DNA methylation is the most extensively studied mechanism for epigenetic gene regulation (1). Recent studies have shown that DNA methylation plays an important role in a number of physiological processes as well as common diseases such as cancer and neurodegenerative disorders (2,3). In mammals, DNA methylation occurs at the C-5 position of cytosine in CpG dinucleotide sequences (Figure 1) (1), which are mainly concentrated in regions known as CpG islands. Methylations within CpG islands usually leads to gene silencing. More recently, DNA methylation in regions located up to 2 kb from known CpG islands (called CpG island shores) has also shown a strong correlation with gene expression (4).

At present, the vast array of platforms available to study DNA methylation present a challenge for scientists who wish to enter this field (5). Among the methods for studying DNA methylation in candidate regions, PCR-based approaches have several advantages (6). Here we provide a practical overview of experimental design and analysis for the most common PCR-based DNA methylation techniques: bisulfite sequencing PCR (BSP), methylation specific PCR (MSP), MethylLight, and methylation-sensitive high resolution melting (MS-HRM). These techniques do not require expensive specialized equipment and could be implemented in a typical molecular genetics laboratory.

Bisulfite conversion
The first step in almost all protocols for studying DNA methylation is bisulfite conversion of the DNA sequence of interest. Bisulfite conversion occurs through a number of chemical reactions (e.g., sulfonation, deamination, and desulfonation) on the DNA that transform non-methylated cytosines into uracils. Methylated cytosines remain unconverted (Figure 1). Classical DNA conversion protocols are time-consuming, often requiring more than 16 h to complete (7), and require multiple tube changing steps that increase the risk of contamination and human error. Classical protocols also risk losing more than 75% of the starting DNA (8,9) during purification and through single-strand breaks that occur during long incubation steps (7,9).

Commercially available bisulfite conversion kits improve recovery of the converted DNA by using shorter incubation steps and alternative purification procedures (9). These kits also facilitate efficient implementation of the conversion reaction, thereby improving downstream results with PCR-based techniques. Thus, kits are highly recommended, especially for those unfamiliar with this field of study. There are many considerations for selecting a kit, including cost, yield, efficiency, and time. A comparison of the main features of available DNA conversion and methylation control kits is included in Tables 1 and 2.

Controls for DNA bisulfite conversion
Evaluation of the quality of converted DNA is recommended when beginning a DNA methylation study; this step is especially important for quantitative PCR-based methods such as MethylLight and MS-HRM. Since bisulfite-treatment can result in DNA fragmentation, thus reducing the number of molecules available for PCR amplification, it is best to test the bisulfite-converted DNA with primer sets that amplify a range of differently sized products. From these products, the ideal amplicon length for downstream analysis can be determined (10), providing information that will aid in primer design.

Incomplete bisulfite conversion will adversely affect the reliability and accuracy of DNA methylation measurements by PCR-based methods (11,12).
Non-methylated cytosines are transformed to thymines. Without Mass Spec
Rapidly Quantify 5-hmC
Without Mass Spec

Therefore, it is necessary to evaluate the efficiency of conversion using commercially available primer sets to amplify the converted DNA (e.g., DAPK1 Catalog #D5014–2, Zymo Research, Orange, CA) (Table 1). The resulting DNA product can be sequenced to verify the efficiency of conversion for all non-CpG cytosines. Alternatively, converted DNA may be amplified with primers designed for the non-converted DNA sequence. In this case, the absence of a PCR amplicon suggests a complete conversion reaction.

Converted DNA must also be quantified prior to downstream PCR applications. The amount of DNA may be determined by spectrophotometric measurements using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) (13) with settings for single-stranded DNA, or agarose gel electrophoresis and classical UV spectrometric analyses (5). Other more specific methods, such as qPCR (including MethyLight control assays) or PicoGreen may be more reliable and better suited for measuring limited amounts of DNA (14).

Designing primers for PCR-based DNA methylation analysis
Designing primers against a region of interest (ROI) is the most critical step in obtaining adequate DNA methylation results using PCR-based methods. Several software platforms such as Methyl Primer Express (Applied Biosystems, Foster City, CA), MethPrimer (15), BiSearch (16), MethMaker (17), and MSPprimer (18) have been developed for this purpose. All of these programs allow users to customize primer length, amplicon length, and Tm (melting temperature) differences, as well as enable searches for CpG islands in the input sequence, and identify possible stable primer-dimer or hairpin structures that should be avoided. The advantages and disadvantages of each program are compared in Table 3. Primers should not bind to regions containing common SNPs (19), which can be identified easily using the UCSC Genome Browser (http://genome.ucsc.edu).

Because bisulfite treatment decreases DNA sequence complexity, primers have an increased tendency to bind multiple target sequences in converted DNA (18). Therefore, in silico evaluation of primer specificity is a key step during primer design for bisulfite-converted DNA methods. BiSearch software is unique in terms of its ability to find the number of potential matches, including partial matches, for each individual primer in the bisulfite-converted methylated or unmethylated genome and to perform in silico PCR on the bisulfite-converted human genome using any primer pair (16). In our laboratory, we have observed greater success using primers with less than 3000 matches.

At present, the software available for primer design does not account for PCR bias (20–22). When faced with bias, it is important to use additional tools to review the ROI sequence, highlight the CpG and non-CpG cytosines, and design adequate primers. BioWord is a free Microsoft Word plugin that allows manipulation, editing, and processing of DNA sequences and has proven useful for working with sequences prone to PCR bias (23). Another available option is a shareware version of the licensed software FastPCR, which includes a tool for in silico bisulfite conversion of non-CpG cytosines (24).

PCG-based techniques
Bisulfite sequencing PCR
Bisulfite sequencing PCR (BSP) was the first technique described for analyzing DNA methylation status using PCR (25). The technique consists of PCR amplifying a bisulfite-converted DNA ROI, followed...
by Sanger sequencing of the product either directly or after cloning into a suitable vector. Direct-BSP: By comparing sequencing results with the respective reference genomic DNA sequences, direct sequencing of PCR products provides information on the average methylation status for each CpG dinucleotide. Direct-BSP is the shortest form of BSP, but holds several technical challenges inherent in sequencing, such as poor signal quality and artifacts in cytosine signals that may affect electropherogram analysis; it also has a low sensitivity (26). Because of these difficulties, BSP with cloning is more common. Cloning-based BSP: In cloning-based BSP, PCR products are cloned into a vector and transformed into competent *E. coli* cells. After expansion and purification of the plasmids, the PCR product inserts are sequenced. The CpG methylation status for each CpG dinucleotide in the ROI is determined by sequencing each expanded clone (27,28). The resulting averages are referred to as DNA-methylation haplotypes. Cloning-based BSP requires at least six sequencing reactions to obtain a sensitivity higher than direct BSP (29), making this an expensive and labor-intensive option that is especially cumbersome for population-based studies. Digital (single-molecule) BSP: Another BSP option for producing DNA-methylation haplotypes is digital-BSP (22,30). This method requires serial dilution of a DNA template to optimize conditions for PCR amplification of a single converted DNA molecule per reaction tube (via the Poisson distribution), thus avoiding both PCR bias and cloning. Digital-BSP is considered the gold standard for detecting the methylation status of specific loci (22). However, this method is inefficient because 87% of the reactions cannot be analyzed, and 3% are control reactions; thus, useful information is only obtained from the remaining 10% (22). An alternative approach is to use MS-HRM to select the clones for sequencing (31).

**Primer design considerations:** Methylation-independent PCR (MIP) primers should be designed to allow the amplification of bisulfite-converted DNA regardless of methylation status. Primers

---

**Table 1. Comparison of commercially available kits for bisulfite conversion (single column format)**

<table>
<thead>
<tr>
<th>Provider (References)</th>
<th>DNA Input</th>
<th>DNA Output (1)</th>
<th>Time (2)</th>
<th>Additional notes</th>
<th>Link (3)</th>
<th>Reactions/ Cost (4)</th>
<th>Citations (5) search words</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymo DNA Methylation Gold Kit (D5005 &amp; D5006)</td>
<td>500 pg – 2 µg (Optimal 200 – 500 ng)</td>
<td>≥10 µl &gt;99% &gt;75%</td>
<td>4</td>
<td>Modified DNA can be stored at −20°C for 1 month</td>
<td>goo.gl/zAwu1</td>
<td>50 rxns (2.7) 200 rxns (2.3)</td>
<td>1,380 Cells-to-Cpg-Bisulfite Conversion</td>
</tr>
<tr>
<td>Qiagen EpiTect Bisulfite Kit (59104)</td>
<td>1 ng – 2 µg</td>
<td>≥20 µl &gt;99% NA</td>
<td>6</td>
<td>Protocol for FFPE Samples</td>
<td>goo.gl/rNNeu</td>
<td>48 rxns (4.4)</td>
<td>1,020 EpiTect- Bisulfite-Kit Qiagen</td>
</tr>
<tr>
<td>Zymo DNA Methylation Direct Kit (D5020 &amp; D5021)</td>
<td>50 pg - 2 µg (Optimal 200 - 500ng)</td>
<td>10 µl &gt;99.5% &gt;80%</td>
<td>5</td>
<td>Protocol for FFPE Samples</td>
<td>goo.gl/UJnDI</td>
<td>50 rxns (3.6) 200 rxns (2.5)</td>
<td>95 Zymo DNA Methylation-Conversion-Kit Qiagen</td>
</tr>
<tr>
<td>Zymo EZ DNA Methylation-Lightning Kit (D5030 &amp; D5031)</td>
<td>100 pg - 2 µg (Optimal 200 - 500ng)</td>
<td>10 µl &gt;99.5% &gt;80%</td>
<td>3</td>
<td>For long-term use store at −70°C</td>
<td>goo.gl/zAwu1</td>
<td>50 rxns (3.6) 200 rxns (2.5)</td>
<td>0 MethylLightening-kit Zymo</td>
</tr>
<tr>
<td>Epigentek BisulFlash DNA Modification Kit (P-1026-050)</td>
<td>0.2 ng – 1 µg (Optimal 200–500 ng)</td>
<td>&gt;90% &gt;99.9%</td>
<td>2.5</td>
<td>Protocol for DNA input 0.2 ng/50 cells</td>
<td>goo.gl/TU79j</td>
<td>50 rxns (2.9)</td>
<td>2 Epigentek BisulFlash DNA Modification Kit</td>
</tr>
<tr>
<td>Chemicon (Millipore) CpGenome Turbo Bisulfite Modification Kit (P7847)</td>
<td>500 pg-1 mg (Optimal 1 ng -1 mg)</td>
<td>25 µl &gt;99.9% NA</td>
<td>3</td>
<td>Modified DNA can be stored at −20°C for 2 months</td>
<td>goo.gl/XMNDz</td>
<td>50 rxns (3.3)</td>
<td>1 CpGenome Bisulfite Modification-Kit-Millipore-fast DNA</td>
</tr>
<tr>
<td>Chemicon (Millipore) CpGenome Fast DNA Modification Kit (P7824)</td>
<td>1 ng - 1 mg (500 pg of DNA can be used)</td>
<td>&gt;35-45µL NA NA</td>
<td>24</td>
<td>Modified DNA can be stored at −20°C for 2 months</td>
<td>goo.gl/5sem</td>
<td>T25rxns (8.0)</td>
<td>3 CpGenome Bisulfite fast Modification-Kit-Millipore</td>
</tr>
<tr>
<td>Epigentek Methlyamp Modification Kit (P-1001-1 &amp; P-1001-2)</td>
<td>1 ng-1 µg (Optimal 50-200 ng)</td>
<td>8-18 µL &gt;99.5% &gt;90%</td>
<td>4</td>
<td>Modified DNA can be stored at −20°C for 2 months</td>
<td>goo.gl/7EIOc</td>
<td>40 rxns (2.7) 80 rxns (2.5)</td>
<td>67 Methylamp-DNA-Modification-Kit-Coupled-DNA Epigentek</td>
</tr>
<tr>
<td>Epigentek ¥ Methylamp DNA Isolation &amp; Modification Kit (P-1002-40)</td>
<td>1 ng-1 µg (Optimal 50-200 ng)</td>
<td>8-18 µL &gt;99.9% &gt;90%</td>
<td>3</td>
<td>Protocol for adhesive cells, plasma, serum, and body fluids</td>
<td>goo.gl/I0Ovy</td>
<td>40 rxns (5.0)</td>
<td>1 Methylamp-Coupled Epigentek</td>
</tr>
<tr>
<td>New England Biolabs EpiMark Bisulfite Conversion Kit (E31385)</td>
<td>50 ng-2 mg</td>
<td>40 µl</td>
<td>4</td>
<td>Modified DNA can be stored at −20°C for up to 2 months</td>
<td>goo.gl/kkRlk</td>
<td>48 rxns (2.8)</td>
<td>8 EpiMark Bisulfite-Conversion-Kit</td>
</tr>
<tr>
<td>Sigma-Aldrich Imprint Bisulfite DNA Modification Kit (M050)</td>
<td>10 ng to 1 mg (Optimal 10 ng -1 mg)</td>
<td>8-20 µL &gt;99% &gt;90%</td>
<td>3</td>
<td>50 pg / 20 cells DNA NA</td>
<td>goo.gl/U1hOl</td>
<td>50 rxns (4.3)</td>
<td>46 Imprint-DNA Modification Kit Sigma</td>
</tr>
<tr>
<td>Invitrogen Cells-to-Cpg Bisulfite Conversion Kit (4445555)</td>
<td>50 pg-5 µg (Optimal 100 ng - 1 µL)</td>
<td>10 µL-40 &gt;99.5% NA NA</td>
<td>7</td>
<td>Protocol for blood, 10 - 100 cells (optimal 5000 -100 cells)</td>
<td>goo.gl/upJ1A</td>
<td>50 rxns (5.0)</td>
<td>1 Cells-to-Cpg-Bisulfite-Conversion-kit</td>
</tr>
<tr>
<td>Human Genetic Signatures MethyEasy Xceed Human Genetic Signatures</td>
<td>50pg - 5 mg (12 µL -100 µL)</td>
<td>&gt;50 µl &gt;99,9 &gt;99%</td>
<td>2.5</td>
<td>Protocol for input equivalent to 8 human cells</td>
<td>goo.gl/4JuMl</td>
<td>40 rxns (6.5)</td>
<td>52 MethyEasy “Xceed”</td>
</tr>
<tr>
<td>Diagenode MagBisulfite Magnetic bisulfite conversion kit (AF-106-0024)</td>
<td>&gt;1 ng (Optimal 100ng -1µg)</td>
<td>&gt;50 µl &gt;99% &gt;90%</td>
<td>4.5</td>
<td>Magnetic bead purification</td>
<td>goo.gl/Q2JHN</td>
<td>24 rxns (13.5)</td>
<td>0 MagBisulfite + Diagenode</td>
</tr>
<tr>
<td>Clontech EpiXplore Methyl Detection Kit (631968)</td>
<td>50 pg-5 µg</td>
<td>12-100 µl NA NA</td>
<td>2.5</td>
<td>&lt;50 ng input DNA NA</td>
<td>goo.gl/miigf</td>
<td>10 rxns (17.5)</td>
<td>1 EpiXplore-Methyl-Detection Kit</td>
</tr>
</tbody>
</table>

1. Top: Output volume; Middle: Expected conversion efficiency; Bottom: Expected recovery. 2. Approximate time length of conversion process in hours. 3. Weblink shortened using Google URL shortener service. 4. Number of reactions for each kit and costs for reaction in U.S.-Dollars (in parentheses), derived from provider’s web page (currency conversion if required). 5. Number of citations found using Google Scholar (using the references as search words). ¥ Substitute of Epigentek kit, old version no longer available. Abbreviation: rxns: Reactions. NA: No available information found online.
should also not bind regions containing CpG dinucleotides (Figure 2A) (25) and should flank a sequence of converted DNA containing as many thymines originating from the conversion of non-CpG cytosines as possible (25). Guidelines for designing BSP primers were initially published by Clark et al. in 1994 (25).

Recently, several more techniques have been developed using primers based on the same principles (32). One variant of the direct-BSP method uses two rounds of nested PCR with primers designed by standard methods for the first round and primers with a GC-rich tag at the 5’ end for the second round. This primer modification is intended to reduce non-specific amplification during direct-BSP and compensate for the frequent artifacts seen in direct-BSP results (33,34).

Bias in BSP: Several studies using MIP primers have shown bias toward unmethylated or methylated alleles (8,20), likely due to sequence differences between methylated and unmethylated alleles (34). For example, Warnecke et al. found a 33-fold amplification bias in BSP: Primers to bind the opposite DNA strand promoter (20). In some cases, adjusting concentrations or redesigning the primers to bind the opposite DNA strand may be sufficient to resolve this bias (20,34).

Shen et al. found that, in some instances, adjusting the annealing temperature may correct this bias (35). Wojdacz et al. developed a new approach to primer design that allows the use of annealing temperature changes to adjust for amplification bias (36,37). These new guidelines for bias compensation are described in detail in the MS-HRM section of this article.

Another potential source of error occurs in cloning-based BSP methods. Cloning biases may skew the reliability of results generated from BSP assays (22,34). There is evidence that amplicons without cytosines may be more difficult to clone efficiently (8). Although direct-BSP has low sensitivity, it provides more accurate detection of differences as low as 20% in methylation status in a single CpG (29).

Data analysis: Quantification of methylation levels is determined by comparing the relative peak heights of cytosine and thymine (or adenine and guanine in cases of complementary strand sequences) (25) in each CpG position in the electropherogram (Figure 2B). A qualitative analysis of bisulfite sequencing results can be performed if a clear single peak is present for each CpG cytosine position. In that case, a thymine peak would be interpreted as a non-methylated CpG, and a cytosine peak would represent a methylated CpG. Analysis of raw sequence data from direct-BSP is often difficult, but correction algorithms aid data interpretation. The Epigenetic Sequencing Methylation Software (ESME) program includes an algorithm to analyze direct-BSP sequencing results and provides a quality control filter (Table 4) (29).

ESME may also be used to analyze cloning-based BSP electropherograms (29). In cloning-BSP or digital-BSP, satisfactory sequencing results belonging to the same sample should be averaged to determine the level of methylation for each CpG position. This task is facilitated by BiQ-Analyzer and BISMA (38,39) (Table 4).

BSP selection: Different BSP methodologies are optimal for different methylation studies, depending on the particular conditions of a study and other parameters, including cost, research question, and available samples. For example, the study initially validating digital MethyLight is a case where digital-BSP was the best choice, since it allowed accurate validation of another single-molecule technique and the use of automated PCR-processing for a large number of sequencing reactions (30). In many other cases, cloning-BSP is preferred because it is the only option for determining DNA-methylation haplotypes in general laboratories (34). Direct-BSP was selected to assess DNA methylation of raw sequence data from direct-BSP is often difficult, but correction algorithms aid data interpretation. The Epigenetic Sequencing Methylation Software (ESME) program includes an algorithm to analyze direct-BSP sequencing results and provides a quality control filter (Table 4) (29).

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Table 2. Comparison of commercially available kits for DNA methylation controls

<table>
<thead>
<tr>
<th>Provider (Reference)</th>
<th>Amount / Cost (1)</th>
<th>Features</th>
<th>Notes</th>
<th>Link (2)</th>
<th>Citations (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millipore S8001 (S8001M &amp; S8001U)</td>
<td>5µg / 20µL $212</td>
<td>Methylated DNA HCT116 DKO cells DNA methylated by M.SssI DNMT (EC 2.1.1.37) &gt;95% of CpGs methylated</td>
<td>Storage: -15°C to -25°C in aliquots protected from freeze-thaws for up to 2 years Do not freeze and thaw the same &gt;20°C aliquot more than 3x for best results</td>
<td>goo.gl/6DMntf goo.gl/tFRPx</td>
<td>67 40</td>
</tr>
<tr>
<td>Millipore Chemicon-CpGenome Universal Methylated / Unmethylated DNA (S7821 &amp; S7822)</td>
<td>10 µg $299</td>
<td>Methylated DNA Enzymatically methylated human male genomic DNA</td>
<td>Storage: -15°C to -25°C in aliquots protected from freeze-thaws for up to 2 years Do not freeze and thaw the same &gt;20°C aliquot more than 3x (70°C for longer storage)</td>
<td>goo.gl/tkmBz goo.gl/AYQBA</td>
<td>238</td>
</tr>
<tr>
<td>Qiagen † Human control DNA set (Converted methylated and unmethylated) (596965)</td>
<td>100 µL each of a 10 ng/µl solution $358</td>
<td>Non-Methylated DNA HCT116 DKO gDNA DNMT3a (-/-) Methylated DNA In vitro methylation of HCT116 gDNA</td>
<td>The control non-converted DNA is shipped at room temperature and should be stored at -20°C immediately upon receipt (at least 6 months without reduction in performance) Bisulfite converted DNA is stored in EB Buffer in a ready-to-use 10 ng/ml solution</td>
<td>goo.gl/D9z8K</td>
<td>53</td>
</tr>
<tr>
<td>Clontech Unmethylated HCT116 DKO gDNA 10 µg 3521 EpiScribe® Methylated HCT116 gDNA 10 µg</td>
<td>$153.00 10 µg</td>
<td>Non-Methylated DNA NA Methylated DNA In vitro methylation of the control DNA using SssI methylase</td>
<td>3520 EpiScope Methylated HeLa gDNA</td>
<td>goo.gl/RsCTk</td>
<td>2</td>
</tr>
<tr>
<td>Zymo Human Methylated &amp; Non-methylated DNA Set DNA / primers (D5014, D5014–1, D5014–2) D5014</td>
<td>5+5 µg/20+20 µL ($ 402</td>
<td>Methylated DNA HCT116 DKO cells DNA enzymatically methylated by C6g Methylase Non-Methylated DNA HCT116 DKO cells DNMT1 (/-) DNMT3b (-/-) &lt;5% of CpGs methylated</td>
<td>Recommends using hot-start DNA polymerases for amplification of bisulfite-treated DNA</td>
<td>goo.gl/7Z6L</td>
<td>40 33 16</td>
</tr>
</tbody>
</table>

1. Cost in U.S. Dollars from provider’s web page. 2. Weblink shortened using Google URL shortener service. 3. Number of citations found using Google Scholar. NA: No available information found online. †Qiagen portfolio of controls includes additional choices, however the mentioned 59695 is a set that includes the other references (59655, 59665, 59568).
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Methylation Specific PCR
Methylation specific PCR (MSP), first described by Herman et al. in 1996, determines the methylation status of an ROI through selective amplification of methylated and unmethylated alleles. The two-tube approach employs two primer sets: one binding specifically to the methylated sequence and another binding to the unmethylated sequence (11,41) (Figure 2C). A two-round variant of MSP, referred to as nested-MSP (N-MSP), has been described and can be used in special cases (42).

MSP is a simple method that requires resources commonly available in a molecular genetics laboratory and, once standardized, is effective for detecting methylated or unmethylated alleles without quantification. Processing up to 24 samples for both primer sets using conventional MSP requires about 4 h. Commercially available PCR master mixes for MSP (EpiTect MSP Kit, Qiagen, Hilden, Germany) are available; however, only conventional PCR reagents, including Hot-Start Taq polymerase, are required for the setup of MSP (43). While MSP assay kits are not commercially available, the MethPrimerDB database is available for help in selecting MSP primers (44).

Several real-time PCR adaptations of MSP also have been developed, including MethylQuant, a common option based on the measurement of increased fluorescence from SYBR Green I (45), and a real-time MSP approach combining conventional qPCR measurements with an additional melting step to detect amplicons associated with incomplete DNA conversion (46) or to distinguish the methylation status of individual alleles by comparison with standards of known allelic methylation status for an SNP located in the amplicon region (47).

Primer design considerations:
As described by Herman et al., both methylated and unmethylated MSP primer sets should be designed to anneal to the same CpG containing region. MSP primers should include abundant CpG sequences at the primer binding sites to provide maximal discrimination between the methylated and unmethylated alleles. For the same reason, these CpGs should be as close as possible to the 3’ region of the primer (11) (Figure 2C). Additionally, a high number of thymines derived from non-CpG cytosines should be included to ensure specificity for converted DNA. MSP primer design is facilitated by the software listed in Table 3.

Data analysis:
An amplification product of the correct molecular weight on an electrophoresis gel can be interpreted as methylated or unmethylated, depending on the specific primers used (11). The presence of amplification products using both sets of primers indicates a sample with both methylated and unmethylated DNA in the ROI (Figure 2D). However, a band from a reaction with methylated-specific primers might be a false positive. To avoid misinterpretation, inclusion of unmethylated DNA, non-converted DNA, and no-template negative controls is required (46,48). Likewise, the absence of an amplicon could be due to issues with the PCR reaction and must be controlled for as well (49).

The primary limitation of this technique is that it is qualitative (11). In general, well-standardized MSP assays provide information restricted to three possible outcomes: (i) presence of a methylated allele, (ii) presence of an unmethylated allele, or (iii) presence of both alleles. In assays intended to test MSP sensitivity, several ratios of the methylated and unmethylated DNA were used as templates. The results showed no clear correspondence between band intensity and dilution ratio, with many cases exhibiting very similar bands even for disparate levels of DNA methylation (50). On the other hand, several MSP assays demonstrated high sensitivity, detecting methylation percentages (MP) as low as 0.1% (50 pg of methylated DNA out of 50 ng of total DNA) or 1% (0.1 ng of methylated DNA out of 10 ng of total DNA) in different studies (11,43,50).

Possible challenges:
Low quality DNA is associated with a decrease in reproducibility (51). As mentioned above, it is critical to avoid amplification of non-converted DNA using MSP primers (11,52). Kristensen et al. identified false positive MSP results due to incomplete bisulfite-conversion, which is particularly problematic if only four or fewer non-CpG cytosines are included in the primer binding region (52). This issue has been associated with the apparent low reproducibility of numerous MSP assays (46,53). On the other hand, even after PCR amplification, MSP results can be validated by means of pyrosequencing to confirm the full conversion of every non-CpG cytosine (49). In MSP, PCR for methylated-specific or unmethylated-specific primer sets can frequently be standardized with non-identical PCR conditions (for example, different annealing temperatures) (11), possibly through inherent differences in sequence composition between primer sets. Therefore, identical PCR conditions for both MSP primer sets are not required for accuracy (11).

Real time PCR-based methods
MethyLight
Dual TaqMan labeled probes were developed for genotyping studies several years ago (54). Eads et al. subsequently introduced the use of TaqMan technology to determine DNA methylation status in specific genomic regions, a technique that was named MethyLight (55). Peter Laird’s group defined four types of MethyLight reactions, depending on which oligonucleotides are designed to discriminate the methylation status: (i) only the primers, (ii) only the TaqMan probe, (iii) both primers and probe, or (iv)...

Table 3. Freely available software for primer design for methylation analysis

<table>
<thead>
<tr>
<th>Tool</th>
<th>MSP / BSP design</th>
<th>Design for bias compensation</th>
<th>Primer thermodynamic evaluation</th>
<th>Additional advantage</th>
<th>Additional disadvantage</th>
<th>In silico PCR on bisulphite treated DNA</th>
<th>Link</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi-Search</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>Able to test the primer specificity</td>
<td>It does not inform the cause of primer rejection in in-silico PCR</td>
<td>yes</td>
<td>bisearch.enriim.hu</td>
</tr>
<tr>
<td>MethPrimer</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>Simple and flexible</td>
<td>No automatic function to search for primers reverse complementary to the input sequence</td>
<td>no</td>
<td><a href="http://www.urogenie.org/methprimer/">www.urogenie.org/methprimer/</a></td>
</tr>
<tr>
<td>Methyl Primer Express (Applied Biosystems)</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>Many customizable features</td>
<td>No very intuitive</td>
<td>no</td>
<td><a href="http://www.expbiosystems.com">www.expbiosystems.com</a></td>
</tr>
<tr>
<td>Perlprimer</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>Customable CpG island definition</td>
<td>Bugs on the copy-paste function</td>
<td>no</td>
<td>perlprimer.sourceforge.net/</td>
</tr>
<tr>
<td>MSPPrimer</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>Primer design with higher specificity Nested MSP design included</td>
<td>User registration is necessary</td>
<td>No</td>
<td><a href="http://www.mspprimer.org">www.mspprimer.org</a></td>
</tr>
</tbody>
</table>
Table 4. Multi-purpose software for DNA methylation test design and analysis of results.

<table>
<thead>
<tr>
<th>Tool</th>
<th>Method</th>
<th>Function</th>
<th>Web link</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioWord</td>
<td>Editing, replacing assistance</td>
<td>For all PCR-based DNA methylation techniques</td>
<td><a href="http://sourceforge.net/projects/bioword/">http://sourceforge.net/projects/bioword/</a></td>
</tr>
<tr>
<td>FastPCR†</td>
<td>In-silico analysis of several designed primers and probes</td>
<td>Bisulfite conversion of non-CpG cytosines</td>
<td><a href="http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm">www.biocenter.helsinki.fi/bi/programs/fastpcr.htm</a></td>
</tr>
<tr>
<td>MethGraph</td>
<td>Automatic genomic representation of the designed primers</td>
<td>In-silico evaluation of oligonucleotide sequence similarities to bisulfite modified genome sequences</td>
<td><a href="http://mefile.ugent.be/methgraph/">http://mefile.ugent.be/methgraph/</a></td>
</tr>
<tr>
<td>MethBlast</td>
<td>In-silico evaluation of oligonucleotide sequence similarities to bisulfite modified genome sequences</td>
<td>Generate possible assays for the supported experimental methods in the ROI</td>
<td><a href="http://methmarker.mpj-inf.mpg.de/">http://methmarker.mpj-inf.mpg.de/</a></td>
</tr>
<tr>
<td>MethMaker</td>
<td>Primer/probes design and primer evaluation</td>
<td>Primer design and primer evaluation</td>
<td><a href="http://www.premierbiosoft.com/molecular_beacons/index.html">www.premierbiosoft.com/molecular_beacons/index.html</a></td>
</tr>
<tr>
<td>Beacon Designer software‡</td>
<td>Primer/probes design and primer evaluation</td>
<td>Primer/probes design and primer evaluation</td>
<td><a href="http://www.premierbiosoft.com/molecular_beacons/index.html">www.premierbiosoft.com/molecular_beacons/index.html</a></td>
</tr>
<tr>
<td>ESME</td>
<td>Direct BSP</td>
<td>Analysis of results</td>
<td><a href="http://www.epigenome.org/index.php/page">www.epigenome.org/index.php/page</a> = download</td>
</tr>
<tr>
<td>BiQ Analyzer</td>
<td>Cloning-based BSP</td>
<td>Analysis of results</td>
<td><a href="http://biq-analyzer.bioinf.mpi-inf.mpg.de/download.php">http://biq-analyzer.bioinf.mpi-inf.mpg.de/download.php</a></td>
</tr>
<tr>
<td>Poland</td>
<td>MS-HRM</td>
<td>Tm Calculation for PCR amplicon</td>
<td><a href="http://www.biophys.uni-duesseldorf.de/local/Poland/poland.html">www.biophys.uni-duesseldorf.de/local/Poland/poland.html</a></td>
</tr>
<tr>
<td>OligoCalc</td>
<td></td>
<td></td>
<td><a href="http://www.basic.northwestern.edu/biotools/oligocalc.html">www.basic.northwestern.edu/biotools/oligocalc.html</a></td>
</tr>
</tbody>
</table>

The software displayed in this table are freely available online with two exceptions:
† Shareware with full functionality available only for a limited time.
‡ Licensed software, demo version with limited functionality.

Methylation-sensitive high resolution melting

In the DNA double helix, a cytosine and a guanine of complementary strands are linked by a triple hydrogen bond while a thymine and an adenine are joined by a double hydrogen bond (36). Therefore, base composition can directly influence the thermodynamic behavior of DNA in a melting analysis. Tm is defined as the temperature at which the PCR product dissociates into two single strands and a sharp drop in fluorescence of a DNA intercalating dye is observed (37). This basic principle can be used to discriminate between methylated and unmethylated alleles following bisulfite conversion. Distinction between alleles is achieved through Tm analysis of the MIP-PCR products in the ROI, in which the methylated allele usually has a higher Tm than the unmethylated allele (60) (Figure 3D).

Initially, Worm et al. [61] described an in-tube melting protocol for analyzing DNA methylation prior to the development of high resolution melting (HRM) technology. After technical improvements in melting assays, Wojdacz et al. developed a DNA associated with colorectal cancer (59). Here, the assay was focused on clinical applications of cancer detection. It should be noted that the cost of using TaqMan probes can be higher than other real-time PCR methods that utilize cheaper intercalating dyes (46). This is of particular importance if the sample size of the proposed study is large, or if a significant number of ROIs is to be assessed.

Example of MethyLight selection: MethyLight is the technique of choice when a study requires accurate quantitative assessment of DNA methylation. It has been used in a number of cancer associated DNA methylation studies, including the development of an assay to measure the presence of methylated alleles in three genes associated with colorectal cancer (59). Here, the assay was focused on clinical applications of cancer detection. It should be noted that the cost of using TaqMan probes can be higher than other real-time PCR methods that utilize cheaper intercalating dyes (46). This is of particular importance if the sample size of the proposed study is large, or if a significant number of ROIs is to be assessed.

Equation 1.

\[
\begin{align*}
\text{ROI signal of methylated status (Sample)} & - \text{Target ROI gene MethyLight STATUS (Fully methylated standard)} \\
\text{MIP (DNA input amount normalizing) (Sample)} & - \text{MIP input amount normalizing gene (Fully methylated standard)} \\
\end{align*}
\]

\[\times 100\]
methylated DNA. Non-converted DNA has the highest number of triple hydrogen bonds and therefore presents the highest melting peak. On bottom: schematic plot of the differences for the normalized signal of the standard curves. The plot also presents a curve for an illustrative sample that is located between the 10% and 20% standard dilution curves. (In this type of melting curve, 0% of methylation is used as the cluster of reference.) Conventions as in Figure 2.

**PCR bias:** As for other MIP based amplifications, potential PCR bias for MS-HRM was estimated during development of the technique (21). MS-HRM showed a strong amplification bias toward unmethylated sequences when the classic recommendations for primer design stated by Clark et al. were followed (25). In contrast, using the recommendations of Wojdacz et al. for primer design (21), variations of the annealing temperature in the PCR cycling step allowed for control of PCR bias (21). Monitoring of real-time PCR amplification establishes an additional quality control step for MS-HRM experiments (13). Similar to digital-BSP, digital MS-HRM is also useful for reducing PCR bias (62). Considering the possibility of PCR bias, it is important to highlight that quantitative methylation analysis with MS-HRM is based on the assumption that methylation levels of CpG sites between the primers is the same as methylation levels of CpG sites covered by the primers.

**Primer design considerations:** MS-HRM primer design follows the same general principles of classic MIP design as previously detailed by Clark et al. (25). However, in order to compensate for PCR bias, there are new recommendations for MS-HRM primer design that advise inclusion of one or two CpG annealing sites (located as far as possible from the 3' end of the primers to avoid methylation specific amplification) (Figure 3C) (60). Currently, there are no programs for MS-HRM primer design that incorporate the new recommendations to compensate for PCR bias. Finally, several programs such as OligoCalc, Poland, and MELT (Table 4) can predict the melting curves of the PCR products.

**Figure 3. Real time PCR assays: MethyLight and MS-HRM.** (A) Schematic lollipop graph of MethyLight subtypes. The most used approach consists of primers and probes designed for converted methylated DNA sequences and uses MIP primers to normalize the DNA input (top). Another choice is to design a set of primers and probes specific for methylated DNA and another set specific for non-methylated DNA (middle). In these cases, the sum of both signals is used to normalize the individual signals. Similar to classic MSP, one primer set is specific for methylated DNA and the other set for unmethylated DNA (bottom). One probe, designed to bind DNA independent from its methylation state, is used with both primer sets. The normalization procedure is similar to the one for the middle section. (B) Amplification curve of the most used MethyLight subtype (top A). The RFU value determination for 100% methylated control DNA allows calculation of the percentage of methylated molecules in the evaluated sample. The MIP signals allow an adequate control of DNA input amount for both the evaluated sample and 100% Methylated DNA. (C) Simplified outline of an MS-HRM amplicon for the analysis of methylation status. The triple hydrogen bond of G and C is represented. (D) On top: schematic graph of the negative first derivative of the melting-curve. DNA that is 0% methylated has a lower melting temperature peak in comparison to 100% methylated DNA. Non-converted DNA has the highest number of triple hydrogen bonds and therefore presents the highest melting peak. On bottom: schematic plot of the differences for the normalized signal of the standard curves. The plot also presents a curve for an illustrative sample that is located between the 10% and 20% standard dilution curves. (In this type of melting curve, 0% of methylation is used as the cluster of reference.) Conventions as in Figure 2.
Review

Table 5. Comparison of PCR-based DNA methylation techniques

<table>
<thead>
<tr>
<th>Basics</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct BSP</td>
<td>• MIP primers to amplify the region of interest (ROI) (for the population of cells that conform the sample) and sequencing</td>
<td>• Information of methylation at CpG resolution level</td>
</tr>
<tr>
<td></td>
<td>• MIP primers to amplify the ROI (for the population of cells that conform the sample), cloning and sequencing reaction for each clone</td>
<td>• One sequencing reaction for sample</td>
</tr>
<tr>
<td>Cloning-based BSP</td>
<td>• Information of methylation at CpG resolution level</td>
<td>• No PCR bias</td>
</tr>
<tr>
<td></td>
<td>• Information at single molecule level</td>
<td>• No cloning bias</td>
</tr>
<tr>
<td></td>
<td>• Reduced noise in the sequences reaction</td>
<td></td>
</tr>
<tr>
<td>Single molecule (digital) direct BSP</td>
<td>• MIP primers to amplify the ROI using serial dilutions of template to obtain single molecule PCR products</td>
<td>• Quantitative information of methylation at CpG resolution level</td>
</tr>
<tr>
<td></td>
<td>• Sequencing the positive amplifications to obtain a number of single molecule sequences</td>
<td>• Information at single molecule level</td>
</tr>
<tr>
<td></td>
<td>• Quantitative information of methylation at CpG resolution level</td>
<td>• Reduced noise in the sequences reaction</td>
</tr>
<tr>
<td></td>
<td>• No PCR bias</td>
<td>• No cloning bias</td>
</tr>
<tr>
<td>Classical MSP</td>
<td>• MSP primers, PCRs and electrophoresis</td>
<td>• High sensitivity</td>
</tr>
<tr>
<td></td>
<td>• CpG site at or near the 3´ primer allow amplification of only methylated or unmethylated DNA</td>
<td>• May be very cost-effective in settings where the objective is detection of any degree of methylation at primer binding region</td>
</tr>
<tr>
<td></td>
<td>• Stringent annealing temperatures to avoid amplification of unconverted DNA</td>
<td>• No sequencing reaction required</td>
</tr>
<tr>
<td>Real Time Based MSP</td>
<td>• Feasibility of a semiquantitative approach</td>
<td>• No quantitative (semiquantitative approach) and low specificity</td>
</tr>
<tr>
<td></td>
<td>• Very high sensitivity</td>
<td>• Two primer sets, each set used in one separated tube reaction (possibility of different amplification efficiencies)</td>
</tr>
<tr>
<td></td>
<td>• Information of methylation at primer binding region</td>
<td>• No information of methylation at CpG resolution level</td>
</tr>
<tr>
<td></td>
<td>• No sequencing reaction</td>
<td>• No information at single molecule level, unless using digital approach</td>
</tr>
<tr>
<td></td>
<td>• Methylation index $M_{M+U}$</td>
<td></td>
</tr>
<tr>
<td>MS-HRM</td>
<td>• MIP / primer set for compensation of PCR bias level</td>
<td>• No information of methylation at CpG resolution level</td>
</tr>
<tr>
<td></td>
<td>• One step PCR and HRM</td>
<td>• High sensitivity, a quantitative approach</td>
</tr>
<tr>
<td></td>
<td>• Information of methylation at regional level</td>
<td>• One primer set in a closed tube assay</td>
</tr>
<tr>
<td></td>
<td>• Immediate detection of false-positive amplification and PCR bias</td>
<td>• Information of methylation at CpG resolution level</td>
</tr>
<tr>
<td>Methylight</td>
<td>• Different kinds of Methylight approaches.</td>
<td>• Very high sensitivity</td>
</tr>
<tr>
<td></td>
<td>Refer to text for particular advantages and disadvantages of each kind of Methylight</td>
<td>• Closed tube assay</td>
</tr>
<tr>
<td></td>
<td>• Quantitative approach</td>
<td>• Quantitative information of methylation at CpG resolution level</td>
</tr>
</tbody>
</table>

Data Analysis: Wojdacz et al. (36,37) proposed a method for estimating methylation levels by comparing the melting patterns of standard templates with known proportions of methylated and unmethylated DNA controls to the melting patterns found in a sample. The semi-quantitative estimate is based on similarities in HRM patterns without a mathematical approach for calculating the DNA methylation percentage. More recently, Tse et al. (2011) implemented an MS-HRM approach to quantify the methylation status of each sample with high reproducibility. Peak-height and area under-the-curve from the normalized, temperature-shifted difference curves were used to generate linear standard curves (13) (Figure 3D). Quantitative data were obtained by interpolation of the first derivative of the normalized melt curves, generated by the linear regression analysis of the standard curve (13). When heterogeneous DNA methylation patterns are present in a sample, HRM analysis will identify such heterogeneity by the complex shape of the melting curves; however, in such cases quantitative HRM measurement is not possible (62). The presence of SNPs in the amplicon region could generate additional variations in the melting profiles (37).

Examples of MS-HRM selection: MSP-based assays only evaluate DNA methylation for CpG sites present in the primer binding region (usually <25 bp per primer). In contrast, MS-HRM evaluates all of the CpGs banked by the primers (usually >80 bp), regardless of the methylation status of CpGs within the primer binding site (36). Therefore, MS-HRM provides the ability to evaluate a larger genomic region when compared with MSP-related techniques (Figure 4) (11). MS-HRM is a good choice for quantitative determination of DNA methylation levels, when sequence level detail is not required (63). A good example of the usage of MS-HRM is a colorectal cancer study where the authors distinguished different stages of the disease and their correlations with the quantity of DNA methylation (64).

Proper controls for PCR-based DNA methylation analysis

In addition to the controls used in conventional PCR assays, other steps should be taken to verify the accuracy of DNA methylation data generated in PCR-based assays. Unconverted genomic DNA is an essential control that should be included in all optimization processes for PCR-based DNA methylation assays; it provides information on the amplification of
Figure 4. CpG coverage of PCR-based DNA methylation techniques. A simplified lollipop schema shows the CpG dinucleotides as circles. The methylation status is not represented. Grey circles represent the non-evaluated CpGs in each corresponding technique. Red circles represent the CpG dinucleotides evaluated in each method. In the MethyLight assay, the green oval represents the fluorescent molecule and the black oval represents the quencher molecule.

non-converted DNA with primers that are specific for the converted DNA. For specific assays, amplification from bisulfite-treated DNA should show a clear difference from any possible result using non-converted DNA.

In one of the pioneering MSP studies, Herman et al. verified primer specificity for the bisulfite modified p16 sequence using untreated DNA in reactions with either methylated-specific or unmethylated-specific primers (11). As expected, no amplification was found when non-converted DNA was used as a template. Nonetheless, several reports of MSP standardization did not include or report this kind of control (65,66).

Similarly, the use of non-converted DNA is also recommended when using the MS-HRM technique during assay optimization (37). This type of control is the easiest to include but, paradoxically, is the control most commonly omitted or not reported (63). It allows experimental verification of the specificity of the assay for converted DNA. In most cases, there should be no amplification products; however, in some instances products will be amplified that can be easily identified when compared with the converted DNA (37).

Use of fully methylated and unmethylated DNA is a critical experimental control as well. It should be noted that DNA considered to be fully unmethylated comes from a variety of different sources. The practice of using DNA obtained from peripheral blood mononuclear cells (PBMC) as a fully unmethylated DNA control is valid in cases where the samples are indeed completely unmethylated at the loci of interest. Several reports have focused on detecting DNA methylation status in peripheral blood, showing biologically important methylation levels for multiple genes (52,67). For example, low level methylation of many cancer-relevant genes may be found in the PBMCs from normal individuals. Therefore, the indiscriminate use of DNA from PBMCs as a negative control in sensitive assays for DNA methylation detection may be particularly problematic (52).

Manufacturers of commercially available DNA controls have different strategies for providing fully methylated and unmethylated DNA. For example, fully non-methylated kits from Zymo and Millipore use DNA from cells that contain genetic knockouts of 2 key DNA methyltransferases, thus reducing methylation levels by more than 95% (68).

Fully methylated DNA can be obtained from M.SssI-methylated DNA from, among many sources, double knockout cells for DNMT1 and DNMT3b (Table 2). Another alternative is to use the product from whole genome amplification (WGA) with kits such as REPLI-g (Qiagen), which does not reproduce the DNA methylation pattern and has a theoretical methylation level of less than 10^-6. However, this amplification approach may carry the risk of reduced representation of the loci of interest (69). Therefore, the use of identical amounts of methylated and unmethylated controls derived from the same class of template (genomic DNA or WGA-products) could guarantee that equivalent amounts of effective templates are included.

Discussion

Studying DNA methylation for a candidate ROI using PCR-based methods is a topic of present and future importance. There are many advantages of genome-wide platforms; however, PCR-based techniques permit detailed analysis of specific regions of the genome, including CpG island shores. In addition, the associated costs of implementing and executing PCR-based techniques are lower, allowing the initial study of several candidate ROIs. PCR-based approaches also offer the advantage of a lower burden of false discoveries and the ability to confirm a large number of ROIs identified in genome-wide screening of a few samples (70).

DNA methylation analysis using pyrosequencing is a quantitative approach that does not require a cloning step, but presents the risk of PCR bias, similar to BSP. More importantly, pyrosequencing instrumentation is not commonly available in a general laboratory. For interested readers, comparisons and discussions of pyrosequencing techniques are available elsewhere (71).

This article highlights several considerations for PCR-based DNA methylation studies. The approaches reviewed here have different advantages and disadvantages that should be evaluated before starting any DNA methylation study. Similarly, it is clear that the different PCR-based techniques discussed here have differences in CpG coverage and possibility for quantitation (Figure 4). A comparison of all PCR-based DNA methylation techniques is presented in Table 5.

Several considerations concerning PCR-based methods for DNA methylation analysis will be crucial for the consolidation of the field of molecular epigenetics. Current needs in this field include (i) detailed experimental comparisons of results obtained with different PCR-based techniques (72), (ii) the availability of a large number of predesigned PCR-based DNA methylation assays to facilitate broad use, (iii) the implementation of minimum reporting guide-
lines for manuscripts describing results of PCR-based analyses of DNA methylation, including details of experimental conditions such as controls, primer sequences, and programs used for primer design (73), (iv) the further development of additional PCR-based techniques that allow DNA methylation measurements in a more quantitative and reproducible way (5), and (e) the implementation of automatic and multiplexed protocols for DNA methylation using currently available techniques to improve efficiency and reduce costs (59).

For readers interested in genome-wide DNA methylation analysis, we recommend two available review articles (74,75). Finally, it is critical to keep in mind that the results of PCR-based DNA methylation methodologies are reliable only in an experimental setting with adequate methodological controls.

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Competing interests.
The authors declare no competing interests.

References

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