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Review

PCR-Based Methods for Detecting Single-Locus DNA Methylation Biomarkers in Cancer Diagnostics, Prognostics, and Response to Treatment

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BACKGROUND: DNA methylation is a highly characterized epigenetic modification of the human genome that is implicated in cancer. The altered DNA methylation patterns found in cancer cells include not only global hypomethylation but also discrete hypermethylation of specific genes. In particular, numerous tumor suppressor genes undergo epigenetic silencing because of hypermethylated promoter regions. Some of these genes are considered promising DNA methylation biomarkers for early cancer diagnostics, and some have been shown to be valuable for predicting prognosis or the response to therapy.

CONTENT: PCR-based methods that use sodium bisulfite–treated DNA as a template are generally accepted as the most analytically sensitive and specific techniques for analyzing DNA methylation at single loci. A number of new methods, such as methylation-specific fluorescent amplicon generation (MS-FLAG), methylation-sensitive high-resolution melting (MS-HRM), and sensitive melting analysis after real-time methylation-specific PCR (SMART-MSP), now complement the traditional PCR-based methods and promise to be valuable diagnostic tools. In particular, the HRM technique shows great potential as a diagnostic tool because of its closed-tube format and cost-effectiveness.

SUMMARY: Numerous traditional and new PCR-based methods have been developed for detecting DNA methylation at single loci. All have characteristic advantages and disadvantages, particularly with regard to use in clinical settings.

In human cells, DNA methylation occurs almost exclusively at the carbon-5 position of cytosine residues of the CpG dinucleotide, which is particularly abundant in the promoter region of many genes. These regions of high CpG density are referred to as CpG islands and are generally unmethylated in healthy cells. Nevertheless, DNA methylation is important in healthy cells: It is involved in X chromosome inactivation in females *(1)*, imprinting *(2)*, and inactivation of germ line genes, such as those in the *MAGE*[2] (melanoma antigen) gene family *(3)*. Furthermore, methylation of CpG dinucleotides is believed to protect healthy cells from inappropriate transcription of repetitive elements, such as long interspersed nuclear elements (LINEs)[3] and Alu repeats *(4)*, and methylation of CpG dinucleotides is thought to help maintain chromosomal stability *(5, 6)*. In fact, 70%–80% of all CpG dinucleotides of the typical genome are methylated *(7)*.

In cancer, many typically unmethylated promoter regions of tumor suppressor genes undergo de novo methylation and transcriptional silencing at an early stage of tumor development, often despite an overall hypomethylation of the cancer genome *(8)*. Many important genes undergo silencing in human malignancies, and all cellular pathways related to cancer can be affected *(9, 10)*. DNA methylation and silencing may affect one or both of the alleles of tumor suppressor genes, and the unmethylated allele may be inactivated by genetic events such as mutation, deletion, or loss of heterozygosity.

Thus, aberrant methylation of the promoters of a number of genes shows great promise as biomarkers for early detection *(11, 12)* and predicting prognosis *(13–17)*. Furthermore, tumor-derived circulating DNA from apoptotic cancer cells can often be detected in the serum and other body fluids obtained from can-

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[2] Human genes: *MAGE*, melanoma antigen family; *MGMT*, *O*-6-methylguanine-DNA methyltransferase; *H19*, H19, imprinted maternally expressed transcript (non-protein coding).
[3] Nonstandard abbreviations: LINE, long interspersed nuclear elements; MIP, methylation-independent PCR; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis; MS-SnuPE, methylation-sensitive single-nucleotide primer extension; SIRPH, SnuPE ion pair reversed-phase HPLC; MCA, melting curve analysis; MS-MCA, methylation-sensitive MCA; dsDNA, double-stranded DNA; HRM, high-resolution melting; MS-HRM, methylation-sensitive HRM; McMSP, melting curve MSP; SMART-MSP, sensitive melting analysis after real-time MSP; MS-FLAG, methylation-specific fluorescent amplicon generation.

cer patients *(18)*. The most studied body fluids are nipple aspirate in breast cancer, sputum and bronchoalveolar lavage in lung cancer, urine in prostate cancer, and plasma and serum in multiple cancers *(12)*. In these types of samples, however, tumor-derived DNA is difficult to detect because it is often present at very low concentrations and has been contaminated substantially with DNA from healthy cells. Thus, methods with excellent detection capabilities are often needed to identify aberrantly methylated tumor-derived DNA in body fluids *(19)*.

For some applications, tumor-derived material is tested directly and does not require the use of methods with low detection limits. This applies to one of the only DNA methylation biomarkers in clinical use, methylation of the promoter of the *MGMT* gene (*O*-6-methylguanine-DNA methyltransferase), which predicts a favorable outcome in glioblastoma patients treated with alkylating agents *(20)*. Although many promising DNA methylation biomarkers have been identified, their still limited use in clinical settings is often due to the lack of sufficient diagnostic sensitivity and specificity required for a diagnostic test. The diagnostic sensitivity of a biomarker is the proportion of individuals with confirmed disease who test positive for the particular biomarker assay, whereas diagnostic specificity is the proportion of healthy control individuals who test negative. Otherwise, one uses analytical sensitivity, which we define in this review as the smallest detectable proportion of methylated template in a background of unmethylated template *(21)*.

The vast majority of DNA methylation assays are based on a PCR that uses sodium bisulfite–treated DNA as a template. Two different strategies have been used in the design of primers for such reactions. Methylation-independent PCR (MIP) primers are used in most of the available PCR-based methods, which are designed for proportional amplification of methylated and unmethylated DNA; however, methods that provide the highest analytical sensitivity generally use methylation-specific PCR (MSP) primers, which are designed for the amplification of methylated template only.

We review a number of the most promising MIP- and MSP-based methods and discuss the relative advantages and disadvantages with regard to their clinical applicability; however, no method is universally superior, because it is impossible to obtain all of the following objectives with a single method: quantitative accuracy, high analytical sensitivity, low false-positive and false-negative rates, high throughput, assessment of single CpG sites, low risk of PCR contamination (closed-tube assay), easily interpretable results, no need for specialized equipment, and cost-effectiveness. Table 1 provides an overview of the methods discussed and compares them with respect to several parameters that are important for clinical applicability.

Sodium Bisulfite Treatment

Epigenetic information is lost during the PCR because the DNA polymerase does not distinguish between methylated and unmethylated cytosines; thus, the polymerase incorporates guanine and subsequently unmethylated cytosines in both situations. After the PCR, any originally methylated alleles will be diluted to undetectable concentrations; therefore, the DNA must be modified in a way that allows the methylation information to be preserved. Treatment with sodium bisulfite, which deaminates cytosine to uracil *(22)* is the method of choice in most laboratories for this purpose. Because the rate of deamination of 5-methylcytosine to thymine is much slower than the conversion of cytosine to uracil, it is assumed that the only cytosines remaining after sodium bisulfite treatment are derived from 5-methylcytosines. Thus, during subsequent PCRs, uracil residues are replicated as thymine residues, and 5-methylcytosine residues are replicated as cytosines (Fig. 1). The protocol described by Frommer and colleagues *(23)* has been widely used for sodium bisulfite treatment, and a variety of commercial kits are now available for this purpose. When the sodium bisulfite treatment is performed under appropriate conditions, the expected conversion rate of unmethylated cytosines is about 99% *(24)*. Despite this high conversion rate, however, it is possible that a small subset of the DNA copies have a substantially lower conversion rate *(25)* and that the distribution of unconverted sites is nonrandom; thus, some promoter regions are more prone to incomplete conversion. The conversion rate may also depend on DNA quality *(25)*. This possibility is especially important to keep in mind when looking for low levels of methylation with methods based on MSP primers.

The sense and antisense strands are no longer complementary after sodium bisulfite treatment. Thus, MIP or MSP primers are designed for either strand.

Methods Based on MIP Primers

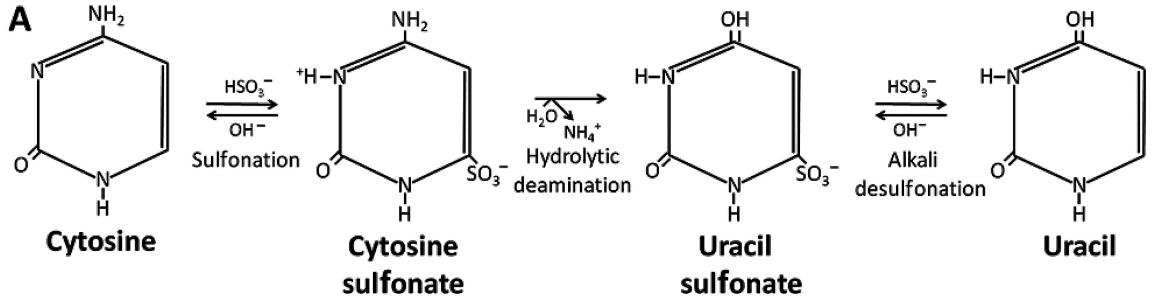
It can be difficult to amplify a target independent of its methylation status. In most situations, there is a PCR bias toward amplification of unmethylated DNA *(26)*, which can be explained by differences in GC content after bisulfite modification of the DNA. Different solutions to this problem have recently been proposed. First, the inclusion of a limited number of CpG sites in the MIP primer sequences has been reported to allow the bias to be controlled via manipulation of the an-

Table 1. Methods discussed in this review compared for several parameters important for their clinical applicability.[a]

Method	Analytical sensitivity	Throughput	Closed tube	Quantitative accuracy	Advantage	Disadvantage	References
Direct bisulfite sequencing	Low	Medium	No	Low	The methylation status of individual CpG sites can be resolved	Cloning procedures or digital PCR is necessary if single molecules are to be sequenced	Frommer et al. *(23)*
Pyrosequencing	Medium	Medium	No	High	Quantitative data on individual CpG sites can be obtained	Cloning procedures or digital PCR is necessary if single molecules are to be sequenced	Collela et al. *(35)*, Uhlmann et al. *(36)*, Tost et al. *(37)*
COBRA	Medium	Medium	No	High	Cost-effective	Restriction enzymes recognizing a given sequence context may not be available	Xiong and Laird *(42)*
MS-SnuPE	Medium	Medium	No	High	Multiplexing is possible	Radioactive labeling required	Gonzalgo and Jones *(44)*
MS-MCA	Medium	High	Yes	Medium	Not much labor required	Interpretation can be difficult if too many CpG sites occur between the primers	Worm et al. *(49)*
MS-HRM	High	High	Yes	Medium	Reversal of PCR bias	Interpretation can be difficult if too many CpG sites occur between the primers	Wojdacz and Dobrovic *(31)*
MALDI-TOF	Medium	Ultrahigh	No	High	Quantitative data on individual CpG sites can be obtained	Expensive equipment required	Ehrich et al. *(58)*
HeavyMethyl	High	High	Yes	High	Low false-positive rate	Many oligonucleotides are used	Cottrell et al. *(32)*
MSP	High	Medium	No	Low	Cost-effective	False positives	Herman et al. *(63)*
MethyLight	High	High	Yes	High	Not much labor required	A control assay is needed	Eads et al. *(68)*
McMSP	High	High	Yes	Low	Cost-effective	False positives	Akey et al. *(73)*
SMART-MSP	High	High	Yes	High	Low false-positive rate	A control assay is needed	Kristensen et al. *(61)*
MS-FLAG	High	Medium	No	High	Multiplexing is possible	Gel electrophoresis is used for post-PCR analysis	Bonanno et al. *(75)*
Next-generation sequencing	High	Ultrahigh	No	High	The methylation status of individual CpG sites can be resolved	Expensive equipment required	Taylor et al. *(24)*, Cokus et al. *(79)*, Korshunova et al. *(80)*

[a] Analytical sensitivity and quantitative accuracy are dependent on the specific assay and parameters such as the concentration and quality of input DNA and PCR conditions. For this reason, we have not defined absolute values for these parameters but have evaluated the methods relative to each other.

nealing temperature *(27)*. In some situations, however, increasing the annealing temperature may be enough to neutralize the bias despite the use of traditional MIP primers without CpG sites *(28)*. Amplification of single molecules has been shown to overcome the PCR bias phenomenon *(29, 30)*. Another limitation of MIP-based methods is the relatively low analytical sensitivity, although this sensitivity can be increased by



B

	Original sequence	Sequence after bisulfite treatment
Unmethylated DNA	A-T-**C**-**G**-G-T-C-A-T-**C**-**G**-C-A-T	A-T-**U**-**G**-G-T-U-A-T-**U**-**G**-U-A-T
Methylated DNA	A-T-**C**-**G**-G-T-C-A-T-**C**-**G**-C-A-T	A-T-**C**-**G**-G-T-U-A-T-**C**-**G**-U-A-T

Fig. 1. Sodium bisulfite treatment of genomic DNA.

(A), The procedure is based on the chemical reaction of single-stranded DNA with sodium bisulfite (HSO_3^-) at low pH and high temperatures. The chemistry of each reaction step is as follows: sulfonation at the carbon-6 position of cytosine, irreversible hydrolytic deamination at the carbon-4 position to generate uracil sulfonate, and, finally, subsequent desulfonation under alkaline conditions to generate uracil. Methylation at the carbon-5 position impedes sulfonation at the carbon-6 position in the first reaction step. Although 5-methylcytosine can react with bisulfite, this reaction is extremely slow, and the equilibrium favors 5-methylcytosine rather than thymine (the deaminated product of 5-methylcytosine). Of note is that subsequent purification is necessary to remove bisulfite salts and other chemicals used in the procedure. (B), The sodium bisulfite treatment converts unmethylated cytosines of the original DNA sequence to uracil, whereas methylated cytosines remain as cytosine. The CpG dinucleotide is the methylation target in human cells (bold).

introducing CpG sites into the primers *(31)* or through the use of oligonucleotide blockers *(32)*.

BISULFITE GENOMIC SEQUENCING

The gold standard in DNA methylation analysis has traditionally been the sequencing of bisulfite-modified and PCR-amplified DNA *(23)*, because this approach provides information at the level of individual CpG sites. Sequencing is mostly done with MIP primers but can be used to confirm MSP results as well. PCR products can be sequenced directly or as single clones. Sequencing of cloned PCR products provides information on individual molecules, whereas direct sequencing provides an estimate of the average methylation status of each CpG site in all of the molecules. When MSP primers are used, all of the cloned molecules are expected to be methylated, whereas the ratio of methylated to unmethylated molecules in a sample can be determined if MIP primers are used and enough clones are sequenced. Unfortunately, sequencing of single clones is too time-consuming and expensive to be used in routine clinical settings *(33)*. Bisulfite genomic sequencing of single clones can be affected by a cloning bias, and it can be problematic to accurately sequence the longer stretches of thymine that are often encountered in bisulfite-modified DNA *(34)*. Recently, a digital bisulfite-sequencing approach that allows sequencing of single molecules without cloning procedures has been described *(30)*. This approach requires both multiple reactions of the sample and sample dilution to a critical level to minimize the occurrence of more than 2 PCR template molecules per reaction well. At least 96 reactions of the sample should be performed so that a reasonable number of positive wells are available for subsequent sequencing. Nevertheless, this approach saves time and labor compared with subcloning procedures for isolating single bisulfite-converted DNA molecules.

PYROSEQUENCING

An attractive alternative to the traditional dideoxy sequencing approach (Sanger sequencing) is pyrosequencing, which is based on the detection of pyrophosphate. This method, which has also been adapted to

methylation analysis with bisulfite-modified DNA, yields quantitative information on single CpG sites *(35–37)*. Pyrosequencing is based on the detection of emitted light during synthesis of the complementary strand by an exonuclease-deficient DNA polymerase. When nucleotides are incorporated, pyrophosphate is released and converted to ATP by the enzyme ATP sulfurylase. The ATP molecules provide energy for the enzyme luciferase to oxidize luciferin in a reaction that generates light. The 4 different nucleotides are added sequentially to enable base calling.

The instrumentation required for pyrosequencing can be used for many applications *(38)*; however, the quantitative accuracy and reliability of the data decrease with the distance of the CpG from the 3′ end of the forward primer, a feature that limits the number of bases/CpG sites that can be analyzed in a single sequencing reaction *(39)*. The long stretches of thymine often found in bisulfite-modified DNA are also likely to affect reproducibility. Pyrosequencing is usually carried out with MIP products; however, it can also be used to identify false-positive results in MSP assays *(40)*.

COMBINED BISULFITE RESTRICTION ANALYSIS

Digestion of PCR products with certain restriction enzymes can be used to distinguish between methylated and unmethylated DNA *(41)*. The differences in sequence between methylated and unmethylated DNA after bisulfite modification can lead to the creation of new methylation-dependent restriction sites or the maintenance of restriction sites in a methylation-dependent manner. This property was exploited in the development of a quantitative method termed "combined bisulfite restriction analysis" (COBRA) *(42)*, which relies on the separation of digested PCR products by agarose or polyacrylamide gel electrophoresis and subsequent quantitative hybridization. The feature that most limits this technology is that many CpG sites cannot be analyzed because the restriction enzymes that would be appropriate for recognizing the sequence context are not available. Furthermore, apart from the PCR bias phenomenon *(26)*, accurate quantification may be compromised by the formation of heteroduplexes between strands containing the restriction site and strands that do not and by incomplete conversion of unmethylated cytosines during the bisulfite modification. The method is relatively labor-intensive but is cost-effective.

METHYLATION-SENSITIVE SINGLE-NUCLEOTIDE PRIMER EXTENSION (MS-SnuPE)

The SnuPE assay was originally developed for detection of single-nucleotide mutations *(43)* but was later applied to DNA methylation studies *(44)*. The product generated by the MIP is subsequently isolated via gel electrophoresis and annealed to an internal primer that terminates immediately 5′ of the single nucleotide to be assayed (i.e., the cytosine of a CpG site in methylation studies). The internal primer is then extended with a DNA polymerase that uses ^{32}P-labeled dCTP or dTTP. Reaction products are separated on polyacrylamide gels for visualization, and the relative amounts of the 2 nucleotides present in the MIP product can be quantified with phosphor-imaging analysis. A relatively high throughput is possible, especially when multiple internal primers are included in a single primer-extension reaction *(45)*. The internal primers should not anneal to sequences that originally contained CpG sites to prevent the introduction of a bias at this step, but achieving this goal can be difficult in CpG-dense regions. The method is quite labor-intensive and has the disadvantage of requiring the use of radioactive materials.

Alternatively, the SNaPshot technology from Applied Biosystems can be used as a detection platform, thereby omitting radioactive labeling *(46)*. Another variant of MS-SnuPE that uses denaturing HPLC instead of radioactivity for separation and quantification of the extended primer products has also been described *(47)*. This approach, SnuPE ion pair reversed-phase HPLC (SIRPH), was recently evaluated for detecting *MGMT* promoter methylation but was not recommended over COBRA and pyrosequencing *(33)*. Finally, a microarray-based version was recently introduced as a semiquantitative high-throughput method *(48)*.

METHYLATION-SENSITIVE MELTING CURVE ANALYSIS

The sequence differences between methylated and unmethylated DNA obtained after sodium bisulfite treatment can be exploited by melting curve analysis (MCA), because the higher GC content of methylated DNA makes it more resistant to melting *(49)*. In this approach, methylation-sensitive MCA (MS-MCA), the MIP is performed in the presence of a fluorescent dye that intercalates double-stranded DNA (dsDNA). The melting properties of the product are examined immediately after the PCR in a closed-tube system by continuous monitoring of the fluorescence while the temperature is increased. Most small PCR products melt mainly within a limited temperature window, producing an abrupt decrease in fluorescence as the dye is released. When the results are viewed as derivative melting curves, this major transition in fluorescence can be seen as peaks indicating the melting temperature of the product (Fig. 2). In methylation analysis, the target sequence may be fully methylated, fully unmethylated, or heterogeneously methylated. When a mixture of fully methylated and fully unmethylated mole-

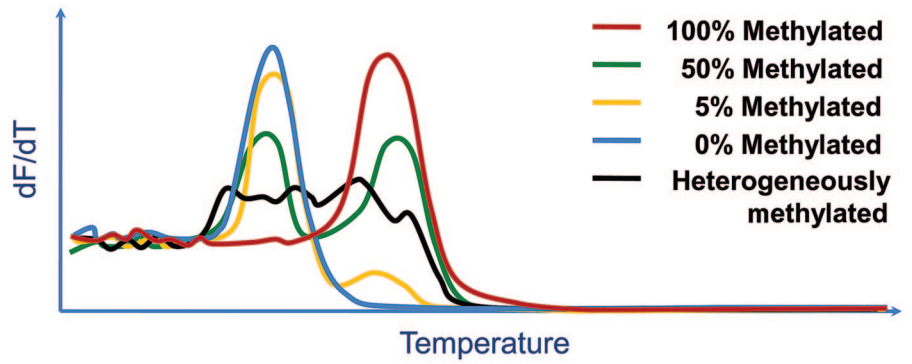


Fig. 2. Principle of melting analysis for methylation detection.

Methylated DNA is more GC rich after sodium bisulfite treatment than unmethylated DNA. The PCR product of a methylated template is thus more resistant to melting and melts at higher temperatures (red curve). If the proportion of methylated and unmethylated molecules is equal and there is no PCR bias, the melting profile will consist of 2 peaks (green curve), one peak corresponding to methylated molecules and the other to unmethylated molecules. The detection limit, which is dependent on assay design, may be about 5% methylated DNA in an excess of unmethylated DNA (yellow curve) when MIP primers without CpG sites are used. If the target sequence is heterogeneously methylated, a complex melting pattern will result (black curve). This figure is for illustration only. dF/dT, the derivative of fluorescence with respect to temperature.

cules is amplified, 2 distinct melting peaks are observed, and interpretation is easy. When heterogeneously methylated molecules are amplified, however, the melting pattern can be complex and difficult to interpret (Fig. 2).

METHYLATION-SENSITIVE HIGH-RESOLUTION MELTING (MS-HRM)

In general, the development of the HRM technology *(50)* created several methodologic advantages. First, because the HRM approach acquires more data points, the melting peaks are sharper, and subtle differences within the amplicons can be detected. Second, the temperature variations produced with HRM instrumentation are generally extremely small, and a relatively high throughput is possible, depending on the instrument used *(51)*. Third, most of the software provided with the instruments permits normalization for end-level fluorescence *(52)*, temperature shifting, and use of internal oligonucleotide calibrators *(53)*. HRM is often performed with a dye that can be used at saturating concentrations without inhibiting the PCR. Nonsaturating dyes such as SYBR Green I can also be used, and although this dye does not detect heteroduplexes well, this feature may be an advantage in methylation studies because heteroduplexes unnecessarily complicate the melting pattern. Sequence-specific binding of SYBR Green I has been reported *(54)*, indicating a potential problem in methylation studies that use melting techniques.

HRM has been used for methylation analysis with traditional MIP primers in the analysis of an imprinted locus *(55)*, with MIP primers that include a limited number of CpG sites to correct for PCR bias and to increase the analytical sensitivity, for the analysis of *MGMT* promoter methylation, and for methylation changes in *H19* [H19, imprinted maternally expressed transcript (non-protein coding)] *(31, 56)*. This approach, MS-HRM, involves including CpG sites in the primer sequences, which pushes the PCR bias toward the methylated allele, and optimization of annealing temperatures has significantly increased the analytical sensitivity *(31)* by making the reaction more MSP-like. It has recently been shown that studies of promoters that tend to be heterogeneously methylated can be improved with a digital MS-HRM approach *(57)*.

MALDI-TOF MASS SPECTROMETRY WITH BASE-SPECIFIC CLEAVAGE AND PRIMER EXTENSION

The use of MALDI-TOF mass spectrometry for DNA methylation analysis has several advantages. The methodology is relatively sensitive, and a methylation level of 5% can be detected without including any CpG sites in the MIP primer sequences. Furthermore, very high throughputs are possible, and the methodology is quantitatively accurate *(58)*.

The experiments to be performed prior to mass spectrometry analysis can be based on base-specific cleavage or primer extension *(59)*. The base-specific cleavage strategy involves amplification with one

primer tagged with a T7 promoter sequence to allow in vitro transcription of the PCR product into a single-stranded RNA transcript. Subsequent base-specific cleavage by an endoribonuclease such as RNase A produces different cleavage patterns for methylated and unmethylated CpG sites, depending on the use of non-cleavable nucleotides. The cleavage products are then analyzed by MALDI-TOF mass spectrometry, which permits the relative amounts of methylated and unmethylated DNA to be determined via comparisons of signal intensities.

When a primer-extension strategy is used, a post-PCR primer-extension reaction is performed with a primer designed to anneal immediately adjacent to the CpG site under investigation. The primer is then extended with a mixture of 4 different terminators, such as dideoxy NTPs. Depending on the methylation status of the CpG site, the primer-extension reaction will terminate on different nucleotides and generate distinct signals when analyzed by MALDI-TOF mass spectrometry. Multiplexing of up to 25 different primer-extension reactions is feasible without compromising the quantitative results if primers are designed carefully *(59)*. The base-specific cleavage strategy is recommended for purposes requiring the analysis of larger regions of unknown methylation content, whereas the primer-extension strategy should be used in routine analyses of a relatively small number of well-characterized informative CpG sites.

The main disadvantages of this methodology are that the required equipment is expensive and the complexity of the methodology can make it challenging to use. For instance, the presence of unexpected single-nucleotide polymorphisms can lead to misinterpreted results because no actual sequence information is given. Coolen and colleagues recently addressed this problem *(60)* in a publication that also demonstrated that MALDI-TOF mass spectrometry can determine whether the detected methylation is allele specific.

HEAVYMETHYL

In the HeavyMethyl methodology *(32)*, oligonucleotide blockers are used to discriminate between methylated and unmethylated alleles. The MIP primers are designed to hybridize next to a CpG-rich sequence, for which blockers have been designed to hybridize only to unmethylated DNA. Thus, if the DNA is methylated, the blockers cannot hybridize and leave the primer-binding site accessible for the primers to bind. Amplification will then occur. Amplification is detected with a probe that contains CpG sites, a fluorophore label, and a quencher. When the exonuclease activity of the polymerase cleaves the probe, the fluorophore is released from the quencher, and light is emitted (Fig. 3). The emitted light is proportional to the amount of amplicon in the test tube, allowing accurate quantification of the methylation level.

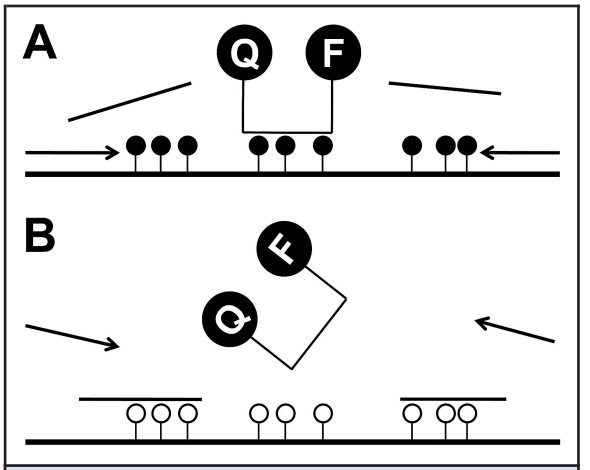


Fig. 3. Principle of HeavyMethyl.

(A), When the target sequence is methylated, the blocker oligonucleotides (denoted as solid lines) are unable to bind, allowing the MIP primers (denoted as arrows) to bind to the target. The amplification of the target is monitored with a fluorescent probe designed to be specific for methylated DNA only. When the primers and the probe have hybridized correctly, the probe is cleaved by the exonuclease activity of the polymerase, causing the fluorophore (F) to be released from the quencher (Q) and light to be emitted. The emitted light is proportional to the amount of target DNA molecules in the test tube and can be monitored with the appropriate equipment. (B), When the target sequence is unmethylated, the blocker oligonucleotides are capable of binding and prevent the MIP primers from binding to the target. Furthermore, the probe will not bind to unmethylated DNA, and no amplification will occur. The probe will not be cleaved, and no light will be emitted. Modified from [Cottrell et al. *(32)*].

The use of blocker molecules significantly increases the analytical sensitivity, which is comparable to methods that use MSP primers. HeavyMethyl allows a high throughput and is a closed-tube method; however, the main advantage relative to conventional MSP (discussed below) may be that false-positive rates are extremely low. These rates are low because the blockers provide methylation specificity at every cycle of the PCR, whereas in MSP, a false-priming event needs to happen only once to get the amplification going. Another advantage of HeavyMethyl is that the flexibility of primer and blocker design may allow detection of heterogeneous methylation.

Methods Based on MSP Primers

MSP primers are designed to amplify methylated DNA only, and thus the PCR bias phenomenon associated with MIP-based methods is not an issue. This specificity is achieved by including many CpG sites in the primer sequences, preferably at or close to the 3′ end. In concert with stringent PCR conditions, only amplification of methylated DNA will occur. MSP assays are generally associated with high false-positive rates *(40, 61, 62)*, however, especially with a high number of PCR cycles, which can be necessary for obtaining highly analytically sensitive assays. False-priming events (in which amplification happens despite the mismatches of the primer sequences with the template) and incompletely bisulfite-converted DNA molecules within the test tube may be responsible for false-positive results. False-priming events can be detected with an appropriate negative control and prevented by limiting the number of cycles and/or using a higher annealing temperature. Incompletely converted molecules may mimic methylated sequences, because MSP primers contain multiple cytosines derived from CpG sites. Although this feature makes such primers highly selective for methylated templates, it also facilitates amplification of incompletely converted sequences in the bisulfite-treated DNA. Having multiple non-CpG cytosines within the MSP primers may limit this problem.

METHYLATION-SPECIFIC PCR

In the traditional MSP methodology *(63)*, a second set of primers is often designed (in addition to the MSP primers) for the amplification of unmethylated DNA so that the presence of suitable template can be confirmed after the sodium bisulfite treatment. Gel electrophoresis is used for detecting the PCR products, and in situations in which both unmethylated DNA and methylated DNA are present in the test tube, a comparison of band strengths allows a very approximate estimate of relative methylation levels. Methylation levels can be estimated more accurately with real-time PCR (see below), but quantitative information can also be obtained with fluorescently labeled amplicons analyzed by a genetic analyzer *(64, 65)*. MSP is very cost-effective, but any of the false-positive results mentioned above cannot be detected. Furthermore, opening of PCR tubes should be avoided, especially in clinical settings, to reduce the risk of PCR contamination. No specialized equipment is needed, however, and the method is simple to use. For these reasons, MSP, which can be performed in almost any laboratory, is the most widely used method for the analysis of DNA methylation at specific loci.

MSP is known for its high analytical sensitivity. In the original publication *(63)*, MSP was reported to detect 0.1% methylated template in an excess of unmethylated DNA, but it may detect as little as 0.0002% when a nested approach is used *(66)*. The analytical sensitivity of MSP assays is also influenced by primer design, the number of PCR cycles, and annealing temperature. Use of fewer cycles gives fewer false-positive results but also decreases the analytical sensitivity of the assay, whereas the use of appropriately designed primers and a high annealing temperature prevents false-priming events. Thus, many published MSP assays vary tremendously in analytical sensitivity *(66, 67)*.

QUANTITATIVE MSP: METHYLIGHT

MSP was first made quantitative by the use of fluorescent hydrolysis probes that enabled real-time detection of the MSP amplification *(68–70)* (Fig. 4). This approach, which is most often referred to as MethyLight, overcomes most of the problems associated with MSP. First, amplification is observed only when the probe has hybridized between the primers, thus eliminating any signal from nonspecific amplification, such as primer dimer formation. For this reason, the fact that gel electrophoresis is not required makes MethyLight a closed-tube method capable of high throughput. Second, the additional CpG sites within the probe sequence make false-priming events less likely, and false-positives due to incomplete conversion can be limited by having many non-CpG cytosines within the probe sequence. The introduction of a probe complicates assay design, however, and can cause MSP to miss heterogeneously methylated sequences that it would otherwise detect because of the requirement for the probe to hybridize correctly before a signal is observed. The analytical sensitivity of MethyLight is similar to that of MSP but has recently been shown to be increased to at least 0.05% when a digital approach for amplifying single molecules is used *(30)*. As with all quantitative MSP-based methods that use sodium bisulfite–modified DNA, a control gene is used to normalize for DNA input.

QUANTITATIVE MSP: SYBR GREEN–BASED

Quantitative MSP with fluorescent dyes that intercalate dsDNA has circumvented the need for fluorescent probes (Fig. 4). This change was first achieved with the dye SYBR Green I in a methodology that uses gel electrophoresis for evaluating the PCR product *(71)*. Because all dsDNA within the test tube is detected, primer dimers may compromise the quantitative accuracy *(72)*. This possibility also implies that post-PCR analysis methods such as gel or capillary electrophoresis are required to determine whether the amplification was specific.

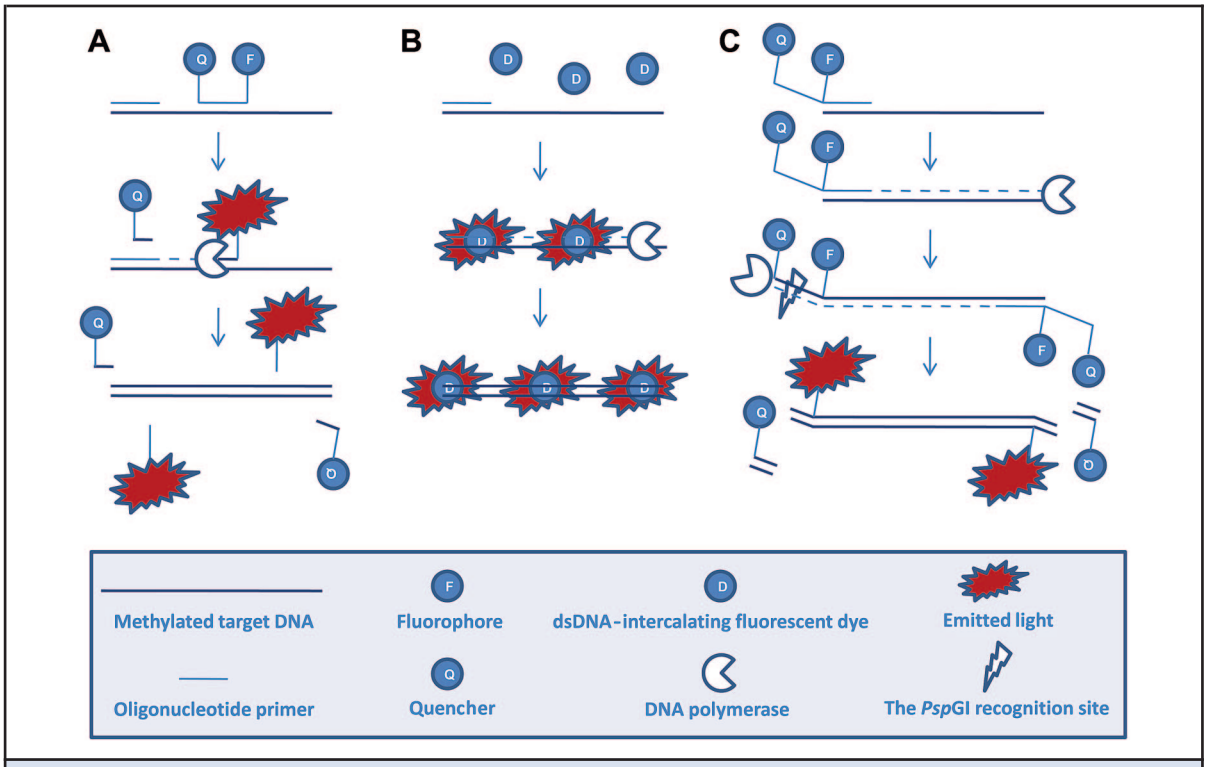


Fig. 4. Three different ways of creating real-time fluorescence signals in quantitative MSP analysis.

(A), In the MethyLight technology, the fluorescence signal is created by cleavage of a hydrolysis probe designed to hybridize between the MSP primers via the exonuclease activity of the DNA polymerase. This probe is labeled with a fluorophore and a quencher molecule. After cleavage, the fluorophore is released from the quencher, and light is emitted. The emitted light is proportional to the amount of amplicon within the test tube, and primer dimers are not detected. (B), In the SMART-MSP methodology and with SYBR Green–based detection, the use of dsDNA-intercalating fluorescent dyes enables the creation of a signal in real time. The dye molecules emit light only when intercalated into dsDNA; thus, the magnitude of the signal is proportional to the amount of dsDNA within the test tube. Dyes that can be used at saturating conditions without inhibiting the PCR are preferred. (C), In the MS-FLAG methodology, the cleavage of labeled primers by the thermostable endonuclease *Psp*GI enables signal creation in real time. The recognition site of this enzyme is created only after the DNA polymerase has extended the target DNA. As in the MethyLight method, the quencher molecule is separated from the fluorophore, and the emitted light is proportional to the amount of dsDNA within the test tube.

SYBR Green I has also been used for post-PCR melting analysis in the nonquantitative melting curve MSP (McMSP) methodology to avoid the use of gel electrophoresis *(73)*; however, the limited resolution of the post-PCR melting analysis did not allow additional information not provided by gel electrophoresis to be obtained. Thus, these melting analyses may be associated with false-positive results, as with conventional MSP, but McMSP has the advantage of being a closed-tube method, allowing a high throughput.

QUANTITATIVE MSP: SENSITIVE MELTING ANALYSIS AFTER REAL-TIME MSP (SMART-MSP)

The SMART-MSP methodology *(61)* takes advantage of the additional resolution of the HRM technology for the detection of false-positive results. Thus, SMART-MSP is also based on dsDNA-intercalating fluorescent dyes (Fig. 4). The real-time MSP provides quantitative data that may be analyzed with the relative $2^{-\Delta\Delta Ct}$ quantification approach if the PCR efficiencies for the gene and the control are approximately equal *(74)*. The use of an HRM step after the PCR has improved probe-free quantitative MSP analysis in several ways. This validation step provides information that cannot be obtained by gel electrophoresis, and false-positive results caused by false-priming events or incomplete conversion can often be detected, depending on how the amplicon is designed *(61)*. Notably, HRM analysis may be suitable only for detecting false-positive results, however, and not for situations in which incomplete con-

version leads to slight overestimation of methylation levels. This is because the signal from the methylated and fully converted molecules will be much stronger in such situations than the signal derived from incompletely converted molecules.

SMART-MSP can detect 0.1% methylated template *(61)*. A high throughput is possible, and the risk of PCR contamination is low because of the closed-tube format of the method.

QUANTITATIVE MSP: METHYLATION-SPECIFIC FLUORESCENT AMPLICON GENERATION (MS-FLAG)

Another quantitative MSP approach that circumvents the need for additional probes has recently been introduced. In MS-FLAG *(75)*, the fluorescence signal is created by cleavage of the MSP primers by the thermostable endonuclease *Psp*GI. The primers contain an oligonucleotide 5′ tail carrying a fluorophore and a quencher separated by the recognition site of the endonuclease. The double-stranded recognition site is not created until the primers have annealed and the polymerase has created a new copy of the target (Fig. 4). The quantitative accuracy of the MS-FLAG methodology can be compromised by primer dimer formation and has to be avoided through optimal design of the primers. Thus, post-PCR analysis is required to confirm that the amplification was specific. Because melting analyses are not compatible with this approach, gel electrophoresis is used for this purpose.

The analytical sensitivity of MS-FLAG is comparable to that of other MSP-based methods but is less cost-effective than SMART-MSP because of the use of fluorescently labeled primers and the thermostable endonuclease. Because additional probes are not required, however, primers can be labeled differently, and so it is possible to design multiplex MS-FLAG assays to limit costs and labor *(75)*.

Discussion

Early detection of cancer often improves the clinical outcome. Methods for relatively early detection exist for breast and prostate cancer. On the other hand, many imaging and cytology-based strategies have failed to achieve early detection of lung and other cancers *(76)*. Thus, there is a need for new molecular methods to detect preneoplastic and small malignant lesions *(19)*. A number of specific loci are potential candidates as DNA methylation biomarkers especially directed toward early cancer detection.

There are many reasons for cancer-specific methylated loci being suitable as biomarkers for cancer detection *(19)*. First, DNA is a stable molecule that can easily be isolated from body fluids and tissues, in contrast to the RNA needed for reverse-transcription PCR assays. Second, DNA containing methylation information can be isolated from formalin-fixed and paraffin-embedded tissues and be used in most PCR-based methods for detecting DNA methylation. Third, the methylation signal to be detected is positive, in contrast to the loss of heterozygosity or changes in gene expression, which can be difficult to detect in the presence of an excess of nonaffected DNA.

Much more work is required to validate the clinical use of many DNA methylation biomarkers, however, because existing markers often lack the diagnostic sensitivity and specificity required for a diagnostic test. Lack of diagnostic sensitivity and specificity may be in part a technological problem, because false-positive results will produce a low diagnostic specificity and false-negative results will produce a low diagnostic sensitivity. Nevertheless, numerous new genes that undergo cancer-specific methylation have recently been identified, and the creation of panels of markers with higher diagnostic sensitivity and specificity for particular purposes is ongoing *(76)*.

The choice of CpG sites to be analyzed is also important, because the methylation status of some sites may prove better than others for distinguishing healthy tissue from malignant tissue *(33)*. Furthermore, the degree of interindividual variation is still unknown *(77)*, and it is therefore more difficult to define what is typical with respect to DNA methylation, in contrast to genetic events, for which we have a reference sequence.

Because tumor-derived material in body fluids often is difficult to detect, highly sensitive methods with low limits of detection are needed for many applications. These methods are generally based on MSP primers; however, they are also associated with false-positive results. The false positives can be limited through the use of fluorescent probes in the MethyLight methodology *(68)* or by HRM in the SMART-MSP methodology *(61)*. Furthermore, these methods are quantitative. The quantitative data can be used to set a threshold for the methylation level to provide the highest diagnostic sensitivity and specificity for a given biomarker. When the threshold is lowered, the diagnostic sensitivity typically will increase, and the diagnostic specificity will decrease.

Very sensitive detection of DNA methylation at specific loci is not limited to MSP-based methods. HeavyMethyl *(32)* is a probe-based methodology that uses oligonucleotide blockers to obtain an acceptable analytical specificity for methylated DNA. The use of blockers dramatically increases the analytical sensitivity. Furthermore, because the blockers provide analytical specificity in every cycle of the PCR, the false-positive rate of this approach is very low. MS-HRM is another MIP-based method that provides a high analytical sensitivity. In addition, HeavyMethyl and MS-

HRM are quantitative methods that do not require the PCR tubes to be opened. Therefore, the clinical applicability of these methods is comparable to that of MethyLight and SMART-MSP (see Table 1).

It is desirable that the results obtained be confirmed by more than one method; however, because of the innate differences among the various methods, the results obtained with different methods cannot be expected to be identical. Sequencing of a limited number of samples is often used to confirm results in both MIP- and MSP-based methods, but sequencing all samples is too time-consuming and too expensive for most laboratories.

Conclusion and Future Perspectives

A more detailed analysis of individual CpG sites in the studied CpG islands may be necessary to identify the most informative sites (i.e., those providing the highest diagnostic sensitivity and specificity). Currently, there is a tendency in the literature for CpG sites to be chosen for their optimal primer location, depending on the specific assay, because detailed studies of the CpG sites of individual molecules have thus far been expensive and time-consuming. Emerging massively parallel sequencing methods *(78)*, often referred to as "next-generation" sequencing, promise to make the enormous amount of information required for this purpose easily obtainable. Currently, 3 platforms are available: the Genome Sequencer FLX system/454 sequencing (named "454" by 454 Life Sciences, now acquired by Roche), the Genome Analyzer system (named "Solexa" by Solexa, now acquired by Illumina), and the SOLiD system (named "SOLiD" by Applied Biosystems). The first of these new methods to be applied to sodium bisulfite–modified DNA was the 454 system based on pyrosequencing. A detailed methylation study of 25 gene-related CpG islands in 40 samples from different nonmalignant and malignant blood cells has been performed *(24)*. This pilot study confirmed the utility, robustness, and superiority of the method with bisulfite-modified DNA as the template. In addition to the ultrahigh throughput this approach offers, it is capable of providing accurate sequences, even at the long stretches of thymine often found in bisulfite-modified DNA. This approach eliminates the bias often encountered when PCR products are subcloned in bacteria *(34)*, and far more individual "clones" (molecules) can be analyzed relative to the few clones typically analyzed in bisulfite sequencing *(23)*. Thus, the 454 approach has the potential to identify the most informative CpG sites within a given CpG island rapidly and with high accuracy and thereby provide guidelines for the development of analytically sensitive and more cost-effective assays, such as MethyLight, SMART-MSP, HeavyMethyl, and MS-HRM, with sufficient robustness for clinical use.

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