Nested multigene MSP/DHPLC method for analyzing promoter hypermethylation status in clinical samples

Kevin K. Divine, Kieu C. Liechty, Kevin C. Crume, and Steven A. Belinsky
 Lovelace Respiratory Research Institute, Albuquerque, NM, USA

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DNA methylation, the addition of a methyl group to the 5' position of cytosine in CpG dinucleotides, is essential for normal development and function of mammalian cells. Alterations in DNA methylation have been implicated in numerous diseases including cancer, where aberrant gene promoter hypermethylation is associated with transcriptional silencing of genes (1). It is an early event in neoplastic progression found in virtually every type of human cancer and can be detected in biological fluids prior to symptomatic disease (2,3). Identifying cancer in its earliest stages could greatly reduce morbidity and mortality. Biomarkers based on gene promoter hypermethylation currently are being developed and evaluated as molecular markers for cancer detection and therapeutic efficacy. Methylation-specific PCR (MSP) is the most widely used technique to study CpG island methylation. It is a sensitive and specific assay relying on bisulfite modification (BSM) to differentiate methylated and unmethylated CpGs. BSM converts unmethylated cytosines in genomic DNA to uracils, while methylated cytosines remain unchanged. MSP typically can detect one methylated allele in 1000 unmethylated alleles (4). We previously developed a nested MSP method that increased MSP sensitivity approximately 50-fold (5). First-stage PCR primers in nested MSP recognize a bisulfite-modified template but do not discriminate between methylated and unmethylated alleles. Multiple genes can be amplified in a first-stage nested MSP (6,7). In the second stage, two PCRs are conducted. One uses primers

specific for an unmethylated template, and the other uses primers specific for a methylated template. Agarose gel electrophoresis is used to separate and visualize the PCR products. The presence of a stage 1 product or a stage 2 unmethylated product verifies target sequence amplification. We have used this approach to detect aberrant tumor-specific gene promoter hypermethylation in sputum DNA from patients prior to clinical diagnoses of lung cancer (5). However, currently only the stage 1 PCR of nested MSP is multiplexed, separate stage 2 MSPs are required to determine the methylation status for an individual gene. This approach is both time-consuming and costly, thus limiting the efficiency of conducting population-based screening studies. We have addressed these limitations by developing a nested MSP method that simultaneously assays the methylation status of multiple gene promoters and combined it with nongel-based detection of the PCR products. The nongel-based analysis relies on denaturing high-performance liquid chromatography (DHPLC) using the WAVE® instrument (Transgenomic, Omaha, NE, USA). Method set up is straightforward, semi-automated, allows simultaneous examination of the promoter methylation status for multiple genes, uses the same DNA quantity as a nested single-gene MSP analysis, and can utilize formalin-fixed paraffin-embedded DNA from multiple tissue sources.

A description of the nested multigene MSP/DHPLC method is provided, and the results are compared with those obtained with the nested

single-gene MSP/agarose gel analysis method. The gene promoter hypermethylation status of *p16*, *RASSF1A*, and *DAPK* was analyzed in 40 sputum samples from current and former smokers at risk for lung cancer and in 20 formalin-fixed paraffin-embedded breast tumor samples. Each of the genes is a tumor suppressor affecting different cellular pathways: (i) *p16*, cell cycle control; (ii) *DAPK*, cell adherence and metastasis processes; and (iii) *RASSF1A*, apoptosis (8). All have important roles in carcinogenesis and are silenced by aberrant promoter methylation (8,9). To demonstrate the utility of this method, the samples chosen were predominately methylated, and thus, the methylation prevalence is not indicative of that normally detected for these genes in either tissue. Primer sequences for the nested three-gene MSP method are listed in Table 1. Stage 1 and stage 2 unmethylated PCR conditions consisted of a 10-min 94°C denaturation step, followed by 40 cycles of a 30-s 94°C denaturation step, a 30-s 60°C annealing step, a 30-s 72°C extension step, and a final elongation step of 72°C for 10 min. In the stage 2 methylated PCR, times for the second denaturation step, the annealing step, and the extension step were decreased to 15 s. All PCRs were conducted in a final volume of 50 µL. The first stage PCR used approximately 150 ng bisulfite-modified DNA, the second stage unmethylated PCR used 1 µL of a 1:50 dilution of stage 1 PCR product, and the second stage methylated PCR used 5 µL of a 1:50 dilution of stage 1 PCR product as a template. Differences in reaction conditions for stage 2 methylated PCR between the nested single-gene MSP and the nested three-gene MSP method involve alterations in primer, *Taq* DNA polymerase, and MgCl₂ concentrations. Specifically, *p16* and *DAPK* primer concentrations were reduced by 50% to 10 µM, the amount of AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Foster City, CA, USA) was doubled to 5 U, and an additional 7.5 nmol of MgCl₂ were added.

DHPLC analyses were conducted using a DNASep® HT column (Transgenomic) under nondenaturing conditions at a temperature of 50°C

with a 1.5 mL/min flow rate and UV detection. Under these conditions, PCR product separation is based on size not sequence. Resolution is limited to approximately 1% of the PCR product size. DHPLC analysis conditions for stages 1 and 2 nested three-gene MSP assays are listed in Table 2. PCRs were

loaded onto the WAVE autosampler, and 5-μL injections were used for each analysis. DHPLC analysis of both stage 1 and 2 products required <6 min/sample. Typical DHPLC detection of stages 1 and 2 unmethylated MSP products are shown in Figure 1. It should be noted that given the heterogeneity of biological samples, verification of target gene amplification is normally accomplished by the presence of a stage 1 PCR product. Stage 2 unmethylated primers are typically used only when analyzing extensively degraded DNA as found in some formalin-fixed paraffin-embedded tissue samples.

One-hundred percent concordance was observed in the detection of methylated stage 2 MSP products between the nested single-gene MSP/agarose gel electrophoresis method and the nested three-gene MSP/DHPLC method. An example of MSP product separation using each method is shown in Figures 2, A–F. DHPLC-based separation has better resolution and increased sensitivity for detecting methylated PCR products compared with agarose gel electrophoresis (Figure 2). For example, sputum samples 5 and 8 are methylated for both *p16* and *DAPK*. Resolving the 8-bp size difference between stage 2 PCR products is difficult using agarose gel electrophoresis, while this difference is clearly discerned by DHPLC. The increased resolution of DHPLC makes it easier to separate and identify methylated MSP products. This is essential in accurately determining the promoter methylation status of individual genes in a multiplex reaction. Additionally, as demonstrated in Figure 2, E and F, DHPLC analysis of MSP products obtained using a formalin-fixed paraffin-embedded DNA template do not present added difficulties. Compared with conventional nested single-gene MSP, a nested multigene MSP/DHPLC method requires less time, reagents, and sample DNA to analyze the gene promoter methylation status of clinical samples from a variety of tissues. The ability to identify methylated MSP products using UV detection also eliminates the cost associated with fluorescence probes required for other nongel-based MSP methods such as TaqMan®.

Table 1. Nested Multiplex MSP Primers for *p16*, *RASSF1A*, and *DAPK* Genes

Gene	Nucleotide Sequence	PCR Product Size (bp)
Stage 1 Primer Sequences		
p16	Forward 5'-GAGGAAGAAAAGAGGAGGGGTTG-3'	274
	Reverse 5'-ACAAACCTCTACCCACCTAAATC-3'	
RASSF1A	Forward 5'-GGAGGGAAGGAAGGGTAAGG-3'	260
	Reverse 5'-CAACTCAATAAACTCAAACCTCCC-3'	
DAPK	Forward 5'-GGTTGTTTYGGAGTGTGAGGAGG-3'	236
	Reverse 5'-CTAAAACTCCCCRATCCCT-3'	
Stage 2 Primer Sequences Unmethylated		
p16	Forward 5'-TTATTAGAGGGTGGGGTGGATTGT-3'	145
	Reverse 5'-CAACCCCAAACCAACCATAA-3'	
RASSF1A	Forward 5'-GGTTTTGTGAGAGTGTGTTTAG-3'	172
	Reverse 5'-ACACTAACAAACACAAACCAAAC-3'	
DAPK	Forward 5'-GGAGGATAGTTGGATTGAGTTAATGTTT-3'	80
	Reverse 5'-CAAATCCCTCCCAAACACCAA-3'	
Stage 2 Primer Sequences Methylated		
p16	Forward 5'-GAGGGTGGGGCGGATCGC-3'	143
	Reverse 5'-GACCCCGAACCGCGACCG-3'	
RASSF1A	Forward 5'-GGGGGTTTTGCGAGAGCGC-3'	203
	Reverse 5'-CCCATTAAACCCGTACTTCG-3'	
DAPK	Forward 5'-ATAGTCGGATCGAGTTAACGTC-3'	152
	Reverse 5'-AAAATAACCGAAACGACGACG-3'	
MSP, methylation-specific PCR.		

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Figure 1. Denaturing high-performance liquid chromatography (DHPLC) chromatogram of stage 1 and stage 2 unmethylated methylation-specific PCR (MSP) products. Elution order proceeds from smallest to largest MSP product. (A) Stage 1 elution times are: *DAPK*, 2.33 min; *RASSF1A*, 2.73 min; and *p16*, 3.04 min. (B) Sputum (SP) sample #8 (SP#8) stage 2 unmethylated DHPLC chromatogram. Elution times are: *DAPK*, 2.07 min; *p16*, 2.64 min; and *RASSF1A*, 2.83 min. (C) Table listing the retention times and corresponding peak heights in millivolts of the DHPLC analysis of SP#8 stage 2 unmethylated nested three-gene stage 2 MSP products.

Figure 2. Detection of stage 2 methylated products. (A) Agarose gel electrophoresis analysis of methylated nested three-gene stage 2 methylation-specific PCR (MSP) products. MSP product sizes are listed on the right of the gel, and corresponding gene identities are listed on the left side of the gel. WB represents the water blank. H2009, MCF7, and Calu6 are cell line controls for the different stage 2 MSP gene products. Sputum samples (SP) #1–#8 are from smokers at risk for lung cancer. Breast tumor (BT) samples BT#1 and BT#2 are from formalin-fixed paraffin-embedded breast tumors. (B) SP#2 denaturing high-performance liquid chromatography (DHPLC) chromatogram illustrating resolution of methylated *p16* and *RASSF1A* MSP products. (C) SP#5 DHPLC chromatogram demonstrating resolution of *p16* and *DAPK* MSP products of similar size and similar MSP product quantity. (D) SP#8 DHPLC chromatogram demonstrating resolution of *p16* and *DAPK* MSP products of similar size with large differences in MSP product quantity. (E) BT #1 DHPLC chromatogram illustrating resolution of *p16* and *RASSF1A* MSP products obtained from formalin-fixed paraffin-embedded tissues. (F) BT #2 DHPLC chromatogram illustrating resolution of *DAPK* and *RASSF1A* MSP products obtained from formalin-fixed paraffin-embedded tissues. (G) Compilation of DHPLC results from an analysis of methylated nested three-gene stage 2 MSP. The same SP samples were analyzed using both agarose gel electrophoresis and DHPLC. The table lists retention times and corresponding peak heights in millivolts.

Savings realized are proportional to the number of genes present in the nested multigene MSP and/or the number of gene products analyzed per DHPLC chromatogram. To further increase throughput and savings, we are modifying the multigene MSP/DHPLC method to simultaneously detect promoter methylation of four genes (*Pax5 α* , *Pax5 β* , *GATA4*, and *GATA5*) (Figure 3). The *Pax5* and *GATA* gene families encode transcription factors implicated in carcinogenesis (10,11). All four genes are silenced by aberrant promoter methylation in lung cancer (10,12). The conditions for the nested four-gene MSP/DHPLC method are available in the supplementary material available online at www.BioTechniques.com.

We have described a nested multigene MSP/DHPLC method to simultaneously examine gene promoter hypermethylation of three genes. Although this method was developed to detect multiple gene methylation events in complex biological samples, quantitation of methylation levels can be accomplished by determining the ratio of methylated to unmethylated DNA as reflected by individual peak heights expressed in millivolts (Figures 1B and 2C). This method will aid in the rapid and economical assembly and validation of gene panels involved in cancer initiation, progression, and malignant transformation. Once validated, biomarkers based on these gene panels can be used in the early detection, diagnosis, and prognosis of multiple types of cancer.

Table 2. DHPLC Analysis Gradients

Stage 1 PCR Products			Stage 2 Unmethylated PCR Products			Stage 2 Methylated PCR Products		
Time	%A	%B	Time	%A	%B	Time	%A	%B
0.0	47	53	0.0	58	42	0.0	53	47
0.5	42	58	0.5	52.9	47.1	0.5	49	51
2.8	40	60	1.1	46.6	53.4	2.0	41	59
3.8	38	62	1.9	42.6	57.4	2.6	39	61
3.9	47	53	2.6	39.3	60.7	2.7	53	47
			3.3	37	63			
			3.4	58	42			

DHPLC, denaturing high-performance liquid chromatography. Time is in minutes. %A, % buffer A (0.1 M triethylammonium acetate); %B, % buffer B (0.1 M triethylammonium acetate, 25% acetonitrile).

Figure 3. Detection of nested three-gene and four-gene stage 2 methylation-specific PCR (MSP) methylated products. (A) Composite denaturing high-performance liquid chromatography (DHPLC) chromatogram containing three methylated stage 2 MSP products (*p16*, *DAPK*, and *RASSF1A*) obtained from the nested multigene MSP method. The sample was produced via mixing methylated cell line stage 2 MSP products. (B) DHPLC chromatogram containing four methylated stage 2 MSP products (*Pax5 β* , *GATA4*, *Pax5 α* , *GATA5*) obtained from the nested multigene MSP method using DNA from the MCF7 cell line. Elution times are: *Pax5 β* , 2.00 min; *GATA4*, 2.26 min; *Pax5 α* , 2.51 min; and *GATA5*, 2.87 min.

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COMPETING INTERESTS STATEMENT

Dr. Belinsky is a consultant to OncoMethylome Sciences. Under a licensing agreement between Lovelace Respiratory Research Institute (LRRRI) and OncoMethylome Sciences, nested MSP was licensed to OncoMethylome Sciences, and Dr. Belinsky is entitled to a share of the royalties received by LRRRI from sales of the licensed technology. LRRRI, in accordance with its conflict-of-policies, is managing the terms of these arrangements. The remaining authors declare no competing interests.

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Address correspondence to Kevin K. Divine, Lovelace Respiratory Research Institute, 2425 Ridgecrest Dr. SE, Albuquerque, NM 87108-5127, USA. e-mail: kdivine@LRRI.org

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