Methylation-sensitive high-resolution melting

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Published online 20 November 2008; doi:10.1038/nprot.2008.191

The base composition of PCR products derived from sodium bisulfite-modified templates is methylation dependent. Hence, methylated and unmethylated, PCR products show different melting profiles when subjected to thermal denaturation. The methylation-sensitive high-resolution melting (MS-HRM) protocol is based on the comparison of the melting profiles of PCR products from unknown samples with profiles specific for PCR products derived from methylated and unmethylated control DNAs. The protocol consists of PCR amplification of bisulfite-modified DNA with primers designed to proportionally amplify both methylated and unmethylated templates and subsequent high-resolution melting analysis of the PCR product. The MS-HRM protocol allows in-tube determination of the methylation status of the locus of interest following sodium bisulfite modification of template DNA in less than 3 h. Here, we provide a protocol for MS-HRM, which enables highly sensitive, labor- and cost-efficient single-locus methylation studies on the basis of DNA high-resolution melting technology.

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INTRODUCTION

Epigenetics is the study of somatically heritable changes of gene expression that occur without a change in the primary DNA sequence. Covalent histone modifications and changes of DNA methylation are the most widely investigated epigenetic mechanisms altering gene expression.

The methylation of cytosines occurs typically in CpG dinucleotides. CpG sites are non-randomly distributed throughout the human genome, with higher concentrations in the promoter and the first exon of the protein-coding genes. The regions with relatively higher CpG dinucleotide content are referred to as CpG islands (CGI). Up to 60% of protein-coding genes contain CGIs in the promoter region, and the methylation status of promoter CGIs generally correlates inversely with the transcriptional status of the gene[1].

Hypermethylation of the promoter CGIs of tumor suppressor genes has been recognized as an alternative mechanism in Knudson's two-hit theory of tumor suppressor gene inactivation, and the methylation of specific genes has been correlated with the outcome of different cancer types[2,3]. Many methylation changes have been shown to be cancer type specific and occur very early in carcinogenesis[4]. Moreover, the presence of differentially methylated sequences in body fluids, e.g., plasma and sputum, has been shown to be detectable long before clinical manifestation of the neoplastic disease[5].

Methylation changes are thus potentially powerful prognostic and predictive markers in cancer diagnosis and treatment. Furthermore, the methylation changes at imprinted loci have been shown as causative factor of many imprinting disorders, e.g., Beckwith Wiedemann[6], and age-dependent changes of methylation in particular are increasingly being associated with the pathology of many disorders[7].

In conclusion, new labor- and cost-efficient technologies are needed to allow high-throughput assessment of single-locus methylation changes and introduction of methylation tests into diagnostic settings.

PCR-based protocols are most widely used in the investigation of single-locus methylation changes[8,9]. Because methylation marks are removed from genomic DNA by DNA polymerase and not replicated during PCR amplification, the DNA template has to be chemically modified with the use of sodium bisulfite to preserve methylation information before PCR amplification. Sodium bisulfite changes unmethylated cytosines into uracil, whereas 5-methylcytosines are resistant to this modification. Subsequent amplification of bisulfite-modified template results in different amplicons from methylated and unmethylated templates: a relatively GC-rich PCR amplicon originating from methylated templates where methylated cytosines are preserved, and a GC-poor amplicon originating from unmethylated templates where all the cytosines are changed into uracils.

The techniques utilizing PCR amplification of bisulfite-modified DNA can be divided into two groups depending on the PCR primers used. One group utilizes primers that specifically amplify methylated (or unmethylated) templates, e.g., methylation-specific PCR (MSP) or quantitative MSP[10,11]. The second group is based on primers that allow amplification of the template regardless of its methylation status for post-PCR methylation analyses and include bisulfite sequencing[12,13], restriction digestion[14], single-strand conformation analysis[15], melting curve analysis[16] and high-resolution melting[17]. The proportional amplification of methylated and unmethylated templates is critical for this group of analyses, and preferential amplification of one of the templates (PCR bias) can lead to misinterpretation of the results. PCR bias is sequence dependent and has been shown to lead to under-amplification of the sequences originating from methylated templates[18–20]. We have addressed the PCR bias issue in methylation studies by developing a new primer design system that enables compensation for PCR bias and significantly increases the sensitivity of high-resolution melting-based methylation detection (see Experimental design). Our new primer design protocol can be used as an alternative to previously published guidelines for primer design[13] when PCR bias is encountered in the experiments.

The use of melting analyses in methylation studies was first reported by Guldberg and colleagues[16]. The recent development of new generation of melting instrumentation (HRM-capable fluorimeters), fluorescent dye chemistries and the new approach to primer design allowed the development of MS-HRM[6,17].

High-resolution melting technology was initially developed for genotyping studies and is based on the comparison of the melting profiles of sequences that differ in base composition and has been

shown to have a sensitivity superior to direct sequencing[21]. Each double-stranded DNA molecule is characterized by a melting temperature or, for longer molecules, a melting profile. The melting temperature is defined as the specific temperature at which the DNA helix dissociates into two single strands and is sequence dependent. The melting temperature of a PCR product can be investigated by subjecting it to an increasing temperature gradient in the presence of a DNA intercalating dye, which emits fluorescence when bound (intercalated) to double-stranded DNA. The fluorescent dye will bind to double-stranded DNA emitting high levels of fluorescence until the temperature reaches the melting temperature of the PCR product. At the melting temperature, the PCR product dissociates into two single strands and the dye can no longer bind and fluoresce, and a sharp drop in the fluorescence is observed. The changes of fluorescence levels across a denaturing gradient describe an amplicon's melting profile. The PCR product originating from the methylated allele will have different sequence composition (GC content) from the PCR product derived from unmethylated variant of the same locus. As a consequence, both products have distinct melting temperature and melting profiles. Hence, to investigate the methylation status of an unknown locus, the melting profile of the PCR product derived from that locus has to be compared with the profiles of methylated and unmethylated controls.

The melting analyses do not allow detailed information about the methylation of single cytosines within the sequence of interest to be obtained. These can be assessed only by DNA sequencing technologies. Nevertheless, MS-HRM analysis can distinguish fully and partially methylated samples. Therefore, MS-HRM can identify those samples for which further sequence information would be of interest by selecting them from the samples that are clearly unmethylated and thus do not require sequencing.

The MS-HRM protocol provides a high-throughput platform for cost- and labor-efficient screening for methylation changes and has the potential to be the method of choice in both research and diagnostic settings.

Experimental design

Design of primers allowing correction for PCR bias. The proportional amplification of bisulfite-modified methylated and unmethylated templates can be compromised by PCR bias due to the fact that they have different base compositions. PCR bias is described as the preferential amplification of one DNA template. The PCR bias in methylation studies has long been recognized and is principally directed toward the unmethylated (GC-poor) strand[18]. The relatively disproportionate amplification of the unmethylated strand may result in misinterpretation of the final results and the underestimation of methylation levels (**Fig. 1**). The widely adopted primer design guidelines for post-modification methylation analysis protocols generally advise not to include any CpG nucleotides into the primer sequence and if it is not possible to do so, to use a mismatched C from CpG dinucleotide with T to avoid the preferential binding of the primer to methylated templates[13]. We have shown that in most cases, inclusion of a limited number of CpGs into the primer sequence is necessary to compensate for PCR bias[17,19]. Our primer design system is thus based upon the inclusion of a limited number of CpG dinuclotides into the primer sequence enabling the conditionally selective binding of the primer to the methylated sequence and therefore enabling compensation for PCR bias that would otherwise normally favor the amplification of the unmethylated template[22]. Furthermore, reversal of PCR bias to favor methylated sequences increases the sensitivity of the melting assays, allowing detecting methylation levels as low as 1–0.1%, which is a level of sensitivity similar to MSP (T.K.W., Tanni Bargbo and L.L.H., unpublished data).

Our primer design guidelines are outlined below (for examples, see **Table 1**).

1. Primers should usually contain one or two CpG dinucleotides each.
2. The CpGs should be placed as close as possible to the 5′-end of the primer.
3. The melting temperature of the primers should be matched, preferably within 1 °C.
4. The 3′-end of the primer should contain one or more Ts corresponding to non-CpG Cs, to ensure amplification of only bisulfite-converted template.

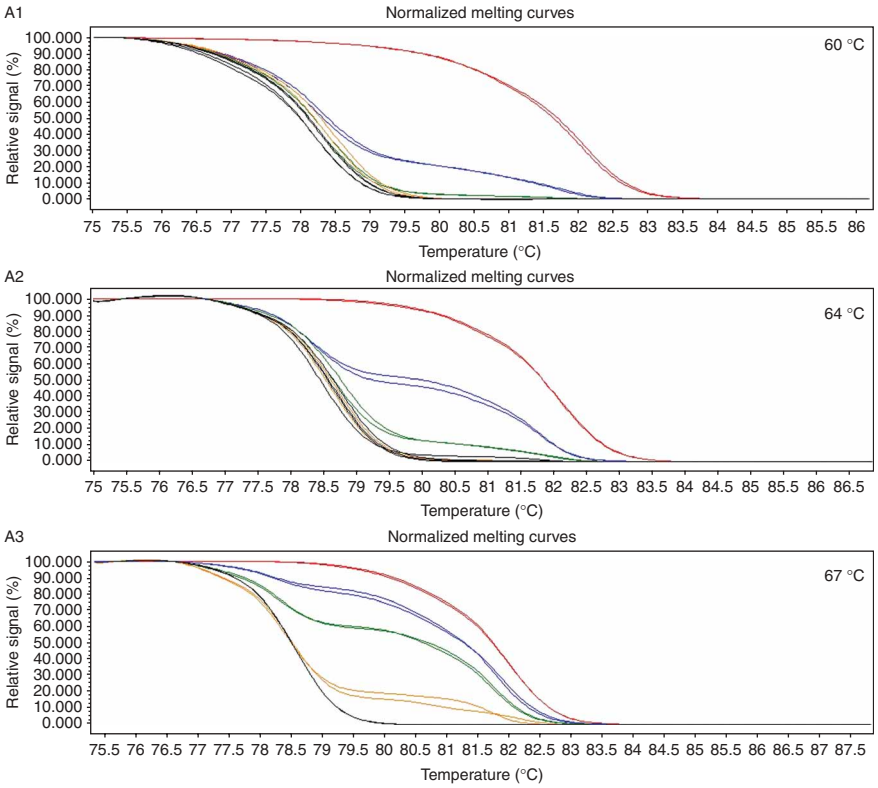

Figure 1 | The effect of the PCR annealing temperature on the sensitivity of the ATM MS-HRM assay. The dilutions of methylated and unmethylated templates are indicated as follows: 100% methylated-red, 10% methylated-blue, 1% methylated-green and 0.1% methylated-brown and unmethylated-black. At 60 °C, there is extensive PCR bias leading to only the 10% dilution resolving from the unmethylated control. At 67 °C, robust amplification of methylated sequence from the 0.1% dilution point is observed, indicating annealing temperature-dependent reversal of PCR bias to favor methylated sequences.

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TABLE 1 | The primers designed to target the promoter sequences of three tumor methylation markers.

Assay name	Primer sequences 5′–3′	Locus (Genome browser UCSC March 2006)
ATM	F-CGAAGAGGGTGGGTGAGAGTTT	chr11:107,598,748–107,598,832
MS-HRM	R-ACGCCATATCCACCAATAACCAAC	
RARβ2	F-CGAGTTGTTTGAGGATTGGGATGT	chr3:25,444,840–25,444,928
MS-HRM	R-AATACGTTCCGAATCCTACCCC	
GSTP1	F-GAGAAGTACGAGATGTGGGGAT	chr11:67,108,431–67,108,498
MS-HRM	R-TACACTCCTAACCCCTCCCC	

MS-HRM, Methylation-sensitive high-resolution melting. ATM, Ataxia Telangiectasia Mutated (gene).

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5. The primers should meet standard parameters for primer design, e.g., secondary structure, primer dimer formation.
6. The preferred length of the amplified sequence should be around 100 bp to reduce the complexity of the melting profile.

DNA template preparation. Any reliable method for DNA extraction can be used to prepare template for MS-HRM analyses.

Choice of equipment for melting analysis. A real-time PCR thermocycler coupled with a HRM-capable fluorimeter is the ideal platform for MS-HRM experiments. To date, five HRM systems are available: the LightCycler480 (Roche), the RotorGene6000 (Corbett Research), the Applied Biosystems 7500 real-time PCR system (Applied Biosystems), the LightScanner System and the HR-1 instrument (Idaho Technologies). The LightScanner and HR-1 instruments contain only an HRM module. Earlier real-time instruments with a melting module can be used in melting-based methylation studies. Nevertheless different systems have different readout parameters, and therefore the data quality obtained from those platforms may vary significantly and has to be evaluated by the user in a platform-specific manner. In our experience, HRM systems showed marked superiority over the first-generation real-time systems with melting modules in which the low sensitivity and narrow range of fluorescence detection did not allow unambiguous distinction between methylated, unmethylated and partly methylated profiles. Furthermore, the first-generation real-time systems are not compatible with new data analysis algorithms invented to handle high-resolution fluorescence data (software supplied with the HRM instruments).

Controls. The methylated reference for melting experiments can be obtained by treating genomic DNA with M.SssI. enzyme (NEB, cat. no. M0226L). Complete methylation of DNA is hard to achieve without repeated rounds of incubation. 'Fully' methylated genomic DNA can also be purchased from commercial suppliers (e.g., Millipore, CpGenome Universal Methylated DNA, cat. no. S7821, or EpiTect Control DNA methlyated (100), cat. no. 59655). Nevertheless the methylation status of the locus of interest in commercial control may be different from that expected and needs to be confirmed before performing experiments. Unmethylated reference DNA from any tissue in which the sequence of interest does not show methylation can be used as a source of unmethylated reference. Unmethylated genomic DNA is also available from commercial suppliers (Millipore, CpGenome Universal Unmethylated DNA, cat. no. S7822, or EpiTect Control DNA unmethlyated (100), cat. no. 59665). Some laboratories used whole genome-amplified DNA as the unmethylated control. Many genes show low-level methylation in certain tissues. The methylation status of the DNA chosen as the unmethylated control has to be investigated before performing analyses. The assay optimization experiments should also include non-bisulfite-treated DNA as negative control to ensure no amplification of gDNA.

The methylated and unmethylated template dilution series. A dilution series of relevant methylated template in unmethylated template background has to be used to estimate the extent of PCR bias, sensitivity of the assay and methylation levels in screened samples.

Bisulfite modification of genomic DNA. This can be performed as described[10]. Nevertheless, currently commercially available kits for bisulfite modification ensure highly efficient template conversion, reduction of the time needed for conversion and high bisulfite-modified template recovery rates. Bisulfite-modified template is susceptible to rapid degradation. The bisulfite-modified sample should be used up soon after bisulfite modification or, if required for a longer period of time, the DNA should be stored at −80 °C. Repeated freezing and thawing of the template should also be avoided.

DNA saturating dyes. These are used as PCR additives as they do not interfere with the polymerase performance in saturating concentrations. High-resolution PCR Master mixes containing all PCR reagents and the saturating dye can be used for increased convenience of setup. Extensive evaluation of DNA intercalating dyes has been described[23].

Optimization of the PCR mix. The concentration of Mg^{+2} supplied in the PCR buffers is normally not sufficient to ensure efficient amplification of bisulfite-modified template. An increase in the concentration of Mg^{+2} to 2.5–3.0 mM significantly enhances the amplification. Increased Mg^{+2} concentration enhances non-specific amplification and therefore has to be optimized for each primer set.

Detection of PCR bias and optimization of the annealing temperature of PCR amplification. To test primers for the extent of PCR bias, the reaction mix from Step 3 should be run with the 50:50 methylated and unmethylated template mix. An equal amount of the PCR product (indicated by the equal heights of the melting peaks) has to be seen originating from each template to rule out the presence of preferential amplification of unmethylated sequence. The primers containing CpGs are able to reverse PCR bias at the specific annealing temperature. To test the annealing temperature at which the primers are able to compensate for

PCR bias, run the PCR mix across a range of the temperatures with the mixes of methylated and unmethylated template. The analysis of the HRM scans will show at which annealing temperatures the preferential amplification is eliminated (**Fig. 1**). We suggest that the initial PCR amplification be run at the annealing temperature, i.e., 5 °C below the melting temperature of the primers.

Predicting melting behavior of methylated and unmethylated PCR products. PCR amplicons may contain many melting domains, which are defined as subregions of the amplicon with specific melting temperatures. An amplicon with several melting domains may display a complex melting profile (with many drops in fluorescence), which may be hard to interpret. Amplicons for MS-HRM analyses should preferably include only one melting domain, with one melting temperature resulting in a sharp melting curve. An estimate of the amplicon's melting properties can be made using algorithms such as POLAND (http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html) or the MELT94 algorithm (available from http://web.mit.edu/osp/www/melt.html). These algorithms can also be used to investigate the difference in overall melting temperature between methylated and unmethylated amplicons, which depends on the number of CpGs within the amplicon, and its base sequence. The difference has to be big enough to allow unambiguous distinction of both amplicons. This is of special importance when non-HRM melting modules are used in experiments and the melting temperature differences have to be bigger to compensate for lower sensitivity of the fluorescence detection. In our experience, 6–8 CpGs in the amplicon ensure unambiguous melting profiles. For HRM platforms, the differences can be as low as one CpG. Nevertheless the empirical examination of the amplicon's melting properties is necessary for conclusive results.

Data analysis: derivative peaks. The melting curve data can be transformed into peaks by plotting the negative derivative of fluorescence over temperature. After this transformation, the top of the peak represents the sharpest drop in the florescence from the melting curve slope and therefore indicates the melting temperature of the amplicon. Both HRM and the real-time instruments with melting modules use this data analysis.

Data analysis: normalized melting curves and estimation of methylation levels. New algorithms have been developed to analyze HRM data. On HRM scans, the replicates of a sample after amplification display different amounts of PCR product. The algorithms developed for analyses of HRM data (the software packages are supplied with the machines) allow the normalization of the differences in fluorescence between samples. Therefore, in final analyses, only the shapes of the melting curves are taken into account. The shapes of the curves display the ratio of methylated and unmethylated PCR products in the sample and allow direct comparison of the two samples on the bases of similarities of their HRM profiles. Hence the estimation of the proportion of methylated to unmethylated template can be performed if the melting profile of unknown sample is compared with the melting profile derived from the sample with known proportion of methylated to unmethylated templates.

MATERIALS

REAGENTS

- Bisulfite modification kit (e.g., EpiTect Bisulfite Kit, Qiagen, cat. no. 59104, Human Genetic Signatures, MethylEasy Xceed, cat. no. ME002, Zymo, EZ DNA Methylation Kit, cat. no. D5001, ABI, methyISSEQr, cat. no. 4374710) ▲ CRITICAL EpiTect Bisulfite Kit in our hands is the most robust in our laboratory settings in regard to time of use and high post-bisulfite DNA recovery rates.
- DNA intercalating dye (e.g., LCGreen I and LCGreen plus, Idaho Technology Inc., cat. no. BCHM-ASY-0003 and BCHM-ASY- 0005, respectively; Syto9, Invitrogen, cat. no. S-34854; LightCycler480 ResoLight Dye, Roche, cat. no. 04909640001; Eva Green, Biotium Inc., cat. no. 00013) ▲ CRITICAL There are differences in performance parameters of different dyes. Comprehensive comparison of differed dyes performance was described in ref. 23.
- *Taq* polymerase (e.g., FastStart Taq DNA Polymerase, Roche, cat.no. 12032902001)
- Master mixes with PCR reagents and intercalating dye (e.g., LightCycler 480 HRM Master, Roche, cat. no. 04909631001; SensiMixHRM, Quantace Ltd, cat. no. QT805-02)
- Mg^{+2} (included in the polymerase or PCR master mix kit; see Experimental design)
- Methylated reference (see Experimental design for details)
- Unmethylated reference (see Experimental design for details)
- DNA primers (standard RP-column-purified primers)

EQUIPMENT

HRM fluorimeter ideally coupled with a real-time PCR cycler (e.g., LightCycler 480 System, Roche, cat. no. 04545885001), Rotor-Gene 6000 (Corbett Research, cat. no. 6600), the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, cat. no. 4351104), LightScanner and HR-1 instrument (Idaho Technologies, cat. no. LSCN-ASY-0011 and HR01-ASY-0001, respectively). ▲ CRITICAL The LightCycler 480 and Rotor-Gene 6000 are in standard use in our laboratories. Both systems have comparable performance but different formats. Very convenient from automation point of view, 96-well plate format of LightCycler 480 and single tube to 100 wells Gene disk format of Rotor-Gene 6000 allow the choice of the most convenient format for individual laboratory setting.

PROCEDURE

1| Extract DNA template through any 'good practice' method (see Experimental design).

2| Modify DNA using sodium bisulfite, as described in ref. 11. When a commercially available kit is chosen, follow the supplier's protocol (see also Experimental design).

3| Prepare the following reaction mix to amplify bisulfite-modified DNA:
Bisulfite-converted template from Step 2 (3–10 ng): 2 µl
Primers 250 nM of each forward and reverse: 0.5 µl
Mg^{+2} (3 mM final concentration): 2.4 µl
LightCycler 480 HRM Master: 10 µl
H_2O: 5.1 µl

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4| Amplify DNA in thermocycler using the following cycling conditions:
10 min at 95 °C (depends on the type of polymerase used and HotStart protocols are advised here).
50 cycles of:
5 s at 95 °C
5 s at primer specific annealing temperature
5 s at 72 °C.

▲ CRITICAL STEP The amplification of sufficient amount of PCR product from bisulfite-modified template normally requires more cycles than a standard PCR amplification. Initial experiments should be run with 50 cycles and the number of the cycles should subsequently be adjusted.

? TROUBLESHOOTING

5| Allow reannealing of the PCR products and acquire HRM scans using the following conditions:
1 min at 95 °C (to denature all amplified PCR product)
Cool down to 70 °C
1 min at 70 °C (to allow all PCR products to hybridize).

▲ CRITICAL STEP The default settings of fluorescence acquisition and the temperature ramp rates suggested by the equipment supplier should be used during the initial HRM scans. A temperature gradient from 70 to 95 °C is advised. The ranges of the temperatures can subsequently be adjusted for each assay to cover the melting of temperatures of the methylated and unmethylated products.

? TROUBLESHOOTING

6| The HRM data can be subsequently analyzed by the software supplied with the instruments. In principle, MS-HRM technology investigates the methylation status of the unknown sample by comparing its melting profile with the profiles derived from of the samples with methylated and unmethylated controls. For details, see Experimental design and ANTICIPATED RESULTS.

● TIMING

Step 2, bisulfite modification: 5–16 h depending on the protocol used
Steps 3 and 4: PCR amplification: 1.5–2 h
Step 5, HRM analyses: up to 30 min
Step 6, data analysis: 15–60 min

? TROUBLESHOOTING

Step 4

Problem. Low-efficiency PCR amplification.
Solution. Test the primers on control methylated/unmethylated control template, optimize the PCR chemistry and parameters, repeat the PCR with new reagents, test the bisulfite conversion protocol for losses of the template during purification step, and when no HRM master mixes are used, decrease the dye concentration.
Problem. Primer dimers in the PCR product.
Solution. Decrease the primer and/or the Mg^{+2} concentration, and increase the annealing temperature of PCR amplification.

Step 5

Problem. Non-reproducible results.
Solution. Repeat bisulfite modification of the sample (the problem may arise from incomplete bisulfite conversion of the template), and decrease the DNA input for bisulfite modification. Redesign the primers to contain 5′-CpGs.
Problem. No unambiguous peaks are obtained in HRM analyses.
Solution. Redesign primers to include more CpGs into the amplified sequence and to include only one melting domain. Note that DNA sequence variations, like point mutations, insertions deletions and SNPs, can induce changes in melting profiles.
Problem. Presence of nonspecific peaks on the HRM scans.
Solution. Redesign the primers, optimize the PCR chemistry and increase the annealing temperature of PCR amplification.
Problem. Amplification using primers without CpGs shows PCR bias toward unmethylated sequence.

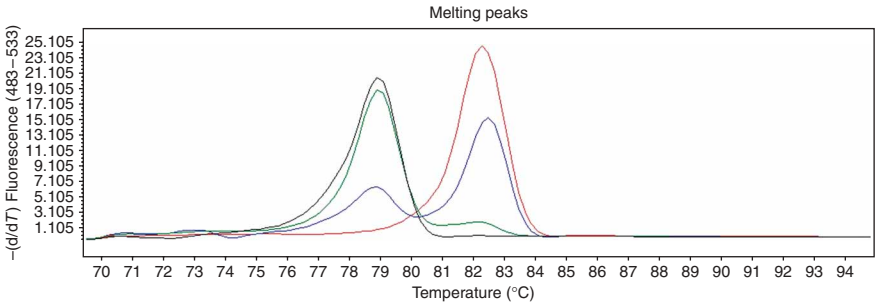

Figure 2 | Derivative curve-based analysis of HRM results of RARβ2 gene. Melting curves for each methylated and unmethylated template dilution point were transformed to peaks by calculation of $-(dF/dT)$. Two different peaks are present for the PCR product derived from methylated (red) and unmethylated (black) templates. The samples with the mixes of methylated and unmethylated DNA display two peaks (blue and green).

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Solution. Optimize annealing temperature and Mg^{+2} concentration of the PCR amplification, and redesign primers to include limited number of CpGs (see Experimental design).

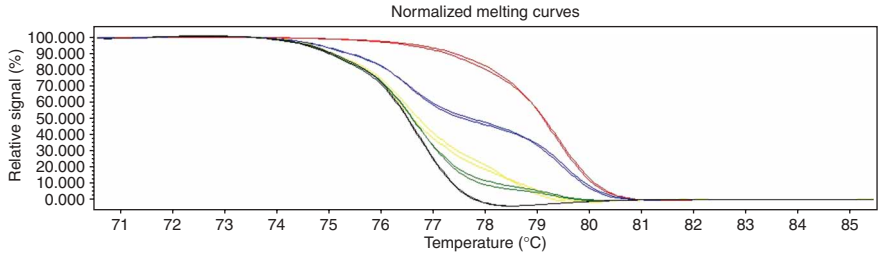

Figure 3 | Normalized HRM curve-based estimation of methylation levels for GSTP1 gene. The PCR product HRM curve derived from unknown sample (yellow) was plotted against HRM profiles of PCR product derived from standards with known concentration of methylated to unmethylated template. The results show that the methylation of the samples is in the range of 1–10% (100% red, 10% blue, 1% green, 0%/ unmethylated template black).

ANTICIPATED RESULTS

Derivative peaks

When the derivative peaks data analyses format is used, for each of the unmethylated and methylated reference samples, an unambiguous peak should be obtained. The unknown sample can be scored on the bases of the similarities to one of the two reference profiles. The samples containing PCR product derived from both methylated and unmethylated templates will display two peaks similar to the methylated and unmethylated references (**Fig. 2**).

Normalized melting curves

Normalized HRM profiles allow estimation of methylation levels of unknown samples if they are run along with the standards representing different mixes of methylated and unmethylated templates. The methylation levels of an unknown sample can be estimated by comparing their melting profiles with the melting profiles of PCR products derived from controls with known methylated to unmethylated template ratio[17] (**Fig. 3**).

Heterogeneously methylated samples

A number of loci in the human genome do not undergo full methylation but are variably methylated at the CpGs, which is known as heterogeneous methylation. Therefore, the sequences that are heterogeneously methylated give rise to a mixture of PCR products with Ts at some CpG sites and Cs at others. The PCR products with minor differences in the sequence can cross-hybridize and form heteroduplexes. Heteroduplexes are less stable than homoduplexes in denaturing conditions and therefore display different melting temperature from the fully methylated and unmethylated references. The HRM melting profiles of the PCR products derived from the samples with heterogenously methylated templates show a characteristic complex melting pattern, which allows for their ready identification, especially when first derivative curves are analyzed. The heterogeneous pattern of methylation can be investigated in detail by sequencing-based methodologies.

ACKNOWLEDGMENTS We thank the Lundbeck, Toyota and Harboe foundations (grants to T.K.W. and L.L.H.), the National Health and Medical Research Council of Australia and US Army Medical Research and Materiel Command (grants to A.D.) for the support of the research leading to this publication.

AUTHOR CONTRIBUTIONS A.D. and L.L.H. contributed equally to this work.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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