described. Whole genome amplification and enzymatic methylation were performed to generate un-methylated and methylated DNA. The generated DNA were confirmed using methylation sensitive/dependant enzymes, and methylation specific PCR. Control reaction assays confirmed the generated methylated and un-methylated DNA.

Key words: Epigenetics; DNA methylation; Control DNA

INTRODUCTION

DNA methylation occurs by adding methyl group to carbon 5́ of the cytosine base in the CpG dinucleotides in CpG islands. The CpG islands are located in 60% of the promoters of mammalian genes, and have a higher CpG ratio greater than 60%. These regions are very important in gene expression regulation by chromatin rearrangements, in which the methylation of these promoter regions could lead to heterochromatin formation and gene silencing [1]. Previous studies have shown that aberrant methylation is considered to be a hallmark of malignant transformation in different human cancers, genomic imprinting, and in different developmental processes [2-3].

Many methods summarized in recent literature have been developed for DNA methylation analysis using different strategies [4]. These methods have considerably relied on methylation sensitive restriction digestion, immunopreciptation, and sodium bisulfite

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conversion of desired DNA. Positive methylated and negative un-methylated controls are required to confirm methylation evaluation assays. Many different DNA sets for methylation controls are commercially available; however, a cost-efficient and reproducible method for generating DNA sets is needed. In this work we describe a simple method for generating whole genome CpG methylated and un-methylated DNA controls that could performed as positive and negative controls for DNA methylation analysis studies.

MATERIALS AND METHODS

Preparation of whole genome un-methylated DNA: Whole Genome Amplification (WGA) of genomic DNA was performed by Improved Primer Extension Pre-amplification PCR (I-PEP-PCR) [5]. In this method, a degenerate random 15-mer primer (5́-NNNNNN NNNNNNNNN-3́) was performed to amplify the whole genome sequence using the following PCR temperature profile: an initial dissociation temperature of 94ºC for 2 min, and 50 cycles of 94ºC for 60 sec, 28 ºC (ramping to 55ºC) for 3 min, 55 ºC for 4 min, 68ºC for 30 sec, and a final extension temperature of 68ºC for 8 min. The amplification was performed using Expand Long Template PCR System (Roche, Mannheim, Germany) in a Mastercycler® pro thermal cycler (Eppendorf, Hamburg, Germany). The amplified products were visualized on 1% agarose gel electrophoresis and Sybr Green dye. The amplified products were treated by McrBC, an endonuclease that cleaves DNA containing methylcytosine on one or both strands. McrBC does not act upon un-methylated DNA and degrades initial methylated template DNA. Subsequently, the phenol-chloroform separation technique was performed to purify the amplified product from PCR and other reagents.

Preparation of whole genome methylated DNA: The purified whole genome amplified PCR product was used as template for the methylation of all cytosine residues at CpG dinucleotide sites using CpG Methyltransferase M.SssI and S-adenosyl methionine (SAM) as a methyl donor (NEB, UK).

Quality Control Assays

Enzymatic control: *HpaII*/*McrBC* (NEB, UK) methylation sensitive and dependent enzymes were used to control methylated and un-methylated DNA. For this reason, digestion with these enzymes was used to determine the efficiency of methylation reaction; the digestion reaction results being visualized on 1% agarose gel electrophoresis.

Bisulfite conversion and methylation specific PCR (MSP) control: In support of our assays, the sodium Bisulfite conversion of un-methylated and methylated DNA was carried

out using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The methylation specific PCR (MSP) was performed using methylated and un-methylated specific primer sets. The primer sets for an intron 1 of FAS gene were extracted from previously published work [6], and were used for discrimination between methylated and un-methylated samples.

RESULTS

Whole genome amplification and methylation of DNA: Whole genome amplification was done using an I-PEP-PCR protocol in order to generate un-methylated PCR product. Besides, a non-template control (NTC) reaction was set to determine non-template amplification, showing no amplification in the NTC reaction (Figure 1). The I-PEP-PCR product was digested by the *McrBc* enzyme and re-purified by the phenol-chloroform method. Afterwards, the template was methylated by the MsssI methyltransferase enzyme.

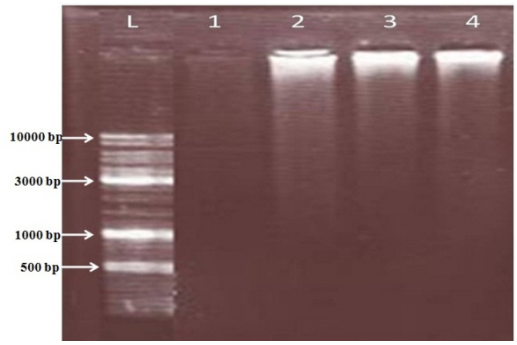

Figure1. Agarose gel electrophoresis of whole genome amplified DNA. The peripheral blood DNA was amplified using I-PEP-PCR protocol and visualized on 1% agarose gel (wells 2, 3, and 4) along with a non-template control (well 1).

Methylation Quality Controls: The generated un-methylated and methylated templates were digested using *HpaII* methylation sensitive and *McrBc* methylation dependant enzymes and visualized on 1% agarose gel electrophoresis (Figure 2). The figure shows that *McrBc* digested the methylated DNA and left the un-methylated DNA intact (wells 1 and 2), while *HpaII* digested the un-methylated DNA and did not affect the methylated DNA (wells 3 and 4).

Methylation specific PCR: Methylation specific PCR (MSP) was performed on sodium bisulfite converted DNA using methylated and un-methylated primer sets. The results confirmed the generation of methylated and un-methylated DNA (Figure 2).

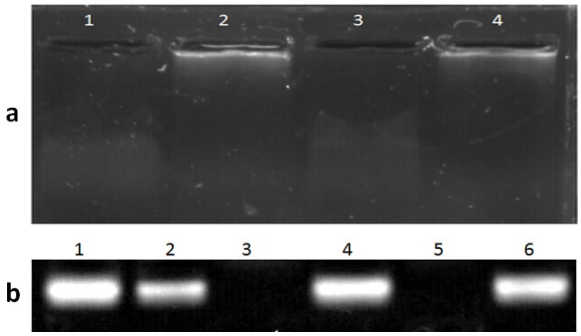

Figure 2: Control assays for generated DNAs (a) Visualization of *HpaII* and *McrBc* incubated methylated and un-methylated DNA. The wells 1 and 2 show digested methylated and intact un-methylated DNA by *HpaII* enzyme, while wells 3 and 4 show digested methylated and un-methylated DNA by *McrBc* enzyme. (b) Methylation Specific PCR (MSP) for confirmation of generated methylated and un-methylated DNA. The wells 1 and 2 show methylated and un-methylated controls provided by Qiagen. The wells 3 and 4 show MSP on un-methylated DNA using methylated and un-methylated specific primer sets respectively. The wells 5 and 6 show MSP on methylated DNA using un-methylate and methylated specific primer sets.

DISCUSSION

In this paper we have described a convenient method for generating control methylated and un-methylated DNA, which can be used for proper assessment of DNA methylation in many DNA samples. As an important part of epigenetics, DNA methylation, is involved in many biological processes such as tissue specific gene regulation, genomic imprinting, and X-chromosome inactivation. Moreover, DNA methylation modifications have been reported in many human diseases such as cancers [7]. Many techniques have been developed for DNA methylation assessment, and most of them take advantage of restriction digestion and sodium bisulfate conversion of DNA [8]. In conclusion, our results showed that the described method for generating whole genome methylated and un-methylated control sets is reliable and easy to use to produce methylation control DNA sets in many methylome studies on other creatures.

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Conflict of Interest: Author has no financial or any non-financial competing interests.

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