Calibration Improves Methylation Sensitive High Resolution Melting Analysis

ABSTRACT

Methylation-sensitive (MS) Hi-Res Melting is a sensitive detection method that requires no post-PCR sample manipulation. Analysis is fast and simple by comparing melting temperature (Tm) and melting curve shape. Multiple CpGs can be simultaneously analyzed. This technique is sensitive enough to show single methylation event within a group of multiple CpGs. High discrimination is possible because of the exquisite sensitivity of Hi-Res Melting. We amplified a 152-bp genomic fragment of a proposed regulatory element to miRNA-195 containing 5 CpGs distributed throughout the amplicon. We were able to differentiate overall methylation from 10% to 100% as well as see small melting differences between 39% and 40%, individual CpG site above or below melting curve shape as data for overall methylation. The post-PCR calibration step was implemented to overcome common challenges. First, there can be variation between replicates conversions of the same sample. Commercial kits appear to minimize this problem. Second, it is commonly believed that bisulfite-treated DNA rapidly degrades, necessitating testing within hours or days of treatment. Our results demonstrated sample stability for 3 months at 4°C in TE buffer. Third, small sample-to-sample temperature, volume, and PCR buffer differences can confound the interpretation of results. The use of internal melting temperature calibrators during amplification calibration mitigated these issues and provided for more consistent sample interpretation. MS-Hi-Res Melting is a powerful tool for epigenetic research.

BACKGROUND

High-resolution instrumentation and advanced dyes such as LCGreen® Plus can be used to generate a highly detailed picture of methylation within a given PCR fragment. The post-PCR technique of Hi-Res Melting can be performed on bisulfite treated genomic template. This converts 5-methylcytosine to uracil, which base pairs with adenine during PCR, and ultimately converts to T. To overcome these differences, PCR is usually done on the bisulfite amplified template to maximize the absolute Tm. Hi-Res Melting is excellent at detecting sequence heterogeneity. This is because heteroduplexes created from sequence heterogeneity broaden the shape of the curve. The CpG position relative to the position of these small amplicons also affects shape. Both Tm and small sample-to-sample temperature differences are calibrated and validated for discrimination in sequence heterogeneity.

MATERIALS AND METHODS

Standard DNA Abstraction was performed using a commonly available kit. Following manufacturer’s instructions, bisulfite conversion on sample DNA was performed using the EpiTect™ kit (Qiagen, Valencia, CA, USA). For hypermethylated controls, we pre-purified the CpGenome™ Universal Methylated DNA (catalog # S7821) (Millipore, Billerica, MA, USA). We also obtained DNA from Raji cells using standard kit extraction. PCR was performed using High Sensitivity Mastermix (Idaho Technology, Salt Lake City, UT, USA) containing LCGreen™ Plus, oligonucleotide calibrators, dNTPs, hot start enzyme, and buffers. Oligonucleotide primers were synthesized and used in the reaction at 300 nM each. PCR was performed in a 25-µL reaction of high white PCR-grade, CA USA) with an initial 2-min. denaturation at 95°C followed by 45 cycles of 30-sec. holds at 94°C and 66°C. Heteroduplex generation was performed by a final (95°C, 2min), and anneal at 40°C. Hi-Res Melting was performed between 50°C and 95°C (LightScanner, Idaho Technology, Salt Lake City, UT, USA). Calibration was performed and data was analyzed in the small amplicon module using Call-IT® algorithms in the LightScanner analysis software, according to directions. After melting, products were column-purified and sequenced using standard techniques.

RESULTS

Average methylation was calculated by estimating the relative CT peak heights in sequencing electropherograms. Total percentages for the forward and reverse sequencing reactions from each sample were summed and divided by 5 (the number of CpG sites within the amplicon) resulting in the average methylation. Colors indicate sample identities and are consistent throughout this poster.

FIGURE 1

Distribution of the average methylation found across samples used in this study. A 152-bp amplicon from the miRNA-195 gene containing 5 CpG sites was used.

Methylation in each sample

RESULTS 1

Tm is explained by methylation. Calibration improves the relationship between Tm and overall methylation. Tm data is presented from a 152 bp amplicon of miRNA-195 containing 5 CpG sites. On the top two panels, uncalculated derivative melting curves (A) and the observed correlation (B) between Tm and average methylation are seen. On the bottom two panels, calculated derivative melting curves (C) and the observed correlation (D) between Tm and average methylation are seen. Data is improved with calibration. Average methylation data is as follows: Pink (100%), dark green (40%), light green (40%), orange (39%), dark blue (10%), and light blue (8%). Notice the relative position of the orange samples, which have more similar Tm relative to green samples as they are calibrated (C). This is in agreement with the actual average methylation calculated from sequencing.

DISCOVERY 1

Amplification Tm increases with average methylation.

DISCOVERY 2

The use of oligonucleotide-based calibration improves the Tm correlation with average methylation. This enables more accurate sample measurement and comparison.

FIGURE 3

Uncalculated derivative melting curves of several tumor samples and hypermethylated control. Average methylation across 5 CpG sites of the 152 bp miRNA-195 amplicon is as follows: Pink (100%), dark green (40%), light green (40%), and dark blue (10%). As can be seen in panel (A), both the hypermethylated pink and the hypermethylated dark blue samples have sharper peaks—explained by little to no heteroduplex content. Light green (sample P3B2) has a smaller low-temperature shoulder relative to dark green (sample P3B20). This results from slightly more heteroduplex content caused by an increase in the hypermethylated site #2 and a decrease in hypermethylated sites #4 and #5 (B).

DISCOVERY 3

Derivative melting curve shape broadens with increasing heteroduplex content, which is a function of original 5-methylcytosine heterogeneity. This can be seen in Figure 3.

DISCOVERY 4

Repeatable between bisulfite conversions on the same good using Qiagen EpiTect kit. In addition, three (3) methyl stability of bisulfite converted DNA (measured by similar melting curves) is shown. Melting profiles do not appear to depend on conversion or storage under our conditions (Figure 4). In addition, there is no relationship between crossing point and sample storage time, indicating good sample stability.

CONCLUSIONS

Hi-Res Melting in the presence of internal oligonucleotide calibrators results in excellent qualitative and quantitative information from Tm and shape. We obtained good sample reproducibility between bisulfite-converted template DNA. In addition, sample stability of bisulfite-converted template at 5 ng-15 ng µL appears to be adequate beyond 3 months at 4°C. Using this technique, it is feasible to obtain rapid, cost-effective epigenetic scanning across multiple CpG sites.

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