Calibration Improves Methylation Sensitive High Resolution Melting Analysis

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ABSTRACT

Methylation-sensitive (MS) Hi-Res Melting is a sensitive detection method that requires no post-PCR sample manipulation. Analysis is fast and simple and achieved by comparing melting temperature (Tm) and melting curve shape. Multiple CpGs can be simultaneously analyzed. This technique is sensitive enough to show a 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 sites appear to affect melting curve shape as does the average methylation. We also investigated ways to overcome common challenges. First, there can be variation between replicate 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 amplicon denaturation mitigated these limitations 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 adenosine during PCR, and ultimately converts to T. Percentage differences in GC are thus transferred into the amplicon altering Tm. Hi-Res Melting is excellent at detecting sequence heterogeneity. This is because heteroduplexes created from sequence heterogeneity broaden the shape of the curves. The CpG position relative to the position within the amplicon can also affect shape. Both Tm as well as curve shape can be used in combination for epigenetic studies. Methylation is inherently heterogeneous and LightScanner instrumentation and software with calibration are ideally suited to discriminate fine differences in sequence heterogeneity.

MATERIALS AND METHODS

Standard DNA extraction was performed using commonly available kits. Following manufacturer's instructions, bisulfite conversion on sample DNA was performed using the EpiTect™ kit (Qiagen, Valencia, CA, USA). For hypermethylated controls, we purchased 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 included in the reactions at 0.25 µM each. PCR was performed on iCyclers (Bio-Rad, Hercules, 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 denature at 95°C/30 sec. and anneal at 28°C/30 sec. 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 C:T 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.

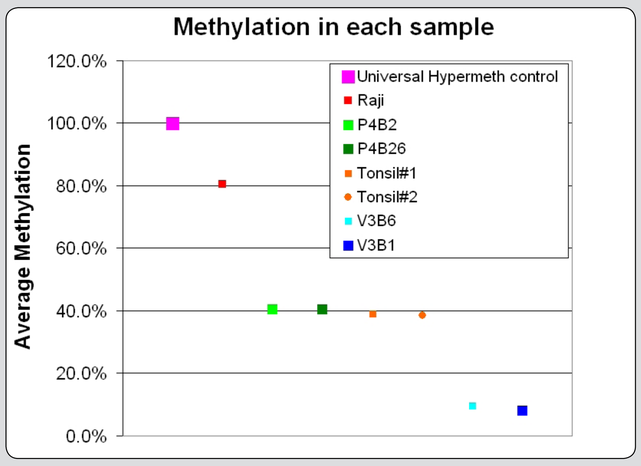
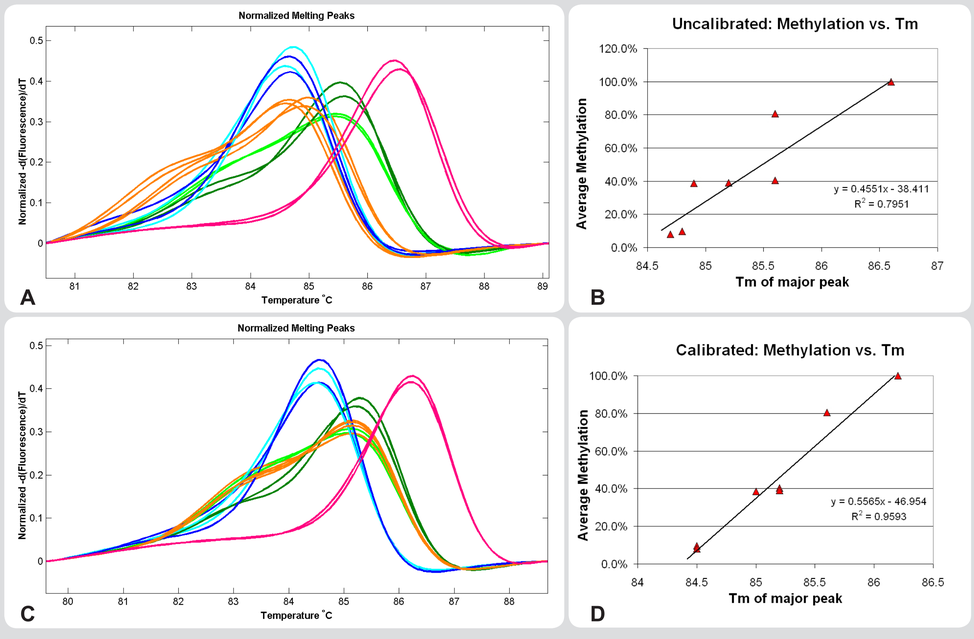


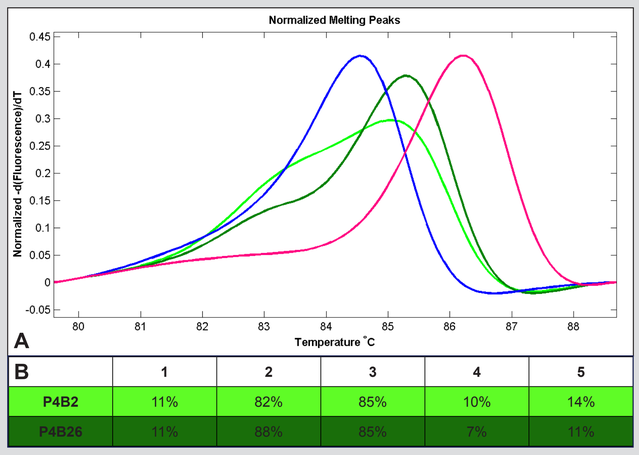
FIGURE 2
Tm is explained by methylation. Calibration improves the relationship. Data is presented from a 152 bp amplicon of miRNA-195 containing 5 CpG sites. On the top two panels, uncalibrated derivative melting curves (A) and the observed correlation (B) between Tm and average methylation are seen. On the bottom two panels, calibrated derivative melting curves (C) and the observed correlation (D) between Tm and average methylation are seen. Data is improved with calibration. Average methylation 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
Amplicon Tm increases with average methylation.

DISCOVERY 2
The use of internal oligonucleotide-based calibration improves the Tm correlation with average methylation. This enables more accurate sample measurement and comparison.

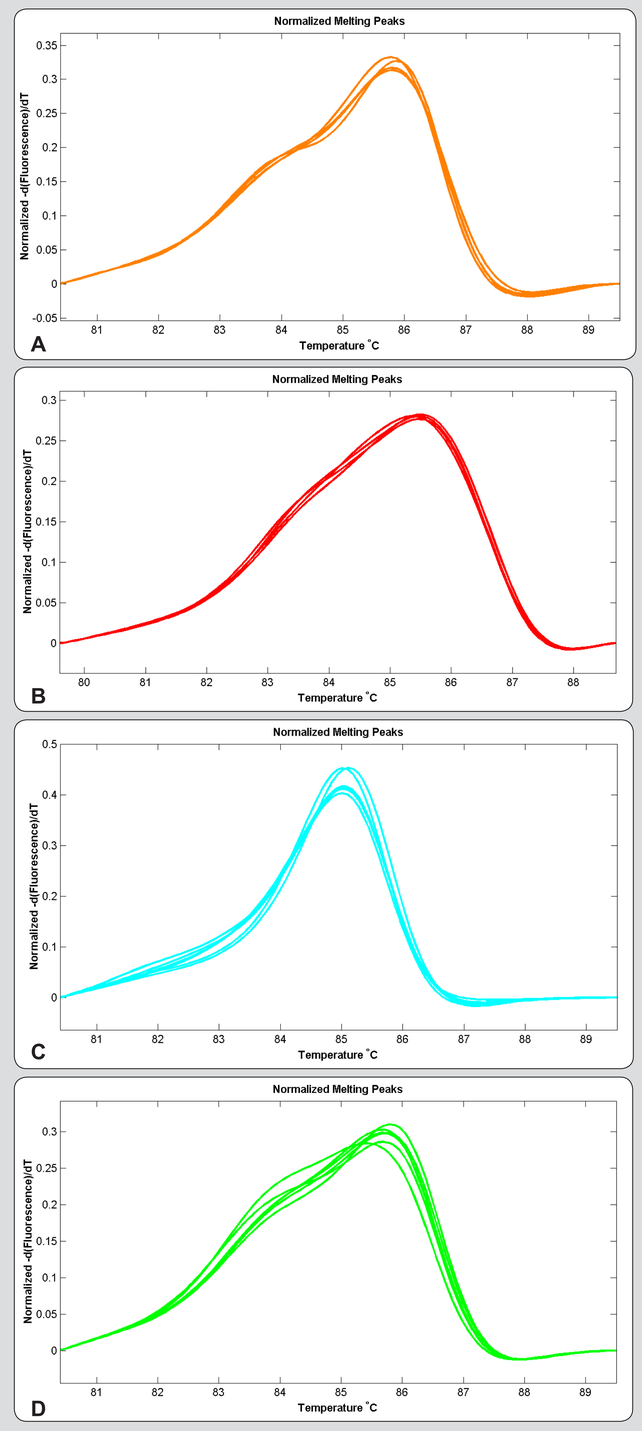
FIGURE 3
Uncalibrated derivative melting curves of several tumor samples and hypermethylated control. Average methylation across 5 CpG sites of the 152 bp miRNA-195 amplicon is as follows: Pink (100%), dark green (40%), light green (40%), and dark blue (10%). As can be seen in panel (A), both the hypermethylated pink and the hypomethylated dark blue samples have sharper peaks—explained by little to no heteroduplex content. Light green (sample P4B2) has a bigger low-temperature shoulder relative to dark green (sample P4B26). This results from slightly more heteroduplex content caused by an increase in the hypermethylated site #2 and a decrease in hypomethylated sites #4 and #5 (B).



B	1	2	3	4	5
P4B2	11%	82%	85%	10%	14%
P4B26	11%	88%	85%	7%	11%

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.

FIGURE 4
Melting curves are invariant of bisulfite conversion replicates or storage time. On the top panels are derivative melting curves from tonsillar (A) and Raji (B) samples showing tight melting reproducibility across 2 independent bisulfite treatments (and 2 PCR replicates per treatment) each. On the bottom two panels, are representative melts from samples V3B6 (C) and P4B2 (D) across 3 independent bisulfite conversions spanning 3 months time. Sample stability post bisulfite treatment with a commercial kit (EpiTect) appears to be good for Hi-Res Melting.



DISCOVERY 4
Repeatability between bisulfite conversions on the same sample is good using Qiagen EpiTect kits. In addition, three (3) month 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, real-time PCR results showed no relationship between crossing point and sample storage time, indicating good template 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 repeatability between bisulfite-treated 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.

High Sensitivity Mastermix (part numbers HRLS-ASY-0008 & HRLS-ASY-0009) can be purchased from Idaho Technology, Inc. or your distributor. For more information visit http://www.idahotech.com

RELATED PUBLICATIONS

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- Gundry CN, Dobrowolski SF, Martin YR, Robbins TC, Nay LM, Boyd N, Coyne T, Wall MD, Wittwer CT, Teng DH (2008) Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons. Nucleic Acids Res. 2008 Apr 29. [Epub ahead of print] PMID: 18448472 [PubMed - as supplied by publisher]