Protocol

Methylated CpG Island Amplification and Microarray (MCAM) for High-Throughput Analysis of DNA Methylation

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INTRODUCTION

This protocol describes the use of methylated CpG island amplification (MCA) in combination with a microarray platform to analyze genome-wide DNA methylation in a high-throughput fashion. In this approach, termed MCAM, methylated CpG islands are selectively targeted using oligonucleotide adaptors after two rounds of digestion with a combination of methylation-sensitive and methylation-insensitive nucleases. They are then amplified using PCR. The resulting amplicons, representing the methylated fraction of the genome, are labeled with fluorochromes. Subsequently, a comparative hybridization of reference and test samples (typically normal and tumor DNA specimens) is done on a microarray platform.

RELATED INFORMATION

This protocol has been tested in cancer cell lines and primary tumors (Estécio et al. 2007). Validation experiments by bisulfite-PCR (considered to be the gold standard) of fifteen genes in three cancer lines and eleven primary colorectal carcinomas revealed sensitivity and specificity of 88% and 96%, respectively. Additionally, MCAM could detect methylation differences of as little as 10% between tumor and normal adjacent paired colon samples.

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

Reagents

Agarose gel (1.5%)
Cot-1DNA (human; Invitrogen)
Cy3-dCTP and Cy5-dCTP (GE Healthcare)
<I>DMSO (see note at Step 10)
dNTP mix (25 mM)
Ethanol (prechilled to 4°C for Step 23)
Genomic DNA

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Klenow fragment of DNA polymerase I

The best source for this exonuclease at 40 units/μL is the Bioprime DNA Labeling System (Invitrogen).

<R>LowC dNTP mix (10X)

<R>MCA reaction buffer (10X)

<R>MCAM wash solution 1

MCAM wash solution 2

<R>SSC (20X) is diluted 100-fold in H₂O to prepare this wash solution, which is 0.2X SSC.

Oligonucleotide primers:

We have developed two different sets of primers (RMCA and RXMA) that differ in CG content and represent slightly different subsets of CpG islands. On average, the RMCA primers amplify smaller and more CG rich fragments than RXMA. Some probes work well using either condition, but others work better (or exclusively) using only one of the sets of primers. In our experience, the use of either set of primers for MCAM resulted in similar sensitivity and specificity. For this reason, one set of primers per experiment can be successfully used to reveal global patterns of methylation.

Octomers (random, 8N)

RMCA Primers (RMCA24 : 5'-CCACCGCCATCCGAGCCTTTCTGC-3'; RMCA12 : 5'-CCGGGCAGAAAG-3')

RXMA Primers (RXMA24 : 5'-AGCACTCTCCAGCCTCTCACCGAC-3'; RXMA12 : 5'-CCGGGTCGGTGA-3')

PCR Purification kit (QIAquick kit; Qiagen)

<R>Phenol:chloroform (1.5:1 [v/v]; pH 9.0)

<R>Prehybridization solution for MCAM

<R>React2 buffer (10X)

Restriction enzymes: SmaI and XmaI

SDS hybridization solution (2X; Genisphere)

Sodium acetate (3 M)

T4 DNA ligase and 10X ligase buffer

Taq DNA polymerase

<R>TE buffer (pH 8.0)

Equipment

Beaker (large)

Centrifugal filter unit (e.g., Microcon YM-30; Millipore)

Centrifuge (benchtop)

Coverslips

Dishes (slide staining)

Electrophoresis equipment for 1.5% agarose gel

Hot plate

Hybridization chamber (e.g., HybChamber Mica, Genomic Solutions)

Ice

Incubator preset to 42°C

Microarray slides

Microcentrifuge

PCR equipment

Rack (for slides)

Rocker platform

Scanner and software (e.g., Genepix 4000B scanner and GenepixPro 6.0 software; Axon Instruments)

Spectrophotometer

Tubes (1.7-mL microcentrifuge)

Vortexer

Water bath preset to 60°C

Water baths or heat blocks preset to 16°C, 25°C, 37°C, 65°C, 95°C, and 100°C

METHOD

Figure 1 presents an overview of the MCAM method.

Preparation of MCA Amplicons

This method was adapted from an original protocol by Toyota et al. (1999). An online version is also available at <http://www.mdanderson.org/departments/methylation/>.

1. Digest 5 μ g of genomic DNA using 100 units of SmaI for 16 h at 25°C.
2. Add 20 units of XmaI and incubate the reaction for 6 h at 37°C.
3. Bring the reaction volume to 500 μ L with H₂O and add one volume of 1.5:1 phenol:chloroform (pH 9.0). Vortex the mixture for 1 min, centrifuge for 10 min at 13,000 rpm, and transfer the supernatant to a 1.7-mL microcentrifuge tube.

FIGURE 1. Schematic diagram of the MCAM method. Enrichment for methylated DNA and reduction of genome complexity is achieved by serial digestion with SmaI (methylation sensitive) and XmaI (methylation sensitive) restriction enzymes, followed by ligation of adaptors and PCR amplification. The resulting amplicons, representative of the methylated fraction of tumor and normal cells, are labeled and cohybridized in a microarray platform. Image acquisition and data analysis allow identification of methylated and nonmethylated genes by comparing intensity values of Cy5 and Cy3 dyes for each pair of tumor and control samples.

4. Precipitate the DNA by adding 1/10th volume of 3 M sodium acetate and two volumes of 100% ethanol.
5. Incubate the sample at -70°C for 1 h, and centrifuge for 30 min at 13,000 rpm. Pour out the ethanol and air dry the DNA pellet.
6. Resuspend the DNA in 20 μL of TE and determine the DNA concentration using a spectrophotometer.
7. Prepare the adaptor mixture as follows:
 - i. Dilute the RXMA (or RMCA) primers to 100 μM .
 - ii. Combine 50 μL of RXMA24 with 50 μL of RXMA12 (or RMCA24 and RMCA12).
8. Incubate the adaptor mix for 3 min at 65°C , and cool it to room temperature over 60 min.
This mixture can be stored for up to 6 mo at -20°C .
9. Ligate the adaptors to the digested DNA as follows:
 - i. Combine the following reagents:

500 ng	Digested DNA (Step 6)
10 μL	Adaptor mixture (Step 8)
3 μL	10X ligase buffer
400 units	T4 DNA ligase
to 30 μL	H_2O
 - ii. Incubate the ligation reaction for 16 h at 16°C .
10. Prepare the MCA reactions using the following reagents:

10 μL	10X MCA reaction buffer
100 pmol	RXMA24 (or RMCA24) primer
15 units	<i>Taq</i> DNA polymerase
1.2 μL	dNTP mix (25 mM)
5 μL	DMSO (RMCA primers only)
3 μL	Ligation mixture (Step 9.ii)
to 97 μL	H_2O

DMSO is not needed with RXMA primers.
Typically, four MCA reactions per tested sample are necessary to generate enough PCR product for labeling.
11. Incubate the reaction for 5 min at 72°C , and perform PCR as follows:

Number of cycles	Temperature	Time
20	95°C	1 min
	72°C (for RXMA) or	3 min
	77°C (for RMCA)	
1	72°C (for RXMA) or	10 min
	77°C (for RMCA)	
12. Analyze 10 μL of the PCR products on a 1.5% agarose gel to check the quality of the amplification.
A relatively strong smear, ranging from 300 bp to 2 kb, should be visible for RXMA primers. Expect a smear ranging from 200 bp to 1 kb when using RMCA primers.

Labeling Amplicons and Slide Hybridization

This hybridization protocol is optimized for the Human CpG-island 12K Array (HCGI12K) from University Health Network (www.microarrays.ca).

13. Combine PCR products of replicate samples and purify them using the QIAquick PCR Purification kit according to the manufacturer's recommendation. Elute the samples in 30 μL of 1X TE buffer.
14. Quantify the DNA yield using a spectrophotometer.

15. Label the PCR products.
 - i. Assemble each labeling reaction as follows (35- μ L total volume):

25 μ L (5 μ g)	MCA product (Step 11) in H ₂ O
10 μ L (7.5 μ g/ μ L)	Random octomer primer (8N)
 - ii. Denature the nucleic acids by incubating at 100°C for 5 min, and then immediately transfer the tubes to ice.
 - iii. Add the following reagents to the nucleic acids (to a 50- μ L total volume):

5 μ L	10X React2 buffer
5 μ L	10X LowC dNTP mix
3 μ L	Cy3-dCTP (or Cy5-dCTP)
2 μ L	Klenow fragment (stock 40 units/ μ L)

Typically, Cy5-dCTP mix is used for tumor DNA and Cy3-dCTP is used for normal (reference) DNA.
 - iv. Incubate the reactions for 2 h at 37°C.
16. Increase the reaction volume to 100 μ L with H₂O, and purify each sample using a PCR purification kit (QIAquick) as follows:
 - i. Add 500 μ L of PBI buffer to the column in the PCR purification kit, let it stand for 5 min, centrifuge the column for 1 min at 13,000 rpm, and discard the flow-through.
 - ii. Wash the column with 700 μ L of PE buffer, centrifuge for 1 min at 13,000 rpm, discard the flow-through, and centrifuge once again at 13,000 rpm for 1 min.
 - iii. Place the column in a clean 1.7-mL microcentrifuge tube, and elute twice with 50 μ L of H₂O, waiting 2 min between centrifugations.
17. Read the samples in a spectrophotometer at the following wavelengths (use the full volume of labeled sample): Cy3= 550 nm; Cy5= 650 nm.
18. Calculate the concentration of incorporated dyes as follows:

$$\text{Cy3} = A_{550}/0.15 = \text{pmol}/\mu\text{L of Cy3 dye incorporated}$$

$$\text{Cy5} = A_{650}/0.25 = \text{pmol}/\mu\text{L of Cy5 dye incorporated}$$

Optimal amounts for hybridization are between 250 pmol (minimum) and 400 pmol (maximum) of incorporated dyes.
19. Prehybridize the slides for 45 min at 42°C in prehybridization solution for MCAM.
20. Combine equal picomole amounts of labeled tumor and normal amplicons and bring to 500 μ L with H₂O. Reduce the volume to a 15- μ L concentrate using a centrifugal filter unit (Microcon YM-30) as follows:
 - i. Centrifuge the column for 12 min at 14,000g.
 - ii. Invert the column in a new microcentrifuge tube and centrifuge it for 3 min at 5000 rpm to collect the sample.
 - iii. Transfer the sample to a new microcentrifuge tube and bring the volume to 15 μ L with H₂O, if necessary.

This step typically takes 12 min at 14,000g.
21. Heat a large beaker of H₂O to boiling on a hot plate.
22. Rinse microarray slides in room temperature H₂O.
23. Place the slides in boiling H₂O for 1 min and in 4°C ethanol for another 1 min.
24. Immediately centrifuge the microarrays for 6 min at 600 rpm in a benchtop centrifuge.
25. Add 10 μ L of human Cot1 DNA (total 10 μ g) and 25 μ L of 2X SDS hybridization solution to the sample from Step 20. Mix gently.

26. Denature the DNA (from Step 25) for 2 min at 95°C, and hold the tubes in a 60°C water bath.
27. Place the microarray slides in a hybridization chamber. Add 50 µL of MCAM wash solution 2 underneath each side of the slide.
28. Transfer the labeled DNA onto the array area and place a coverslip over the array.
29. Close the hybridization chamber tightly and incubate it in the dark for 16 h in a 60°C water bath.
The hybridization chamber should be submerged in water, but direct contact with the bottom of the water bath should be avoided.
30. Warm two 500-mL bottles of MCAM wash solution 1 and 1 500-mL bottle of MCAM wash solution 2 to 60°C.
31. Place the slides, with coverslips facing down, in a slide-staining dish containing MCAM wash solution 1 at 60°C. Keep the slides at a 45° angle to the bottom of the dish to allow the coverslips to detach from the microarrays.
32. Place a slide rack in a slide-staining dish filled with prewarmed MCAM wash solution 1. Transfer the slides into the dish and place it on a rocker platform set to 100 rpm for 10 min.
33. Transfer the slides to a new dish filled with prewarmed MCAM wash solution 2 and place it on a rocker platform set to 100 rpm for 10 min.
34. Rinse the slides by submerging the slide rack 10-20 times in H₂O.
35. Immediately centrifuge the slides for 6 min at 600 rpm in a benchtop centrifuge.
36. Scan the slides.

DISCUSSION

The MCAM protocol was optimized using an array containing 12,192 CpG-island clones from the Sanger Institute (Cross et al. 1994; Heisler et al. 2005). However, this method can be applied to other array platforms, such as oligo, tiling, and custom-made arrays with larger gene representation. Our method compares favorably to other published methylation microarray protocols because it provides reproducible results with a high validation rate and demonstrated use in clinical samples for clustering cases into distinct molecular groups. In our experience, the sensitivity of methods to isolate DNA using 5-mC antibodies and methyl binding domain proteins is low, which limits their application in genome-wide studies (Weber et al. 2005; Rauch et al. 2006). The use of methylation-sensitive enzymes with frequent cutting sites, for example, HpaII/MspI (Hatada et al. 2006), results in a high genome fraction for amplification. In such highly complex circumstances, PCR efficiency is compromised and likely favors amplification of non-CpG island DNA. In comparison, our MCAM technique simultaneously reduces complexity and increases specificity by targeting methylated CpG islands before amplification.

A limitation of our method is that the generation of MCA amplicons depends on the presence of two SmaI sites in relatively close proximity (no more than 1-2 kb apart), which occurs in around 80% of the promoter CpG islands, as calculated from *in silico* digestion of the human genome. Also, differences in CG content make certain sequences more difficult to amplify in MCA and decrease hybridization efficiency, which may explain the presence of false-positive and false-negative data in MCAM. Despite these limitations, the low rate of false-positive data makes MCAM valuable in large-scale investigations.

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