Precision and Performance Characteristics of Bisulfite Conversion and Real-Time PCR (MethyLight) for Quantitative DNA Methylation Analysis

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Assays to measure DNA methylation, which are important in epigenetic research and clinical diagnostics, typically rely on conversion of unmethylated cytosine to uracil by sodium bisulfite. However, no study has comprehensively evaluated the precision and performance characteristics of sodium bisulfite conversion and subsequent quantitative methylation assay. We developed quantitative real-time polymerase chain reaction (MethyLight) to measure percentage of methylated reference (PMR, ie, degree of methylation) for the *MGMT*, *MLH1*, and *CDKN2A* (p16) promoters. To measure the precision of bisulfite conversion, we bisulfite-treated seven different aliquots of DNA from each of four paraffin-embedded colon cancer samples. To assess run-to-run variation, we repeated MethyLight five times. Bisulfite-to-bisulfite coefficient of variation (CV) of PMR ranged from 0.10 to 0.38 (mean, 0.21), and run-to-run CV of PMR ranged from 0.046 to 0.60 (mean, 0.31). Interclass correlation coefficients were 0.74 to 0.84 for the three loci, indicating good reproducibility. DNA mixing study with methylated and unmethylated DNA showed good linearity of the assay. Of 272 colorectal cancers evaluated, most showed PMR either <1 or >10, and promoter methylation (PMR >4) was tightly associated with loss of respective protein expression ($P < 10^{-16}$). In conclusion, sodium bisulfite conversion and quantitative MethyLight assays have good

precision and linearity and can be effectively used for high-throughput DNA methylation analysis on paraffin-embedded tissue. (J Mol Diagn 2006; 8:209–217; DOI: 10.2353/jmoldx.2006.050135)

Transcriptional inactivation by promoter CpG island methylation in tumor suppressor genes is an important mechanism in human carcinogenesis, and methylated cytosine has been called “the fifth base.”^{1–3} Aberrant CpG island methylation has been shown to occur in a nonrandom manner, and the pattern of methylation is tumor-specific.⁴ Given the importance of DNA methylation in cancer and various other diseases, assays to measure DNA methylation may be very useful in both research and clinical practice. DNA methylation of some tumor suppressor genes in breast cancer has been shown to be predictive of responsiveness to tamoxifen therapy.⁵ Recently, methylation and silencing of the *MGMT* gene in glioblastoma has been associated with an increased benefit from temozolomide treatment.⁶ DNA methylation may be a useful marker for cancer diagnosis, screening, surveillance in high-risk individuals, monitoring of minimal residual disease, and determining optimal therapeutic options.^{2,7–12} Therefore, there are increasing demands for reliable assays to measure DNA methylation, particularly for formalin-fixed paraffin-embedded tissue.

A number of methods to determine DNA methylation status in tumor tissues have been developed. For formalin-fixed, paraffin-embedded tissue, methylation-specific polymerase chain reaction (MSP) after sodium bisulfite conversion¹³ is widely used. However, because of the qualitative nature of the assay, MSP cannot reliably distinguish low levels of methylation from high levels of methylation. Furthermore, MSP does not appear to be highly reproducible for some samples that may have low

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levels of methylation, and it is difficult to assess performance characteristics. Thus, clinical use of qualitative MSP is a major concern in terms of quality control and assurance. Quantitative measurement of methylation is important because low levels of methylation (below the threshold of transcriptional silencing) may not be biologically important. Hence, a variety of quantitative assays to measure DNA methylation have been developed, including combined bisulfite restriction analysis,¹⁴ restriction ligation-mediated polymerase chain reaction (PCR),¹⁵ methylation-sensitive single nucleotide primer extension (Ms-SNuPE),¹⁶ ion-pair reverse-phase high performance liquid chromatography,¹⁷ denaturing high performance liquid chromatography,¹⁸ pyrosequencing,^{19–21} MALDI-TOF,²² and real-time PCR.^{5,23–31} Most of these methods use DNA treated with sodium bisulfite. Therefore, sodium bisulfite treatment is a critical step in the measurement of DNA methylation. However, reproducibility of the sodium bisulfite conversion step has not been well investigated. Sodium bisulfite treatment presents a harsh environment for DNA molecules, and previous data suggest that ~84 to 96% of DNA is degraded during 4 hours of bisulfite treatment at 55°C.³² Assurance of reproducibility in this critical bisulfite treatment step is essential in applying quantitative methylation assays to clinical practice.

In this study, we evaluated the precision and performance characteristics of sodium bisulfite treatment and MethyLight, a real-time PCR assay.^{5,26,28,33} Our data indicate that both sodium bisulfite conversion and MethyLight assays have good reproducibility and precision and can be effectively used to quantify DNA methylation in paraffin-embedded tumor tissue.

Materials and Methods

Tissue Specimens

Tissue collection and analysis in this study were approved by the Dana-Farber Harvard Cancer Center and Brigham and Women's Hospital Institutional Review Boards. Formalin-fixed, paraffin-embedded tissue samples of four cases (designated as case 1 through case 4) of colon cancer were anonymized after collection from the archival file of Department of Pathology, Brigham and Women's Hospital. For the correlation study of methylation and immunohistochemistry, formalin-fixed, paraffin-embedded tissue samples of colorectal cancer were collected from participants of the Nurses' Health Study and Health Professional Follow-up Study cohorts.^{34,35} Informed consents from all participants were obtained. Tumors were selected from these cohorts, based on availability of adequate tumor tissue samples and assay results at the time of this study, resulting in the inclusion of a total of 274 cases of colorectal cancer.

Sodium Bisulfite Treatment and DNA Extraction

Hematoxylin and eosin (H&E)-stained slides of the tumors were reviewed, and areas of tumor were marked, to exclude pure normal tissue and enrich tumor DNA. H&E-

stained tumor sections (10 $\mu\text{m} \times 14$ sections) were scraped off slides from each of case 1 through case 4 and suspended in 140 μl of tissue lysate solution (100 mmol/L Tris, pH 8, 10 mmol/L ethylenediaminetetraacetic acid, pH 8, 1 mg/ml proteinase K, and 0.05 mg/ml tRNA). The lysate solutions were incubated overnight at 50°C. The lysate was aliquoted into seven tubes, each containing 18 μl of tissue lysate, and stored at -20°C until sodium bisulfite modification was performed. For methylation and immunohistochemistry study (using cohort colorectal cancer cases), H&E-stained tissue sections (depending on tissue and tumor size, in average, large tumor tissue 10 $\mu\text{m} \times 1$ section) from each case were scraped off slides, suspended in 20 μl of the tissue lysate, and incubated at 50°C overnight. The tissue lysate was then stored at -20°C until sodium bisulfite modification was performed.

Sodium metabisulfite (1.9 g) was dissolved in mixture of 3.2 ml of 0.44 mol/L NaOH at 50°C. Then, 0.5 ml of 1 mol/L hydroquinone was added to the dissolved sodium bisulfite mixture. An 18- μl aliquot of the tissue lysate was denatured at 100°C for 10 minutes and chilled on ice. Then, 2 μl of 3 mol/L NaOH was added and incubated at 42°C for 20 minutes. The bisulfite solution (120 μl) was added (total volume of 140 μl) and incubated at 50°C for 15 hours in the dark. The bisulfite-converted DNA was recovered using a Qiagen QIAamp viral RNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with some modifications. Buffer AVL/carrier RNA (560 μl) was added to the 140 μl of bisulfite-converted DNA sample and incubated at room temperature for 10 minutes. Ethanol (560 μl) was then added, and after extensive mixing, the mixture was loaded onto the provided spin columns in consecutive 630- μl aliquots. After each loading, the columns were centrifuged at full speed (21,000 $\times g$) for 1 minute. Both the filtrate and spin column were saved, and both filtrates were passed through the column a second time in the same manner to increase the yield of recovery. The spin column was then washed with 500 μl of buffer AW1, followed by centrifugation at 21,000 $\times g$ for 1 minute. Buffer AW2 (500 μl) was then added to the column, and the column was centrifuged at 21,000 $\times g$ for 4 minutes to wash the column and eliminate possible buffer AW2 carry over. DNA in the spin column was eluted by the addition of 40 μl of buffer AVE, followed by a 1-minute incubation at room temperature and centrifugation at 7600 $\times g$ for 1 minute. This elution step was repeated with a second 40- μl volume of buffer AVE. Fifty μl of 0.2 mol/L NaOH was added to the 80- μl pooled eluate for 15 minutes at room temperature to desulphonate the sample, and then 10 μl of 1 mol/L HCl was added to for neutralization. Buffer AVL/carrier RNA (560 μl) was then added to the 140- μl sample mixture, and the recovery procedure was repeated with a new spin column. The eluted DNA (80- μl volume) was then used for MethyLight analysis.

Quantitative Real-Time PCR (MethyLight)

Real-time PCR assays to measure DNA methylation (MethyLight) have been described.^{5,26,28} Briefly, three

Figure 1. Precision study for sodium bisulfite conversion and MethyLight. Bisulfite conversion was performed on seven different aliquots from each of the four cell lysate samples (cases 1 through 4), starting on 7 different days. Each sample was assessed for PMR at each locus by the MethyLight assay. MethyLight assays were repeated for each bisulfite-treated DNA sample on 5 different days, and PMR values were again determined.

sets of primers and probes designed specifically for bisulfite-converted DNA were used: a set for the gene of interest and two sets for *ACTB* and *COL2A1* to normalize for the amount of input DNA. We used an ABI 7300 real-time PCR instrument (Applied Biosystems, Foster City, CA). Primers and probes for *CDKN2A*, *MGMT*, *ACTB*, and *COL2A1* were previously described.⁵ The *MLH1* forward primer is 5'-AGG AAG AGC GGA TAG CGA TTT-3', the *MLH1* reverse primer is 5'-TCT TCG TCC CTC CCT AAA ACG-3', and the *MLH1* probe is 6FAM-5'-CCC GCT ACC TAA AAA AAT ATA CGC TTA CGC G-3'-BHQ1. The amount of methylated DNA (PMR, percentage of methylated reference³⁶) at a specific locus was calculated by dividing the *GENE:ACTB* (or *COL2A1*) ratio of a sample by the *GENE:ACTB* (or *COL2A1*) ratio of SssI-treated human genomic DNA (presumably fully methylated) and multiplying by 100. Reactions using SssI-treated DNA were used to normalize for any difference in amplification efficiencies between *GENE* and *ACTB* (or *COL2A1*).

Measuring the Precision of Sodium Bisulfite Conversion and MethyLight Assay

Figure 1 shows our strategy to measure precision and reproducibility of bisulfite conversion of DNA and quantitative MethyLight assay. We performed bisulfite conversion on seven different aliquots from each of the four cell lysate samples (case 1 through case 4), starting on 7 different days. Thus, from each cell lysate, we have seven separate bisulfite-converted DNA samples (designated as B1 through B7), which ideally should give at least similar values for methylation at a specific locus. We measured PMR for each locus by the MethyLight assay on each of these seven separate bisulfite-DNA samples. For each case, we measured the coefficient of variation (CV) of these seven PMR values on B1 through B7, which would primarily depend on variations in bisulfite conversion. We repeated the MethyLight assays five times (des-

Figure 2. DNA mixing study to assess linearity of MethyLight. Human peripheral blood leukocyte DNA (Promega) was first treated with M.SssI to methylate all CpG sites (near complete methylation and no loss of DNA was assumed). Mixtures of SssI-treated DNA:untreated DNA were prepared in triplicate (each containing 1 µg of template DNA) as shown. One hundred percent SssI-treated DNA served as methylated reference DNA. Each DNA mixture was bisulfite-treated and assayed in duplicate by MethyLight. Linear regression analysis was performed to assess assay linearity.

ignated as M1 through M5) and determined PMR values on the exact same set of bisulfite-converted DNA samples (B1 through B7) on 5 different days. We calculated the CV of these five PMR values (M1 through M5) on each one of the seven bisulfite-converted DNA samples (B1 through B7). Thus, these seven CVs would primarily depend on day-to-day variations of each MethyLight assay.

We also evaluated reproducibility of standard curves, which affects run-to-run precision. We examined five standard curves in five MethyLight repeats (M1 to M5 in Figure 1). To obtain PMR variations solely due to variations of standard curves, we also performed a separate MethyLight run with five standard curves in a single plate and calculated five different PMR values for each sample-marker combination, using each one of the five different standard curves.

DNA Mixing Study to Assess Assay Linearity

We performed a mixing study to assess linearity of sodium bisulfite treatment and quantitative MethyLight assays (Figure 2). We first treated human peripheral blood leukocyte DNA (Promega, Madison, WI) with SssI to methylate all CpG sites. Assuming near complete methylation and no loss of DNA during SssI treatment, we prepared DNA mixtures in triplicates (each containing 1 µg of template DNA) for SssI-treated DNA:untreated DNA as follows: 50:50, 30:70, 20:80, 10:90, and 5:95 (Figure 2). We used 100% SssI-treated DNA as control reference DNA to calculate PMR as described above. We treated DNA mixtures in triplicates with sodium bisulfite and duplicated MethyLight assays, resulting in six data points of PMR values for each dilution. Linear regression analysis was performed to assess assay linearity.

Immunohistochemistry

Immunohistochemistry for MLH1, p16 (CDKN2A), and MGMT was previously described.³⁷ Immunohistochemistry was evaluated while blinded from methylation data.

Table 1. Ct Values of Case 2 in MethyLight for *CDKN2A* (p16)

		Different MethyLight runs					Mean of Ct values	SD(M)
		M1	M2	M3	M4	M5		
Different bisulfite treatments	B1	31.35	31.15	31.02	31.17	31.20	31.18	0.12
	B2	30.86	30.79	30.49	30.82	30.75	30.74	0.15
	B3	31.41	31.19	31.42	31.33	31.22	31.31	0.11
	B4	31.05	31.04	30.95	31.07	30.67	30.96	0.17
	B5	31.07	30.94	31.07	31.15	31.18	31.08	0.09
	B6	30.46	30.49	30.54	30.75	30.53	30.55	0.11
	B7	30.41	30.22	30.09	30.18	30.33	30.25	0.13
Mean of Ct values		30.94	30.83	30.80	30.92	30.84		
SD(B)		0.39	0.36	0.45	0.39	0.36		

Each of B1 through B7 represents each bisulfite-converted sample, and each of M1 through M5 represents each of MethyLight assay results on 5 different days. SD(M) refers to MethyLight-to-MethyLight SD within each sample, and SD(B) refers to bisulfite-to-bisulfite SD within each MethyLight run.

Statistical Analysis

For statistical analysis, paired *t*-test to compare means, *F*-test to assess the equality of variances³⁸ and linear regression analysis to assess assay linearity were performed using the Microsoft Excel 2000 Analysis ToolPak. For *F*-test, raw PMR values were log-transformed to make distributions close to normal. Interclass correlation coefficients were calculated to assess assay reproducibility using the SAS program (version 9.1; SAS Institute, Cary, NC). To assess the association between promoter methylation and loss of protein expression, Fisher's exact test was performed using the SAS program. All *P* values were two-sided.

Results

Precision of Sodium Bisulfite Conversion and Quantitative Real-Time PCR (MethyLight)

We measured the threshold cycle (Ct) in our MethyLight assay for each of the bisulfite-converted samples (cases 1 through 4, samples B1 through B7) in each of the five MethyLight runs (M1 through M5) (Figure 1). Case 1 showed positive reaction (Ct <40) in *MLH1*, cases 2 and 3 showed positive reaction in *CDKN2A* and *MGMT*, and case 4 showed positive reaction in *CDKN2A* and *MLH1*. Table 1 shows representative Ct values (case 2, samples

B1 through B7) for each of the five different MethyLight analyses (M1 through M5) for *CDKN2A*. We designated SD(B) as bisulfite-to-bisulfite SD (SD) (within-run variation in seven bisulfite-treated samples B1 to B7) in a particular MethyLight run, and SD(M) as run-to-run SD (within-sample variation by five runs M1 to M5) on a particular sample (Figure 1 and Table 1). Table 2 shows SD(B) and SD(M) for all positive markers and control genes *ACTB* and *COL2A1* in cases 1 through 4, which appeared comparable with SDs of Ct values that have been described in various real-time PCR assays.^{39–41} SD(B) and SD(M) of Ct values for *COL2A1* on the four cases were significantly smaller than those for *ACTB* (*P* = 0.0004 by paired *t*-test).

We obtained the PMR based on standard curves in each run, Ct of a gene of interest (GENE), Ct of *ACTB* (or *COL2A1*) as a control reaction to normalize for the amount of input DNA, and reactions for both GENE and *ACTB* (or *COL2A1*) of Sssl-treated DNA to normalize for any difference in PCR efficiencies between GENE and *ACTB* (or *COL2A1*). Representative PMR values are listed in Table 3 (case 3, samples B1 to B7) in the five different MethyLight runs (M1 to M5) for *MGMT*. We designated CV(B) as bisulfite-to-bisulfite CV, ie, within-run variation in seven bisulfite-treated samples B1 to B7, and CV(M) as MethyLight-to-MethyLight run-to-run CV, ie, within-sample variation by five runs M1 to M5.

As described previously, PMR values of ≤4 indicated methylation-negative.³³ In our analysis, negative markers

Table 2. Standard Deviations (SD) of Ct Values in MethyLight

<i>CDKN2A</i>		<i>MLH1</i>		<i>MGMT</i>		<i>ACTB</i>		<i>COL2A1</i>	
Mean of SD	Range	Mean of SD	Range	Mean of SD	Range	Mean of SD*	Range	Mean of SD*	Range
Case 1 SD(B)		0.41	0.21–0.67			0.39	0.24–0.82	0.26	0.20–0.37
SD(M)		0.96	0.58–1.1			0.45	0.14–0.93	0.19	0.095–0.44
Case 2 SD(B)	0.39	0.36–0.45		0.48	0.31–0.75	0.40	0.20–0.54	0.33	0.19–0.46
SD(M)	0.12	0.093–0.17		0.56	0.37–0.86	0.61	0.44–0.78	0.27	0.15–0.48
Case 3 SD(B)	0.51	0.40–0.65		0.21	0.10–0.31	0.53	0.39–0.76	0.19	0.14–0.32
SD(M)	0.51	0.36–0.67		0.17	0.076–0.29	0.50	0.27–0.74	0.19	0.13–0.21
Case 4 SD(B)	0.35	0.34–0.36	0.38	0.23–0.62		0.33	0.14–0.55	0.18	0.15–0.22
SD(M)	0.17	0.089–0.31	0.48	0.23–0.97		0.46	0.28–0.67	0.16	0.052–0.26

*Paired *t*-test, *P* = 0.0004.

Blanks indicate negativity for methylation in a particular case-marker combination.

Abbreviations: SD(B), bisulfite-to-bisulfite SD within each run; SD(M), run-to-run SD within each bisulfite-treated sample.

Table 3. PMR Values of Case 3 in MethyLight for *MGMT* Using *COL2A1* as a Reference

		Different MethyLight runs					Mean of PMR	SD	CV(M)
		M1	M2	M3	M4	M5			
Different bisulfite treatments	B1	36.75	28.71	33.57	43.92	22.69	33.13	8.04	0.24
	B2	39.52	35.63	40.11	42.61	31.90	37.95	4.21	0.11
	B3	35.53	36.22	30.21	44.78	29.74	35.30	6.07	0.17
	B4	36.16	40.01	33.42	42.43	41.46	38.70	3.80	0.10
	B5	39.08	37.83	33.56	61.04	37.44	41.79	10.96	0.26
	B6	31.62	36.00	34.49	46.19	32.56	36.17	5.85	0.16
	B7	30.26	34.54	40.80	51.03	36.73	38.67	7.89	0.20
Mean of PMR		35.56	35.56	35.16	47.43	33.22			
SD		3.50	3.50	3.86	6.67	6.10			
CV(B)		0.10	0.10	0.11	0.14	0.18			

Abbreviations: CV(B), bisulfite-to-bisulfite coefficient of variation (CV) within each run; CV(M), run-to-run CV within each bisulfite-treated sample.

typically showed $\text{PMR} \leq 1$ and were most frequently undetected ($\text{Ct} > 45$ and $\text{PMR} = 0$). There were a total of five case-negative marker combinations (case 1, *CDKN2A*; case 1, *MGMT*; case 2, *MLH1*; case 3, *MLH1*; and case 4, *MGMT*). Within a total of 175 PMR results (five case-marker combinations times seven bisulfite-treated samples times five MethyLight runs) for these negative reactions, when using *COL2A1* as a control, 160 runs (91%) showed PMR values of 0, 14 runs (8%) showed a $\text{PMR} < 1$, and only one run (0.6%) showed $\text{PMR} > 1$ ($\text{PMR} 1.01$). However, when using *ACTB* as a control, 4 of 175 negative reactions (2.3%) showed $\text{PMR} > 1$ (PMR ranging from 1.10 to 3.59). Notably with either reference, none of these 175 presumed negative reactions showed $\text{PMR} > 4$.

Mean and variance of PMR values in MethyLight using *ACTB* as a control were compared to those using *COL2A1* as a control (Table 4). For each case-marker combination, we obtained PMR values 35 times (seven different bisulfite-treated samples times five MethyLight runs) using each one of *ACTB* and *COL2A1* as a control. Distributions of PMR values became closer to normal distributions after log transformation of raw PMR values. Thereafter, we assessed the equality of variances in the 35 repeated runs. PMR variances using *ACTB* were significantly larger than those using *COL2A1* in four of seven case-marker combinations ($P < 0.003$) (Table 4), indicating *COL2A1* as a superior control. CVs of PMR values in MethyLight using *COL2A1* as a control are shown in

Table 5. Both bisulfite-to-bisulfite variation [CV(B)] and run-to-run variation [CV(M)] were similar, ranging from 0.05 to 0.6. Therefore, MethyLight assay showed fair reproducibility in terms of both bisulfite-to-bisulfite variations [CV(B)] and run-to-run variations [CV(M)]. To assess within-sample variation of PMR values, interclass correlation coefficients were calculated as 0.84, 0.74, and 0.79 for *CDKN2A*, *MLH1*, and *MGMT*, respectively, indicating fair reproducibility of MethyLight assays.

To examine variations of standard curves, which contribute to run-to-run variations, we made five standard curves in a single plate. For each sample-marker combination, we calculated five different PMR values, using each one of the five different standard curves. Slopes of the five standard curves ranged -3.23 to -3.57 (mean, -3.41 ; SD, 0.14), intercepts ranged 31.63 to 31.88 (mean, 31.79; SD, 0.10), and correlation coefficients (r) ranged 0.9928 to 0.9971 (mean, 0.9953; SD, 0.0025). CVs of the five different PMR values for each sample-positive marker combination ranged 0.00368 to 0.136 (mean, 0.0629; median, 0.0515). These data indicate that run-to-run PMR variations were partly due to variations in standard curves, which were small. In MethyLight runs M1 through M5, slopes of standard curves ranged -3.19 to -3.78 (mean, -3.52 ; SD, 0.20), intercepts ranged 31.56 to 32.96 (mean, 32.25; SD, 0.44), and correlation coefficients (r) ranged 0.9857 to 0.9990 (mean, 0.9934; SD, 0.0041). These data indicate good reproducibility in standard curves.

Table 4. Mean and CV of PMR Values in MethyLight

		<i>CDKN2A</i>		<i>P</i> value, <i>F</i> -test for the equality of variances	<i>MLH1</i>		<i>P</i> value, <i>F</i> -test for the equality of variances	<i>MGMT</i>		<i>P</i> value, <i>F</i> -test for the equality of variances
Reference gene		Mean of PMR*	CV of PMR*		Mean of PMR*	CV of PMR*		Mean of PMR*	CV of PMR*	
Case 1	<i>ACTB</i>				122.05	0.48	$P = 0.08$			
	<i>COL2A1</i>				48.27	0.47				
Case 2	<i>ACTB</i>	941.16	0.71	$P = 0.0004$				140.69	0.64	$P = 0.2$
	<i>COL2A1</i>	345.7	0.35					56.07	0.42	
Case 3	<i>ACTB</i>	8.95	0.60	$P = 0.002$				64.92	0.61	$P = 8 \times 10^{-8}$
	<i>COL2A1</i>	5.25	0.30					37.39	0.19	
Case 4	<i>ACTB</i>	369.79	0.52	$P = 4 \times 10^{-6}$	32.87	0.33	$P = 0.9$			
	<i>COL2A1</i>	115.11	0.21		11.22	0.31				

Blanks indicate negativity for methylation in a particular case-marker combination.

*The mean and CV of PMR values are based on 35 different results (seven different bisulfite-treated samples times five different MethyLight runs) for each case-marker combination.

Table 5. CV of PMR Values in MethyLight Using *COL2A1* as a Reference

		<i>CDKN2A</i>		<i>MLH1</i>		<i>MGMT</i>	
		Mean of CV	Range	Mean of CV	Range	Mean of CV	Range
Case 1	CV(B)			0.23	0.11–0.32		
	CV(M)			0.51	0.47–0.57		
Case 2	CV(B)	0.18	0.12–0.23			0.30	0.21–0.35
	CV(M)	0.36	0.29–0.48			0.44	0.30–0.60
Case 3	CV(B)	0.29	0.23–0.38			0.13	0.10–0.18
	CV(M)	0.22	0.046–0.48			0.18	0.10–0.26
Case 4	CV(B)	0.17	0.15–0.20	0.21	0.062–0.31		
	CV(M)	0.17	0.085–0.26	0.31	0.16–0.52		

Abbreviations: CV(B), bisulfite-to-bisulfite CV within each run; CV(M), run-to-run CV within each bisulfite-treated sample.

Assay Linearity

We performed repeated measurements of PMR values on DNA mixtures containing 50%, 30%, 20%, 10%, and 5% of methylated (M-SssI-treated) DNA (Figures 2 and 3). Correlation coefficients and *P* values for the test of a linear association were as follows: $r = 0.962$, $P = 3 \times 10^{-17}$ for *CDKN2A*; $r = 0.913$, $P = 2 \times 10^{-12}$ for *MLH1*; and $r = 0.904$, $P = 10^{-9}$ for *MGMT*. Thus, our MethyLight assay showed fair to good linearity for all three genes.

Distribution of PMR Values and Correlation with Loss of Protein Expression

We performed MethyLight assays for the *CDKN2A*, *MLH1*, and *MGMT* promoters and immunohistochemistry to assess protein loss in 274 colorectal cancer cases. Among these, 272 cases (99.2%) demonstrated successful amplification in MethyLight assays, and only two cases (0.8%) failed to amplify. More than half of the tumors showed PMR = 0 (no methylation) for each marker, and most methylation-positive cases showed relatively high PMR values (Table 6). There were only rare cases with PMR values between 3 and 5. As such, the MethyLight assay for each gene appears to distinguish

two distinct subsets of colorectal cancer, reflecting methylation-negative and -positive tumors. Our previous MethyLight analyses have defined methylated promoters as those with PMR >4 and unmethylated promoters as PMR ≤4.³³ The bimodal distributions of PMR values in Table 6 appear to support a cutoff of 4.

We compared promoter methylation status defined by PMR values for *CDKN2A*, *MLH1*, and *MGMT* to loss of protein expression assessed by immunohistochemistry (Table 6). Notably, intact protein expression was observed in 96% (156 of 163), 99% (224 of 227), and 86% (120 of 139) of samples with PMR ≤4 for the promoters of *CDKN2A*, *MLH1*, and *MGMT*, respectively. Loss of protein expression was observed in 62% (45 of 73), 96% (43 of 45), and 66% (69 of 104) of samples with PMR >4 for *CDKN2A*, *MLH1*, and *MGMT*, respectively. Using a PMR cutoff of 4, promoter methylation was significantly associated with loss of respective protein expression ($P = 1 \times 10^{-21}$ for *CDKN2A*, $P = 6 \times 10^{-44}$ for *MLH1*, and $P = 1 \times 10^{-17}$ for *MGMT*).

Discussion

Promoter CpG island methylation and transcriptional inactivation of tumor suppressor genes are thought to be important carcinogenic mechanisms. Assays to measure DNA methylation may be potentially very useful in clinical practice, because many tumor suppressor genes have been shown to be methylated and functionally silenced in a variety of human neoplasias.^{2,3} DNA methylation may be a useful marker for predicting prognosis and monitoring efficacy of adjuvant therapy in cancer patients^{10,11} and for evaluating risks for tumor recurrence in surveillance of high- or low-risk individuals.^{8,9,12} A variety of assays to measure DNA methylation have been developed for paraffin-embedded tissue, and many of these methods rely on sodium bisulfite treatment of genomic DNA from tumor tissue.⁷ However, precision and performance characteristics of sodium bisulfite treatment and measurement of methylation have not been comprehensively evaluated.

In this study, we evaluated precision of sodium bisulfite conversion as well as subsequent real-time PCR (MethyLight) assays for the *CDKN2A* (p16), *MLH1*, and *MGMT* promoters. Advantages of MethyLight technology include its quantitative and high-throughput nature

Figure 3. DNA mixing study and linear regression analysis to assess linearity of MethyLight. DNA mixing assays were performed as described in Materials and Methods and Figure 2. The 100% SssI-treated DNA sample served as methylated reference DNA to calculate PMR. Linear regression analysis to assess assay linearity was performed.

Table 6. Distributions of PMR Values of *CDKN2A* (p16), *MLH1*, and *MGMT* in Colorectal Cancer and Loss of Protein Expression

PMR (methylation)	CDKN2A expression			MLH1 expression			MGMT expression		
	Loss	Present	Total	Loss	Present	Total	Loss	Present	Total
0	5	139	144	3	215	218	17	108	125
0-1	1	12	13	0	6	6	1	6	7
1-3	1	5	6	0	1	1	1	3	4
3-4	0	0	0	0	2	2	0	3	3
4-5	0	0	0	0	0	0	0	1	1
5-10	0	3	3	3	2	5	3	0	3
10-50	4	7	11	27	0	27	10	7	17
>50	41	18	59	13	0	13	56	27	83
Total	52	184	236	46	226	272	88	158	243

Notes: The distributions of PMR values support PMR cutoff of 4. *P* values for statistical significance in association of PMR >4 with loss of expression are: $P = 1 \times 10^{-21}$ for *CDKN2A*, $P = 6 \times 10^{-44}$ for *MLH1*, and $P = 1 \times 10^{-17}$ for *MGMT*.

and relatively simple assay procedures, which do not require opening of tubes containing PCR products. All of these features are extremely beneficial in clinical molecular diagnostics. Any of these properties is not a feature of qualitative MSP, which has been widely used for methylation detection. As for disadvantages of MethyLight, it cannot quantify methylation at the individual nucleotide level; rather, it can assess methylation levels at the primer sites and/or a probe site as a whole. On the other hand, bisulfite-sequencing, such as bisulfite-pyrosequencing,¹⁹⁻²¹ can achieve resolution at the individual nucleotide level. However, in contrast to MethyLight, pyrosequencing involves end-point analysis of PCR products and requires procedures that need opening of PCR tubes.

To measure the relative amount of methylated DNA, we divided the measured amount of a methylated gene of interest by the amount of *COL2A1* (or *ACTB*) for input DNA. One should also be aware of PCR bias in quantitative molecular assays,⁴² so we further normalized for a potential difference in PCR efficiencies between the gene of interest and *COL2A1* by dividing the gene:*COL2A1* ratio in a sample of interest by that ratio in SssI-treated DNA (presumably fully methylated). For all three genes tested, we showed good precision in threshold cycle (Ct) values in terms of both bisulfite-to-bisulfite variation (among seven bisulfite-treated aliquots B1 to B7) in the same run and MethyLight-to-MethyLight run-to-run variation (among five repeated MethyLight runs M1 to M5) in the same bisulfite-treated DNA sample. *COL2A1* reactions, as well as PMR values based on *COL2A1* reactions, showed much smaller variances than *ACTB*. Thus, *COL2A1* is a superior control gene to *ACTB* in terms of precision of MethyLight assays.

We also demonstrated that run-to-run CVs of PMR values were larger than bisulfite-to-bisulfite CVs of PMR values. This likely reflects the introduction of other sources of variations when we measured run-to-run CVs of PMR, including different standard curves. Thus, we also examined variations of standard curves in a single plate as well as between different MethyLight runs. We demonstrated that variations of standard curves were small and acceptable. Other sources of run-to-run variations are independent control reactions (*COL2A1* or

ACTB) in different MethyLight runs. A gene that showed small variation in repeated amplification reactions, such as *COL2A1*, would be suitable for a control. Normalizing methylation measurement for input DNA by a control reaction is necessary to compare quantitative methylation data across samples. Quality and quantity of DNA and the degree of bisulfite conversion can vary from sample to sample.

To assess acceptability of the precision of sodium bisulfite conversion and subsequent MethyLight assays, we measured PMR values (degrees of methylation) for the *CDKN2A*, *MLH1*, and *MGMT* promoters in 272 cases of colon cancer. We demonstrated that overall distributions of PMR values for all three loci were bimodal (either $\text{PMR} \leq 1$ or >10) with only rare cases showing PMR values between 3 and 5. For most cases, CVs of PMR were sufficiently low to definitively differentiate methylation-positive tumor from methylation-negative tumor, with a PMR cutoff of 4. Thus, our data indicate that precision of sodium bisulfite conversion and quantitative MethyLight assays is acceptable, and we can reliably determine the status of methylation for most cases by a single MethyLight run.

In addition, for each of the three genes tested, promoter methylation was strongly associated with loss of respective protein expression. Low PMR values between 0 and 4 were principally unrelated to loss of expression and, therefore, seemed to represent biologically insignificant low levels of methylation. There were infrequent cases with loss of expression without promoter methylation and cases with intact protein expression despite PMR values >10 . The former may reflect different mechanisms of gene silencing, including gene deletion or mutation, and the latter may reflect partial methylation or mono-allelic methylation. Difficulty in interpreting immunohistochemistry, particularly for *MGMT* and *CDKN2A* (p16), may also contribute to some of the discrepant results.

In conclusion, sodium bisulfite conversion is reproducible, and subsequent quantitative real-time PCR methylation assays have acceptable precision. Quantitative promoter methylation data are highly correlated with loss of protein expression. Carefully validated quantitative

MethylLight assays will be useful in both research and clinical molecular diagnostics.

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Note Added in Proof

We have shown that MethylLight assays are useful for molecular classification of colorectal cancer, in particular for the determination of CpG island methylator phenotype, using a large number of samples.⁴³

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