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Methylated-CpG Island Recovery Assay

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Abstract

Alterations in DNA methylation patterns are implicated in playing a major role in the development of cancer, thus highlighting the need to continually develop new technologies to analyze epigenetic marks. Methylated-CpG Island Recovery Assay (MIRA), based on the high affinity of the MBD2b/MBD3L1 complex for double-stranded methylated DNA, allows for the recovery of methylated DNA without the use of bisulfite conversion or antibody recognition. MIRA is capable of detecting low-density methylation of a single methylated CpG nucleotide. This technique can be used in conjunction with microarrays or next-generation sequencing to analyze recovered methylated DNA on a genome-wide scale.

Keywords

DNA methylation; Methylated-CpG Island Recovery Assay; methyl-CpG binding protein; microarray; next-generation sequencing

1. Introduction

DNA methylation at CpG dinucleotides is an important epigenetic modification in mammalian cells. Epigenetic modifications describe the heritable changes in gene expression that occur without a change in the primary DNA sequence (1). Regions of DNA where a large density of CpG dinucleotides exist are known as CpG islands which are found in the proximal promoter region in nearly half of the genes in the mammalian genome (2). In normal cells, hypermethylated promoters serve to maintain genes in an inactive state that are not required for that specific cell type. During oncogenesis, genome-wide hypomethylation combined with site-specific hypermethylation leads to alterations in the expression of genes associated with tumor development (3, 4). Because changes in DNA methylation have been implicated in playing a central role in tumorigenesis, it is important that techniques be available to readily analyze changes in DNA methylation at CpG islands (5, 6). While numerous methods exist for analyzing DNA methylation, the Methylated-CpG Island Recovery Assay (MIRA) is of high interest. It is important to note that MIRA is sensitive enough to detect low-density methylation of a single methylated CpG nucleotide; however, it is the spacing between individual methylated CpG nucleotides that possibly limits the sensitivity of MIRA (7). This technique can be used in combination with a variety of microarrays to analyze changes in the methylome (8).

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In 1975, the first assay was developed to analyze DNA methylation at specific nucleotide sequences using methylation-sensitive restriction enzymes called isoschizomers. Isoschizomers are capable of cutting the same unmethylated DNA sequence; however, only one of the two restriction endonucleases will cut the nucleotide sequence if it is methylated. Thus, the presence of DNA methylation at specific cytosine residues can affect the results obtained using restriction endonucleases. Methylation of a specific genomic sequence can be observed through Southern blot hybridization (9).

Since the initial attempt of locating DNA methylation in the mid- 1970's, a wide variety of other methods have been created. Although many techniques for detecting methylated CpG islands exist, sodium bisulfite treatment of genomic DNA has become the preferred method for studying DNA methylation. Bisulfite treatment of genomic DNA converts unmethylated cytosines to uracils by deamination and leaves methylated cytosines unaffected. After the area of interest is amplified through PCR, it is usually cloned and sequenced. Using PCR, this technique analyzes only a small region of the genome, obviously presenting a large limitation of the procedure (10, 11). In addition, the loss of a base through bisulfite sequencing compromises the complexity of data generated and requires comparison to the original sample sequence. Although bisulfite treatment of DNA has been incredibly valuable over the last few decades, the inability to compare DNA methylation density between large discrete genomic regions at the single nucleotide level has limited its usefulness in the post-genomic era.

The first next-generation assay for studying genome-wide DNA methylation is the methylated DNA immunoprecipitation microarray (MeDIP) technique. MeDIP is capable of detecting DNA methylation at CpG islands across the entire genome. MeDIP relies on antibodies that recognize 5-methylcytosine (5mC) to immunoprecipitate single-stranded DNA that contains heavily methylated CpG sites. The methylated DNA can then be further analyzed with DNA microarrays or parallel sequencing techniques by simply quantifying the regions that were recovered. Because MeDIP relies on genomic regions that are heavily methylated, regions of DNA with a low CpG dinucleotide density may not be accurately analyzed by MeDIP despite the fact that DNA methylation changes may exist. In addition, antibodies can be expensive and rather variable in their recognition response (12).

The latest and most efficient means of analyzing genomic DNA methylation patterns is MIRA. MIRA does not rely on bisulfite conversion of DNA or antibody recognition of single-stranded DNA. MIRA can analyze DNA methylation patterns in double-stranded DNA and has a higher sensitivity and specificity than the aforementioned methods. Mammalian genomes encode several proteins that are capable of binding to methylated CpG dinucleotides and serve to repress transcription of the downstream gene (13, 14). While the first methyl-CpG binding (MBD) protein discovered was MeCP2, MIRA is based on the recognition of methylated CpG dinucleotides by the methyl-CpG-binding domain protein-2b (MBD2b). The binding of MBD2b to methylated DNA is sequence-independent, making it a more attractive candidate for isolating methylated DNA than MeCP2 (15, 16). MBD2b has one of the highest affinities for binding methylated DNA; however, the binding affinity is further increased by the addition of the methyl-CpG-binding domain protein 3-like-1 (MBD3L1) (7, 15, 17). MBD3L1 binds with MBD2b *in vivo* and *in vitro* to promote the formation of methylated-DNA-binding complexes (17). This complex is the central core that allows MIRA to successfully function.

The first step in MIRA is to fragment double-stranded DNA to an average length of 0.35 Kb by either sonication or by restriction endonuclease digestion. Methylated DNA fragments are then bound by the glutathione-S-transferase (GST)- tagged recombinant MBD2b protein/His-tagged MBD3L1 complex. The DNA-protein complex is then captured using

glutathione beads in an affinity column. Following capture, DNA is eluted from DNA-protein complexes and amplified with PCR for site-specific analysis (7). See **Fig. 1** for an overview of the MIRA procedure.

MIRA has several advantages over other current DNA methylation analysis techniques and may be applicable in a variety of clinical and diagnostic situations, including characterization of DNA methylation in tissues or body fluids. Unlike MeDIP, MIRA works on double-stranded DNA and does not require the use of antibodies against 5-methylcytosine that is specific to single-stranded DNA. In addition, MIRA does not require the occurrence of methylation-sensitive restriction sites within the targeted sequence. Because it is not extremely laborious, MIRA is useful in genome-wide methylation analysis in cancers. MIRA may also be used to detect cell-type dependent differences in DNA methylation for a large number of genes through subsequent microarray analysis. In addition, the number of false-positives generated by the MIRA method is extremely low, making it an even more attractive candidate for analyzing DNA methylation at CpG dinucleotides (7, 8).

Recently, Active Motif has developed a commercially available kit (*MethylCollector Ultra*) based on the MIRA assay. DNA is sheared and incubated with the His-tagged MBD2b/MBD3L1 protein complexes. The protein/DNA complex is captured with nickel-coated magnetic beads. Unmethylated DNA and protein are washed away and methylated DNA is eluted and ready for analysis. Active Motif has also developed an *UnMethylCollector* kit that recovers only unmethylated DNA. The combination of these two kits provides a very powerful tool able to analyze genome-wide DNA methylation patterns. The use of *UnMethylCollector* further validates experimentation results obtained by *MethylCollector Ultra*. Instead of assuming the status of DNA is unmethylated by negative identification with the *MethylCollector Ultra* kit, *UnMethylCollector* provides a positive method of ensuring collected data is unmethylated.

Multiple commercially available arrays exist for the analysis of recovered DNA fragments; this chapter will focus on describing methods to analyze the products of MIRA and current trends in epigenetic research.

2. Materials

- 2.1 1 µg purified Glutathione-S-transferase-tagged recombinant Methyl-CpG-binding domain (MBD) protein-2b (GST-MBD2b) (See **Note 4.1**)
- 2.2 1 µg purified His-tagged MBD3L1 (See **Note 4.1**)
- 2.3 Glutathione sepharose CL-4B matrix (Amersham Biosciences)
- 2.4 Binding Buffer (10 mM Tris-HCL (pH 7.5), 50 mM NaCl, 1 mmol/L EDTA, 1 mM DTT, 3 mM MgCl₂, 0.1% Triton-X100, 5% glycerol, 25 µg/mL, bovine serum albumin, and 1.25 µg sonicated JM110 (*dcm* minus) bacterial DNA)
- 2.5 Wash Buffer (Binding buffer containing 700 mM NaCl)
- 2.6 Guanidinium hydrochloride-containing Elution Buffer
- 2.7 Qiaquick PCR purification kit
- 2.8 Genomic DNA

^{4.1}Plasmids expressing both of the recombinant MBD proteins used in the MIRA assay are available upon request by contacting the original authors (see Ref. 7).

- 2.9 Ultrasonic Homogenizer with a microtip
- 2.10 Benchtop microcentrifuge

3. Methods

3.1. DNA preparation

- 3.1.1 Genomic DNA from cell cultures or tissue samples should be prepared using any of the standard protocols.
- 3.1.2 Shear genomic DNA using Ultrasonic homogenizer to an average length of 0.35 Kb by sonicating each sample 5 times for 10 sec at an output setting of 30%. Sonicate samples in an ice bath to prevent overheating. See **Note 4.2** for additional information regarding shearing and linker ligation for microarray analysis.

3.2. Bead preparation

- 3.2.1 Centrifuge 100 μ l of GST-sepharose slurry for 5 min at 500g. See **Note 4.3** for additional information bead preparation.
- 3.2.2 Carefully remove supernatant.
- 3.2.3 Wash beads 3 times with 500 μ L of binding buffer.
- 3.2.4 Resuspend beads in 400 μ L of binding buffer.

3.3. Pre-incubation

- 3.3.1 Combine 1 μ g of GST-MBD2b and 1 μ g of MBD3L1 with GST sepharose beads prepared in the previous step and incubate for 20 min at 4°C on a rotating platform. (See **Note 4.4**)

3.4. MIRA reaction

- 3.4.1 Combine 500 ng of sonicated DNA with binding reaction from the previous step and incubate for 4 h at 4°C on a rotating platform. (See **Note 4.5**)
- 3.4.2 Pellet beads by centrifugation at 500g for 5 min.
- 3.4.3 Wash beads 3 times with 500 μ L of Wash Buffer. For each wash, incubate at room temperature (RT) for 5 min on a rotating platform followed by centrifugation at 500g for 5 min.

3.5. Elution and DNA purification

- 3.5.1 Following the third and final wash, resuspend beads in 100 μ L of Elution Buffer and incubate at 50°C for 1 h.
- 3.5.2 Pellet beads by centrifugation at 500g for 5 min.
- 3.5.3 Carefully remove supernatant and transfer to a fresh tube.

^{4.2}If microarrays are to be used to identify methylated genomic regions, genomic DNA should be digested with MseI and linker DNA ligated. Ligation of linker DNA allows for amplification of recovered DNA prior to microarray hybridization.

^{4.3}GST-beads are shipped in an ethanol slurry and bead preparation is performed to remove all traces of ethanol that may interfere in the MIRA reaction.

^{4.4}The high binding affinity of GST-MBD2b and MBD3L1 allows formation of the MBD2b-MBD3L1 complex *in vitro*. This step is critical to the recovery of methylated DNA and steps should be taken to ensure this part of the procedure is performed properly.

^{4.5}To ensure even mixing, samples should be lightly mixed by hand at each hour interval during the course of the 4 h incubation.

- 3.5.4** Purify methylated genomic DNA using the *Qiaquick PCR Purification Kit* following the manufacturer's protocol.

3.6. Amplification of Recovered DNA

- 3.6.1** To analyze a specific region of genomic, amplify with PCR using gene primers.
- 3.6.2** For microarray analysis, amplify all recovered DNA using primers specific to linker DNA. (See **Note 4.6**)

3.7. Analyzing the Genomic Products of MIRA

The MIRA-chip method can be used to determine the DNA methylation status of large numbers of genes and chromosomal regions of both normal and cancerous tissues. Rauch and colleagues characterized the DNA methylation status of the human HOX gene clusters by combining the MIRA method with genome-wide CpG island tiling arrays (**18**). Site-specific PCR amplification can also be used in conjunction with MIRA to analyze DNA methylation changes at a particular gene of interest.

It was originally assumed that functionally important DNA methylation changes occurred in promoter regions and CpG islands. However, as recently noted by Feinberg and colleagues, many methylation alterations occur in sequences up to 2 Kb from CpG islands; such areas are termed CpG island shores. It is methylation changes at CpG shores that have been shown to discriminate normal tissue from cancerous tissue more so than CpG island methylation status (**19**). Thus, a distinct limitation exists in using only CpG island tiling arrays in conjunction with MIRA to analyze DNA methylation changes as all possible sites where methylation may occur are not included in the array.

This problem is partially solved through the use of a human promoter array, which allows the user to analyze promoter DNA from over 25,000 genes. The Affymetrix product covers 7.5 Kb upstream and 2.45 Kb downstream of the 5' transcriptional start site. For over 1300 cancer-associated genes, the promoter coverage was expanded to include 10 Kb upstream through 2.45 Kb downstream. Thus, the ability to analyze DNA methylation at CpG shores is possible; however, it does not test for all genomic regions, including exonic and intronic DNA.

Of further interest, however, is whole-genome tiling arrays (WGAs) which interrogate an entire genome in an unbiased fashion through the use of non-overlapping or partially overlapping probes spaced at regular intervals. The technique is unbiased due to the ability to include as yet unidentified genes. WGAs have never been used in conjunction with MIRA but have been used with MeDIP to map the *Arabidopsis thaliana* methylome (**20**). After immunoprecipitation with an anti-5-methyl cytosine antibody, the complex is hybridized to a tiling array to map the methylome. However, the extremely high costs of WGAs make them an impractical choice for use in mammalian genomes. In addition, because WGAs have such a high level of sensitivity and assume no underlying gene models or annotations, results may contain an overlap of noise and signal (**21**). It is important to note that Rauch and colleagues recently used MIRA in combination with whole-genome tiling arrays to characterize the entire B cell methylome of an individual human at 100-bp resolution (**22**). Although progress is being made on current microarray technologies, most study of DNA methylation patterns in mammalian genomes is currently limited to CpG islands and promoters.

^{4.6}For genome wide analysis of recovered DNA, a much greater amount of DNA is required. Linker DNA that is ligated following enzymatic digestion can be used to amplify all recovered DNA.

Several techniques have been developed to analyze DNA methylation patterns on a genome-wide scale. Current methods involve either cleavage by methylation-sensitive restriction endonucleases (3), bisulfite sequencing (23) or precipitation of methylated DNA with an antibody (12). However, these microarray methods are not capable of providing a high-resolution DNA methylation map of the mammalian genome.

Next-generation sequencing (NGS) allows for the sequencing of short DNA fragments at a cost much lower than the traditional Sanger method of sequencing. It is capable of single-nucleotide resolution and consequently is challenging microarrays as the tool of choice for analyzing the genome. Known as ChIP sequencing (ChIP-seq), NGS has been combined with chromatin-immunoprecipitation (ChIP) to sequence the genomic DNA fragments bound by transcription factors (24). In addition, MIRA-seq provides extremely quantitative data about genome-wide methylation. MIRA enriched fragments undergo bisulfite conversion and analysis by NGS (25). Thus, NGS has future implications of further analyzing epigenetic marks of the genome.

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Figure 1. Schematic of the steps necessary to complete MIRA

The products of DNA shearing and the bead/protein complex are combined to perform the MIRA reaction. After the methylated DNA is recovered, purified and amplified with PCR, it can be analyzed via a variety of assays.