Technical Advance

MethySYBR, a Novel Quantitative PCR Assay for the Dual Analysis of DNA Methylation and CpG Methylation Density

Pang-Kuo Lo,* Hanano Watanabe,*†
Pi-Chun Cheng,*† Wei Wen Teo,*
Xiaohui Liang,* Pedram Argani,‡ Ji Shin Lee,§
and Saraswati Sukumar*

From the Breast Cancer Program, Department of Oncology, and the Department of Pathology,† Johns Hopkins University School of Medicine, Baltimore, Maryland; the Molecular and Cellular Biology Program, Biology Department,‡ Johns Hopkins University, Baltimore, Maryland; and the Department of Pathology,§ Chonnam National University Medical School and Research Institute of Medical Sciences, Gwangju, Republic of Korea*

Development of facile, sensitive, specific, and economical assays for the analysis of methylated alleles is crucial to the use of methylated biomarkers for cancer detection. We hereby report a novel method, MethySYBR, a SYBR green-based PCR assay for the dual analysis of DNA methylation and CpG methylation density. MethySYBR begins with multiplex PCR to enable the simultaneous amplification of many discrete target alleles in a single reaction using as little as 3 pg of bisulfite-converted DNA. In the second round of PCR, the specific methylated target is quantified from multiplex products using both nested methylation-independent and methylation-specific primer sets. Moreover, the use of SYBR green dye during quantitative PCR enables melting curve analysis of target amplicons to determine the methylation density of CpG sites on target alleles. To establish proof of principle, two cancer-specific methylated genes, *RASSF1A* and *OGDHL*, were assessed by MethySYBR. We demonstrated that MethySYBR sensitively detected methylated alleles in the presence of a 100,000-fold excess of unmethylated allele. Furthermore, MethySYBR was shown to be capable of analyzing minute amounts of DNA from paraffin-embedded tissue. Therefore, the MethySYBR assay is a simple, highly specific, highly sensitive, high-throughput, and cost-effective method that is widely applicable to basic and clinical studies of DNA methylation. (J Mol Diagn 2009, 11:400–414; DOI: 10.2353/jmoldx.2009.080126)

Aberrant methylation of CpG island-containing gene promoters is one of the most common alterations seen in a large variety of neoplasms and frequently correlates with gene silencing.^{1–3} A wide range of tumor suppressor genes have been found to be inactivated through the promoter-hypermethylation mechanism.^{2,3} It has been shown that some changes in DNA methylation can be an early event in carcinogenesis and are present in the precursor lesions of various cancers.^{4,5} Therefore, the detection of methylated genes has been recognized as a potentially promising method for early detection of cancer.^{6,7}

Currently, several widely applicable methods such as methylation-specific PCR (MSP)⁸ and combined bisulfite restriction analysis⁹ are based on sodium bisulfite-mediated modification of the unmethylated cytosine, but not methylated cytosine, to uracil, generating sequence differences between methylated and unmethylated loci. Among these techniques, MSP is the most widely used method for methylation analysis.⁸ MSP, a gel-based qualitative technique, has been improved by the use of fluorescent TaqMan probes, which enables quantitative analysis of MSP products (eg, MethyLight).^{10,11} Development of fluorescent probe-based quantitative MSP allows sensitive and high-throughput detection of DNA methylation. A derivative technique developed in our laboratory, called Quantitative Multiplex Methylation-Specific PCR (QM-MSP), involves a multiplex PCR in the pre-amplification step before two-color real-time quantitation of methylated and unmethylated gene alleles, enabling the use of minute amounts of DNA specimens to analyze multiple methylated genes or multiple loci of a gene.^{12–14} However, for clinical tissue samples containing heteroge-

Supported by grants from the National Institutes of Health (Breast Cancer SPOR, grant number P50 CA 88843); and The Avon Foundation Breast Cancer Prevention Initiative to S.S.

Accepted for publication April 9, 2009.

Supplemental material for this article can be found on <http://jmd.amjpathol.org>.

Address reprint requests to Saraswati Sukumar, Ph.D., Breast Cancer Program, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans St., CRBI-143, Baltimore, MD 21231. E-mail: saras@jhmi.edu.

Table 1. Primer and Probe Sequences Used in MethySYBR and MethyLight Assays

Assay type	Primer or probe	Primer or probe sequence	Size (bp)*	Orientation	Status†
MethySYBR	RASSF1A-EXT-F1	5'-GGGTTTATAGTTTGTATTAGGTT-3'	201	Forward	Indep
	RASSF1A-EXT-F2	5'-TTTATAGTTTGTATTAGGTTTATTG-3'	198	Forward	Indep
	RASSF1A-EXT-R	5'-CAACTCAATAAACTCAAACCTCCC-3'		Reverse	Indep
	RASSF1A-FM	5'-GCGTTGAAGTCGGGGTTC-3'	75	Forward	M
	RASSF1A-RM	5'-CCCGTACTTCGCTAACTTTAAACG-3'	75	Reverse	M
	OGDHL-EXT-F1	5'-GGGGTTAGGGTTTAG-3'	245	Forward	Indep
	OGDHL-EXT-F2	5'-GGGTTTAGGGTTTAGTTT-3'	244	Forward	Indep
	OGDHL-EXT-R	5'-AAAACACAAATCAAAAACCTAC-3'		Reverse	Indep
	OGDHL-FM	5'-TGGGTCGTTAGTATCGTGGATAGC-3'	116	Forward	M
	OGDHL-RM	5'-CCTAAATCACGTAACGCAACCG-3'	116	Reverse	M
MethyLight	RASSF1A-FM	5'-GCGTTGAAGTCGGGGTTC-3'	75	Forward	M
	RASSF1A-RM	5'-CCCGTACTTCGCTAACTTTAAACG-3'	75	Reverse	M
	RASSF1A-M probe	VIC-ACAAACGCGAACCAGAACCAACCA-TAMRA		Reverse	M
	OGDHL-FM	5'-TGGGTCGTTAGTATCGTGGATAGC-3'	116	Forward	M
	OGDHL-RM	5'-CCTAAATCACGTAACGCAACCG-3'	116	Reverse	M
	OGDHL-M probe	VIC-TCGTAACGTCTCCATAAATACCCGC-TAMRA		Reverse	M
	ACTB-F	5'-TGGTGATGGAGGAGGTTAGTAAGT-3'	133	Forward	Indep
	ACTB-R	5'-AACCAATAAACCTACTCCTCCCTTAA-3'	133	Reverse	Indep
	ACTB probe	6FAM-ACCACCACCCACACACAATAACAAACACA-TAMRA		Reverse	Indep

*The size of amplicons.

†Indep, methylation-independent; M, methylated.

neously methylated sequences, complex fluorescent probe design is required for these fluorescent probe-based MSP methods to detect heterogeneously methylated alleles,¹⁰ which increases the cost and execution of the assay. Therefore, further improvements in quantitative MSP are still required.

Here we present a novel SYBR green dye-based DNA methylation assay referred to as MethySYBR. The rationale for us to develop a SYBR green dye-based assay is that, besides its low cost, as compared with expensive fluorescent probe-based methods, it can incorporate melting curve analysis to examine the melting temperature of endpoint PCR products, which can be used to determine the methylation density of CpG sites on methylated alleles. Our new technique adopts the two-step PCR strategy to improve the PCR specificity and allow it to multiplex many genes for DNA methylation analysis by using a minute amount of bisulfite-converted DNA. We used this new technique to establish quantitative and methylation-density analyses of two cancer-specific methylated genes, *RASSF1A* and *OGDHL*, in breast cancer. We show that this new technique is high-throughput, highly sensitive, specific and cost-effective, rendering it suitable for large-scale precise DNA methylation analysis of clinical biopsies like paraffin-embedded tissue.

Materials and Methods

DNA Extraction from Cell Lines, Tissue Samples

The breast cancer cell lines, MDA-MB-231, MDA-MB-468, MCF7, T47D, and ZR-751 were obtained from ATCC (American Type Culture Collections, Rockville, MD). Human mammary epithelial cells (HMEC) were from Clonetics (Walkersville, MD). Reduction mammoplasty specimens from normal women, and breast tumors were obtained from the Johns Hopkins Hospital (Baltimore, MD). All of the samples were collected in accordance

with institutional guidelines for protection of human subjects. Samples containing >50% tumor cells were selected after microscopic examination of representative tissue sections from each tumor. We isolated genomic DNA from cell lines or primary breast tumor tissues (either from fresh frozen tissues or from paraffin-embedded tissue sections) using standard techniques as described.^{15,16} Fully methylated MDA-MB-231 genomic DNA (Sss1-231 DNA) was prepared by treating MDA-MB-231 DNA with Sss1 DNA methyltransferase (New England Biolabs, Ipswich, MA) according to manufacturer's instructions.

Primers and Probes for MethySYBR and MethyLight

The *RASSF1A* and *OGDHL* promoter and gene sequences, obtained from the Database of Transcriptional Start Sites (DBTSS, <http://dbtss.hgc.jp>, March 16, 2008) and the GenBank database of National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>, March 18, 2008), were used to design primers and fluorescent probes for methylation studies. The sequences of primers and probes used for MethySYBR and MethyLight are shown in Table 1. Five discrete MethySYBR primers for a gene were designed, including external forward primer (EXT-F1), external nested forward primer (EXT-F2), external reverse primer (EXT-R), nested methylation-specific forward (FM) and reverse primer (RM). Sequences of both the external and external nested primers (RASSF1A-EXT-F1, F2 and R; OGDHL-EXT-F1, F2 and R) contain no CpG dinucleotides and are complementary to the flanking sequences outside the CpG-rich gene DNA region. For designing the nested methylation-specific primers, at least three CpG sites were included in the primer sequence, and the cytosine from one of these CpG sites was always placed at or adjacent to the 3' end of the primer sequence to render the primers as specific to methylated templates as

possible. The nested methylation-specific primer set was also used in MethyLight. Two methylation-specific TaqMan probes for detection of *RASSF1A* and *OGDHL* were designed for the MethyLight assay. The probe sequences cover at least three CpG sites. The MethyLight primers and probe for β -actin (*ACTB*) gene were described in Fackler et al¹² For the quantitative accuracy of MethyLight, the sizes of methylated *RASSF1A* and *OGDHL* amplicons were designed to be similar to the size of *ACTB* amplicons. Primers were obtained from Invitrogen Corporation (Carlsbad, CA) and labeled gene-specific probes were obtained from Applied Biosystems (Foster City, CA).

Sodium Bisulfite Treatment of Genomic DNA

Sodium bisulfite treatment of genomic DNA basically followed the method developed by Herman et al⁸ with some modifications as described previously.¹⁷ This process converts unmethylated cytosine residues to uracil, whereas methylated cytosines remain unchanged. Bisulfite-modified DNA samples were aliquoted and stored at -80°C .

PCR and Melting Curve Conditions for the MethSYBR Assay

The MethSYBR procedure requires two sequential steps of PCR reactions (Figure 1). The initial pre-amplification PCR reaction (the multiplex step) was performed in 25 μL

Figure 1. Schematic diagram of MethSYBR. Bisulfite-converted genomic DNA is used as a template for the one-step and two-step MethSYBR assays. In the two-step MethSYBR assay, gene-specific external primers (Ext-F1 and Ext-R) (methylation status independent) are used in the first pre-amplification step to enrich the specific gene DNA. The pre-amplification step can be used to coamplify multiple gene loci (multiplex PCR) by including a combinational panel of their gene-specific primer pairs. In the second real-time quantitative step, endpoint PCR products from round 1 PCR are diluted and subjected to quantitative PCR with SYBR green using two sets of gene-specific primers. The first set of primers, including the external nested forward primer Ext-F2 that also does not encompass CpG dinucleotides and external reverse primer Ext-R, is used to quantify the total input amounts of the target alleles; the second set is the nested methylation-specific primers (M-F and M-R) that are used to quantify the methylated target alleles. In the one-step MethSYBR assay, bisulfite-converted DNA is directly subjected to real-time quantitative PCR using two sets of primers same as the ones in the two-step assay. The melting curve profiles from total and methylated PCR reactions are called Total Melting Curve Profile (TMCP) and Methylated Melting Curve Profile (MMCP), respectively. Examples of assumed TMCP and MMCP profiles for homogenous and heterogeneous DNA samples are shown.

of PCR reaction mixture composed of bisulfite-modified DNA, *RASSF1A*, as well as *OGDHL* external primer pairs (*RASSF1A*-EXT-F1, *RASSF1A*-EXT-R, *OGDHL*-EXT-F1, *OGDHL*-EXT-R; 400 nmol/L for each primer), 1.25 mmol/L deoxynucleoside triphosphates, 16.6 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 67 mmol/L Tris-HCl (pH 8.8), 6.7 mmol/L MgCl_2 , 10 mmol/L β -mercaptoethanol, 0.1% dimethyl sulfoxide (DMSO), and 1.25 units Platinum TaqDNA polymerase (Invitrogen Life technologies, Carlsbad, CA). PCR Conditions were 95°C for 2 minutes, followed by 20 to 35 cycles of 95°C for 30s, 56°C for 30s, and 72°C for 45s, then a final extension cycle of 72°C for 4 minutes. For keeping the final PCR amplification in the log phase, the cycle number for the first step PCR needs to be properly set according to the starting amount of bisulfite-modified DNA. Our tests showed that 20 to 25 cycles of PCR are adequate for DNA amount in the range of 3 pg to 300 ng, and 30 to 35 PCR cycles are needed for DNA from a single paraffin-embedded tissue section. PCR products from the multiplex reaction were 5000-fold diluted in 10 mmol/L Tris-HCl (pH 7.5) containing 50 ng/ml of salmon sperm DNA and then subjected to the second-step real-time PCR reaction. The second-step PCR consists of two separate reactions, an external nested PCR for total DNA amplification and a methylation-specific PCR. The primers (*RASSF1A*-EXT-F2 and *RASSF1A*-EXT-R; *OGDHL*-EXT-F2 and *OGDHL*-EXT-R) used in the external nested PCR reaction amplified total DNA templates of the target gene regardless of their methylation status. This served as a reference control to normalize the amount of methylated target alleles detected by the nested methylation-specific primer pair (*RASSF1A*-FM and *RASSF1A*-RM; *OGDHL*-FM and *OGDHL*-RM) between samples. For the second-step real-time PCR reactions, 1 μL of the diluted first-step PCR product was used in each PCR reaction (the total or methylated PCR reaction). For one-step MethSYBR, 1 μL of bisulfite-converted DNA, instead of the diluted first-step PCR product, was directly used in the real-time PCR reactions. The diluted PCR product was added to the reaction mixture composed of a primer pair (400 nmol/L each) for either the external nested or the nested methylation-specific PCR reaction, 1.25 mmol/L deoxynucleoside triphosphates, 16.6 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 67 mmol/L Tris-HCl (pH 8.8), 6.7 mmol/L MgCl_2 , 10 mmol/L β -mercaptoethanol, 0.1% DMSO, 1.25 units of Platinum TaqDNA polymerase, 1 $\times$ ROX dye (Invitrogen Life technologies, Carlsbad, CA), and diluted SYBR green I (1:10⁵) (Invitrogen Life technologies, Carlsbad, CA) in a final volume of 25 μL . The real-time PCR reactions were performed in a 96-well reaction plate using the ABI 7500 Real Time PCR System (Applied Biosystems, Foster, CA). The PCR reactions for amplification of total and methylated DNA templates from each sample were done in separate wells. All samples were analyzed in duplicate or triplicate. The PCR reaction conditions were as follows: 95°C for 2 minutes, followed by 45 cycles of 95°C for 15s, 60°C for 15s, and 72°C for 45s, with one cycle of the dissociation step that consists of 95°C for 15s, 60°C for 1 minute, 95°C for 15s, and 60°C for 15s. The dissociation step has a fixed ramp rate that is approximately 1% of the standard ramp rate on the 7500 system ($\pm 1.6^{\circ}\text{C}/\text{sec}$).

The fluorescence data from the dissociation step were used for melting curve analysis. Bisulfite-converted Sss1-231 DNA, was included in each run to serve as the 100% methylated reference for calculating the relative methylation percentages of DNA samples based on the relative $2^{(-\Delta\Delta C_T)}$ quantitation approach.¹⁸

Calculation of Methylation Percentage

The principle of SYBR green-based quantitation was based on the notion that the fluorescent signal generated from the intercalation of SYBR Green dye into amplified DNA is directly proportional to the amount of amplified DNA.^{19–21} The detected fluorescent signal was further transformed into the cycle threshold (C_T) value, representing the amount of PCR-amplified DNA products. The C_T value of amplified total DNA was subtracted from the C_T value of amplified methylated DNA to get the sample's ΔC_T and calibrator's (Sss1-231) ΔC_T values. For calculation of methylation % for each sample, the calibrator's ΔC_T value was then subtracted from the sample's ΔC_T value to get the " $\Delta\Delta C_T$ " value, which is then entered into the equation, $2^{(-\Delta\Delta C_T)}$, and multiplied by 100 to give an index of the sample's methylation percentage relative to the 100% methylated control.

The MethyLight Assay

The MethyLight assay was performed as described by Eads et al¹⁰ with some modifications. The real-time PCR was performed using a 96-well reaction plate at a final reaction volume of 25 μ l in the ABI 7500 Real Time PCR System (Applied Biosystems, Foster, CA). In the real-time PCR reaction, 1 μ l of bisulfite-treated DNA was added to 24 μ l of reaction buffer containing 400 nmol/L each of forward and reverse primers for *ACTB* and methylated *RASSF1A* (or *OGDHL*), 200 nmol/L each of 6FAM- and VIC-labeled probes, 1.25 mmol/L deoxynucleoside triphosphates, 16.6 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 67 mmol/L Tris-HCl (pH 8.8), 6.7 mmol/L MgCl_2 , 10 mmol/L β -mercaptoethanol, 0.1% DMSO, 1.25 units of Platinum TaqDNA polymerase, 1 $\times$ ROX dye. The reaction conditions were as follows: 95°C for 2 minutes, followed by 50 cycles of 95°C for 15s, and 60°C for 1 minute. Sss1-231 DNA was also included in each run as the 100% methylated reference. The C_T value of *ACTB* was subtracted from the C_T value of the methylated *RASSF1A* (or *OGDHL*) to calculate the ΔC_T value for normalization between analyzed samples. Calculation of methylation percentages of DNA samples relative to Sss1-231 was also based on the relative $2^{(-\Delta\Delta C_T)}$ quantitation approach.¹⁸

Creation of Differentially Methylated *RASSF1A* DNA Templates Using Fusion PCR

To create a series of 95-bp *RASSF1A* DNA templates with differential densities on 10 CpG sites ranging from 0% to 100% in 20% increments (see Table 2), the two-step fusion PCR was performed. As shown in Table 2, each

RASSF1A DNA template was created using four 35-nt primers, two primers (NF and NR) for the 5'-end half region and the other two primers (CF and CR) for the 3'-end half region. For each pair of primers, 15-nt sequences are complementary overlap between primers. Therefore, the 55-bp DNA fragments were created in the first-step fusion PCR. Given that there are 15-bp-overlap sequences between these two half DNA fragments created from the first-step fusion PCR, their first-step products were pooled together for the second-step fusion PCR to create the full-length *RASSF1A* DNA fragment (95 bp) as shown in Table 2. In the first-round fusion PCR, these two pairs of primers (100 μ mol/L each) were separately annealed together in 25 μ l of the PCR reaction mix (16.6 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 67 mmol/L Tris-HCl [pH 8.8], 6.7 mmol/L MgCl_2 , 10 mmol/L β -mercaptoethanol, 0.1% DMSO) containing 1.25 mmol/L deoxynucleoside triphosphates (dNTP) and 1.25 units Platinum TaqDNA polymerase (Invitrogen Life technologies, Carlsbad, CA), and the two 3'-OH ends of annealed primers were then extended by TaqDNA polymerase using dNTP. The PCR program for annealing and extension was as follows: 98°C for 1 minute, down to 60°C at the rate of $-1^\circ\text{C}/3$ seconds, 60°C for 2 minutes, down to 43°C at the rate of $-1^\circ\text{C}/10$ seconds, 43°C for 1 minute, up to 60°C at the rate of $+1^\circ\text{C}/\Delta\text{T}$ sec (ΔT : the increased temperature difference between the last increased temperature and 43°C, for example, when the temperature is going up from 52°C to 53°C, it takes 10 seconds [53 minus 43] to increase 1°C), and final extension at 60°C for 10 minutes. After the first-round fusion PCR, two separate reaction products (25 μ l each) were pooled together and 1.25 units Platinum TaqDNA polymerase was added additionally for the second-round fusion PCR using the same PCR program as mentioned above. The final reaction products were diluted in 1000-fold for the further analysis. For melting curve analysis, 1 μ l of the diluted fusion PCR product was added to the PCR reaction mixture composed of a primer pair (400 nmol/L each of 5'- and 3'-end primers), 1.25 mmol/L deoxynucleoside triphosphates, 16.6 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 67 mmol/L Tris-HCl [pH 8.8], 6.7 mmol/L MgCl_2 , 10 mmol/L β -mercaptoethanol, 0.1% DMSO, 1.25 units of Platinum TaqDNA polymerase, 1 $\times$ ROX dye (Invitrogen Life technologies, Carlsbad, CA) and diluted SYBR green I (1:10⁵) (Invitrogen Life Technologies, Carlsbad, CA) in a final volume of 25 μ l. The real-time PCR reactions were performed in a 96-well reaction plate using the ABI 7500 Real Time PCR System (Applied Biosystems, Foster, CA). The PCR conditions were as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 15s, 60°C for 15s, and 72°C for 45s. The melting curve profile for each PCR reaction was obtained after the endpoint PCR product was analyzed by the dissociation program of the real-time PCR machine.

Bisulfite Genomic Sequencing

For bisulfite sequencing analysis, the endpoint PCR products from the second-step total PCR reaction amplified by external nested primers were purified with the

Table 2. Primer Sequences Used in Fusion PCR to Create Differentially Methylated *RASSF1A* DNA Templates

Primer name	Primer sequence
100% methylation	GGGTT CG TTTGTGGTTT CG TT CG GTT CG CGTTTGTAG CG TTTAAAGTTAG CG AAGTAC CG GGTTTAAT CG GGTTATG T CG GGGGAGTTTGAGTT
RASSF1A-NF1	5'-GGGTT CG TTTGTGGTTT CG TT CG GTT CG CGTTTGTG-3'
RASSF1A-NR1	5'-T CG CTAACTTTAAAC CG CTAACAAAC CG CGAAC CG AA-3'
RASSF1A-CF1	5'- G TTTAAAGTTAG CG AAGTAC CG GGTTTAAT CG GGTT-3'
RASSF1A-CR1	5'-AACTCAAAC TCCCCCG ACATAAC CCCG ATTAAAC CC -3'
80% methylation	GGGTT CG TTTGTGGTTT CG TT CG GTT CG TGTTTGTAG TG TTTAAAGTTAG CG AAGTAC CG GGTTTAAT CG GGTTATG T CG GGGGAGTTTGAGTT
RASSF1A-NF2	5'-GGGTT CG TTTGTGGTTT CG TT CG GTT CG TGTTTGTG-3'
RASSF1A-NR2	5'-T CG CTAACTTTAAAC CA CTAACAAAC CAC GAAC CG AA-3'
RASSF1A-CF1	5'- G TTTAAAGTTAG CG AAGTAC CG GGTTTAAT CG GGTT-3'
RASSF1A-CR1	5'-AACTCAAAC TCCCCCG ACATAAC CCCG ATTAAAC CC -3'
60% methylation	GGGTT CG TTTGTGGTTT CG TT TG GTT TGTG TTTGTAG TG TTTAAAGTTAG CG AAGTAC CG GGTTTAAT CG GGTTATG T CG GGGGAGTTTGAGTT
RASSF1A-NF3	5'-GGGTT CG TTTGTGGTTT CG TT TG GTT TGTG TTTGTG-3'
RASSF1A-NR3	5'-T CG CTAACTTTAAAC CA CTAACAAAC CACA AAC CA AA-3'
RASSF1A-CF1	5'- G TTTAAAGTTAG CG AAGTAC CG GGTTTAAT CG GGTT-3'
RASSF1A-CR1	5'-AACTCAAAC TCCCCCG ACATAAC CCCG ATTAAAC CC -3'
40% methylation	GGGTT CG TTTGTGGTTT CG TT TG GTT TGTG TTTGTAG TG TTTAAAGTTAG TG AAGTAT TG GGTTTAAT CG GGTTATG T CG GGGGAGTTTGAGTT
RASSF1A-NF3	5'-GGGTT CG TTTGTGGTTT CG TT TG GTT TGTG TTTGTG-3'
RASSF1A-NR4	5'-T CA CTAACTTTAAAC CA CTAACAAAC CACA AAC CA AA-3'
RASSF1A-CF2	5'- G TTTAAAGTTAG TG AAGTAT TG GGTTTAAT CG GGTT-3'
RASSF1A-CR1	5'-AACTCAAAC TCCCCCG ACATAAC CCCG ATTAAAC CC -3'
20% methylation	GGGTT CG TTTGTGGTTT CG TT TG GTT TGTG TTTGTAG TG TTTAAAGTTAG TG AAGTAT TG GGTTTAAT TG GGTTATG T TG GGGGAGTTTGAGTT
RASSF1A-NF3	5'-GGGTT CG TTTGTGGTTT CG TT TG GTT TGTG TTTGTG-3'
RASSF1A-NR4	5'-T CA CTAACTTTAAAC CA CTAACAAAC CACA AAC CA AA-3'
RASSF1A-CF3	5'- G TTTAAAGTTAG TG AAGTAT TG GGTTTAAT TG GGTT-3'
RASSF1A-CR2	5'-AACTCAAAC TCCCCCA ACATAAC CCA ATTAAAC CC -3'
0% methylation	GGGTT TG TTTGTGGTTT TG TT TG GTT TGTG TTTGTAG TG TTTAAAGTTAG TG AAGTAT TG GGTTTAAT TG GGTTATG T TG GGGGAGTTTGAGTT
RASSF1A-NF4	5'-GGGTT TG TTTGTGGTTT TG TT TG GTT TGTG TTTGTG-3'
RASSF1A-NR4	5'-T CA CTAACTTTAAAC CA CTAACAAAC CACA AAC CA AA-3'
RASSF1A-CF3	5'- G TTTAAAGTTAG TG AAGTAT TG GGTTTAAT TG GGTT-3'
RASSF1A-CR2	5'-AACTCAAAC TCCCCCA ACATAAC CCA ATTAAAC CC -3'

The CpG dinucleotide and its unmethylated TpG are shown as bold letters. The CpG dinucleotide that has been changed to unmethylated TpG is indicated by an underline. The nucleotide sequences complementary to CpG or TpG are also shown as bold letters in primer sequences.

PCR purification kit (Qiagen, Valencia, CA) after melting curve analysis. The purified PCR products were cloned into the pGEM-T-Easy TA cloning vector according to the manufacture's protocol (Promega, Madison, WI). For each DNA sample, 10 bacterial clones were randomly selected and their respective plasmids were extracted. The plasmid DNA was subjected to sequencing analysis by an Applied Biosystems automated fluorescent sequencer (Applied Biosystems, Foster, CA) using vector primers according to the manufacturer's instructions.

Calculation of the Methylation Density of CpG Sites on Target Alleles

For calculating the methylation density of CpG sites on amplicons detected in the melting curve profile, bisulfite-converted fully methylated and unmethylated DNA controls were analyzed in parallel with unknown DNA samples to obtain their melting temperatures, including the fully methylated T_m (T_m-FM), unmethylated T_m (T_m-UM), and sample's T_m (T_m-SAM). The methylation density % (MD%) of sample amplicons was calculated as MD% = 100 × [(T_m-SAM - T_m-UM)/(T_m-FM - T_m-UM)]. In a reverse way, the methylation density % of sample DNA,

determined by bisulfite genomic sequencing, could be used to calculate its expected T_m as T_m-SAM = [(MD%/100) × (T_m-FM - T_m-UM)] + T_m-UM.

Results

The Design of the MethSYBR Technology

Problems underlying the use of SYBR green dye-based real-time PCR are false priming of primers and primer dimer formation, which interfere with the accuracy of quantitation and result in overestimation of the amount of target DNA particularly when a very low amount of target DNA is analyzed.^{19,21} Thus significant improvements in the specificity of the SYBR green dye-based MSP assay are needed to be applicable to DNA methylation studies. Nevertheless, the melting curve profile generated from the gradual rise in temperature to 95°C and consequent dissociation of SYBR green dye from endpoint PCR products reports the melting temperature of amplicons. Since bisulfite-converted DNA templates with various degrees of methylation at their CpG sites generate amplicons with distinct melting temperatures, the incorporation of melting curve analysis into the SYBR green-based MSP assay

is useful and effective to assess the methylation density of CpG sites on target alleles in a high-throughput way.

To render SYBR green-based MSP applicable to large scale screening of clinical samples, we developed MethySYBR, summarized in Figure 1. The MethySYBR assay consists of two steps of PCR. The first PCR step, called the pre-amplification or multiplex step, enriches all target templates regardless of methylation status using a set of external primers (Ext-F1 and Ext-R) that are complementary to the non-CpG sequences of the target templates (Figure 1). The second nested PCR step is the real-time quantitative PCR that uses the diluted PCR product from the pre-amplification step as the template (Figure 1). The pre-amplification step enables the analysis of small amounts of DNA samples, and multiple genes can be multiplexed together in this step when corresponding sets of gene-specific primers are used.^{12,13} In addition, this design can significantly reduce the possibility of false priming of primers to non-specific templates and formation of primer dimer in the second-step quantitative PCR, which commonly occur in the classic one-step SYBR green-based real-time PCR.^{19,21} If the pre-amplification step is omitted and bisulfite-converted DNA is directly used as a template in the real-time quantitative PCR step, MethySYBR can also be performed in a one-step manner (Figure 1).

In the second-step quantitative PCR, two separate PCR reactions are performed, including a total PCR reaction using the external nested primer set (Ext-F2 and Ext-R) and a methylation-specific PCR reaction using the nested methylation-specific primer set (M-F and M-R) (Figure 1). In our method, the external nested forward primer (Ext-F2) is specifically designed and used only in the second step PCR along with the external reverse primer (Ext-R), a primer that is used in the pre-amplification step also. This design minimizes the complexity of primer design in the assay. If designing the external nested forward primer is problematic for some genes, an external nested reverse primer can also be designed instead. Thus, the design of external nested primers is flexible and can be either forward or reverse. The cycle threshold (C_T) values obtained from amplification of total and methylated templates from assayed samples are used to calculate the methylation percentage relative to the fully methylated DNA control based on the $2^{(-\Delta\Delta C_T)}$ method¹⁸ described in *Materials and Methods*.

In the MethySYBR methodology, a dissociation step is performed immediately after the PCR cycle program in a closed-tube system for melting curve analysis. The assumed melting curve profiles from the total and methylated amplicons are summarized respectively in Figure 1. We made these assumptions by only considering the completely bisulfite-converted condition. For each DNA sample, two separate melting curve profiles would be obtained from the MethySYBR assay. One (named *Total Melting Curve Profile*, TMCP) is from endpoint PCR products amplified with external nested primers, displaying melting temperatures of total target amplicons (Figure 1). The second (named *Methylated Melting Curve Profile*, MMCP) is from endpoint PCR products amplified with nested methylation-specific primers, displaying the melt-

ing temperature of the most methylated target amplicons (Figure 1). Based on the sources of DNA samples, melting curve profiles are categorized as either homogeneous or heterogeneous (Figure 1). In heterogeneous profiles, multiple melting temperature peaks that represent different PCR products can be displayed by TMCP (Figure 1). Given that sodium bisulfite conversion results in sequence variations between DNA templates with different methylation states (such as fully methylated, partially methylated, and unmethylated states), the melting-temperature information is useful to define methylation states of target genes and determine the methylation density of CpG sites on target alleles.

Optimization of PCR Conditions for the MethySYBR Assay

For proof of principle, two methylated genes, *RASSF1A* and *OGDHL*, were analyzed by the MethySYBR assay. *RASSF1A* is a well-known cancer-specific methylated gene and frequently hypermethylated in breast cancer.²² *OGDHL* is a newly identified methylated gene, hypermethylated in multiple types of cancer, including breast cancer.²³ The primer sets for detecting these two genes were designed and synthesized as shown in Table 1. To optimize PCR conditions for the real-time quantitative step, we used HMEC genomic DNA with unmethylated *RASSF1A* and *OGDHL* alleles as a negative control to identify the optimal annealing temperature for MethySYBR. MDA-MB-231 genomic DNA treated with Sss1, a DNA methyltransferase, served as the positive reference DNA (Sss1-231 DNA) that contained fully methylated *RASSF1A* and *OGDHL* alleles, which was used for calculating the background methylation percentage of HMEC DNA. To test whether MethySYBR is capable of multiplexing many genes, *RASSF1A* and *OGDHL* external primer sets were included together in the pre-amplification PCR reaction to coamplify these two genes for use throughout the rest of the study. Three different annealing temperatures for the second-step quantitative PCR were tested. The highest temperature, 60°C, gave the lowest background methylation percentages for *RASSF1A* (0.00026%) (Figure 2A) and *OGDHL* (0.00053%) (Figure 2B). Annealing temperature above 60°C led to a significant delay of amplification of total input templates that used the external nested primer set compared with amplification of methylated templates using the nested methylation-specific primer set (data not shown). Therefore, 60°C was used as the annealing temperature throughout the rest of validation studies of MethySYBR.

For MethySYBR to be accurate, the amplification efficiencies of the total and methylated amplicons must be equal as possible.¹⁸ As shown in Figure 3, A and B, the external nested primer set amplified *RASSF1A* and *OGDHL* from both fully methylated Sss1-231 DNA and unmethylated HMEC DNA at approximately equal rates (comparing the Sss1-231-T plot to the HMEC-T plot). Moreover, Sss1-231 DNA was also amplified by respective external nested and nested methylation-specific

Figure 2. The effect of the annealing temperature setting on the accuracy of MethySYBR. MethySYBR analysis was performed using HMEC DNA (unmethylated control) to test effects of annealing temperatures (56°C, 58°C, and 60°C) on background amplification by nested methylation-specific *RASSF1A* and *OGDHL* primers as described in *Materials and Methods*. The results for the background amplification of HMEC DNA by primers specific for methylated *RASSF1A* (A) or *OGDHL* (B) DNA at three different annealing temperatures were obtained from three separate PCR reactions. The error bars represent the standard deviations of data. For both *RASSF1A* and *OGDHL* genes, the decreases in the background amplification at 60°C compared with the ones at 58°C were statistically significant (* $P < 0.05$).

primer sets at approximately equal rates (comparing the Sss1-231-T plot to the Sss1-231-M plot) (Figure 3, A and B). These analyses indicate that different primer sets and amplicons with different methylation states had no significant impact on PCR amplification efficiency in the MethySYBR assay. In addition, we found that amplification of HMEC DNA by the nested methylation-specific primers was detectable only at very late PCR cycles (see the HMEC-M plot) (Figure 3, A and B). Their respective melting curve profiles (Figure 3, C–F), methylated and unmethylated *RASSF1A* and *OGDHL* amplicons amplified by two different primer sets, had discrete melting peaks. The TMCP showed that total *RASSF1A* amplicons amplified from Sss1-231 and HMEC DNA had distinct melting temperatures (T_m) at 85.3°C and 81.4°C, respectively (Figure 3C). The melting temperatures for methylated and unmethylated *OGDHL* amplicons were 88.6°C and 82.8°C, respectively (Figure 3D). In addition, the MMCP revealed that endpoint PCR products amplified from HMEC DNA using nested methylation-specific primers had the lower T_m (81.3°C for *RASSF1A* and 83.6°C for *OGDHL*) in comparison with endpoint PCR products from

Sss1-231 DNA with the higher T_m (84.0°C for *RASSF1A* and 88.3°C for *OGDHL*) (Figure 3, E and F). These data indicate that the nested methylation-specific primers could false-prime and amplify unmethylated *RASSF1A* and *OGDHL* templates in HMEC DNA at very late PCR cycles as shown in Figure 3, A and B. Thus, this false priming gave HMEC DNA a methylation background as shown in Figure 2. All endpoint PCR products had been confirmed by gel electrophoresis to make sure that they had an equal size (data not shown).

Inclusion of the Pre-Amplification Step in the MethySYBR Technique Significantly Improves Detection Specificity

To demonstrate that inclusion of the pre-amplification step into the MethySYBR assay can improve the specificity of the second-step real-time PCR, we compared the two-step MethySYBR assay with its one-step counterpart assay. As mentioned above, the advantage of the pre-amplification step that enriches target alleles is to overcome the problems such as primer-dimer formation and false priming in the second-step quantitative MSP, which lead to inaccurate quantitation. The test was performed using three different amounts (30 ng, 3 ng, and 0.3 ng) of bisulfite-treated Sss1-231 DNA. After they were subjected to the one-step and two-step MethySYBR assays for detecting *RASSF1A* alleles, the real-time amplification plots for 30 ng of Sss1-231 DNA showed that in the one-step MethySYBR analysis, amplification of total *RASSF1A* DNA (*RASSF1A*-T, 198 bp) with the external nested primer set appeared four cycles late compared with amplification of methylated *RASSF1A* DNA (*RASSF1A*-M, 75 bp) with nested methylation-specific primer set (Figure 4A). Since genomic DNA has been fragmented to various sizes of small DNA after treatment with sodium bisulfite, this delay of amplification of 198-bp *RASSF1A* DNA was due to the significantly lower levels of DNA that could be amplified by external nested primer set than the levels of DNA that could be amplified by nested methylation-specific primer set. In contrast, there was no delay for amplification of total *RASSF1A* amplicons in the two-step MethySYBR analysis because 201-bp pre-amplified PCR products were used as a template for the second-step real-time PCR (Figure 4B). The TMCP analysis showed that primer dimer was formed (T_m at 73.2°C) when 3 ng and 0.3 ng of DNA were used in the one-step MethySYBR assay (Figure 4C), whereas primer dimer was not observed in any of the two-step MethySYBR analysis performed on the DNA samples (Figure 4D). The melting curve data from the one-step assay were further confirmed by gel electrophoretic analysis of PCR endpoint products, which showed two different sizes of DNA bands, one with an exact size, and the other with a smaller size (data not shown). These results reemphasize the general problem of the specificity of one-step SYBR green-based real-time MSP when a small amount of DNA is analyzed. Therefore, we convincingly demonstrated that the two-step MethySYBR has higher specificity and ac-

Figure 3. MethySYBR analysis of bisulfite-converted HMEC and Sss1-treated MDA-MB-231 DNA. **A, B:** The real-time amplification plots for Sss1-231 and HMEC DNA were obtained from two separate PCR reactions using the respective external nested (T) as well as nested methylation-specific (M) primer sets for *RASSF1A* (**A**) or *OGDHL* (**B**). The annealing temperature 60°C was used in studies for both genes. **C, D:** Total melting curve profile (TMCP) analysis of the endpoint PCR product amplified from Sss1-231 and HMEC DNA using the external nested primer pair for *RASSF1A* (**C**) or *OGDHL* (**D**) gene. **E, F:** Methylated melting curve profile (MMCP) analysis of the endpoint PCR product amplified from Sss1-231 and HMEC DNA using the nested methylation-specific primer pair for *RASSF1A* (**E**) or *OGDHL* (**F**) gene.

curacy to detect target alleles than the one-step MethySYBR assay.

Comparison of Sensitivity, Specificity, and Quantitative Accuracy between MethySYBR and MethyLight Techniques

To evaluate quantitative accuracy, specificity and sensitivity of MethySYBR, we performed spiking experiments. The multiplex of both *RASSF1A* and *OGDHL* was performed in the first step of MethySYBR to reveal whether the two-gene multiplex works in spiking experiments. For the assessment of the capability of MethySYBR to detect minute levels of methylated target alleles in the presence of an excess of unmethylated target alleles, 300 ng of fully methylated Sss1-231 DNA was serially diluted in 10-fold increments with unmethylated HMEC DNA, cre-

ating samples with methylated DNA content ranging from 100% to 0.001%. Besides the two-step MethySYBR assay, the classic one-step MethyLight assay was also performed in parallel for comparison. After the spiked samples were subjected to these two assays, their respective real-time amplification data (see Supplemental Figures S1 and S2 at <http://jmd.amjpathol.org>) were obtained to calculate methylation percentages of *RASSF1A* and *OGDHL*. Their melting curve profiles (see Supplemental Figures S1E and S1F at <http://jmd.amjpathol.org>) were also checked to confirm the specificity of the MethySYBR assay. Calculation of methylation percentage for these two distinct assays was based on methods described in *Materials and Methods*. Linear regression analysis was performed after methylation percentages of *RASSF1A* and *OGDHL* alleles were determined from three independent experiments. The regression analyses dem-

Figure 4. The two-step MethySYBR assay has significantly higher PCR specificity and quantitative accuracy compared with its one-step version for analysis of small amounts of bisulfite-converted DNA. **A, B:** The real-time PCR amplification plots. 30 ng, 3 ng, and 0.3 ng of bisulfite-converted Sss1-231 DNA were subjected to the one-step or two-step MethySYBR assay for detection of methylated *RASSF1A*. For 30 ng of DNA, the real-time PCR amplification plots obtained from one-step and two-step MethySYBR are shown in (**A**) and (**B**), respectively. **C, D:** TMCP analysis of endpoint PCR products generated from the one-step and two-step MethySYBR analyses. The TMCP profiles shown in (**C**) were from one-step MethySYBR, and the ones shown in (**D**) were from two-step MethySYBR.

Figure 5. Comparison of the sensitivity and quantitative accuracy between MethySYBR and MethyLight techniques. Three hundred nanograms of bisulfite-converted fully methylated Sss1-231 DNA was serially diluted in 10-fold increments with bisulfite-converted unmethylated HMEC DNA to make 100%, 10%, 1%, 0.1%, 0.01%, and 0.001% methylated templates. These spiked samples were subsequently analyzed by the two-step MethySYBR and one-step MethyLight assays using primer sets for detecting *RASSF1A*, *OGDHL* and *ACTB* (β -actin) genes. In the pre-amplification step of MethySYBR, two-gene multiplex PCR was performed to coamplify *RASSF1A* and *OGDHL* alleles, which was a test to evaluate the quantitative accuracy of the MethySYBR technique after these two genes were multiplexed together. The diluted pre-amplified PCR products were subjected to the second-step real-time quantitative PCR using primer sets specific for each gene to separately detect total and methylated *RASSF1A* and *OGDHL* alleles. In the MethyLight assay, these spiked DNA samples were directly subjected to real-time PCR for quantifying the amounts of *ACTB* and methylated *RASSF1A* (or methylated *OGDHL*) DNA using the two-color detection method. The measured amount of *ACTB* DNA represented the total amount of input DNA in each dilution. The $2^{(-\Delta\Delta C_T)}$ quantitative approach (described in *Materials and Methods*) was applied to calculate the measured methylation percentage of each spiked DNA sample. The expected methylation percentages ranging from 100% to 0.001% were plotted against their respective measured methylation percentages in a double logarithmic diagram. For each data point, three independent analyses were performed and their standard deviations are indicated by vertical bars. The equation of the linear regression curve and the correlation coefficient (R^2) are indicated on the plotted graph. The plotted MethySYBR data for *RASSF1A* and *OGDHL* are shown in (A) and (B), respectively. The plotted MethyLight data for *RASSF1A* and *OGDHL* are shown in (C) and (D), respectively.

onstrated a strong linear relationship ($R^2 = 0.9972$ for *RASSF1A*; $R^2 = 0.9993$ for *OGDHL*) between the measured and expected methylation percentages for all of spiked DNA samples that were assessed by MethySYBR (Figure 5, A and B). Furthermore, MMCP clearly showed that all PCR reactions were specific and accurate according to the melting temperatures of amplicons (see Supplemental Figures S1E and S1F at <http://jmd.amjpathol.org>). In contrast, the measured data points from MethyLight analysis were out of the linear range when the percentage of analyzed methylated DNA was less than 1%, leading to a decrease in correlation coefficients for both *RASSF1A* and *OGDHL* (Figure 5, C and D). The results from the linear regression analysis convincingly demonstrate that two-gene-multiplex MethySYBR is more sensitive and accurate than classic one-step MethyLight.

To test the detection sensitivity of two-gene-multiplex MethySYBR, spiked bisulfite-converted Sss1-231 DNA

samples ranging from 3 pg to 300 ng in 10-fold increments were subjected to MethySYBR and MethyLight analyses for detection of *RASSF1A* and *OGDHL*. As shown in Supplemental Figures S3, S4, and S5 at <http://jmd.amjpathol.org>, both assays detected *RASSF1A* and *OGDHL* over five log units of template quantities. In addition, the correlation coefficients obtained from linear regression analyses in both assays provided evidence that amplification by each primer set was approximately linear over the entire range of DNA quantity (see Supplemental Figures S3C, S3G, S4C, and S5C at <http://jmd.amjpathol.org>). In addition, there was no significant change in ΔC_T over the whole range of DNA quantity (see Supplemental Figures S3D, S3H, S4D, and S5D at <http://jmd.amjpathol.org>). However, the amplification signals for both total and methylated *RASSF1A* and *OGDHL* were robust even when DNA as low as 3 pg was assessed (see Supplemental Figures S4A, S4B, S5A, and S5B at <http://jmd.amjpathol.org>). In contrast, the MethyLight assay gave weaker amplification signals for *RASSF1A*, *OGDHL*, and *ACTB* (see Supplemental Figures S3A, S3B, S3E, and S3F at <http://jmd.amjpathol.org>) for the same amount of DNA. The melting curve analyses confirmed the specificity of PCR reactions for all of spiked DNA samples (see Supplemental Figures S4E, S4F, S5Em, and S5F at <http://jmd.amjpathol.org>). These results clearly demonstrate that two-gene-multiplex MethySYBR was able to detect and quantify a minute amount of DNA as low as 3 pg.

Assessment of the MethySYBR Technique to Measure the Methylation Density of CpG Sites on Target Alleles

To test whether MethySYBR is capable of measuring the methylation density of CpG sites on target alleles, we used the fusion PCR strategy²⁴ (see the schematic diagram shown in Supplemental Figure S6 at <http://jmd.amjpathol.org>) as described in the *Materials and Methods* to create a series of differentially methylated 95-bp *RASSF1A* DNA templates with 10 CpG sites, ranging from 0% to 100% in 20% increments (listed in Table 2), for melting curve analysis. The 95-bp sequences were derived from -75 to +20 nucleotides relative to the first nucleotide (set as +1) of the translation initiation codon (ATG) in exon 1 of *RASSF1A* gene. These DNA templates were subjected to SYBR green real-time PCR using their respective 5'- and 3'-end primers (Table 2) and melting temperatures of endpoint PCR products (83.3°C for 100%, 82.3°C for 80%, 81.3°C for 60%, 80.7°C for 40%, 80.1°C for 20%, and 79.1°C for 0%) were determined from their respective melting curve peaks (Figure 6A). The detected melting temperatures were used to calculate their measured methylation-density percentages according to the equation described in the *Materials and Methods*. The measured methylation-density data were plotted against expected % values for the linear regression analysis to evaluate correlation. As shown in Figure 6B, there is a strong linearity ($R^2 > 0.99$) between mea-

Figure 6. Assessment of the capability of the MethySYBR technique to measure the methylation density of target alleles. **A:** Melting curve analysis of differentially methylated *RASSF1A* DNA. A series of differentially methylated *RASSF1A* DNA, including 0%, 20%, 40%, 60%, 80%, and 100% methylation on 10 CpG sites, were created by fusion PCR (see Supplemental Figure S6 at <http://jmd.amjpathol.org>) and subjected to real-time SYBR green PCR using their respective primer pairs (shown in Table 2). Their respective endpoint PCR products were analyzed by the dissociation program to obtain their melting curve profiles. **B:** Evaluation of the sensitivity and accuracy of MethySYBR to measure the methylation density of target DNA. The measured methylation density for each differentially methylated *RASSF1A* DNA was calculated according to the equation shown in *Materials and Methods*. The measured methylation densities were plotted against the expected methylation densities. The equation of the linear regression curve and the correlation coefficient (R^2) are indicated on the plotted graph.

sured and expected methylation densities for all of analyzed samples. These tested results demonstrate that the melting curve analysis can accurately measure a 20% (equivalent to 2 CpG sites) change in methylation of 10 CpG sites. Therefore, the MethySYBR incorporating melting curve analysis is capable of determining the methylation density of CpG sites on target alleles.

Comparison between MethySYBR and MethyLight Using Breast Normal and Cancer Samples

To test the ability of MethySYBR to assess homogeneous and heterogeneous bisulfite-converted DNA samples, we analyzed methylation of *RASSF1A* and *OGDHL* alleles in DNA samples derived from a series of breast cancer cell lines (homogeneous samples) and breast tumor tissues (heterogeneous samples), including five breast cancer cell lines, 20 cases of epithelia-enriched organoids prepared from fresh normal breast tissue of healthy women

(ORG), five cases of ductal carcinoma *in situ* (DCIS), 24 cases of invasive ductal carcinoma (IDC), and six cases of invasive lobular carcinoma (ILC). To test the capability of MethySYBR to assess variably processed biopsies, DNA samples from DCIS and IDC were prepared from fresh frozen tissue and each ILC DNA sample from a section of formalin-fixed, paraffin-embedded tissue. For comparison, the MethyLight assay was also performed in parallel. Table 3 summarizes the methylation% data (indicating the methylation level) from both assays and the T_m data (indicating the methylation state) from melting curve analyses. We found that the data consistency between MethySYBR and MethyLight was dependent on levels and methylation states of target alleles in DNA samples. Generally, the DNA samples with significant levels ($\geq 1\%$) of fully or nearly fully methylated target alleles gave better data consistency between these two assays (Table 3). However, for the DNA samples with partially and/or low levels ($< 1\%$) of methylated alleles, MethySYBR gave the higher methylation % compared with the data from MethyLight (Table 3). This discrepancy resulted from the low sensitivity of the fluorescent probe to detect low levels of methylated alleles, which has been shown in the dilution experiment (Figure 5, C and D), and partially methylated alleles. In addition, MethySYBR was found to work well for analysis of very small amounts of ILC DNA samples (see Table 3 and Supplemental Figure S7 at <http://jmd.amjpathol.org>). In contrast, MethyLight Failed to assess these samples due to failure of *ACTB* amplification (see Supplemental Figure S7 at <http://jmd.amjpathol.org>). These test results obviously demonstrate that MethySYBR is more sensitive than MethyLight for analysis of paraffin-embedded tissue samples.

Given that TMCP data show the overall T_m profiles, detection of methylated T_m in the TMCP profile indicates existence of significant levels ($> 1\%$) of methylated alleles in DNA samples. As shown in Table 3, *RASSF1A* TMCP profiles for normal organoid samples containing 0.00%–0.65% of methylated *RASSF1A* gave the T_m range from 81.4°C to 81.8°C versus the methylated T_m range from 84.3°C to 85.3°C of breast tumor samples containing 2.46%–87.86% of methylated *RASSF1A*. The same trend was also seen for *OGDHL* detection. Thus, the information from TMCP profiles faithfully reflected measured methylation percentages of DNA samples. After conversion of the T_m from TMCP into methylation density %, the two-dimensional analysis convincingly showed that positive IDC tumor samples exhibited both high methylation level and density percentages, significantly distinct from organoids with low percentages for both methylation data (Figures 7A and 7B). For the MMCP data, they report the T_m of the most hypermethylated alleles. We were aware that MMCP profiles, not TMCP profiles, sensitively reported partially and nearly fully methylated T_m from a trace of *RASSF1A* or *OGDHL* methylated alleles ($< 1\%$) present in organoid or tumor tissue samples. We also converted the T_m data from MMCP into methylation density data for two-dimensional analysis. The plotted data showed that MethySYBR was able to detect low levels of target alleles with methylation densities $> 30\%$ (Figures 7C and 7D). In addition,

Table 3. Comparison between MethySYBR and MethyLight

Sample Name*	RASSF1A				OGDHL			
	MethyLight†	MethySYBR†	TMCP‡	MMCP‡	MethyLight†	MethySYBR†	TMCP‡	MMCP‡
MDA-MB-231	95.03 ± 2.38	94.85 ± 5.77	85.3°C	83.9°C	74.74 ± 6.99	84.40 ± 9.87	88.3°C	88.2°C
MDA-MB-468	75.58 ± 8.28	83.66 ± 3.62	85.3°C	83.8°C	69.45 ± 4.55	82.73 ± 4.94	88.2°C	88.2°C
MCF7	45.58 ± 6.80	82.38 ± 12.01	84.8°C	83.2°C	0	0.06 ± 0.02	83.3°C	85.2°C
T47D	41.52 ± 8.59	74.83 ± 7.28	84.8°C	83.2°C	0	0	83.3°C	84.1°C
ZR-751	38.93 ± 1.15	83.38 ± 5.35	84.8°C	83.2°C	0	0	83.3°C	84.2°C
HMEC	0	0	81.4°C	81.3°C	0	0	82.8°C	83.6°C
ORG-1	0	0.11 ± 0.02	81.5°C	82.8°C	0	0	83.2°C	84.2°C
ORG-2	0	0	81.5°C	81.5°C	0	0	83.2°C	84.2°C
ORG-3	0	0	81.8°C	81.8°C	0	0	83.3°C	84.1°C
ORG-4	0	0	81.5°C	81.8°C	0	0.05 ± 0.02	83.2°C	85.2°C
ORG-5	0	0	81.8°C	81.8°C	0	0	83.3°C	84.2°C
ORG-6	0	0.07 ± 0.03	81.5°C	83.4°C	0	0	83.3°C	84.3°C
ORG-7	0.03 ± 0.02	0.39 ± 0.10	81.5°C	83.1°C	0	0	83.4°C	84.3°C
ORG-8	0	0	81.4°C	81.5°C	0	0	83.1°C	84.1°C
ORG-9	0.02 ± 0.01	0.28 ± 0.06	81.4°C	83.4°C	0	0	82.8°C	83.7°C
ORG-10	0	0	81.4°C	81.3°C	0	0	83.1°C	83.7°C
ORG-11	0.05 ± 0.02	0.34 ± 0.08	81.8°C	83.1°C	0	0	83.1°C	84.3°C
ORG-12	0	0.45 ± 0.05	81.8°C	82.8°C	0	0.14 ± 0.05	83.1°C	87.2°C
ORG-13	0	0	81.8°C	81.5°C	0	0	83.1°C	84.0°C
ORG-14	0	0.24 ± 0.08	81.8°C	83.4°C	0	0	83.1°C	84.2°C
ORG-15	0	0	81.8°C	81.5°C	0	0	83.6°C	84.0°C
ORG-16	0.10 ± 0.04	0.65 ± 0.14	81.8°C	83.1°C	0	0	83.4°C	84.0°C
ORG-17	0	0	81.8°C	81.8°C	0	0	83.4°C	84.1°C
ORG-18	0	0	81.8°C	81.8°C	0	0	83.2°C	84.2°C
ORG-19	0.11 ± 0.05	0.38 ± 0.08	81.8°C	83.1°C	0	0	83.1°C	83.8°C
ORG-20	0	0.23 ± 0.04	81.8°C	82.8°C	0	0	83.1°C	84.0°C
DCIS-1	0	0	81.5°C	81.4°C	0	0	83.1°C	83.8°C
DCIS-2	8.04 ± 1.75	9.87 ± 1.49	81.6°C, 85.2°C	83.7°C	0	0	82.9°C	83.8°C
DCIS-3	24.85 ± 2.72	30.97 ± 4.31	81.5°C, 85.3°C	83.9°C	0.02 ± 0.01	0.41 ± 0.13	83.6°C	86.6°C
DCIS-4	0	0	81.7°C	81.4°C	0	0	83.0°C	83.7°C
DCIS-5	2.76 ± 0.43	3.56 ± 1.64	81.5°C, 85.2°C	83.7°C	0	0.05 ± 0.03	83.1°C	85.3°C
IDC-1	47.03 ± 5.23	43.33 ± 4.94	81.6°C, 85.0°C	83.7°C	0.04 ± 0.01	0.93 ± 0.21	84.3°C	85.2°C
IDC-2	47.97 ± 3.82	55.08 ± 12.25	81.5°C, 85.2°C	84.0°C	0	0	83.2°C	83.8°C
IDC-3	1.33 ± 0.37	3.43 ± 0.91	81.4°C, 84.9°C	83.6°C	0	0	83.1°C	84.1°C
IDC-4	0	0	81.5°C	81.7°C	2.44 ± 1.19	9.76 ± 1.40	82.9°C, 84.4°C, 87.0°C	86.6°C
IDC-5	11.40 ± 3.79	18.39 ± 5.57	81.5°C, 85.2°C	84.0°C	0	0.17 ± 0.07	83.6°C	86.0°C
IDC-6	15.46 ± 4.10	38.29 ± 4.28	81.5°C, 84.5°C	83.2°C	25.86 ± 2.8	51.82 ± 8.97	82.8°C, 88.0°C	87.8°C
IDC-7	0	0	81.5°C	81.4°C	0	0	83.2°C	83.9°C
IDC-8	13.42 ± 2.73	29.08 ± 3.82	81.4°C, 84.3°C	83.2°C	0	0	83.3°C	83.7°C
IDC-9	23.82 ± 3.36	33.03 ± 7.61	81.5°C, 85.3°C	84.0°C	0	0.05 ± 0.02	83.6°C	85.1°C
IDC-10	63.95 ± 10.17	58.85 ± 4.98	81.4°C, 85.2°C	83.8°C	0	0.03 ± 0.01	83.6°C	86.3°C
IDC-11	41.02 ± 3.16	50.47 ± 12.72	81.6°C, 85.2°C	83.9°C	0	0	83.2°C	83.8°C
IDC-12	0.96 ± 0.23	2.46 ± 1.17	81.4°C, 84.9°C	83.3°C	0	0	83.3°C	84.1°C
IDC-13	0	0	81.8°C	81.4°C	0	0	83.3°C	84.1°C
IDC-14	72.39 ± 5.27	75.86 ± 10.31	85.2°C	83.9°C	0	0	83.3°C	84.0°C
IDC-15	32.94 ± 5.38	52.07 ± 5.83	81.7°C, 85.3°C	83.8°C	0	0	83.3°C	84.0°C
IDC-16	0	0	81.6°C	81.6°C	0	0	82.9°C	83.7°C
IDC-17	84.65 ± 9.72	74.53 ± 2.62	85.3°C	84.1°C	0	0	82.9°C	84.2°C
IDC-18	0	0	81.7°C	81.5°C	5.01 ± 1.30	10.24 ± 1.32	83.1°C, 87.4°C	86.8°C
IDC-19	37.86 ± 3.71	35.43 ± 10.2	81.4°C, 85.2°C	83.7°C	0	0	83.0°C	83.9°C
IDC-20	7.75 ± 0.67	14.15 ± 3.1	81.6°C, 85.2°C	83.8°C	0	0	82.8°C	83.6°C
IDC-21	0	0	81.6°C	81.5°C	0	0	83.0°C	83.6°C
IDC-22	38.65 ± 9.86	53.39 ± 4.18	81.5°C, 85.2°C	83.9°C	0	0	83.0°C	83.6°C
IDC-23	1.27 ± 0.27	5.03 ± 1.55	81.4°C, 84.3°C	83.3°C	0	0	83.2°C	83.7°C
IDC-24	18.02 ± 3.08	25.25 ± 8.81	81.4°C, 85.3°C	84.0°C	0	0	82.9°C	84.0°C
ILC-1	Failed	0	81.6°C	81.5°C	Failed	0	83.1°C	83.9°C
ILC-2	Failed	0	81.8°C	81.4°C	Failed	0	83.2°C	84.1°C
ILC-3	Failed	87.86 ± 9.69	85.3°C	84.0°C	Failed	5.41 ± 1.86	82.9°C, 88.3°C	88.2°C
ILC-4	Failed	0	81.8°C	81.5°C	Failed	0	83.1°C	84.2°C
ILC-5	Failed	82.41 ± 10.8	85.3°C	83.8°C	Failed	0	82.8°C	83.9°C
ILC-6	Failed	26.51 ± 1.96	81.8°C, 85.2°C	83.7°C	Failed	0	82.9°C	84.0°C

*HMEC, human mammary epithelial cells; ORG, organoid; DCIS, ductal carcinoma *in vitro* (DNA extracted from frozen tissue); IDC, invasive ductal carcinoma (DNA extracted from frozen tissue); ILC, invasive lobular carcinoma (DNA extracted from a section of paraffin-embedded tissue).

†The shown data are methylation percentages of DNA samples relative to Sss1-231 (100%). The standard deviation (SD) for each sample data was obtained from the data of three independent PCR experiments. 0% was granted to the DNA samples with methylation % less than 0.01%.

‡The melting temperature of endpoint PCR products determined from the TMCP or MMCP analysis is shown.

the two-dimensional data obviously revealed that some IDC samples contained low levels of methylated *RASSF1A* (or *OGDHL*) that exhibited high CpG methylation densities, indicating that the CpG methylation density was not completely correlated with the methylation level. These data, taken together, show that the MethySYBR assay was able to report both levels and states of methylated alleles no matter from both homogeneous and heterogeneous DNA samples, proving to be more sensitive and useful than the traditional MethyLight assay.

Validation of Melting Curve Results Using Bisulfite Genomic Sequencing

To further validate whether melting curve profiles from the MethySYBR analysis can be used to precisely determine the methylation density of target CpG islands present in tissue DNA samples, bisulfite genomic sequencing was performed on IDC-23 and IDC-4 samples to examine DNA methylation of *RASSF1A* and *OGDHL*, respectively. After analysis by the dissociation program to obtain the

Figure 7. Display of methylation information for organoids versus IDC samples using two-dimensional analysis of the methylation level and density of target alleles. The average percentages of methylation levels of *RASSF1A* and *OGDHL* in organoids (20 cases) and IDC samples (24 cases) listed in Table 3 were plotted against their respective CpG methylation densities calculated from the Tm of amplicons for two-dimensional analysis. The methylation-level data for *RASSF1A* and *OGDHL* plotted against methylation-density data from TMCP are shown in (A) and (B), respectively. The *RASSF1A* and *OGDHL* two-dimensional plots of methylation % vs. methylation density % from MMCP are shown in (C) and (D), respectively. The black triangle and gray circle symbols were used to indicate the organoid and IDC data points, respectively. Some data points are not visible because of superimposing between data points.

TMCP profiles (Figure 8, A and B), the PCR products amplified by the external nested primer set were purified and cloned into plasmid DNA. 10 randomly selected clones for each gene were subjected to sequencing analysis. The number of methylated CpG sites was counted based on the sequencing results, which was used to calculate the expected Tm (shown in parentheses of Figure 8, C and D) of cloned DNA according to the equation described in *Materials and Methods*. Through sequencing analysis, we identified unmethylated *RASSF1A* DNA templates and two types of methylated *RASSF1A* DNA templates. For the methylated DNA clones, their ratios of methylated to total CpG sites were 13/16 and 12/16 (Figure 8C). Their expected melting temperatures (84.5°C, 84.3°C, and 81.4°C) were consistent with the measured melting temperatures (84.3°C and 81.4°C) from the TMCP profile (compare Figure 8A to Figure 8C). For *OGDHL* analysis, six types of methylated *OGDHL* DNA templates were identified by sequencing analysis. Their respective ratios of methylated to total CpG sites were 22/30, 21/30, 17/30, 9/30, 8/30, and 1/30. Excluding the DNA template with the 17/30 ratio, all of their expected melting temperatures (including 87.1°C, 86.9°C, 84.5°C, 84.3°C, and 83.0°C) matched the measured melting temperatures (including 87.0°C, 84.4°C, and 82.9°C) of three distinct melting curve peaks shown in the TMCP profile (compare Figure 8B to Figure 8D). The only exception that could not be detected in the TMCP profile is the *OGDHL* DNA clone 6 with 17 methylated CpG sites (expected Tm = 86.1°C), which might be due to its low amount in IDC-4 DNA since just one clone was identified. Overall these validation experiments

clearly demonstrate that our MethySYBR assay could accurately report the methylation degree of CpG sites on major DNA templates.

Discussion

This study presents a novel SYBR green-based real-time MSP technique named MethySYBR, which combines both quantitative and melting curve profiling analyses. Through analysis of DNA methylation of both *RASSF1A* and *OGDHL* in breast cancer using this new technique, we have demonstrated that MethySYBR is a highly sensitive, specific, accurate, and high-throughput method to quantify the methylation levels and assess the methylation states of methylated genes in cancer tissue specimens. The pre-amplification step of MethySYBR can be used to multiplex multiple distinct DNA templates for simultaneous detection of multiple methylated genes or multiple DNA regions within a gene. This merit enables MethySYBR to assess multiple methylated loci using a minute amount of DNA. Therefore, MethySYBR is extremely useful for analyzing small biopsies like paraffin-embedded tissue sections. Indeed, we have demonstrated that MethySYBR performed better than the conventional one-step MethyLight assay for assessing DNA samples from paraffin-embedded tissue.

A simplified quantitative method used in this new quantitative MSP assay uses external nested primers to measure the total amount of input DNA, represented by the reference C_T . This reference C_T is used to normalize the amount of methylated target alleles. The accuracy of this new quantitative strategy has been validated

Figure 8. Validation of melting curve profile data by bisulfite genomic sequencing. **A:** TMCP analysis of *RASSF1A* amplicons from IDC-23 DNA. Bisulfite-converted IDC-23 tissue DNA was subjected to MethySYBR analysis using *RASSF1A* primer sets. The endpoint PCR products amplified with *RASSF1A*-EXT-F2 and *RASSF1A*-EXT-R primers were analyzed by the dissociation program to obtain its TMCP profile. The melting temperatures for two different PCR products are indicated in the TMCP profile. **B:** TMCP analysis of *OGDHL* amplicons from IDC-4 DNA. Bisulfite-treated IDC-4 tissue DNA was subjected to MethySYBR analysis using *OGDHL* primer sets. The endpoint PCR products amplified with *OGDHL*-EXT-F2 and *OGDHL*-EXT-R primers were analyzed by the dissociation program to obtain its TMCP profile. The melting temperatures for three different PCR products are indicated in the TMCP profile. **C:** Summary of the results from bisulfite genomic sequencing analysis of 16 *RASSF1A* CpG sites from IDC-23 tissue DNA. The endpoint *RASSF1A* PCR products from (**A**) were cloned and the results from sequencing analysis of 10 randomly selected clones are shown under the CpG-site map (the CpG dinucleotide is indicated by a vertical line). The ratio of methylated to total CpG sites for each clone is shown on the right side of sequencing results. The expected melting temperature for each identified *RASSF1A* DNA was calculated according to its methylation density (The calculation method is described in *Materials and Methods*) and is shown in parentheses. **D:** Summary of the results of bisulfite genomic sequencing analysis of 30 *OGDHL* CpG sites from IDC-4 tissue DNA. The endpoint *OGDHL* amplicons were analyzed in the same way as *RASSF1A*. The ratio of methylated to total CpG sites for each clone is also shown on the right side of sequencing results. The expected melting temperature for each cloned *OGDHL* DNA is also shown in parentheses.

by comparing it to the classic MethyLight assay. This new quantitative strategy can avoid underestimation of methylation levels of a target gene when one of its alleles is lost through the chromosomal deletion mechanism. In contrast, other techniques that need another internal reference gene (eg, β -actin gene) for relative quantitation¹⁰ may give inaccurate methylation data when a gene is targeted by both DNA methylation and deletion.¹¹ However, our test results (see Figure 4, A and B) showed that this new quantitative strategy was only suitable for the two-step MethySYBR, not its one-step counterpart method, due to the nature of bisulfite-converted DNA composed of various sizes of small DNA fragments.

Compared with fluorescent probe-based quantitative MSP techniques (eg, MethyLight, QM-MSP)¹⁰⁻¹³ that need expensive fluorescent TaqMan probes, MethySYBR is an economical method suitable for the high-throughput analysis of clinical samples. Our test results show that MethySYBR was capable of detecting methylated alleles in the presence of a 100,000-fold excess of unmethylated alleles, which is more sensitive and accurate than classic MethyLight. Most importantly, we have demonstrated that multiplexing two genes in the pre-amplification step did not interfere with the quantitative accuracy of the MethySYBR assay. These results indicate that there was no PCR bias in the first multiplexed pre-amplification step even when the spiked sample containing as low as 0.001% of methylated DNA was assayed. Furthermore, we proved that the pre-amplification step significantly improved the specificity of the second real-time PCR step

by suppressing the formation of primer dimer and enabled the two-step MethySYBR assay to be more sensitive and specific compared with its one-step counterpart method. Thus, MethySYBR, unlike other previous one-step versions of SYBR green-based MSP, can be applied to analysis of clinical biopsies. Indeed, we have demonstrated that MethySYBR could detect both methylated *RASSF1A* and *OGDHL* alleles present in a minute amount of DNA from paraffin-embedded tumor tissue.

Analysis of clinical samples with heterogeneously methylated alleles remains a challenging issue for DNA methylation study. The general probe design for classic MethyLight only detects fully methylated alleles.¹⁰ For detection of heterogeneously methylated alleles, a complicated and expensive design for probes and primers is needed.¹⁰ In contrast, the MethySYBR technique can easily identify heterogeneously methylated DNA templates simply through melting curve profiling analysis without a increase in the complexity and cost of the assay. MethySYBR can basically be used to define three discrete methylation states of target alleles, including unmethylated, partially methylated, and fully methylated states. MethySYBR provides two separate melting curve profiles, TMCP and MMCP, to display melting temperatures of amplicons amplified from total and the most methylated target alleles, respectively. The TMCP analysis represents methylation states of entire target alleles. The MMCP analysis sensitively shows the methylation state of the most methylated target alleles in a DNA sample. Therefore, in addition to quantitative data, Me-

thySYBR can powerfully display informative melting curve data. We have validated that the T_m -information of amplicons obtained from the melting curve profiling analysis can be used to precisely measure the methylation density of target CpG islands. Compared with sequencing-based techniques (eg, pyrosequencing) with a low sensitivity for analyzing bisulfite-converted DNA,^{25–27} MethySYBR was shown to be a simple and highly sensitive assay for determining the percentage of methylated CpG sites on target loci in a minute amount of DNA. Therefore, MethySYBR may serve as a screening tool to display the melting-curve information of methylated genes in clinical biopsies in a convenient, efficient, and high-throughput way regardless of whether DNA samples are homogeneous or heterogeneous. This application is very helpful to immediately identify true positive samples for further advanced analyses (eg, sequencing analysis of the second-step endpoint PCR products obtained from the MethySYBR analysis).

For translational applications of methylated biomarker genes to diagnosis and prognosis of cancer, it is important for the clinical laboratory to identify the correlation between DNA methylation and tumor aggressiveness. However, the qualitative or quantitative information provided by the current DNA methylation assays are not sufficient to address this correlation. In contrast, the MethySYBR assay enables researchers to examine not only DNA methylation levels of genes but also the extent of CpG methylation on gene promoters. In fact, through MethySYBR analysis, we found that methylated *RASSF1A* and *OGDHL* alleles with low levels in some tumor samples contained the high density of CpG methylation. Therefore, dual analysis of the methylation level and density is required to decipher the correlation between DNA methylation and cancer status. Furthermore, MethySYBR can also benefit detection of early-stage cancer since this assay incorporating melting curve analysis is a highly sensitive and specific assay for detection of low levels of methylated alleles no matter what they are partially or fully methylated, which might be missed by classic MethyLight.

In conclusion, the work presented here describes a novel SYBR green-based quantitative PCR assay for dual analysis of the DNA methylation level and CpG methylation density in cells. These improved features render MethySYBR economical, more accurate, highly sensitive, and less prone to false positives. By incorporating the multiplex PCR technology, MethySYBR is suitable for a large-scale assessment of multiple methylated genes in a wide range of samples, which is especially useful for analysis of clinical biopsies with minute amounts of DNA.

Acknowledgments

We thank Mary Jo Fackler for advice and guidance, and Mary Jo Fackler, Alan Rein, and Vanessa Merino for critically reviewing the manuscript.

References

1. Baylin SB: Mechanisms underlying epigenetically mediated gene silencing in cancer. *Semin Cancer Biol* 2002, 12:331–337
2. Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003, 349:2042–2054
3. Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002, 3:415–428
4. Jones PA, Laird PW: Cancer epigenetics comes of age. *Nat Genet* 1999, 21:163–167
5. Toyota M, Issa JP: Epigenetic changes in solid and hematopoietic tumors. *Semin Oncol* 2005, 32:521–530
6. Laird PW: The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003, 3:253–266
7. Shi H, Wang MX, Caldwell CW: CpG islands: their potential as biomarkers for cancer. *Expert Rev Mol Diagn* 2007, 7:519–531
8. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, 93:9821–9826
9. Xiong Z, Laird PW: COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* 1997, 25:2532–2534
10. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW: MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res* 2000, 28:E32
11. Zeschnick M, Bohringer S, Price EA, Onadim Z, Masshofer L, Lohmann DR: A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. *Nucleic Acids Res* 2004, 32:e125
12. Fackler MJ, McVeigh M, Mehrotra J, Blum MA, Lange J, Lapidus A, Garrett E, Argani P, Sukumar S: Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. *Cancer Res* 2004, 64:4442–4452
13. Swift-Scanlan T, Blackford A, Argani P, Sukumar S, Fackler MJ: Two-color quantitative multiplex methylation-specific PCR. *BioTechniques* 2006, 40:210–219
14. Fackler MJ, Malone K, Zhang Z, Schilling E, Garrett-Mayer E, Swift-Scanlan T, Nayar R, Davidson NE, Khan SA, Sukumar S: Quantitative multiplex methylation-specific PCR analysis doubles detection of tumor cells in breast ductal fluid. *Clin Cancer Res* 2006, 12:3306–3310
15. Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, Niranjan B, Buluwela L, Weitzman SA, Marks J, Sukumar S: Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res* 2001, 61:2782–2787
16. Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, Polyak K, Sukumar S, Argani P: DNA methylation of *RASSF1A*, *HIN-1*, *RAR-β*, *Cyclin D2*, and *Twist* in situ and invasive lobular breast carcinoma. *Int J Cancer* 2003, 107:970–975
17. Lo PK, Mehrotra J, D'Costa A, Fackler MJ, Garrett-Mayer E, Argani P, Sukumar S: Epigenetic suppression of secreted frizzled related protein 1 (SFRP1) expression in human breast cancer. *Cancer Biol Ther* 2006, 5:281–286
18. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta CT)}$ Method. *Methods* 2001, 25:402–408
19. Chan MW, Chu ES, To KF, Leung WK: Quantitative detection of methylated *SOCS-1*, a tumor suppressor gene, by a modified protocol of quantitative real time methylation-specific PCR using SYBR green and its use in early gastric cancer detection. *Biotechnol Lett* 2004, 26:1289–1293
20. Chu DC, Chuang CK, Fu JB, Huang HS, Tseng CP, Sun CF: The use of real-time quantitative polymerase chain reaction to detect hypermethylation of the CpG islands in the promoter region flanking the *GSTP1* gene to diagnose prostate carcinoma. *J Urol* 2002, 167:1854–1858
21. Thomassin H, Kress C, Grange T: MethyQuant: a sensitive method for quantifying methylation of specific cytosines within the genome. *Nucleic Acids Res* 2004, 32:e168
22. Dammann R, Yang G, Pfeifer GP: Hypermethylation of the CpG island of Ras association domain family 1A (*RASSF1A*), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. *Cancer Res* 2001, 61:3105–3109
23. Hoque MO, Kim MS, Ostrow KL, Liu J, Wisman GB, Park HL, Poeta ML, Jeronimo C, Henrique R, Lendvai A, Schuurin E, Begum S, Rosenbaum E, Ongenaert M, Yamashita K, Califano J, Westra W, van

- der Zee AG, Van Criekinge W, Sidransky D: Genome-wide promoter analysis uncovers portions of the cancer methylome. *Cancer Res* 2008, 68:2661–2670
24. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR: Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989, 77:51–59
25. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL: A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 1992, 89:1827–1831
26. Warnecke PM, Stirzaker C, Song J, Grunau C, Melki JR, Clark SJ: Identification and resolution of artifacts in bisulfite sequencing. *Methods* 2002, 27:101–107
27. Tost J, Dunker J, Gut IG: Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *BioTechniques* 2003, 35:152–156