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A refined DNA methylation detection method using MspJI coupled quantitative PCR

Christopher J. Petell^a, Gilbert Loiseau^d, Ryan Gandy^a, Sriharsa Pradhan^c, and Humaira Gowher^{a,b,*}

^aDepartment of Biochemistry, Purdue University, West Lafayette, IN 47907, United States

^bPurdue University Center for Cancer Research, Purdue University, West Lafayette, IN 47907, United States

^cNew England Biolabs Inc., 240 County Road, Ipswich, MA 01938, United States

^dCollege of William and Mary, Williamsburg, VA 23187, United States

Abstract

DNA methylation is a highly conserved epigenetic modification with critical roles ranging from protection against phage infection in bacteria to the regulation of gene expression in mammals. DNA methylation at specific sequences can be measured by using methylation dependent or sensitive restriction enzymes coupled to semi- or quantitative PCR (MD-qPCR). This study reports a refined MD-qPCR method for detecting gain or loss of DNA methylation at specific sites through the specific use of MspJI or *HpaII*, respectively. By employing varying concentrations of DNA with methylation ranging from 0 to 100%, our data provide evidence that compared to *HpaII*, MspJI increases the sensitivity and accuracy of detecting relative DNA methylation gains by MD-qPCR. We also show that the MspJI-coupled MD-qPCR can accurately determine the percent gain in DNA methylation at the *SalI4* enhancer and is more sensitive than *HpaII* in detecting relative gains in DNA methylation at the *Oct4* proximal enhancer during embryonic stem cell (ESC) differentiation. The high specificity and sensitivity of this targeted approach increases its potential as a diagnostic tool to detect relatively smaller gains in DNA methylation at specific sites from limited amounts of sample.

Keywords

MspJI; *HpaII*; MD-qPCR; Restriction enzymes; DNA methylation; Pluripotency gene enhancers

Introduction

DNA methylation takes place at the N4 and C5 position of cytosine and N6 of adenine in a sequence specific manner. In bacteria, DNA methylation is catalyzed by methyltransferases

*Corresponding author. Department of Biochemistry, Purdue University, West Lafayette, IN, 47907, United States. hgowher@purdue.edu (H. Gowher).

Contributions

CJP, GL, and RG performed the experiments. CJP, SP, and HG analyzed the data and CJP and HG wrote the manuscript.

in the restriction-modification (R-M) system that usually recognize sequences equal to or longer than four base pairs. In mammals, DNA methylation occurs largely at the CpG dinucleotide and is maintained post replication [1,2]. DNA methylation has diverse biological functions ranging from serving as a defense mechanism in bacteria to the regulation of gene expression and repression of transposable elements in higher eukaryotes [3]. In multicellular organisms, the maintenance of DNA methylation patterns, particularly at the regulatory elements of cell-type specific genes, is critical for cell identity and homeostasis [4,5]. This is substantiated by evidence that aberrant DNA methylation causes repression of tumor suppressor genes, activation of oncogenes, and genome instability in cancer [6–8]. The mechanism/s by which DNA methylation effects gene expression are not completely understood; however, in many cases the presence of methylated cytosine at binding sequences of transcription factors in the regulatory elements of genes can preclude their binding and affect gene regulation [9,10]. Therefore, an accurate determination of the changes in DNA methylation at specific loci between normal and diseased states has the potential to be used as a biomarker.

Considerable effort has led to the development of various methods with improved speed and accuracy for measuring DNA methylation in its sequence context. The gold standard method for DNA methylation measurement is sodium bisulfite conversion. In this method, sodium bisulfite reacts with unmethylated cytosines, and converts them to uracil, whereas the methylated cytosines are not affected [11,12]. Combined with whole genome sequencing, bisulfite conversion provides a high-resolution map of the DNA methylation at a single cytosine level for the entire genome. Methylation-dependent restriction (MDR) employs the R-M systems of bacteria to detect DNA methylation. In this method, the methylation-sensitive restriction enzyme, such as *HpaII* or *HhaI*, cleaves the unmethylated DNA that is detected as a change in the DNA length by Southern Blot or PCR [13–20]. Hybrid methods, such as Combined Bisulfite Restriction Analysis (COBRA) are used to detect methylation by scoring for the loss of restriction enzyme sites such as *TaqI* and *BsAI* after bisulfite conversion of DNA [21]. These hybrid techniques allow for the quantification of lower amounts of DNA methylation at a specific site of the genome, but are tedious and require long sample preparation time.

Gain or loss of DNA methylation at specific genomic sites can be examined by bisulfite conversion or more conveniently by MD-PCR. Due to its easy sample preparation, MD-PCR affords a simple and readily accessible means of determining the changes in DNA methylation at specific sites across multiple samples [22,23]. This can be particularly improved when combined with quantitative-PCR [24–28]. The most popular enzymes used in MD-PCR, such as *HhaI* and *HpaII*, are blocked by DNA methylation at their recognition sites. Therefore, use of these enzymes to quantify gain of methylation by PCR amplification is potentially limited by the degree of methylation. This is because a small increase in methylation may result in a minor increase in the template concentration, which may not be enough to cause a detectable change in the PCR reaction. The McrBC and Mrr-like family of restriction enzymes that in contrast specifically cleave at sequences containing a methylated cytosine can potentially circumvent this issue [29,30]. To test this experimentally, we performed a comparative study with *HpaII* and *MspJI* enzymes as tools to detect gain of

DNA methylation at specific sites. MspJI is a member of the Mrr-like family of restriction enzymes.

Our data show that compared to *HpaII*, using MspJI to measure DNA methylation increases the detection limit of MD-qPCR by more than a factor of four. This difference between *HpaII* and MspJI was more prominent at limiting template concentrations. Bisulfite sequencing showed that a unique CpG site in the *SalI4* enhancer gained up to 75% DNA methylation during ESC differentiation. This gain in DNA methylation at the *SalI4* enhancer was accurately measured by MD-qPCR with MspJI when compared to a known standard. We further show that as the result of its specificity, MspJI is better suited to detect DNA methylation at *Oct4* proximal enhancer compared to the *HpaII* during ESC differentiation. Together, our data support the use of MspJI in MD-qPCR as the enzyme of choice for quantification of small increases in DNA methylation at specific sites in the genome. This is particularly useful for detection of DNA methylation at CpG sites that are present in regions with lower CpG content, such as enhancers and tissue specific promoters and may influence the binding of specific transcription factors in the vicinity resulting in the gain or loss of gene expression.

Materials and methods

DNA methylation and restriction of substrates

A 100 bp region of the *SalI4* enhancer containing one *HpaII* recognition site (5'-CCGG-3') was amplified by PCR from mouse genomic DNA and used as a substrate for all the assays. Methylation of the substrate DNA was carried out by M. *HpaII* (1 U enzyme per ug DNA) using 100 μ M AdoMet (S-Adenosylmethionine) overnight. The DNA methylation of the fragment was tested by treating it with *HpaII* or MspJI restriction enzymes overnight and visualizing the cleaved product on 9% TBE-PAGE (Fig. 2A). The expected fragmentation pattern is illustrated in Fig. S1. The near absence of cleaved fragments in the *HpaII* reaction and loss of the intact DNA substrate in the MspJI reaction ensured the DNA methylation of the substrate. The unmethylated control DNA was incubated in buffer without enzyme and treated similarly for downstream processing. The substrates were purified using a PCR purification kit (Qiagen). The 100% methylated or unmethylated (0% methylated) substrates were mixed together to generate a constant concentration of methylated DNA ranging from 0% to 100% methylation. The mixtures were split equally, and restriction digests were carried out for 2 h or overnight at 37 °C using 100 nM substrate and 1 U enzyme (*HpaII*, MspJI and a no enzyme control) in 1x CutSmart buffer supplied by the manufacturer (NEB). Care was taken to ensure that the enzyme:DNA ratio was within the published range of activity for MspJI and well below the concentration at which off-target activity can occur [31]. The use of purified DNA substrates facilitated an accurate quantification and therefore an appropriate use of the enzymes in these reactions. For MspJI, use of the supplied activator was necessary to make sure that the reaction proceeded to completion. Restriction digestion for 2 h with MspJI may result in partial cleavage, yielding a double stranded break on only one side of the CpG site, which does not interfere with the detection of methylation by qPCR using primers that flank the CpG site.

MD-qPCR for genomic targets

Genomic DNA from undifferentiated ESCs and from cells 3–9 days post differentiation purified using a standard phenol:chloroform isolation was digested (10 µg) overnight at 25 °C with 40 U of *Cvi*QI restriction enzyme (NEB, R0639L), which cuts outside the region of interest. After phenol:chloroform extraction, these samples were subjected to a second round of cleavage by *Msp*JI (20 U) overnight at 37 °C in the presence of the supplied activator. Purified DNA was quantified by PicoGreen according to the manufacturer's protocol (Life Technologies, P11495) using the NanoDrop 3300 fluorospectrometer (Thermo Scientific). The primers used are listed in Table S1.

Quantitative PCR

Equal amounts of cleaved and uncleaved control DNA were amplified by qPCR, using the qPCR master mix EvaGreen according to the manufacturer's conditions (MidSci, BEQPCR-S). 20–22 bp primers were synthesized by IDT (Integrated DNA Technologies) using standard desalting, which allows 95–98% purity for short oligonucleotides. Briefly, in a 15 µL reaction volume, 100 nM primers were mixed with template (variable concentration) and 1X EvaGreen master mix. The qPCR cycling program was: 1. 95 °C, 10 min; 40 cycles of 95 °C, 15 s and 60 °C, 1 min. The measured C_q value is defined as the quantification cycle based on the MIQE guidelines and is calculated by the Biorad CFX Manager 3.1 [32]. A change in DNA methylation is represented by normalized change in the C_q values as follows:

$$\Delta C_q(\text{Sample } X) = C_q(\text{Sample } X)_{HpaII \text{ or } MspJI} - C_q(\text{Sample } X)_{Control} \quad (1)$$

$$\Delta \Delta C_q(MspJI) = \Delta C_q(\text{Sample } X)_{MspJI} - \Delta C_q(0\% \text{ Methylation})_{MspJI} \quad (2a)$$

$$\Delta \Delta C_q(HpaII) = \Delta C_q(\text{Sample } X)_{HpaII} - \Delta C_q(100\% \text{ Methylation})_{HpaII} \quad (2b)$$

$$\text{Normalized } C_q(\text{Sample } X) = C_q(\text{Sample } X)_{HpaII \text{ or } MspJI} - [\text{Peak } C_q - C_q(\text{Sample } X)]_{Control}$$

(3)

For these equations, *Sample X* refers to one concentration of DNA with methylation between 0% and 100% and *Control* refers to the DNA treated with no enzyme or DNA cleaved by *Cvi*QI only. Note that for Equations (2a) and (2b), to determine the change in DNA methylation using *Msp*JI and *Hpa*II, the data are normalized to the C_q of the substrate with 0% and 100% methylation, respectively. To control for loading errors, the C_q

values (*Sample X*) obtained for control samples with varying DNA methylation but same concentration were subtracted from the highest C_q (*Peak C_q*) value obtained in the range. This is done for the ease of representing the data as positive numbers. The primers used are listed in Table S1.

Statistical analysis

For each figure, data are shown as the average and standard deviation of at least two independent experiments; each replicate has at least four technical replicates. Graphpad Prism 6 was used to analyze the data. The values were fit using linear regression of a non-linear fit from which the slope and standard deviation were determined. Statistical analysis for significance was computed by using the paired Student's *t*-test.

Bisulfite conversion method

At a unique CpG site in the *Sal14* and Oct4 enhancer, the gain of DNA methylation during ESC differentiation was measured using bisulfite sequencing. Genomic DNA was purified from undifferentiated ESCs and cells 5 and 9 days post differentiation. Bisulfite sequencing was performed using the EpiTect Fast Bisulfite Conversion Kit (Qiagen, 59802) using 1 µg of genomic DNA. Bisulfite-converted DNA was amplified using nested primers and Taq polymerase (NEB, M0267L). Briefly, 150 ng of converted gDNA template, 400 nM primers, 200 nM dNTPs, and 2.5 U of Taq polymerase were mixed in a 50 µL PCR reaction. The program for the outer PCR was: 1. 94 °C, 4 min; 2. 55 °C, 2 min; 3. 72 °C, 2 min; 4. Go to 1, 1x; 5. 94 °C, 1 min; 6. 55 °C, 2 min; 7. 72 °C, 2 min; 8. Go to 5, 34x; 9. 72 °C, 7 min; 10. 4 °C hold. The setup for the inner PCR was identical except that the template DNA consisted of 2 µL from the outer PCR. The program for the inner PCR was: 1. 94 °C, 2 min; 2. 94 °C, 1 min; 3. 55 °C, 2 min; 4. 72 °C, 2 min; 5. Go to 2, 34x; 6. 72 °C, 7 min; 7. 4 °C hold (Tremblay et al., 1997). Products from the inner PCR were gel purified (Qiagen, 28704) and used as a template for subsequent PCRs to generate a library for high throughput sequencing. The primers used are listed in Table S1.

Library preparation for multiplex sequencing

Bisulfite sequencing was performed to detect DNA methylation changes between undifferentiated and differentiated cells (ESC, D5, D9). We used multiplex high-throughput sequencing to determine these changes. After bisulfite treatment and PCR, the target site amplicons from ESC, D5 and D9 samples were tagged by a unique identifying index sequence at the 5' and 3' ends that was compatible with the Illumina MiSeq platform. We designed a protocol to index the samples based on the TruSeq primer construction, where an 8 bp sequence served as the unique identifier for each sample (Fig. S3). After bisulfite treatment and sample amplification by inner PCR, a short linker sequence and internal TruSeq index primer binding sites were added to the DNA through a 5-cycle PCR. Briefly, 50 ng DNA, 400 nM primers, 200 nM dNTPs, and 2.5 U of Taq polymerase were mixed in a 50 µL PCR reaction. PCR was carried out as follows: 1. 94 °C, 2 min; 2. 94 °C, 1 min; 3. 55 °C, 2 min; 4. 72 °C, 2 min; 5. Go to 2, 4x; 6. 72 °C, 7 min; 7. 4 °C hold. Using 6 µL of the first PCR reaction as template, a second round of a 5-cycle PCR added an 8 bp unique identifier sequence and a TruSeq i5 (forward) or i7 (reverse) single index sequence (Fig. S3). The unique identifier sequence was used to discriminate and sort the sequencing data for

various samples (ESC, D5, D9) for analysis. The program for the indexing PCR was identical to that of the adaptor PCR. Indexed amplicons for each sample were purified and pooled equally to a final concentration of 10 nM. An Illumina MiSeq 500-cycle run generated paired-end 250-base reads. The reads were then mapped using Bowtie2 and analyzed by Bismark for DNA methylation. The unique identifier was used to assign the reads to specific samples. Instances of methylated and unmethylated CpG were quantified, and the average percent methylation for each sample was calculated with the standard deviation. The primers used are listed in Table S1.

Cell culture and differentiation

E14Tg2A ESCs were maintained in media containing LIF and induced to differentiate by LIF withdrawal followed by retinoic acid addition as described [33,34].

Results

Detection range of MD-qPCR using MspJI and HpaII restriction enzymes

The restriction enzyme MspJI cleaves DNA asymmetrically approximately 15 bp from the 3' end of the recognition site, 5'-^mCNNR(N)₉-3'/3'-GNNY(N)₁₃-5', when the cytosine in its recognition site is methylated [30,31,35,36]. The presence of DNA methylation can be detected by MD-PCR or MD-qPCR using enzymes such as *HpaII* and *HhaI* that cut DNA when their recognition sites are unmethylated. Therefore, in a mixed population with a small proportion of methylated DNA, the larger fraction of DNA that is unmethylated is cleaved by the restriction enzymes. This results in the depletion of the amount of template available for amplification. The delay in amplification is caused by the lag phase in the exponential curve of the PCR or qPCR, limiting the detection of low percentage of methylated DNA. When coupled with amplification of the target site by quantitative PCR, low or no methylation results in high C_q values, which may be at the upper limit of the detection range thus decreasing the accuracy of the measurement [37,38]. On the other hand, MspJI cleaves when cytosine in its recognition sequence is methylated leaving unmethylated DNA intact. This overcomes the limitation posed by the lag phase of qPCR when *HpaII* or *HhaI* are used in these assays. Therefore, when coupled to qPCR, low or no methylation results in low C_q, thus widening the detection range of the measurement. A comparative gain in DNA methylation would therefore be detected as an increase in C_q value (Fig. 1).

A schematic representation of MD-qPCR showing the effect of the restriction enzyme used to detect methylation on its amplification by qPCR. A) Open and closed circles represent unmethylated and methylated CpGs respectively on the substrate DNA and the adjacent arrows represent primer binding sites for PCR. Whereas MspJI cleaves the methylated DNA, *HpaII* cleaves unmethylated DNA. B) Illustration of the PCR amplification curves representing decreasing template concentrations. C_q, quantitation cycle; RFU, relative fluorescence units. Cleavage by MspJI reduces the template concentration with increasing DNA methylation and vice versa for *HpaII* cleavage.

We compared the ability of MspJI and *HpaII* to detect DNA methylation using a 100 bp DNA amplified from mouse *SalI4* enhancer as substrate. The PCR-derived substrate contains

one CpG site in a *HpaII* recognition sequence (5'-CCGG-3') which was methylated *in vitro* using *M. HpaII* methyltransferase. The completion of DNA methylation was tested by resistance to cleavage by *HpaII* and susceptibility to *MspJI* restriction enzymes (Fig. 2A). The expected fragmentation pattern is illustrated in Fig. S1. For the MD-qPCR assay, methylated and unmethylated substrates were cleaved by *HpaII* or *MspJI* restriction enzymes, or a no enzyme control (Fig. 2B). Next, qPCR was performed using the cleaved substrate at concentrations ranging from 100 pM to 1 fM (calculated for MW of the intact substrate). The no template control was used to define the background C_q value (23.5 ± 0.5). For the unmethylated substrate, the C_q values for *HpaII*-cleaved DNA were 2 cycles higher than for the *MspJI*-cleaved substrate and no enzyme controls (Fig. 2B, left). Conversely, for the 100% methylated substrate, the C_q values were 2 cycles higher for *MspJI*-cleaved DNA than the *HpaII* cleaved DNA and no enzyme controls (Fig. 2B, right). This is consistent with the substrate specificity of each endonuclease. We were able to detect a linear change in C_q for template concentrations ranging from 100 pM to 10 fM, after which the C_q value approached that of the no template control (Fig. 2B). Using these data, we determined the differences in the linearity of *HpaII* or *MspJI* coupled MD-qPCR for DNA substrates with varying amounts of DNA methylation.

Detection of varying DNA methylation by *HpaII* and *MspJI* in MD-qPCR

We mixed unmethylated and methylated substrate at varying ratios, ranging from 0% to 100% methylated substrate at constant concentration. This was followed by restriction cleavage by either *HpaII* or *MspJI* or the no enzyme control. To examine the linearity of the qPCR for *HpaII* or *MspJI*-cleaved substrates we used a substrate concentration of 100 fM in MD-qPCR. This concentration is at the high end of the range of genomic DNA concentrations (1–100 fM) typically used to quantify single targets in qPCRs [38]. The changes in C_q calculated as ΔC_q (Equations (1) and (2); see methods) were plotted as a function of percent DNA methylation. The data for both *HpaII* and *MspJI* fit well to linear regression ($R^2 = 0.96$ and $R^2 = 0.97$, respectively) suggesting that both *HpaII* and *MspJI* can detect changes in DNA methylation by MD-qPCR when the target DNA is at high concentrations (Fig. 2C).

A) Representative PAGE showing completion of DNA methylation of the substrate. Briefly, 100 nM unmethylated and fully methylated DNA was cleaved with 1 U of *HpaII* or *MspJI* enzymes overnight as described in the Methods section. M represents the DNA maker. The expected fragmentation pattern is shown in Fig. S1. B) Methylated and unmethylated DNA cleaved with *HpaII* or *MspJI* were serially diluted for qPCR. The C_q values were plotted against the concentration of DNA and fit to linear regression. For both substrates, the slope is linear for DNA concentrations ranging from 10 pM to ~10 fM, after which linearity was lost. C) Change in C_q values were plotted as a function of percent DNA methylation of substrate cleaved by *HpaII* or *MspJI* using 100 fM DNA for qPCR. ΔC_q was calculated by normalizing the C_q for cleaved DNA with those derived from the no enzyme control and to C_q values for 0% and 100% methylation for *MspJI* and *HpaII* respectively (Equations (1) and (2) in methods). Averages $\pm$ the standard deviation are shown ($n = 8$).

Effect of HpaII and MspJI on the sensitivity of MD-qPCR

We predicted that MD-qPCR using MspJI would be more sensitive than using *HpaII* in detecting lower percentage of DNA methylation under limiting DNA concentrations. To test this, we used 0–40% methylated substrate for restriction cleavage by MspJI and *HpaII*. We performed two fold serial dilutions of the cleaved substrate ranging from 100 fM to 3 fM for MD-qPCR. The C_q values normalized to the no enzyme control (Equation (3)), were plotted vs the percent methylation for each substrate dilution (Fig. 3A and Fig. S2). The slopes of the linear regression were plotted against the substrate concentration. The data show that for the *HpaII* treated substrate, the magnitude of the slope approaches zero at a higher concentration of DNA (12 fM) compared to that for the substrate treated with MspJI (Fig. 3B).

Together, these data suggest that when coupled with qPCR, MspJI is better than *HpaII* in detecting DNA methylation gains under limiting concentrations of DNA. This data supports a refined method of detecting gain or loss of DNA methylation at specific sites using MD-qPCR through an alternative use of MspJI or *HpaII*, respectively.

A) 0–40% methylated DNA was cleaved with *HpaII* or MspJI and qPCR was carried out at the DNA concentrations of 100 fM, 50 fM, 25 fM, 12 fM, and 3 fM. Normalized C_q values (Equation (3)) were plotted as a function of percent DNA methylation and fit to linear regression (Plots for 50 fM, 3 fM, and are shown in Fig. S2). The magnitude of the slope (m) represents the change in C_q for DNA substrates from 0% to 40% methylation. B) The values of the slopes (m) for all concentrations were plotted against the DNA concentration of the substrate cleaved with *HpaII* or MspJI. We performed a paired Student's *t*-test of the slopes compared to that of the 100 fM of the substrate; * represents a p-value less than 0.05. The m values approach zero and show a large variability at relatively higher concentrations for the *HpaII* cleaved DNA when compared to the MspJI cleaved DNA. Averages $\pm$ the standard deviation are shown (n = 8).

Detection of the temporal gain in genomic DNA methylation by MspJI-coupled MD-qPCR

We compared the MspJI coupled MD-qPCR to bisulfite sequencing in measurement of the temporal gain of DNA methylation at specific genomic sites. We examined an increase in DNA methylation at the *Sal14* enhancer site, which was previously used for our *in vitro* assays. *Sal14* is a pluripotency gene that is expressed in ESCs and repressed upon induction of differentiation (Boland et al., 2014). This is accompanied by the gain of DNA methylation at its enhancer element [34]. We therefore used the *Sal14* enhancer as a model site to measure DNA methylation of *in vivo* targets. To accomplish this, ESCs were induced to differentiate by LIF withdrawal and addition of retinoic acid (Fig. 4A). Genomic DNA was harvested from undifferentiated (ESC) and differentiated cells at different time points post differentiation (D5, D9). We performed bisulfite sequencing of the ~400 bp *Sal14* enhancer region to determine the temporal gain of DNA methylation at the CpG target site during ESC differentiation. Bisulfite treated DNA from various samples were processed for multiplex sequencing on a MiSeq Illumina high throughput platform (Fig. S3; for details see methods). Based on the number of reads, the percent methylation for all the samples could be determined with high confidence (ESCs: 55,809, D5: 95,646 reads, and D9: 75,894).

DNA methylation at the target CpG site on the *SalI4* enhancer increases during differentiation and was measured at 50% on D5 and 75% on D9 post differentiation (Fig. 4B).

We next examined the gain in DNA methylation using MD-qPCR, and to minimize biological variability, the same genomic DNA preparation was used for both MD-qPCR and bisulfite sequencing assays. Genomic DNA from undifferentiated (ESC) and differentiated cells (D5, D9) was sequentially treated with *Cvi*QI and *Msp*JI restriction enzymes (Fig. 4C). *Cvi*QI recognition sites (5'-GTAC-3') flank the target CpG site and therefore generates DNA fragments in a size range of 0.3–1 kbp thus improving the template quality for qPCR. For MD-qPCR, we first tried a small dilution series of genomic DNA template to determine the concentration that gives the C_q in the range of ~22–24 for both *Msp*JI and *Hpa*II cleaved DNA. Based on our results in Fig. 2B, this range of the C_q are expected to give reliable measurement of DNA methylation. 6 ng (2000 copies) of genomic DNA from ESCs, D5 and D9 post-differentiated cells cleaved with *Hpa*II or *Msp*JI was used as template for qPCR. Changes in C_q were calculated as a proxy for DNA methylation, which was calculated as

C_q (Equation (1); see methods). For both *Msp*JI and *Hpa*II, an increase in C_q was observed which corresponds to an increase in DNA methylation (Fig. 4D). The determined values of C_q , 0.4, 1.1 and 1.67 for 20%, 50% and 75% percent DNA methylation respectively (Fig. 2C) closely match the C_q values of 0.4, 1.05, 1.74 corresponding to the percent methylation gain of 20%, 50% and 75% for ESC, D5 and D9 respectively detected by the bisulfite sequencing at this site. However, for *Hpa*II cleaved DNA, the C_q values of 1.3, 0.6, 0.05 for ESC, D5 and D9 respectively corresponding to 40%, ~75% and ~95% methylation from Fig. 2C. Comparing these estimates to the measurements obtained from the bisulphite sequencing, there is more than 20% error in the detection of DNA methylation by *Hpa*II-coupled MD-qPCR. This data confirm that MD-qPCR using *Msp*JI can reliably detect dynamic gains in DNA methylation at *in vivo* targets and can be used to measure changes in DNA methylation at single sites with the use of appropriate standards.

Compared to *Hpa*II, which cuts at CpG dinucleotides in a 4 bp recognition site, *Msp*JI recognizes nearly all methylated cytosines, because it is more promiscuous in its specificity. In regulatory regions of genome with low to intermediate CpG content, *Hpa*II sites are rare. Given, DNA methylation at various CpG sites is stochastic, methylation at the *Hpa*II sites may not represent the earliest methylation events in these regions. We tested this by measuring the DNA methylation in a region of the *Oct4* proximal enhancer containing 3 CpG sites one of which is a *Hpa*II recognition site. During ESC differentiation, the enhancer of the pluripotency gene *Oct4* is methylated [33]. We used *Hpa*II- and *Msp*JI-coupled MD-qPCR to examine the change in DNA methylation in ESCs, D5 and D9 post differentiation. Fold change in C_q represent change in DNA methylation from undifferentiated (ESC) to differentiated state. Whereas for *Msp*JI-coupled MD-qPCR the fold change in C_q values shows a sequential increase during differentiation, there was no significant fold change for the *Hpa*II-coupled MD-qPCR (Fig. 4E). We determined the methylation level of each CpG in the *Oct4* proximal enhancer using bisulfite sequencing. The data show that the *Hpa*II site gains a maximum of 25% DNA methylation by D9 post differentiation (Fig. 4F). This methylation level is too low to be detected by *Hpa*II-coupled MD-qPCR. The other two CpG sites, which gain up to 85% methylation (Fig. 4F), can be recognized by *Msp*JI, thus easily

reported by the MD-qPCR. These data strongly support MspJI as an enzyme of choice to detect DNA methylation at low CpG content regions.

A) Schematic representation of ESC differentiation by LIF withdrawal and addition of retinoic acid (RA) at day three post-induction of differentiation (D3). The time points used are indicated by day (ESC = Undifferentiated, D3, D5, D9 = days post differentiation). B) DNA methylation analysis of the single CpG site in the *Sal14* enhancer by bisulfite sequencing. Average and standard deviation of two biological replicates is shown. C) Illustration of sample treatment for MD-qPCR analysis of a genomic target using MspJI or *HpaII* coupled MD-qPCR. Open circles represent unmethylated CpG, and closed circles methylated CpG. The purified genomic DNA was digested sequentially by *CviQI* and MspJI or *HpaII* enzymes and the target region was amplified by qPCR. Arrows adjacent to DNA represent primer-binding sites. D) DNA methylation analysis of the *Sal14* enhancer by MspJI-coupled MD-qPCR shows a gain in DNA methylation. C_{q_i} is the C_q of MspJI cleaved DNA normalized to that of the *CviQI* control (Equation (1)). E) DNA methylation analysis of the *Oct4* proximal enhancer by MspJI or *HpaII* -coupled MD-qPCR. Fold change in C_{q_i} is calculated relative to C_q of the undifferentiated (ESC) state [$2(C_{q_i} D5/D9 - C_{qESC})$]. F) DNA methylation analysis of the CpG sites in the *Oct4* enhancer by bisulfite sequencing. Average and standard deviation of two biological replicates is shown. For MD-qPCR, averages $\pm$ the standard deviation are shown (n = 8).

Discussion

Studies showing that differential DNA methylation is associated with the presence and prognosis of disease states support the need to accurately and quickly detect abnormal changes in DNA methylation [39–41]. Due to methylation sensitivity and sequence specificity of restriction enzymes, DNA methylation at specific genomic sites can be examined conveniently by MDR especially when coupled to quantitative PCR (MD-qPCR). Several strategies have been employed to improve the detectability of DNA methylation using MD-qPCR that include MethylScreen and EpiTeckII [25]. These techniques use combination of methylation dependent and methylation sensitive enzymes to reduce background noise and report on the density of DNA methylation. Other methylation dependent qPCR techniques such MethylLight does not use restriction enzymes, instead uses Dual labelled Taqman probe which can specifically bind to methylated or unmethylated sequence [13]. This technique is derived from the methylation specific PCR (MSP) method, which uses specific primer design for selective amplification of methylated and unmethylated regions [42]. In this study, we show that the use of methylation-dependent enzyme MspJI expands the possibilities for MD-qPCR to be used for detection of DNA methylation gains. This modified method has several advantages: 1) it is a simple and cost-effective technique that reproducibly permits an accurate determination of DNA methylation; 2) it is equally applicable to *in vitro* and *in vivo* substrates; and 3) compared to *HpaII*, which has a specific four-base recognition sequence, use of MspJI increases the range of target sites that can be assayed by MD-qPCR. Compared to MethylScreen, which requires two restriction enzymes and four reactions and a complex data analysis, this method involves one restriction enzyme and two reactions and a simpler data analysis.

Our analysis with *in vitro* methylated DNA demonstrates that under limiting DNA concentrations, small gains of DNA methylation were resolved when the substrate was cleaved with MspJI compared to that with *HpaII* and the limit of detection of methylation was four fold higher using MspJI-coupled MD-qPCR. We next examined the utility of using MspJI with MD-qPCR for the detection and measurement of gain of DNA methylation *in vivo* during differentiation of ESCs. We measured the gain of DNA methylation at a CpG site in the *SalI4* enhancer during ESC differentiation using bisulfite sequencing and MD-qPCR. Using the change in C_q values in Fig. 2C as a standard for percent DNA methylation, the C_q in Fig. 4D suggests that post differentiation, DNA methylation is potentially increased to ~50% on D5 and ~75% on D9 at the *SalI4* enhancer CpG site. Strikingly, the values for MD-qPCR are consistent with the increase in DNA methylation evaluated by bisulfite sequencing. These data suggest that MspJI is suitable to quantify gain of DNA methylation *in vivo* with the use of appropriate standards. The data from the standard curve suggests that a minimum of 10% change in DNA methylation can be reliably detected with DNA concentration higher than or equal to 12 fM (CpG sites). Further, MspJI allows the detection of methylation at nearly any cytosine residue compared to *HpaII*, which has a four-base pair recognition sequence. This is demonstrated by our data showing that DNA methylation gain at the *Oct4* proximal enhancer could be detected using MspJI- and not *HpaII*-coupled MD-qPCR. However, since MspJI cleaves DNA ~15 bp away from its recognition site, DNA methylation at multiple CpGs in close proximity may not be detected individually. MspJI also has the ability to cleave at hydroxymethylated sites allowing the use of MD-qPCR to monitor hydroxymethylation states, and addition of glycosylation would allow for the distinction between methylation and hydroxymethylation [43]. Although MspJI is incapable of distinguishing between methylation and hydroxymethylation, our data in Fig. 4 are not affected by this property. This is because we are analyzing the regions in the genome that were previously shown to be repressed by DNA methylation upon ESC differentiation [33,34].

Previous studies have used McrBC combined with qPCR to measure DNA methylation at imprinted loci and repeat elements [24,44]. McrBC recognizes two methylated cytosines residues in (G/A)^mC site, 50–3000 bp apart and cuts approximately 30 bp from either of the two half sites [29]. Therefore, the cleavage efficiency of McrBC is influenced by the density of DNA methylation around the target site, thus affecting the accuracy of the measurement of DNA methylation at the target site [25]. This limits the utility of McrBC to measure methylation only at CpG rich regions like repetitive elements and imprinted loci, which are heavily methylated. Changes in DNA methylation at imprinted loci and high CpG rich tissue specific DMRs has been indeed reported using McrBC [24]. In contrast, MspJI recognizes one methylated cytosine in ^mCNNR sites and cuts about 15 bps 3' end of the site, thus specifically reporting on the methylation of the target site [30]. This allows the enzyme to be equally efficient at CpG rich or CpG poor regions independent of the methylation status of the flanking region. Moreover, MspJI recognizes each methylated cytosine individually in a CpG site on the double stranded DNA, thus making it feasible to determine hemi-versus full methylation using alternative primer design in quantitative PCR. This is because the fragmentation of the DNA with hemi- and fully methylated sites will be different [36]. Whereas at hemi-methylated CpG the DNA will be cut only on one side (3' end) of the site,

MspJI will cut on both sides at a fully methylated CpG releasing a short ~32mer fragment. A primer centered on the recognition site paired with either flanking primers will report on the methylation status of the individual cytosines in CpG site. Other members of the MspJI family including FspEI may also be used for these studies; however, FspEI recognizes C^mC sites thereby restricting its potential [9].

Given its simplicity, this method can be broadly employed in experiments investigating the relationship between the change in 5^mC levels at specific sites and cell states or the influence of 5 mC on the interaction of DNA binding factors with their target site. We have previously used this method to monitor establishment of DNA methylation by Dnmt3a at the enhancers of pluripotency genes [34] and confirmed those data using bisulfite sequencing. The property of MspJI to recognize a single methylation event on a CpG site makes it an enzyme of choice to detect the asymmetric methylation activity of *de novo* DNA methyltransferase Dnmt3a [45]. DNA methylation of cell-type specific enhancers strongly correlates with repression of the associated gene and is implicated in diseased cell state [46–49]. Similarly, loss of tumor-suppressor gene expression is accompanied with aberrant gain of DNA methylation at their promoters that can potentially be detected using this method [50–52]. Particularly, using MspJI in this method will support the detection of DNA methylation from limited amounts of patient tissue available for these assays, thus improving the diagnostic potential of MD-qPCR. In addition, DNA methylation also affects binding of transcription regulators to their recognition sequences [9,53]. A high-throughput analysis of the impact of methylation on DNA binding of transcription factors shows that among the factors that bound to methylated motifs many were classified as oncogenes, tumor suppressors, and those required for development [10]. Determination of DNA methylation changes at these sites by MspJI coupled to MD-qPCR can be informative about the potential binding of transcription factors. We propose that this method will be useful for the detection of changes in methylation at low CpG content regions, especially when detection is limited by substrate availability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ab.2017.06.006>.

Fig. 1.
Detection of DNA methylation by MD-qPCR.

Fig. 2.
 Use of MspJI or *HpaII* in MD-qPCR to detect DNA methylation.

Fig. 3.
Sensitivity of MD-qPCR assay using either MspJI or HpaII.

Fig. 4. Accurate determination of DNA methylation changes at the *SalI4* enhancer using MspJI coupled MD-qPCR.