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ORIGINAL ARTICLE

Region of interest methylation analysis: a comparison of MSP with MS-HRM and direct BSP

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Abstract The aim of this study was to compare and contrast three DNA methylation methods of a specific region of interest (ROI): methylation-specific PCR (MSP), methylation-sensitive high resolution melting (MS-HRM) and direct bisulfite sequencing (BSP). The methylation of a CpG area in the promoter region of Estrogen receptor alpha (*ESR1*) was evaluated by these three methods with samples and standards of different methylation percentages. MSP data were neither reproducible nor sensitive, and the assay was not specific due to non-specific binding of primers. MS-HRM was highly reproducible and a step forward into categorizing the methylation status of the samples as percent ranges. Direct BSP was the most informative method regarding methylation percentage of each CpG site. Though not perfect, it was reproducible and sensitive. We recommend the use of either method depending on the research question and target amplicon, and provided that the designed primers and expected amplicons are within recommendations. If the research question targets a limited number of CpG sites and simple yes/no results are enough, MSP may be attempted. For short amplicons that are crowded with CpG sites and of single melting domain, MS-HRM may be the method of choice though it only indicates the overall methylation percentage of the entire amplicon. Although the assay is highly reproducible, being semi-quantitative makes it of lesser interest to study ROI methylation of samples with little methylation differences. Direct BSP is a step forward as it gives information about the methylation percentage at each CpG site.

Keywords DNA methylation · ROI · MSP · MS-HRM · BSP

Introduction

Epigenetics is the study of alterations of the genome leading to changes in gene expression without affecting the basic nucleotide sequence. It includes histone modification, microRNA, and DNA methylation [1, 2]. DNA methylation takes place on carbon atom number five of cytosine residues in a CpG dinucleotide context. CpG islands (CGIs) are mainly located in the promoter region and in the first exon of many genes, yet they can also be found in repetitive genomic sequences and introns [3, 4]. Hypermethylated CGIs in repetitive genomic sequences are believed to have a critical role in maintaining chromosomal stability and integrity, while methylation of cytosines in promoter regions is implicated in dramatically repressing or even silencing the expression of downstream genes [4]. Gene silencing is either mediated through recruitment of repressor factors to the methylated site, or through blocking the binding of transcription factors to the promoter. For instance, when normally hypomethylated promoters

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become hypermethylated or vice versa, affected genes are downregulated or upregulated respectively, leading to abnormal phenotypes [5].

The role of DNA methylation in pathophysiology of diseases has been of major interest in the past decade. For example in cancerous cells, hypermethylation of tumor suppressor gene promoters, hypomethylation of oncogene promoters and the overall global hypomethylation of repetitive elements downregulates the normal expression of DNA repair and cell cycle checkpoint machineries, upregulates expression of growth factors, and diminishes the stability of chromosomes respectively [2, 4, 6]. In addition, DNA methylation changes evaluated in neurodegenerative disorders, autoimmune diseases, and atherosclerosis among others, enhanced our understanding of the origin and development of these pathologies [7, 8]. The emerging significance of DNA methylation in the pathophysiology of diseases and its role as a potential biomarker for early diagnosis and prognosis and to predict pharmacological outcomes, underscores the need for efficient, sensitive and specific methods to study this important regulatory process [4, 9].

DNA methylation measurement can be broadly divided into two groups: DNA methylation of a region of interest (ROI) and whole genome methylation. Whole genome methylation is the study of methylation status of all or major subsets of CpG sites present in the DNA using complex techniques of which the newest is next generation sequencing [10]. In this article, our focus is on three low throughput methods used in the evaluation of DNA methylation of a specific ROI: methylation-specific PCR (MSP), methylation-sensitive high resolution melting (MS-HRM) and direct bisulfite sequencing (BSP).

MSP is a technique whereby two primer pairs are used to specifically amplify either methylated (M) or unmethylated (U) alleles of a certain ROI after bisulfite conversion [11]. MSP primers are designed to contain CpG Cs and several non-CpG Cs. CpG Cs are meant to differentiate methylated from unmethylated alleles, while non-CpG Cs that are transformed to Ts by bisulfite conversion ensure primer specificity to bisulfite converted DNA. The second method we used, MS-HRM, is a relatively new technique that utilizes bisulfite conversion and real-time PCR to distinguish between methylated and unmethylated cytosines [12]. In the DNA double helix, a C binds to a G in the complementary strand by a triple hydrogen bond, while a T binds to an A with a double bond. The energy needed to melt a triple bond is greater than that needed for a double bond. This thermodynamic difference allows the discrimination between a C-rich-methylated-sequence and a T-rich-unmethylated-sequence after bisulfite conversion. Therefore, a sample clustering analysis can be performed by comparing the melting temperatures to standards of known methylation percentage. As for direct BSP, it is performed by Sanger sequencing of PCR products generated by primers that amplify the bisulfite converted ROI regardless of its methylation. The resulting electropherograms show a C peak for a methylated CpG site, a T peak for an unmethylated CpG site, and a Y peak for a partially methylated site. The percentage of C versus T in the latter can then be computed using readily available softwares or algorithms.

The aim of this study was to compare and contrast the advantages and disadvantages of these three techniques in evaluating a CpG area in the promoter region of *Estrogen receptor alpha* (*ERα* or *ESR1*) gene. Despite the presence of good reviews tackling this issue and advances in higher throughput techniques [3, 13], we believe that our contribution would help many low operation laboratories in optimizing, designing, and analyzing methylation results.

Methods

Choice of *ESR1* ROI

ESR1 gene NCBI reference sequence

NC_000006.11 (http://www.ncbi.nlm.nih.gov/nuccore/NC_000006.11) CGIs were identified using the Methyl Primer Express® Software v1.0 (Applied Biosystems, CA, USA). The gene turned out to have three CGIs, one of which starts in the promoter, 91 bp upstream of the transcription starting site, and extends into exon 1. Primer sets were designed to cover that area because it is located in the promoter of two *ESR1* transcript variants and its methylation status might hence influence the transcription of these variants. The CpGs covered by each of the three methods are shown in Fig. 1.

DNA isolation and bisulfite conversion

DNA was isolated from 300 ml of peripheral blood taken from healthy donors. The kit used for DNA isolation was Flexigene DNA kit (Qiagen, CA, USA). The quantity and quality of DNA were evaluated using nanodrop. 260/280 ratio of the samples used were 1.7 and above. One microgram of DNA was then bisulfite converted using Cell-to-CpG bisulfite conversion kit (Applied Biosystems, CA, USA). Bisulfite conversion is a process that includes a set of chemical reactions leading to transformation of unmethylated cytosines to uracil while methylated cytosines are not altered. A uracil is replaced by a thymine residue during PCR. This technique allows differentiation between methylated and unmethylated Cs by the three methods.

The protocol followed was that recommended by the manufacturer. Briefly, DNA was denatured by means of denaturation buffer and incubation at 50 °C. Next, the

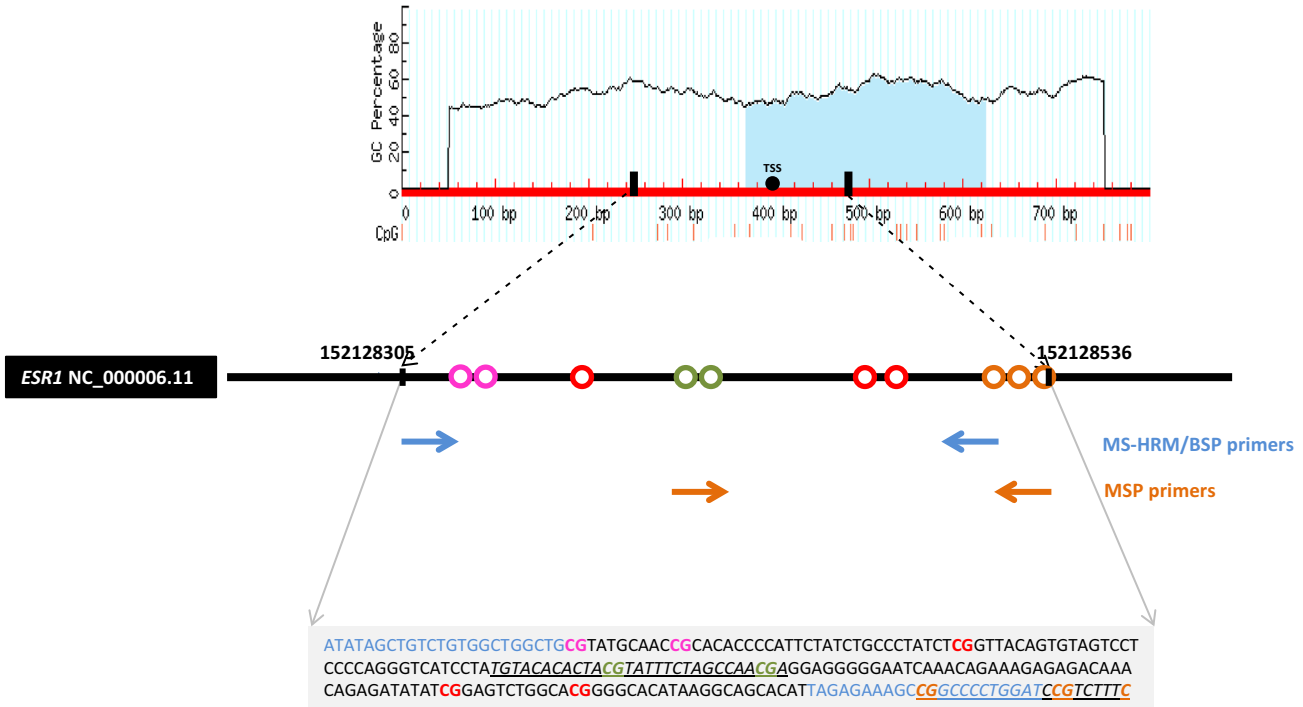


Fig. 1 *ESR1* sequence and CpG sites scanned by each of the three methods MSP, MS-HRM and Sanger sequencing. The *upper figure* shows CpG density of a region in *ESR1* gene (±400 bp from transcription start site-TSS) (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). *Blue arrows* indicate MS-HRM and sequencing primer annealing sites, while *orange arrows* indicate MSP primer annealing sites. *Pink circles* indicate CpGs scanned by MS-HRM only, *orange circles* indicate CpGs scanned by MSP only, *red circles* indicate CpGs scanned by both MS-HRM and BSP, and *green circles* indicate CpGs scanned by MSP, MS-HRM and BSP. CpGs at the beginning of the sequenced amplicon were not included in the BSP analysis, since sequencing is of low quality at the periphery of the amplicon. In the genomic sequence, MS-HRM and sequencing primer annealing sites are *highlighted in blue font*, while MSP primer annealing sites are *underlined*. CpGs in *pink font* were scanned by MS-HRM only, CpGs in *orange font* were scanned by MSP only, CpGs in *red font* were scanned by both HRM and sequencing, and CpGs in *green font* were scanned by the three methods: MSP, MS-HRM and sequencing. (Color figure online)

conversion reagent was added and the samples were incubated in a thermal cycler according to the following protocol: 65 °C for 30 min, 95 °C for 1.5 min, then 65 °C for 30 min and 95 °C for 1.5 min, and a final step of 65 °C for 30 min. After that, desulfonation was done and the converted DNA was finally washed, eluted and stored at −20 °C until used.

Controls

Cells-to-CpG™ methylated and unmethylated gDNA Control Kit (Applied Biosystems, CA, USA) was used to prepare standards of known methylation status. Totally methylated standard DNA (100%) and totally unmethylated standard DNA (0%) were bisulfite converted using the bisulfite conversion kit as described above. Different mixes of 0 and 100% were used to prepare 5, 10, 25, 50 and 75% standards. Non-converted genomic DNA was also used to make sure that primers amplify converted DNA only. A No-Template-Control (NTC) was included in all PCRs.

Methylation-specific PCR (MSP)

We used the two primer pairs shown in Table 1 to amplify the *ESR1* promoter ROI. These were designed using MethPrimer algorithm (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) and purchased from TibMolBiol (Germany). They both included Ts corresponding to non-CpGs in order to prevent amplification of non-bisulfite converted DNA, and CpG site(s) to allow specific amplification of methylated and unmethylated alleles by methylated and unmethylated primers, respectively. The reaction mix contained 1× MeltDoctor HRM Master Mix (Applied Biosystems, CA, USA), 0.3 µM of either M or U primer pairs, 3 µL of converted DNA, and sterile nuclease-free water to a total of 25 µL (Amresco, OH, USA). Although other and cheaper master mixes can be used for MSP, we used MeltDoctor HRM Master Mix to minimize variability among the three methods. PCR was done using the following protocol as described previously [14]: denaturation step at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, annealing temperature (T_{ann}) 55 °C for the methylated

Table 1 Sequences of the primers used for amplification of *ESR1* promoter region for MSP, MS-HRM and direct BSP

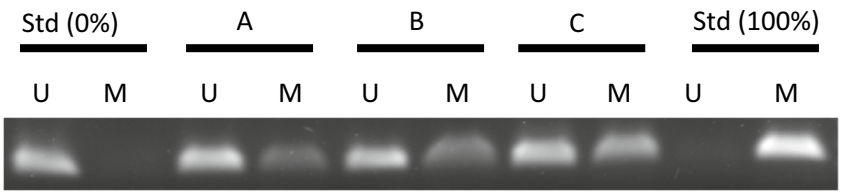
Primer description	Primer sequence	PCR product size (bp)	Melting point (°C)
MSP			
Left methylated primer	5′ TGTATATATTACGTATTTTTAGTTAACGA 3′	136	50.5
Right methylated primer	5′ GAAAAACGAATCCAAAAACCG 3′		56.9
Left unmethylated primer	5′ TGTATATATTATGTATTTTTAGTTAATGA 3′	137	46.2
Right unmethylated primer	5′ CAAAAAACAAATCCAAAAACCAAC 3′		57.6
HRM and direct BSP			
Left primer	5′ ATATAGTTGTTTGTGGTTGGTTG 3′	222	52.6
Right primer	5′ ATCCAAAAACCRACTTTCTCTA 3′		48.7

primer set and 58 °C for the unmethylated primer set for 30 s, and 72 °C for 30 s, then, a final elongation step of 72 °C for 10 min. PCR was first attempted with a range of T_{ann}s, and we selected those that resulted with the most specific PCR bands on gel electrophoresis as shown in Supplementary Fig. 1. The PCR product was visualized on a 1% agarose gel under UV-light using Gel Doc machine (BioRad, CA, USA). Samples were repeated at least twice on different days for validation, and 0 and 100% standards were used to ensure specific amplification (Supplementary Fig. 2). Although not recommended because of previously described poor reliability [3], we used Image Lab software (BioRad, CA, USA) to estimate band intensities and compare methylated and unmethylated allele frequencies.

Methylation-specific high resolution melting (MS-HRM)

Primers were designed for *ESR1* promoter ROI using Methyl Primer Express® Software v1.0 (Applied Biosystems, CA, USA). These amplify the ROI regardless of its methylation status and bind to a minimal number of CpG sites. Similarly to MSP, MS-HRM primers were designed to include Ts corresponding to non-CpGs in order to prevent amplification of non-bisulfite converted DNA. Methylation independent amplification was ascertained by the exclusion of CpG site(s) at the 3′ end. One CpG site was included at the 5′ end of the primers and replaced with a mixed base instead of C (Y for C/T and R for G/A) to

(a)



(b)

Primer pair	Standard	Expected	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	CV[a] (%)
			Methylation (%)						
M	**0**	0	0.3	59.5	46.6	2.4	69.4	0	109.01
U		100	74.2	85.6	0.6	82.9	73.9	85.1	49.17
M	**100**	100	0.2	85.9	0.5	85.6	87.8	87.1	77.00
U		0	9.5	38.4	29.5	2.2	0	0	125.40

a.Coefficient of variation

Fig. 2 Methylation specific PCR (MSP) results. **a** Agarose gel showing amplicons from unmethylated (*U*) and methylated (*M*) primer pairs of *ESR1* gene promoter in samples and standards. The percent methylation of samples *A*, *B* and *C* are based on results from bisulfite sequencing PCR (BSP). **b** Comparison of methylation results of *ESR1* gene promoter using Image Lab software of standards performed on six different days

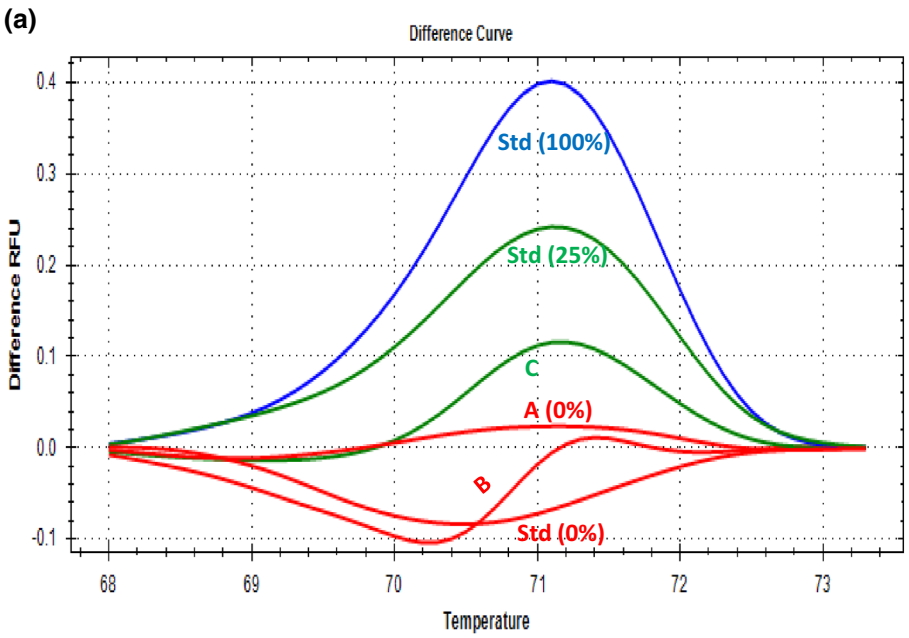


Fig. 3 Methylation sensitive high-resolution melting (MS-HRM) results. **a** Difference *curves* for normalized signals of *ESR1* gene promoter in samples and standards using Precision Melt software. The percent methylation of samples *A, B* and *C* are based on results from bisulfite sequencing PCR (BSP). **b** Comparison of methylation results of *ESR1* gene promoter of serial mixtures[a] of standards performed on 5 different days

(b)

Standard (%)	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	CV[b](%)
	Metling temperature					
0	70.2	70.2	70.2	70	70.2	0.13
2	70.2	70.2	70.2	70	70.2	0.13
5	70.2	70.2	70.2	70	70.2	0.13
10	70.4	70.4	70.4	70.2	70.2	0.15
25	70.6	71.4	71.4	71.2	70.4	0.66
50	71.6	71.6	71.4	71.4	71.6	0.15
75	71.6	71.6	71.4	71.4	71.6	0.15
100	71.6	71.6	71.4	71.4	71.6	0.15

a. Different shades of gray highlight groups of methylated standards that result into the same melting temperature
b. Coefficient of variation

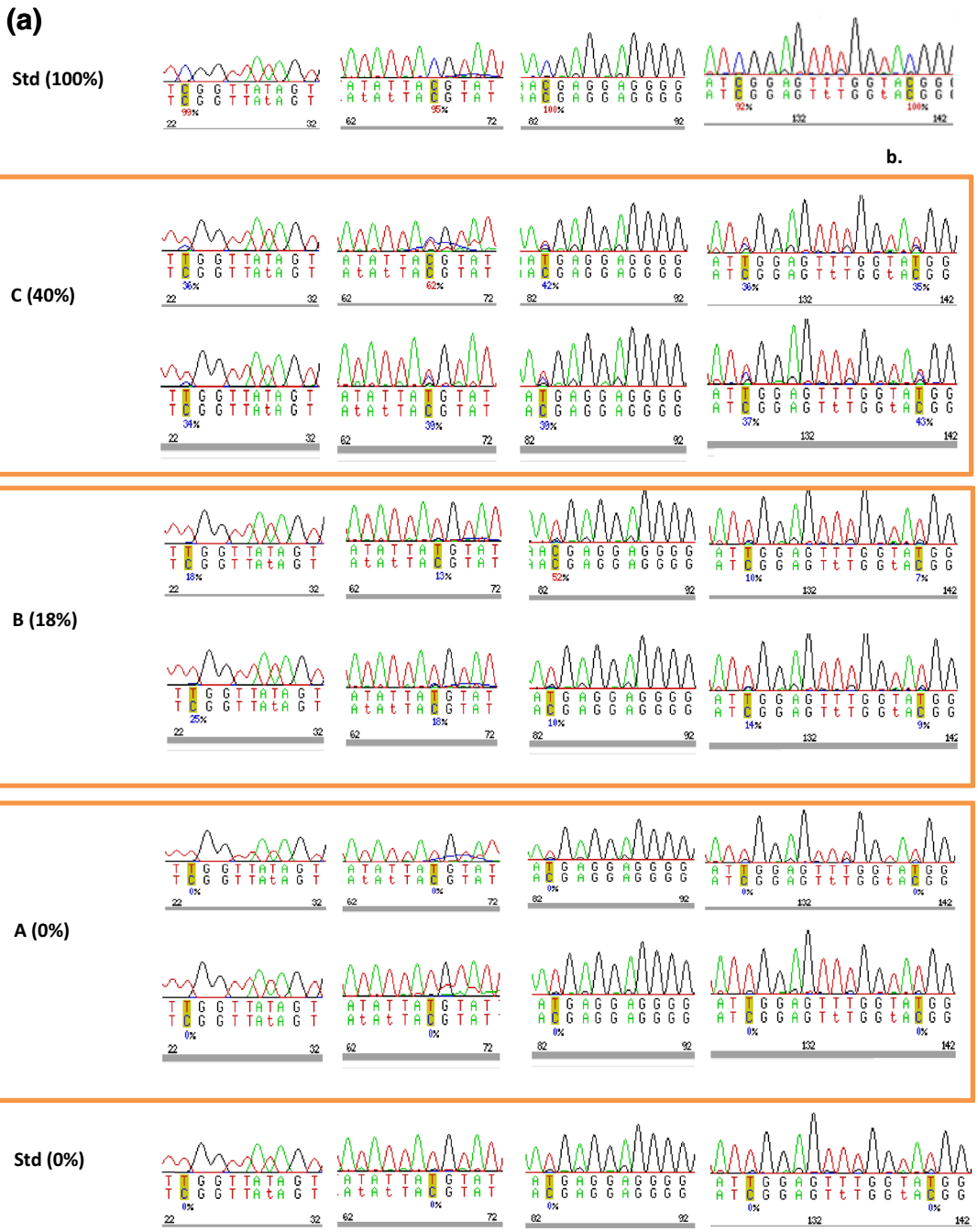
correct for the limited sequence input of the evaluated ROI. We checked for the presence of 1 melting domain in the expected PCR product using POLAND software (http://www.biophys.uni-duesseldorf.de/html/local/POLAND//poland.html). Four primer pairs were initially tested, and the primer pair that produced a specific amplicon on gel electrophoresis was chosen for the rest of the experiments.

PCR reaction mixtures were prepared in the same concentrations and final volume such as with MSP. Real-time PCR and melting analysis were done on a CFX96 real time PCR instrument (BioRad, CA, USA) according to the following protocol: denaturation of the double helix at 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and T_{ann} 61 °C for 1 min. Then the melting curve was generated as follows: 95 °C for 10 s, 61 °C for 1 min, 61 °C for 15 s up to 95 °C with a 0.2 °C increment. Finally, reassembly of the dsDNA was performed by incubation at 61 °C for 15 s. A range of T_{ann}s were first attempted, and we selected the one that resulted in the highest discrimination at low methylation percentages because we expected the peripheral blood samples that we were testing to be hypomethylated (i.e. <50% methylation) (Supplementary Fig. 3) [15]. Samples and standards were tested in duplicates, and those with a standard deviation (SD) >0 were repeated. Methylation analysis was done using the CFX Manager v3.1 and Precision Melt software v1.2 (BioRad, Ca, USA). 96-well plates containing PCR products were preserved at 4 °C for BSP analysis.

Direct bisulfite sequencing PCR (BSP)

PCR products of the MS-HRM experiment were extracted from 1% agarose gel using PeqGOLD DNA Extraction kit (PeQlab, Germany) after cutting the bands from the gel under UV-light as recommended by the manufacturer. Then, 10–14 µL of purified PCR product were sent with

(a)



(b)

	Methylation (%)											
	Standard (0%)						Standard (100%)					
Position	23	68	83	128	140	Average of the 5 positions	23	68	83	128	140	Average of the 5 positions
Assay 1	3	6	0	0	0	1.8	98	94	100	91	94	95.4
Assay 2	0	0	0	0	0	0	74	95	100	89	87	89
Assay 3	0	0	0	0	0	0	99	95	100	92	100	97.2
CV[a](%)						173.2[b]						4.6

a.Coefficient of variation

b. The gray color highlights the 2 methylations % that were measured on day 1 and resulted into a high CV for the 0% standard.

◂**Fig. 4** Bisulfite Sequencing PCR (BSP) results. **a** Sanger sequencing showing methylation percentages of 5 CpG sites of *ESR1* gene promoter in samples with repeats and standards. **b**. Comparison of methylation results of *ESR1* gene promoter of standards performed on three different days at five different positions covered by the primer pair

0.625 µM left primer for sequencing on a Sanger sequencer [16]. About 180 bp including 5 CpG sites were successfully sequenced. Raw sequence data were visualized using Finch TV v1.4.0 software (http://en.freedownloadmanager.org/Windows-PC/FinchTV-FREE.html) and analyzed using Epigenetic Sequencing Methylation (ESME) 3.2.5 software (http://www.epigenome.org/index.php?page=download). This software aligns the sequenced product with the original DNA sequence after in silico bisulfite conversion. The algorithm then compares the heights of C and T peaks at each CpG site and computes the percentage of methylated alleles according to the height of the C peak [17].

Statistical analyses

Data were entered on excel and comparison of methylation results of known standards by different assays within each method was performed by calculating the coefficients of variation (CV) among band intensities, melting temperatures, and % methylation as applicable. Pearson correlation analysis was also performed.

Results

MSP

Figure 2a shows the PCR products of the methylated and unmethylated alleles of *ESR1* promoter for 3 samples A, B and C, in addition to known standards: totally methylated (100%) and totally unmethylated DNA (0%). As expected, methylated primer (M) generated a PCR product with 100% standard, while unmethylated primer (U) gave a product with 0% standard only (Assay 6 from Fig. 2b). Nevertheless, this was not always the case as bands many times appeared with both primer pairs and standards although no bands appeared with the non-converted DNA, suggesting poor primer specificity (Fig. 2b, Supplementary Fig. 2). In addition, the assay was not sensitive in differentiating differential methylation of samples. This is apparent with sample A whereby a band appeared with the methylated primer pair though it was shown to be 0% methylated by BSP. Nevertheless, there was a trend for known hypermethylated samples by BSP to show brighter bands with the M primers when compared to hypo or non-methylated samples as per BSP. The assay was also non-reproducible as shown by the high CV values (Fig. 2b). Note that an additional primer pair was evaluated and gave similar non-specific and non-reproducible results.

MS-HRM

Figure 3a shows the difference curves of three samples A, B, and C and the serial mixtures of standards generated using the Precision melt software. Melt peaks and normalized difference curves are provided in Supplementary Fig. 4. Although MS-HRM optimization showed almost perfect results at the chosen T_{ann} (Supplementary Fig. 3), the curves of samples A (0% methylation by BSP) and B (18% methylation by BSP) both clustered with the 0% standard, while sample C (40% methylation by BSP) clustered with the 25% standard. Besides, groups of serial mixtures of methylated standards (for example 50% and above) resulted into the same melting temperature, and were often shown in the same cluster (example: assays 1, 3 and 5) (Fig. 3b), hence the decreased sensitivity of this assay. Nevertheless, and as shown in Fig. 3b, the assay was highly reproducible with consistently low CV values, and there was a very good correlation between the melting temperatures of the standards within (see repeats in Supplementary Fig. 5) and among PCR plates (for example for assay 1 vs. assay 2: $R^2 = 0.8395$ $P < 0.001$).

Direct BSP

Direct BSP analysis resulted into quantitative data that were as sensitive and reproducible as MS-HRM. For instance, and as shown in Fig. 4a that was generated by ESME software after entering the sequenced data (top sequence) and the reference sequence (bottom sequence), results of the 0 and 100% standards were almost perfect, whereby Cs were completely converted to Ts in the 0% unmethylated standard while Cs of CpGs remained unchanged in the 100% methylated standard (Assay 3 of Fig. 4b). Importantly, no Cs appeared in the normalized sequence trace, thus asserting complete bisulfite conversion.

The method was highly reproducible as shown with the low CV of the 100% methylated standard. Although the CV of the 0% methylated standard was high, this was driven by 2 sites in assay 1 that were slightly higher than zero and hence markedly affected the CV. In addition, reproducibility analysis with the average of methylation at the 5CpG positions showed a statistically significant correlation R of 0.813 ($P < 0.001$) among 32 repeats of peripheral blood samples. Similar results were shown with each individual position (data not shown). Despite these good results, it is important to note that sensitivity was less than perfect. For instance, and as shown with sample repeats (Fig. 4a) and

different assays of known standards (Fig. 4b), the % methylation values were not exactly the same.

Discussion

In the present study, we compare three relatively straightforward, efficient, and cost effective methods performed to evaluate the methylation status of a certain ROI (Table 2). MSP failed to attain any of the quality measures; for instance, data generated by this method were not reproducible, though we were very diligent in managing all variables that might affect the data (DNA concentration, primer concentration, PCR protocol, PCR instrument used…). Moreover, the assay was neither specific nor sensitive due to non-specific binding of primers, a problem that could have been tackled by including more CpGs to the 3′ end of the primers. Moreover, the right unmethylated primer included more than 5 consecutive A nucleotide repeats which may have affected the PCR efficiency. This is in addition to the limitation that only CpGs within the primer binding site are evaluated. MS-HRM was a step forward into categorizing the methylation status of the samples as percent ranges. Using this method, we succeeded to differentiate among un-, moderate, and hypermethylation status, but failed to generate quantitative results and to calculate exact methylation percentages. Though the assay was highly reproducible, being semi-quantitative makes it of lesser interest to study samples with insignificant differences in methylation percentages. It can however be very useful in evaluating methylation differences between samples with large differences in % methylation as shown in the comparative analysis of *ESR1* promoter ROI methylation of the ER negative breast cancer cell line (MDA-MB-231) with the ER positive breast cancer cell line (MCF-7) (Supplementary Fig. 6). Direct BSP was as sensitive and reproducible as MS-HRM, but most informative regarding methylation percentage of each CpG site. ESME software increased confidence in direct BSP data and produced the quantitative aspect of this technique even though this software suffers from few disadvantages. For instance, it is not very user-friendly and needs professional guidance to be installed and function. In addition, only "clean" sequences that do not contain mutations and/or background noise and that are adequately bisulfite-converted can be analyzed.

Each of these three methods can be enhanced to give better results. For MSP assay, designing primers that span a higher coverage of CpG sites upsurges the specificity of the assay. Yet this may not be applicable to many ROIs in the genome among which the ROI assessed in this study due to the low number of CpGs present in the CGI of interest. In addition, and in order to evaluate methylation of all CpGs within the chosen ROI, several primers that span the whole sequence can be designed, yet this is laborious and needs a lot of DNA [14]. A variant of MSP is melting curve analysis MSP (MCA-MSP). This method though has similar limitations to MSP, its sensitivity is improved due to the direct assessment of the amplified product by melting curve analysis rather than gel electrophoresis visualization [19]. Another variant of MSP is the quantitative MSP (qMSP) technique that uses, in addition to a combination of MSP and SYBR green-based technologies, serial mixtures of methylated and unmethylated standards and an internal standard with no CpG sites such as β-actin. Methylation values are calculated using the standard curves for unmethylated and methylated primer pairs, and results are corrected for DNA input with the internal standard. To make the results easier for clinical interpretation, fully methylated and unmethylated DNA portions can be calculated as percentages of methylated reference (PMR) and percentages of unmethylated reference (PUR) [19].

Regarding MS-HRM, both amplicon size and CpG density within the amplicon are important for adequate analysis. In our case, the amplicon was of single melting domain, but the melting curves of unmethylated amplicons often clustered with those of hypomethylated amplicons (of <10% methylation). This might be overcome by reducing the amplicon size and/or including more CpGs in the amplicon. The number of CpGs recommended for successful MS-HRM analysis is amplicon-dependent. The ideal CpG number allows discrimination between the melting profiles of methylated and unmethylated strands while resulting in amplicons of single melting domain and unambiguous melting curves. Prediction of the amplicon melting domains and melting profiles of the methylated and unmethylated strand by available softwares such as POLAND can help in the selection of ROI to be analyzed by MS-HRM. In addition, designing primers that include 1 or 2 CpG sites at the 5′ end was shown to increase MS-HRM sensitivity to methylated amplicons and thereby decrease the PCR bias towards unmethylated ones [15]. So far, and to our knowledge, the available softwares for MS-HRM primer design, such as Methyl Primer Express® Software v1.0 and the freely available MethPrimer 2.0 (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), design primers that do not contain any CpG sites within their sequences so that both methylated and unmethylated DNA are amplified during PCR. However, if CpGs are unavoidable because of a limited sequence input, the software may add a mixed base at the CpG site within the primer sequence (Y for C/T in left primer or R for G/A in right primer) as shown in our right primer. Nevertheless, the guidelines adopted by the software do not correct for the PCR bias towards unmethylated templates. Besides, it does not take the expected product into consideration, it may hence generate primers that amplify amplicons with

Table 2 Comparison of the three evaluated ROI methylation methods

	Methylation-specific PCR	Methylation specific-high resolution melting	Direct bisulfite sequencing PCR
Cost	Low	Moderate	Higher
Turnaround time	6 h	5 h	8 h
Technique basis	Basic molecular techniques (PCR, gel electrophoresis)	Real time PCR requires optimization of annealing temperature to increase sensitivity at low or high methylation ranges	Basic molecular techniques (PCR, gel electrophoresis, extraction of PCR product from gel, and sequencing)
Analysis software	Visual analysis of bands • Straightforward	CFX manager and Precision melt softwares • Straightforward • User-friendly • Data can be exported to excel	ESME software • Needs Linux operating system • Command line tool should be started from a terminal • Data can be exported to excel
DNA amount needed	75 ng × 2	75 ng	25 ng
CpG sites assessed	Within primers only	Within and between primers	Between primers
Amplicon length[a]	• Optimal: 200 bp • Range: 100–300 bp	• Optimal: 100 bp • Range: 100–300 bp	• Optimal: 200 bp • Range: 100–300 bp
Number of CpGs	• Three or more, located at the 3′ end of primers	• Up to 16 • Use POLAND software to check for the presence of a single melting domain and a marked difference in melting properties between methylated and unmethylated amplicons	• No specifications
Results	• Qualitative • Yes or no • Assumes all CpGs exhibit uniform methylation	• Semi-quantitative • Categorizes into % methylation ranges compared to standards	• Quantitative • % Methylation of each individual CpG in the PCR amplicon is evaluated
Specificity	• Diminished specificity	• Not applicable • Primers amplify ROI regardless of its methylation status	• Not applicable • Primers amplify ROI regardless of its methylation status
Sensitivity	• Not sensitive • Unable to discriminate different ranges of methylation percentages	• More sensitive though not perfect • Amplicons with different methylation percentages can show same melting temperatures	• More sensitive though not perfect • Gives the percentages of methylated and unmethylated alleles for tested samples at each CpG site
Reproducibility	Not reproducible	Reproducible	Reproducible

[a]Amplicon length should not exceed 400 bp due to potential DNA degradation during bisulfite conversion

multiple melting domains that cannot be properly analyzed by MS-HRM (see Supplementary Fig. 7 for an example). Therefore, we feel that it is essential to develop a new software that is specifically tailored for MS-HRM primer design. In addition, MS-HRM analysis depends on the melting curve generated by the real time PCR instrument whereby the lower the temperature increment, the higher the resolution. Due to instrument limitation, we were unable to decrease the increment below 0.2 °C in drawing the melting curves [15]. Finally, among the most described difficulties in generating useful quantitative MS-HRM data are heterogeneous methylation and multiple melting domains [20, 21]. Heterogeneously methylated templates yield complex melting curves that are hard to interpret. The complexity is due to heteroduplexes amplified during PCR caused by the presence of different alleles with different methylation status at each CpG site. This can be addressed by digital MS-HRM that is an MS-HRM variant method, in which the sample is diluted in such a way that each duplex of the amplified sample produces a signal, and the sum of the signals is computed allowing the calculation of overall methylation percentage [20].

As for direct-BSP, although the selected ROI was successfully analyzed in standards and samples, it is important to note that this may not always be the case for amplicons with different genomic features. For instance, amplicons with GT stretches may lead to formation of secondary structures that stop the sequencing reaction abruptly. This can be prevented by the addition of DMSO to the sequencing reaction (Supplementary Fig. 8.1). Besides, amplicons with poly T sequences (i.e. greater than eight consecutive Ts) may result in slippage of DNA polymerase leading to a shift in the sequencing reaction and eventually increasing the sequencing errors following the poly T region. Amplification of the reverse complementary strand of the bisulfite modified amplicon may solve this problem (Supplementary Fig. 8.2). Cloning-based BSP can be used as an alternative to direct-BSP in order to improve sensitivity and signal quality of sequencing results, and reduce cytosine-signal artifacts and background noise that affect electropherograms and analysis of direct BSP data [3]. In this method, PCR products of the ROI are cloned into vectors and transformed into competent *E. coli* bacterial cells. Plasmids are expanded, then purified from the bacteria to be sequenced. This method allows determination of allele-specific methylation patterns and percentage of methylation in the ROI, but a minimum number of 10–20 clones is required to detect interindividual variabilities in DNA methylation. Hence, it is expensive, labor and time-intensive, and not feasible for population-based studies. Besides, there are several sources of errors in the clone sequencing results such as bias from multiple copies of the same sequence, errors in reading homopolymer regions, and incomplete bisulfite conversion [22]. Finally, pyrosequencing can be used as an alternative for bisulfite sequencing of ROI. It is far less time consuming than cloning-based sequencing and more sensitive since it generates more accurate reads with each run as compared to cloning-based sequencing that relies on the number of reads performed. However, pyrosequencing data may suffer from some sources of errors secondary to poor DNA quality, low template availability, and the presence of homopolymer regions or secondary structures. In addition, a pyrosequencer instrument may not be available in all laboratories [22].

In conclusion, and based on our results, we recommend the use of either method depending on the research question and target amplicon, though each of the three discussed methods can be enhanced such as by performing MCA-MSP, qMSP, digital MS-HRM, and cloning-based BSP. High throughput methods are also commercially available such as next generation bisulfite sequencing and array-based assays, which provide a comprehensive analysis of the methylation status across the genome. If the research question targets a limited number of CpG sites and simple yes/no results are enough, MSP may be attempted. For short amplicons that are crowded with CpG sites and of single melting domain, MS-HRM may be the method of choice though it only indicates the overall methylation percentage of the entire amplicon. Although the assay is highly reproducible, being semi-quantitative makes it of lesser interest to study ROI methylation of samples with little methylation differences. Direct BSP is a step forward as it gives information about the methylation percentage at each CpG site.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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