Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis

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In mammals, the target of DNA methylation is the C5 position of cytosine at the CpG dinucleotides. About 70% of CpG dinucleotides within the genome are methylated, and most unmethylated CpG dinucleotides are found in GC-rich sequences termed CpG islands (1). A large proportion of CpG islands is found in the promoter region of genes, and DNA methylation at these CpG islands can lead to transcriptional silencing of the associated genes. DNA methylation and associated gene silencing has essential normal functions for cell differentiation, imprinting, X-chromosome inactivation, and the suppression of parasitic DNA sequences (2). Aberrant DNA methylation plays an important role in both cancer initiation and progression, and this process is also implicated in other diseases, including imprinting disorders, diseases with trinucleotide expansions, and aging-related diseases (3–5). The central role of DNA methylation in maintaining cellular function, and the broad implications of DNA methylation in diseases have created a strong need for techniques to detect and measure DNA methylation reliably and quantitatively.

Currently, most approaches for measuring DNA methylation are based on sodium bisulfite treatment, which creates sequence differences by converting unmethylated cytosines to uracils, but leaving methylated cytosine unchanged. The differences can then be detected quantitatively by several techniques, such as sequencing of subclones or PCR products (6), restriction-digestion (COBRA) (7), or pyrosequencing (8). The main concern for PCR-based quantitative DNA methylation analysis is PCR bias, which is due to the fact that methylated and unmethylated DNA molecules sometimes amplify with greatly differing efficiencies. Using bisulfite PCR and restriction enzyme digestion, Warnecke et al. (9) reported a strong bias of amplification for unmethylated DNA in two human genes, *p16* and *Rb*, and this bias occurred specifically for the primers directed to top strand DNA. They hypothesized that this bias could be due to the lower PCR efficiency through secondary structure formation for methylated DNA. However, they were unable to overcome this PCR bias by changing PCR conditions, such as extension time, annealing and denaturation temperature, $MgCl_2$ concentration, and the addition of varying concentrations of secondary structure inhibitors, such as dimethyl sulfoxide (DMSO) and formamide. Recently, we have used bisulfite PCR and pyrose-

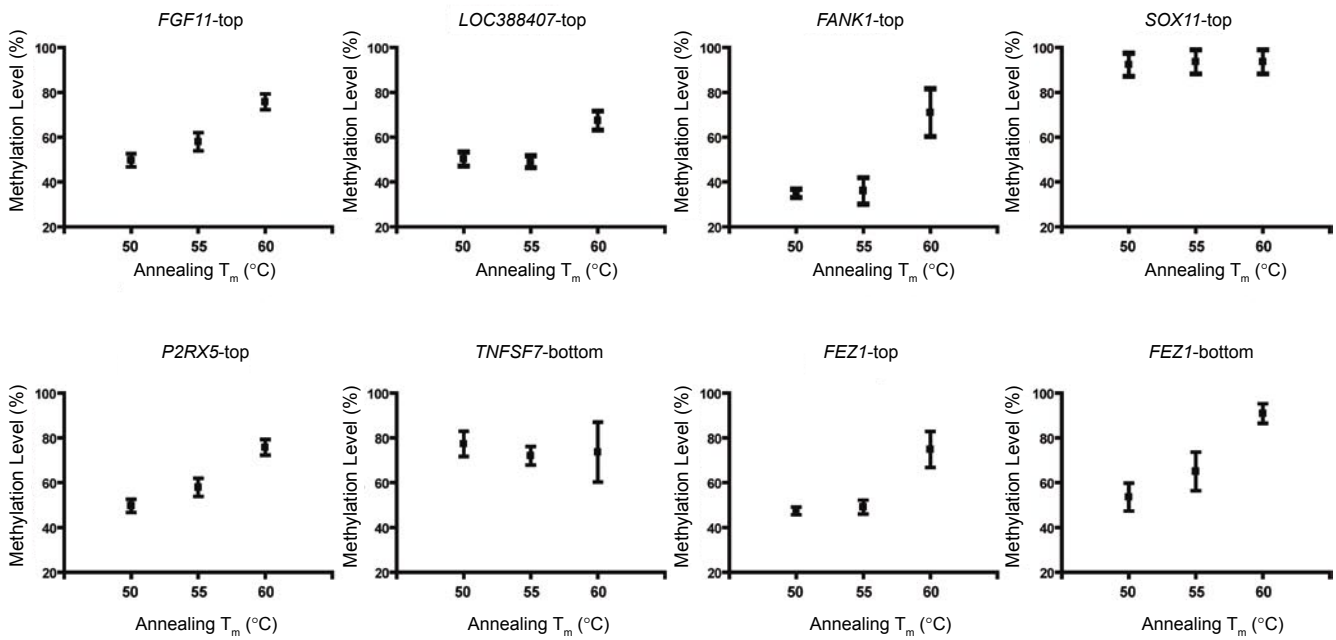

Figure 1. Bisulfite pyrosequencing analysis of six human gene promoters in RKO cell lines. Percent of average methylation at different C sites is shown on the y-axis. Three different annealing temperatures are indicated in x-axis. For four genes (*FGF11*, *LOC388407*, *FANK1*, and *SOX11*), PCR primer sets were designed based on the top strand of the gene. For *P2RX5* and *TNFSF7* genes, PCR primer sets were designed based on the bottom strand of the gene. For *FEZ1* gene, two sets of PCR primers were designed for either top or bottom strand of the gene. T_m, melting temperature.

Table 1. CpG Density, GC Content, and Other Characteristics of PCR Products for Each Assay

Gene	Strand	Nucleotides Between Primers (No.)	CpGs Between Primers (No.)	CpG Density[a]	GC M/UM[b] (%)	CpG Sites Analyzed (No.)	Methylation Affected by T_m (%)
FGF11	Top	123	13	0.11	51/42	5	Y
LOC388407	Top	190	16	0.08	41/34	4	Y
FANK1	Top	169	21	0.12	50/39	6	Y
SOX11	Top	140	19	0.14	51/37	7	ND
P2RX5	Bottom	104	10	0.10	46/38	4	Y
TNFSF7	Bottom	99	7	0.07	43/36	3	ND
FEZ1	Top	233	35	0.15	60/46	12	Y
FEZ1	Bottom	208	33	0.16	49/33	7	Y
ER	Top	66	6	0.09	29/24	6	Y
ECAD	Top	83	12	0.14	38/29	6	Y
CDH13	Top	147	13	0.09	37/30	6	N
MGMT	Top	115	16	0.14	51/41	5	Y

T_m, melting temperature; Y, yes; ND, not determined; N, no.
[a]CpG density was calculated by the number of CpG dinucleotides divided by number of nucleotides between primers.
[b]Percent of (G + C) content within the PCR product, for 100% methylated (M) and 100% unmethylated (UM) DNA.

quencing to study the methylation status at CpG island promoters for several genes. Surprisingly, we find that in most genes we analyzed, the amplification efficiency for the methylated DNA, and thus PCR bias, could be affected by changing annealing temperature for PCR, and this effect was independent of primers designed for top or bottom strand DNA. Using a mixing experiment, we find that much of the bias can be resolved by optimizing annealing temperature.

Bisulfite-pyrosequencing is a recently developed quantitative technique to detect methylation changes; it relies on bisulfite-induced C to T polymorphisms, which can be detected by a pyrosequencer using a sequencing-by-synthesis method. This technique has the advantages of analyzing several methylation sites, introduces an internal control (DNA sequence including a control for unconverted cytosines), and allows accurate quantitation of multiple CpG methylation sites in the same reaction. There are essentially two steps involved in this technique (*i*) PCR following bisulfite treatment and (*ii*) pyrosequencing to measure the degree of methylation at each CpG site within the sequencing region. For primer design, we first identified the region of interest and CpG island for each gene and virtually converted the genomic sequence to the bisulfite-treated sequence by replacing all CG with YG (Y stands for C/T), then all remaining C with T. Based on the converted sequences, we used Pyrosequencing™ Assay Design software (Biotage, Uppsala, Sweden) to design both PCR primers and sequencing primers. We tried to avoid CpGs within the primer sequences. If we could not find suitable primers this way, we included CpGs in each primer, but put them in the 5′ end of the primer and synthesized them as Y (C/T) in the forward strand and R (G/A) in the reverse stand. The primer melting temperature (T_m) for PCR was calculated by the nearest neighbor formula, and the optimal annealing temperature was suggested at 55°C.

We examined methylation patterns at the CpG island promoters of seven genes, *FGF11, LOC388407*, *FANK1*, *SOX11*, *P2RX5*, *TNFSF7*, and *FEZ1* in a colon cancer cell line RKO. Bisulfite treatment of 2 µg genomic DNA was performed as previously described (6). After bisulfite treatment, double-stranded DNA (top and bottom strands) are no longer complementary and can be analyzed separately by designing primers to amplify either top strand sequences or bottom strand sequences. For *FGF11*, *LOC388407*, *FANK1*, and *SOX11* genes, the PCR primers were designed for top strand sequences; for *P2RX5* and *TNFSF7* genes, PCR primers were designed for the bottom strand sequences; and for *FEZ1* gene, we designed two sets of PCR primers for both top and bottom strands. PCR product length, CpG density, and GC content within the PCR product are summarized in Table 1. Primer sequences and sequencing regions are shown in Table 2. For PCR amplification, we used a universal biotinylated primer with hot start PCR as previously reported (8). The PCR was carried out in a 50-µL solution containing 2 µL bisulfite-treated DNA, 1× PCR buffer, 1.25 mM dNTPs, 0.1 µM forward primer, 0.01 µM reverse primer with universal overhang, 0.09 µM universal biotin primer, and 1 U *Taq* DNA polymerase (New England BioLabs, Ipswich, MA, USA). For each assay, three different PCR conditions were performed separately for the same sample by varying annealing temperature at either 50°, 55°, or 60°C. PCR cycling conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, varying annealing temperature for 45 s, and 72°C for 45 s, and a final incubation at 72°C for 4 min. In order to analyze as many CpG sites as possible, we usually divide PCR products for multiple sequencing and use 10 µL PCR product for pyrosequencing.

Pyrosequencing was carried out with Pyro Gold reagents and a PSQ™ HS 96 pyrosequencer (both from Biotage), following the manufacturer's recommendations. The methylation levels at different C sites were averaged to represent the degree of methylation in each gene. All the experiments, including bisulfite treatment, PCR, and pyrosequencing, were done independently at least two times. As shown in Figure 1, at the lowest annealing temperature for PCR (at 50°C), five genes (*FGF11*, *LOC388407*, *FANK1*, *FEZ1*, and *P2RX5*) showed moderate methylation (40%–60%) in RKO, and two genes, *SOX11* and *TNFSF7*, showed heavy methylation (78%–80%) in this cell line. Surprisingly, for the five genes with moderate methylation, we observed substantially higher methylation levels when the annealing temperature for PCR increased from 50° to 55° or 60°C, while for those two genes with heavy methylation, we did not see any changes for the measured methylation levels at different temperatures. Figure 2 shows representative pyrosequencing results for single CpG sites of *FEZ1* gene. When we analyzed all the C sites individually (Figure 2A shows the pyrograms for 12 CpG sites on the top strand, and Figure 2B shows the pyrograms for 7 CpG sites on the bottom strand), we found that the increased methylation measurement by higher annealing temperature affected all C sites equally, suggesting an effect on allele-specific amplification (PCR bias), and we did not find any strand specificity to this effect that occurred to assays designed for top strands or bottom strands. These results indicate that during PCR amplification, methylated and unmethylated DNA has variable efficiency depending on the annealing temperature. These results suggested that we could potentially overcome PCR bias in DNA methylation analyses by optimizing annealing temperature.

In order to test this hypothesis, we set up a series of mixing experiments for four additional genes, estrogen receptor α (*ESR1*), E-cadherin (*CDH1*), O-6-methylguanine DNA methyltransferase (*MGMT*), and H-cadherin (*CDH13*). Mixing experiments using different fractions of methylated and unmethylated genomic DNA prior to bisulfite treatment and PCR is the gold standard to detect PCR bias. We selected these four genes because all have typical CpG islands in their promoter regions, and methylation of these genes has been extensively studied in various diseases (10–13). We carefully designed the primers to amplify 100–200 bp regions that are

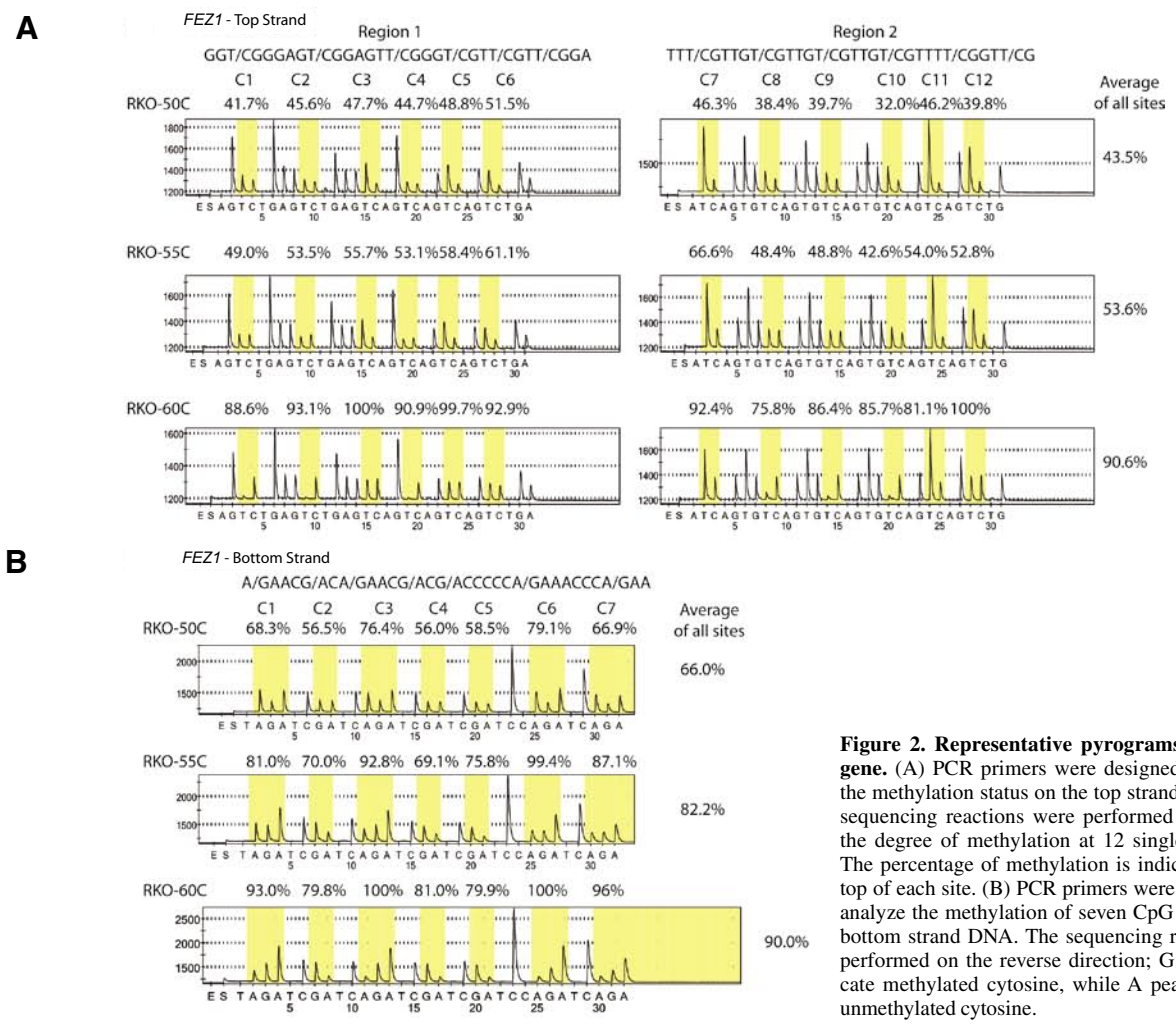

Figure 2. Representative pyrograms for *FEZ1* gene. (A) PCR primers were designed to analyze the methylation status on the top strand DNA. Two sequencing reactions were performed to measure the degree of methylation at 12 single cytosines. The percentage of methylation is indicated on the top of each site. (B) PCR primers were designed to analyze the methylation of seven CpG sites on the bottom strand DNA. The sequencing reaction was performed on the reverse direction; G peaks indicate methylated cytosine, while A peak indicated unmethylated cytosine.

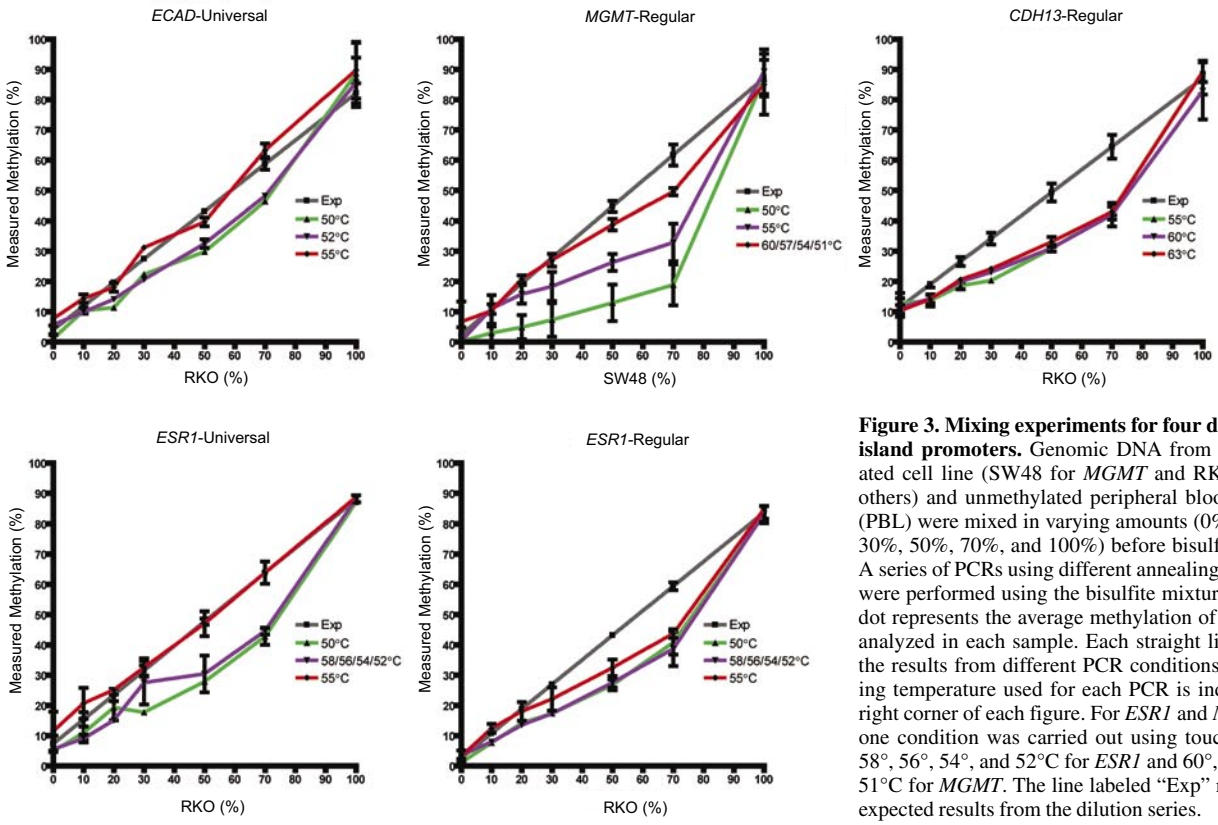

Figure 3. Mixing experiments for four different CpG island promoters. Genomic DNA from fully methylated cell line (SW48 for *MGMT* and RKO for all the others) and unmethylated peripheral blood leukocytes (PBL) were mixed in varying amounts (0%, 10%, 20%, 30%, 50%, 70%, and 100%) before bisulfite treatment. A series of PCRs using different annealing temperatures were performed using the bisulfite mixture DNA. Each dot represents the average methylation of all CpG sites analyzed in each sample. Each straight line represents the results from different PCR conditions. The annealing temperature used for each PCR is indicated in the right corner of each figure. For *ESR1* and *MGMT* genes, one condition was carried out using touchdown PCR, 58°, 56°, 54°, and 52°C for *ESR1* and 60°, 57°, 54°, and 51°C for *MGMT*. The line labeled "Exp" represents the expected results from the dilution series.

close to the transcription start site. We also tried to avoid having CpG sites in PCR primers and sequencing primers, and as shown in Table 1, this was successful except for the *MGMT* gene, for which there was a C site in the 5′ end of the reverse primer, and we synthesized this primer using R (G/A) instead of G in this base. For mixing, we used DNA from peripheral blood leukocytes (PBL) from one healthy male donor as unmethylated DNA and DNA from cancer cell lines that are known to be fully methylated as methylated DNA. We prepared a mixture of unmethylated DNA (PBL) and increasing proportions (0%, 10%, 20%, 30%, 50%, 70%, and 100%) of methylated DNA (from colon cancer cell lines RKO or SW48) before bisulfite treatment. The procedures for bisulfite treatment, PCR amplification, and pyrosequencing were as described before, except for *MGMT* and *CDH13* genes, for which we used directly biotin-labeled reverse primers for PCR. Three different PCR conditions were used for each mixing experiment, and the PCR temperatures for annealing are indicated in Figure 3. As shown in Figure 3, for each set of primers we observed substantial PCR bias at the lowest annealing temperature. Consistent with previous results, in all cases the bias was toward preferential amplification of unmethylated DNA. By increasing the annealing temperature of PCR, we could overcome PCR bias for all genes, except *CDH13*. One condition was carried out by touchdown PCR for *ESR1* and *MGMT* genes, 58°, 56°, 54°, 52°C and 60°, 57°, 54°, 51°C, respectively (Figure 3). The purpose of touchdown PCR is to improve the amplification efficiency for methylated DNA in the first few cycles at a relatively high temperature without decreasing the yield of PCR amplification as the annealing temperature drops later. We found that for *MGMT* gene, touchdown PCR by starting the annealing temperature at 60°C for 5 cycles, gradually decreasing to 57°C for 5 cycles, 54°C for 5 cycles, and finally to 51°C for 35 cycles indeed improved the PCR efficiency for methylated DNA and corrected the PCR bias completely. For *ESR1* gene, the touchdown PCR was performed at 58°C for 3 cycles, 56°C for 4 cycles, 54°C for 5 cycles, and 52°C for 38 cycles, and the PCR bias was still observed at this condition. However, straight PCR annealing at 55°C, we were able to correct the PCR bias completely for this gene, suggesting touchdown PCR at higher starting temperatures may be able to overcome the bias as well. In order to test if the dependence of preferential amplification and annealing temperature is due to the primer system, we also analyzed the *ESR1* gene using a directly biotin-labeled reverse primer and compared the results with universal primer approach (Figure 3, bottom panel). It is clear from the results that regardless of the primer system, there is a strong bias toward the amplification of unmethylated DNA, and increasing annealing temperature for PCR improved the amplification toward methylated DNA. Interestingly, for the same *ESR1* gene in which we were able to overcome the PCR bias using a universal primer approach by PCR at annealing temperature 55°C, we were unable to correct PCR bias completely by performing

Table 2. PCR and Pyrosequencing Primers and Assays for Each Gene

Gene	Primer	Sequence
FGF11	Forward	5′-GTTTTGTGGGTGGGGTAG-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTACRCTTCTACCRAATCAAACTACTAA-3′
	Sequencing	5′-TTTTTGGGGGAGTTTA-3′
	Assay	5′-GYGYGTTTYGGGYGTTTGTYGGTTTGGGGGTGTT-3′
LOC388407	Forward	5′-TGAGGTTTAAGAGGGAATTGT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTATAACTCCRATTCTCCACAACA-3′
	Sequencing	5′-GTGTGAGTGTAGGGGTT-3′
	Assay	5′-TYGAGAGATTTTTAGYGGTTAAGTYGTTYGGATTTTAA-3′
FANK1	Forward	5′-GGGGATGAYGGGGTAAAGT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTAACCCTACCAACAACCCTC-3′
	Sequencing	5′-AGTTAGGTTTATTGGGGA-3′
	Assay	5′-YGATAGYGAYGAYGGYGGYGGTT-3′
P2RX5	Forward	5′-TGTAGGGGTGTTATTGTGTTAG-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTAAATCRAAACRACCTTTTATTAAC-3′
	Sequencing	5′-GAGGAGGAAGAAGAGGTAG-3′
	Assay	5′-YGGTAAGGTYGYGGTTTGAGGTTTYGGTG-3′
FEZ1-Top	Forward	5′-YGGAGGAAGGTTATGAATGTT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTARAATCCCRAAACTCAACC-3′
	Sequencing-1	5′-GGGGAAGGGGTGGGGTGGA-3′
	Sequencing-2	5′-GGTTGGTTGGTTGGTTT-3′
	Assay-1	5′-GGYGGGAGYGGAGTYGGGYGTYGTYGGAGTTG-3′
	Assay-2	5′-TTYGTTGYGTTGYGTTGYGTTTYGGTYGTTT-3′
FEZ1-Bottom	Forward-Universal	5′-GGGACACCGCTGATCGTTTAGYGGATTTYGGGGTTTAGT-3′
	Reverse	5′-AAATAACRTCCAACTCCC-3′
	Sequencing-Reverse	5′-TACCAATTAAATAAATACCC-3′
	Assay	5′-RAACRCRAACRCRCCCCCRAAACCCRAAC-3′
SOX11	Forward	5′-AGYGGTTGYGGTTTAAGTATATGGT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTATTTCTTACTAAAACCCTTAAAAATCT-3′
	Sequencing	5′-TTAGTTAGAGTTTAGAGAAG-3′
	Assay	5′-AGYGYGGTYGGYGGYGGYGGYGGGAG-3′
TNFSF7	Forward	5′-TGAGGGTTGTTTTGGTTTTAT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTACTCTCTATATTTTCTTCCCAACTTTT-3′
	Sequencing	5′-TGTTTTGGTTTTATTGGT-3′
	Assay	5′-YGYGGGTTTGGTGATTTGTTTYGTGGT-3′
ER	Forward	5′-TTTTTGGGTTATTTTTAGTAGATT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTACAAAAAACAACTTCCCTAAACTT-3′
	Reverse-Biotin	5′-CAAAAAACAACTTCCCTAAACTT-3′
	Sequencing-1	5′-GGTTATTTTTAGTAGATTTT-3′
	Sequencing-2	5′-AATTTAGTTTTTATTTAGTA-3′
	Assay-1	5′-YGTGYGTTTTYGTTTTTGGTYGTG-3′
	Assay-2	5′-GYGAYGATAAGTAA-3′
CDH13	Forward	5′-TTGGGAAGTTGGTTGGTTG-3′
	Reverse-Biotin	5′-ACAACCCCTCTTCCCTACCT-3′
	Sequencing	5′-GGAAAATATGTTTAGTGTAG-3′
	Assay	5′-TYGYGTGTATGAATGAAAAYGTYGTYGGGYGTTTTTA-3′
ECAD	Forward	5′-GGAATTGTAAAGTATTTGTGAGTTT-3′
	Reverse-Universal	5′-GGGACACCGCTGATCGTTTATCCAAAAACCCATAACTAACC-3′
	Sequencing	5′-GGAAGTTAGTTTAGATTTTA-3′
	Assay	5′-GTTYGTTTTAGTTYGGTTYGATTYGATYGTATTYGG-3′
MGMT	Forward	5′-TTGGTAAATTAAGGTATAGAGTTTT-3′
	Reverse-Biotin	5′-AAACAATCTACRCATCCT-3′
	Sequencing	5′-GGAAGTTGGGAAGG-3′
	Assay	5′-YGTYGTTYGGTTTGTATYGGTYGAAGGGTTA-3′

Y and R stand for C/T and G/A, respectively.

PCR at the same condition but using a regular biotin-labeled reverse primer, indicating that a higher annealing temperature may be required for the regular primer approach. One possible explanation could be that the universal primer approach (one pair primer contains a tailed universal sequence) provides amplification of tagged fragments, which may reduce primer bias.

The reasons increased annealing temperature can improve PCR efficiency for methylated DNA are unknown. After bisulfite treatment, methylated DNA has a higher GC content than unmethylated DNA, which could favor stable secondary structures that alter amplification efficiency. Raising the annealing temperature of PCR could melt these secondary structures and thus correct the lower amplification efficiency of methylated DNA. Unlike the previous report (9), we find that changing PCR conditions, such as annealing temperature, can overcome PCR bias. Our success may be related to several reasons. Pyrosequencing is able to analyze multiple CpG sites within the sequencing range (in our study, the number of CpG sites we analyzed for each gene ranged from 3 to 12 sites) (Table 1), and it is not gel-based, allowing accurate quantification, which is essential for estimating PCR bias. In addition, pyrosequencing uses three primers (PCR primers and sequencing primer), which ensure that unmodified DNA does not serve as a template. Finally, we used hot start PCR, and our assays were designed for shorter products, ranging from 100–300 bp, which may improve the PCR yield. For *CDH13* and *ESR1* by regular biotin primers, we were unable to correct PCR bias completely by changing the annealing temperature only. It will be interesting to see if we could reduce the bias by modifying other PCR conditions, such as by adding DMSO or betaine. Alternatively, the assay may have to be redesigned. In this study, for positive controls (fully methylated DNA in mixing experiments), we used DNA from two different tumor cell lines instead of *Sss*I-methylated DNA. This is because DNA from cell lines with known complete methylation of a given gene represents a natural positive control that is not subjected to the variability of relying on an enzymatic reaction (*Sss*I) to generate an artificial control. We recognize the potential problem of mixing DNA from different sources, but we are not aware of any study showing that DNA preparations affect methylation. Moreover, we have used two different cell lines as positive controls and found similar results. It has also been shown that PCR bias exists both in DNA from tumor cells and *Sss*I-methylated DNA (9). Based on these findings, we anticipate that similar results should apply for *Sss*I-treated samples as well.

In summary, we found that we can remarkably enhance the amplification efficiency for methylated DNA by increasing the annealing temperature for PCR, and thus overcome PCR bias in quantitative methylation analysis. Based on our results, we highly recommend mixing experiments using varying mixtures of methylated and unmethylated DNA and gradient annealing temperature for PCR to initially set up, evaluate, and calibrate each new assay.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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