

ZYMO RESEARCH
The Beauty of Science is to Make Things Simple

A Method for Quantifying DNA Methylation Percentage Without Chemical Modification

Michael Karberg, Ron J. Leavitt, Danice Anne A. Cabaya, Marc E. Van Eden & Xi Yu Jia
Zymo Research Corporation, Orange, CA

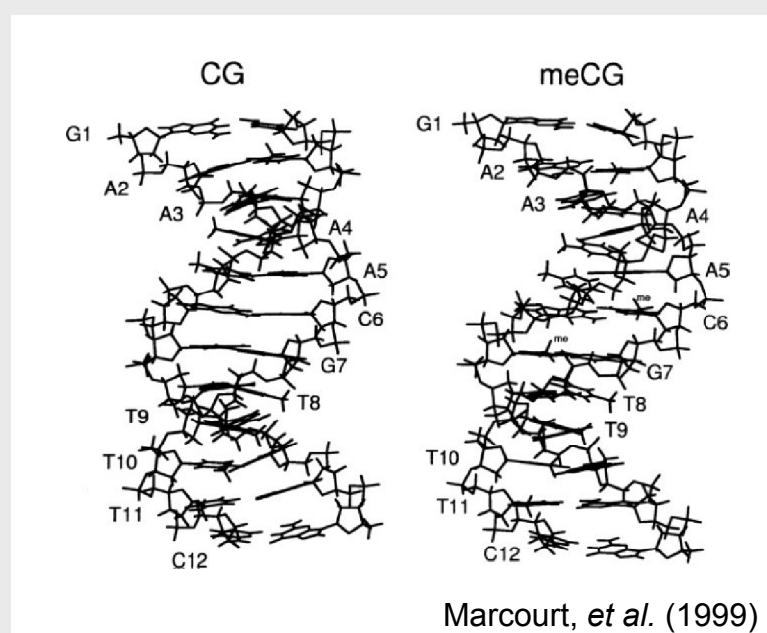
5147

Abstract:

Altered DNA methylation patterns have been associated with aberrant gene expression and the onset of some diseases, including cancer. The ability to quantify DNA methylation in normal and diseased cells would help to monitor changes in DNA methylation, potentially allowing the opportunity to prevent and possibly correct any changes prior to the maturation of the disease. Current methods used to measure DNA methylation, including bisulfite sequencing, Methyl-DIP, MALDI-TOF, and HPLC, are capable of measuring single-nucleotide or multi-locus methylation, but also require proprietary machinery that may be cost-prohibitive for most researchers. Other methods (such as MS-PCR, COBRA, MS-SNuPE, etc.) are limited by sequence-specific restriction enzymes and/or unique primer sets for each locus. In order to differentiate between methylated and non-methylated DNA, most methods require an initial treatment of DNA with sodium bisulfite, which deaminates non-methylated cytosines to uracils but does not affect 5-methylcytosines. Analytical methods that require bisulfite-treated DNA are also negatively affected by incomplete deamination, low DNA yields due to degradation during treatment, and inherently poor DNA stability due to its single-stranded character.

We have developed a simple and cost-effective method to quantify the total cytosine methylation within a DNA sample. Our method takes advantage of the thermodynamic and structural differences between methylated and non-methylated DNA combined with recent improvements in high-resolution fluorescence-based DNA melting detection. Additionally, our method does not require treatment with sodium bisulfite or any other chemical prior to analysis, which means that the output DNA is unmodified and can be used directly in most other applications such as cloning, PCR, and sequencing, etc.

Background:

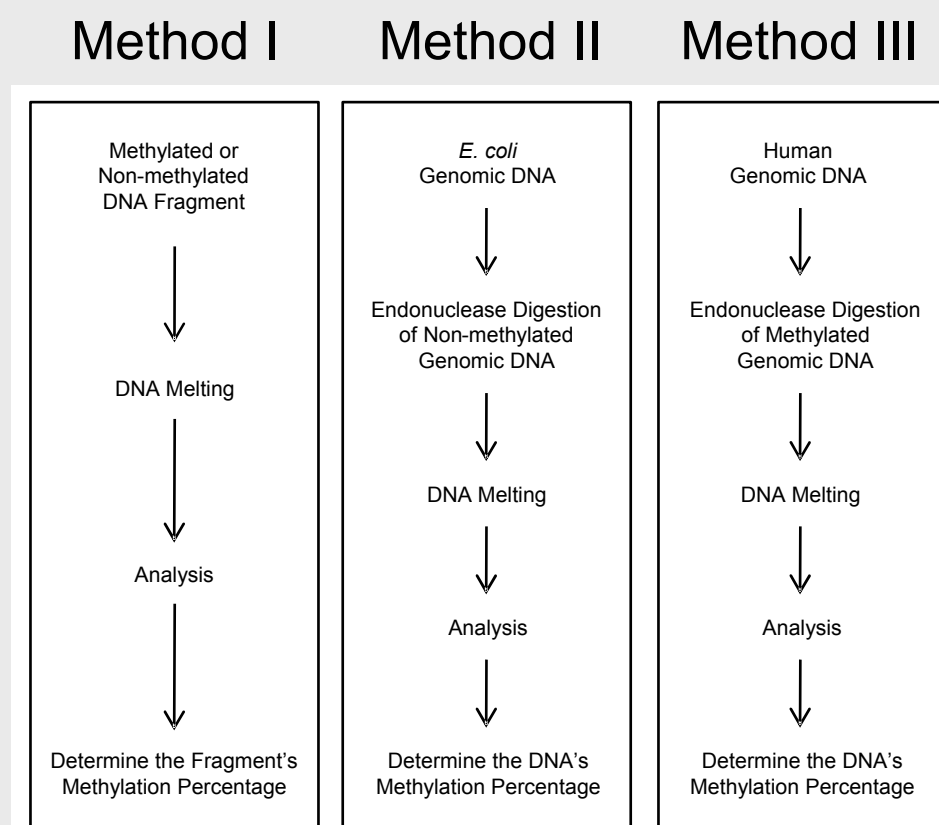


In many animals and plants, cytosines in the CpG dinucleotide context are methylated by the addition of a methyl group to the fifth-position carbon of the cytosine pyrimidine ring. Regions of DNA with high CpG dinucleotide content are identified as CpG islands and are often found within gene promoters or overlapping nearby transcription start sites. Aberrant methylation of this region has been associated with altered gene expression and a cancerous phenotype (Stirzaker, *et al.* 1997). Recent evidence has suggested that the regions of DNA surrounding the CpG islands, or "shores", and within the gene "body" can also be methylated in a manner associated with cancer (Irizarry, *et al.* 2009; Ball, *et al.* 2009). The development of methods to measure DNA methylation has therefore become a focus of the scientific community.

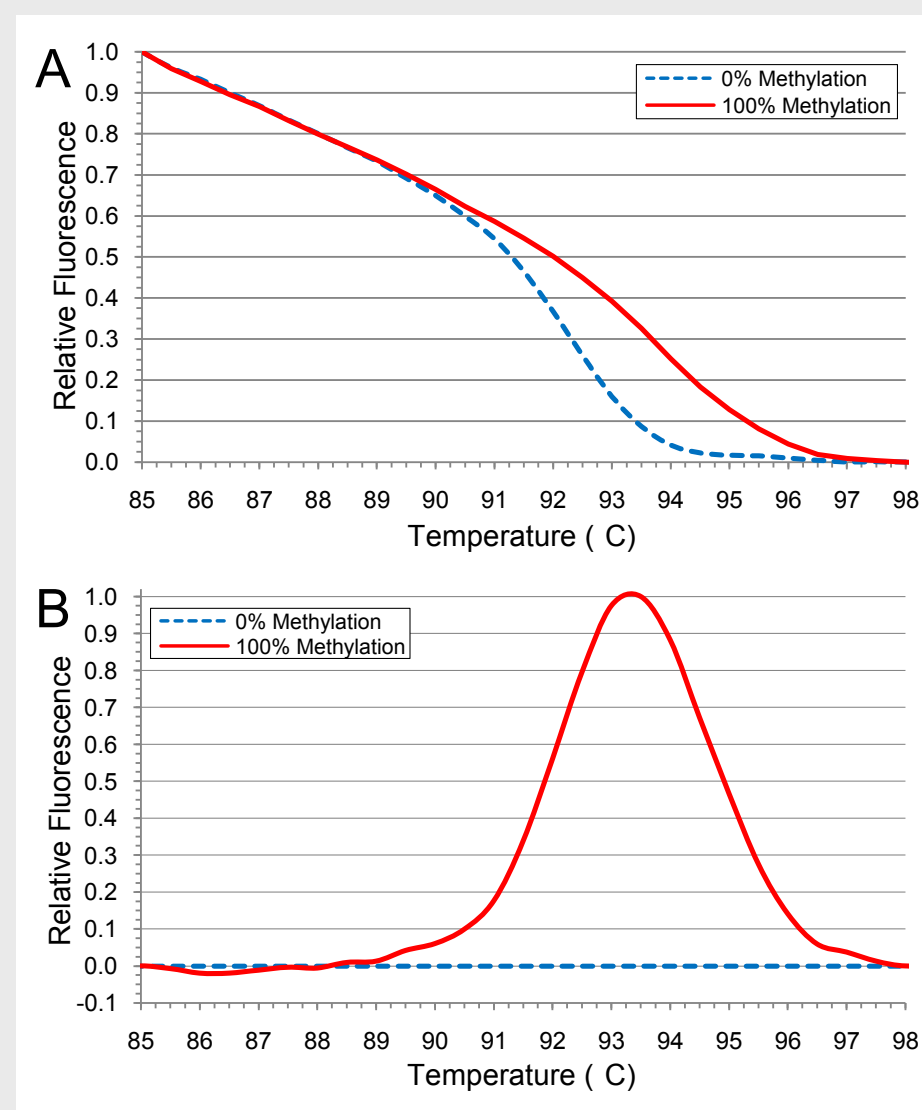
The method presented here is based upon the observation that DNA containing methylated cytosines can have different structural and thermodynamic characteristics (See Figure above; Marcourt, *et al.* 1999). Marcourt, *et al.* found that in some sequence contexts, the minor groove for DNA helices containing methylated cytosines in the CpG context are "pinched" in comparison to the non-methylated counterpart. Our hypothesis was that the structural and thermodynamic differences are amplified when comparing long stretches of methylated and non-methylated DNA, as found in CpG islands. We further hypothesized that this difference could be detected using machines capable of measuring high-resolution melting (HRM), or a machine capable of sensitive real-time PCR, which has become standard equipment in many laboratories.

HRM has been used to measure the methylation status of single CpG residues, or a grouping of CpG residues, by the hybridization of a fluorescently-labeled probe to bisulfite-converted DNA, then determining the methylation status by recording the temperature at which the probe dissociates from the template (Wojdacz & Dobrovic, 2007). These previous methods rely on knowledge about the DNA sequence and bisulfite conversion of the DNA template, which is the chemical conversion of non-methylated cytosine to uracil (methylated cytosine remains represented as cytosine; Frommer, *et al.* 1992). Here, we present a new method using HRM, coupled with a double-stranded DNA-binding dye that fluoresces when bound to DNA, which does not require specific sequence knowledge or bisulfite conversion of the DNA template. The method can differentiate between methylated and non-methylated samples based simply on their melting temperature. Because there is no chemical modification of the DNA, the samples can be used directly in many common downstream applications, such as cloning, PCR, sequencing, or bisulfite conversion. Treatment of the DNA template with methylation-sensitive or insensitive enzymes prior to performing HRM can amplify the differences in melting temperature and help to focus the experiment to a specific region of interest within the DNA. We also show that the method can predict the total methylation of DNA samples within ~8.5% of the actual methylation content.

Results:

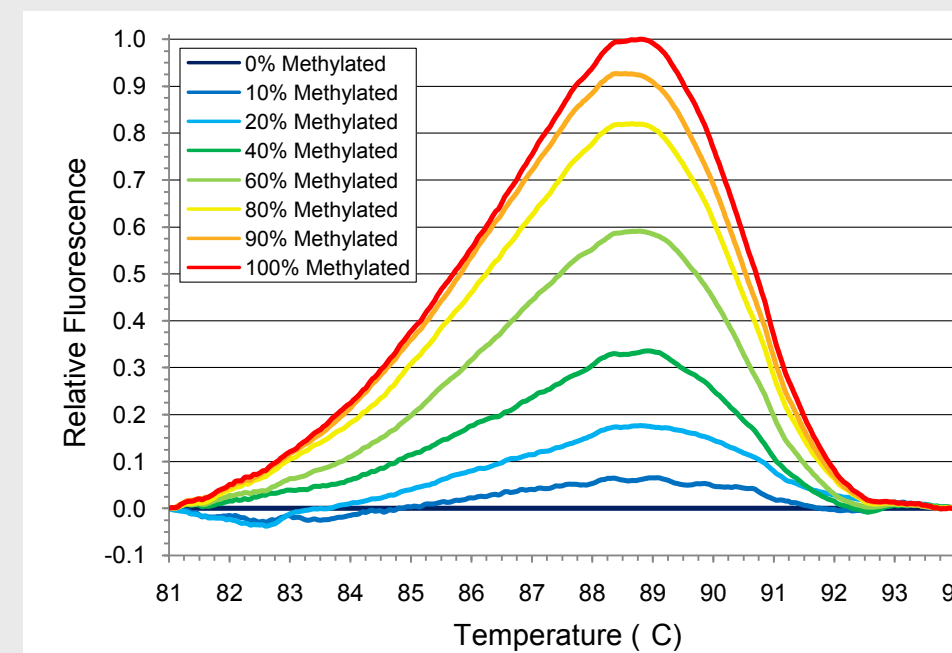


Method I



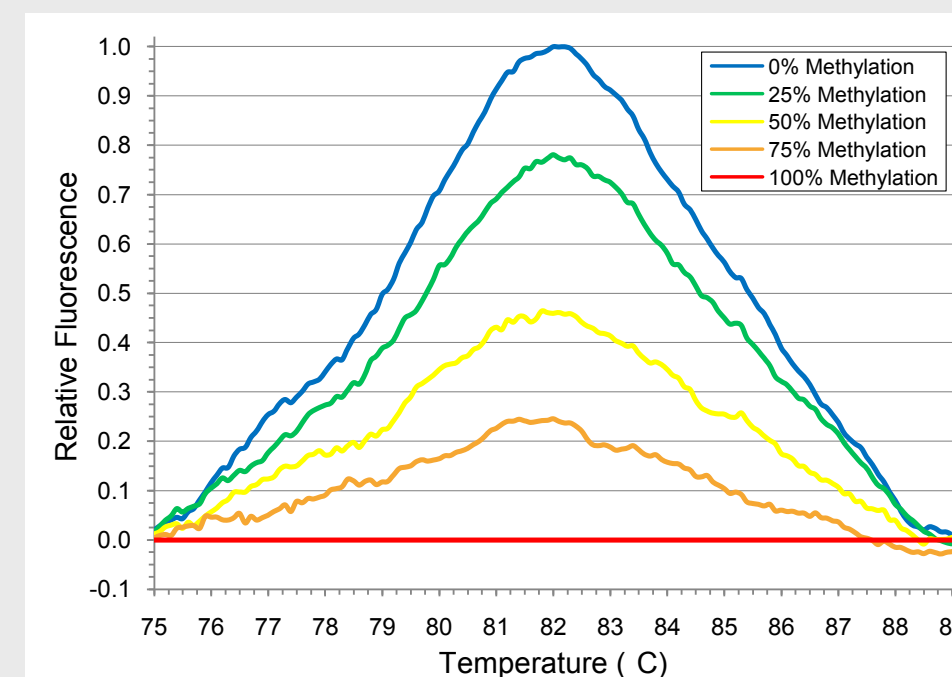
Methylated DNA melts at a higher temperature than non-methylated DNA. High resolution melting (HRM) analysis was performed using a double-stranded DNA-binding dye on non-methylated and artificially methylated 297 bp PCR fragments containing 48 CpGs in order to demonstrate a proof of concept for detecting differences between methylated and non-methylated DNA. A) The fluorescence data from the HRM analysis (normalized to the same scale) shows that the melting temperature of the 0% methylated PCR fragment (~92.5 °C) is lower than that of the 100% methylated PCR fragment (~93.5 °C). B) An alternative way to visualize the data from "A", where the normalized fluorescence of the 0% methylated sample is subtracted from the normalized fluorescence of the 100% methylated sample. The positive fluorescence in this "difference graph" indicates that there is more double-stranded DNA present in the 100% methylated sample than in the 0% methylated sample over the shown temperature range.

Method II



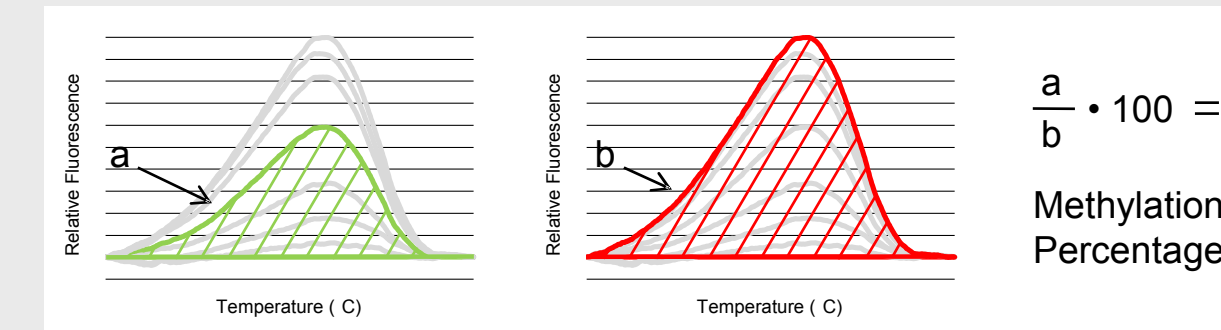
Samples containing a mixed ratio of methylated/non-methylated E. coli DNA have a melting profile that corresponds to the percentage of methylation in the samples. The chromosomal DNA from a methylation-negative *E. coli* strain ER2925 was isolated and enzymatically treated with M.SssI methyltransferase to generate DNA samples of specific methylation state (0% and 100%). Next, the DNA samples were mixed to generate samples with increasing amounts of methylated DNA (0%, 10%, 20%, 40%, 60%, 80%, 90%, and 100%), followed by digestion with HpaII restriction endonuclease, which cuts only non-methylated DNA. Finally, HRM analysis was performed using a double-stranded DNA-binding dye and the data was normalized with the 0% methylated DNA sample, which was set as the baseline for comparison. The average size of the enzymatically-digested DNA is small and melts at a relatively low temperature when compared to undigested DNA. Likewise, the 100% methylated DNA sample has the highest relative fluorescence because it has the lowest amount of fragmentation. The average size of the DNA fragments is larger, thereby melting at a relatively higher temperature. Comparison of the total area under the peak for each sample correlates to the known methylation percentage (data shown in the graph to the right).

Method III

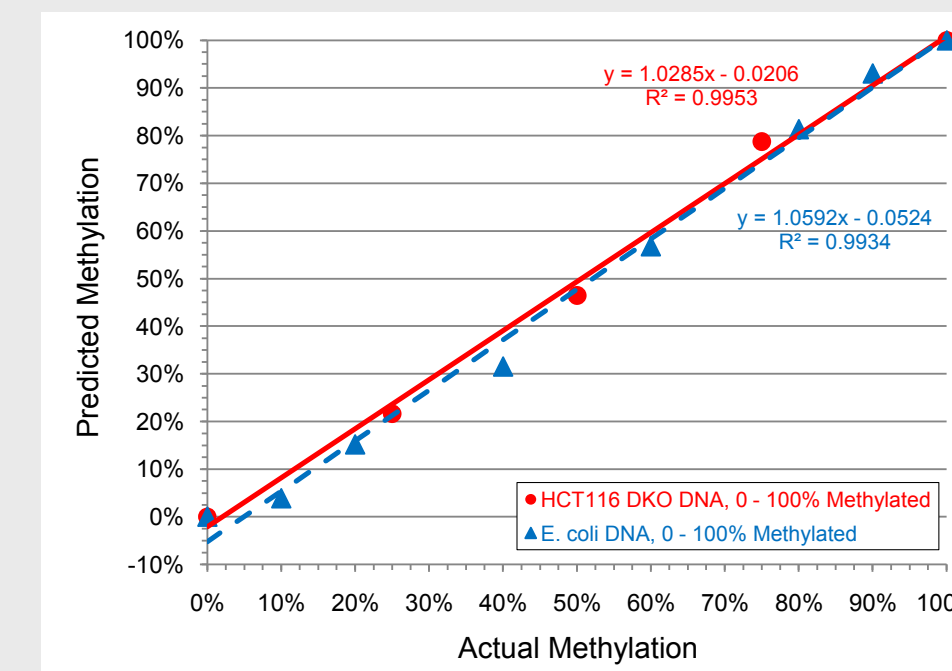


Samples containing a mixed ratio of methylated/non-methylated human DNA have a melting profile that corresponds to the percentage of methylation in the samples. Chromosomal DNA was isolated from HCT116 DKO cells which contain the genetic knockouts DNMT1 (-/-) and DNMT3b (-/-) (Rhee, *et al.* 2002), and enzymatically treated with M.SssI methyltransferase to generate DNA samples of specific methylation state (0% and 100%). Next, the DNA samples were mixed to generate samples with increasing amounts of methylated DNA (0%, 25%, 50%, 75%, and 100%), followed by digestion with McrBC restriction endonuclease, which cuts only methylated DNA. Finally, HRM analysis was performed using a double-stranded DNA-binding dye and the data was normalized with the 100% methylated DNA sample, which was set as the baseline for comparison. Similar to the previous figure, the HRM analysis of these mixed samples show that the differences in the melting profiles correspond to the differences in the methylation percentage. The 100% methylated DNA sample has the lowest relative fluorescence within the range of 75 °C to 89 °C because it has the highest amount of fragmentation by McrBC. The average size of the enzymatically-digested DNA is small and melts at a relatively low temperature when compared to undigested DNA. Likewise, the 0% methylated DNA sample has the highest relative fluorescence because it has the lowest amount of fragmentation. The average size of the DNA fragments is larger, thereby melting at a relatively higher temperature. Comparison of the total area under the peak for each sample correlates to the known methylation percentage (data shown in the graph to the right).

Validation of Methylation Prediction:



Calculating the methylation percentage. The predicted methylation percentage was determined by calculating the sum of the differences in relative fluorescence between each sample and a reference sample (which is set as zero, or subtracted as baseline fluorescence) over a given temperature range. Next the values of each sample were compared as a ratio of the maximum fluorescence difference (which is set as 1). This calculation corrects for samples that may have peak fluorescence differences at different melting temperatures. Alternatively, the predicted methylation percentage could also be calculated by measuring the ratio of the relative fluorescence values at the peak melting temperature difference, with a value of 1 set equal to 100% methylation for the *E. coli* samples (or 0% methylation for the human samples). Data obtained using either calculation is similar (not shown).



The DNA methylation percentage is predicted within 8.5% of the actual methylation percentage. The predicted methylation percentage, as determined by DNA melting, is plotted on the y-axis against the actual methylation percentage determined from the known mixture of 0% and 100% methylated *E. coli* (blue) and human (red) DNA samples, plotted on the x-axis. The near-linear relationship shows the accuracy of predicting methylation percentage by analyzing DNA melting.

Conclusions:

- Here, we present a new method to determine the percentage of total methylation within a DNA sample using HRM, coupled with a double-stranded DNA-binding dye that fluoresces when bound to DNA.
- The method can differentiate between methylated and non-methylated samples based simply on their melting temperature.
- Since there is no chemical modification of the DNA required for the HRM-based method, the samples can be used directly in many common downstream applications, such as cloning, PCR, sequencing, or bisulfite conversion.
- Treatment of the DNA template with methylation-sensitive or insensitive enzymes prior to performing HRM can amplify the differences in melting temperature and help to focus the experiment to specific regions of interest within the DNA.
- The method(s) can predict the total methylation of DNA samples within ~8.5% of the actual methylation content.

References:

1. Stirzaker, *et al.* (1997) Cancer Res 57, 2229-2237.
2. Irizarry, *et al.* (2009) Nature Genet 41, 178-186.
3. Ball, *et al.* (2009) Nature Biotechnol 27, 361-368.
4. Marcourt, *et al.* (1999) European J Biochem 265, 1032-1042.
5. Rhee, *et al.* (2002) Nature 416, 552-556.
6. Wojdacz & Dobrovic (2007) Nucleic Acids Res 35, e41.
7. Frommer, *et al.* (1992) Proc Natl Acad Sci USA 89, 1827-1831.