Direct Genotyping of Single Nucleotide Polymorphisms in Methyl Metabolism Genes Using Probe-Free High-Resolution Melting Analysis

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Abstract

High-resolution melting (HRM) shows great promise for high-throughput, rapid genotyping of individual polymorphic loci. We have developed HRM assays for genotyping single nucleotide polymorphisms (SNP) in several key genes that are involved in methyl metabolism and may directly or indirectly affect the methylation status of the DNA. The SNPs are in the 5,10-methylenetetrahydrofolate reductase (MTHFR; C677T and A1298C), methionine synthetase (MTR; 5-methyltetrahydrofolate-homocysteine methyltransferase; A2756G), and DNA methyltransferase 3b (DNMT3b; C46359T and C31721T) loci. The choice of short amplicons led to greater melting temperature (Tm) differences between the two homozygous genotypes, which allowed accurate genotyping without the use of probes or spiking with control DNA. In the case of MTHFR, there is a second rarer SNP (rs4846091) close to the A1298C SNP that may result in inaccurate genotyping. We masked this second SNP by placing the primer over it and choosing a base at the polymorphic position that was equally mismatched to both alleles. The HRM assays were done on HRM capable real-time PCR machines rather than stand-alone HRM machines. Monitoring the amplification allows ready identification of samples that may give rise to aberrant melting curves because of PCR abnormalities. We show that samples amplifying markedly late can give rise to shifted melting curves without alteration of shapes and potentially lead to misclassification of genotypes. In conclusion, rapid and high-throughput SNP analysis can be done with probe-free HRM if sufficient attention is paid to amplicon design and quality control to omit aberrantly amplifying samples. (Cancer Epidemiol Biomarkers Prev 2008;17(5):1240–7)

Introduction

Abnormal methylation patterns are one of the hallmarks of cancer. Methylation of CpG islands in the promoter region of many genes, including tumor suppressor genes such as the cell cycle inhibitor p16INK4a, the DNA repair genes BRCA1, MLH1, and MGMT, and the p53 regulator p14ARF, has been shown to shut down their expression (1, 2). It is still incompletely understood what underlies this alteration of methylation patterns and which susceptibility factors are involved. Much effort has been put into solving these questions, but they still remain largely unanswered.

Common variants in genes involved in the metabolism of the methyl group are likely candidates for the variation underlying propensity to methylation in normal tissues as well as in tumors (3, 4). A sufficient supply of the methyl group donor 5-adenosyl methionine (SAM) is important to maintain a normal methylation pattern (5, 6). Because the polymorphisms of the 5,10-methylenetetrahydrofolate reductase (MTHFR), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), and DNA methyltransferase 3b (DNMT3b) genes studied here either influence or are influenced by the levels of SAM, they are of particular interest, especially because they have been reported to modify the risk of getting different types of malignancies. However, further investigations are needed. Not all forms of cancers have been investigated in this regard, and some results need validation. Interestingly, some of the variants have been shown to be associated with an increased risk of getting some cancers and a decreased risk of getting others (7-9).

SAM is synthesized using dietary methionine or methionine generated from homocysteine. MTR methylates homocysteine to generate methionine and thus influences the cellular levels of SAM. MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the carbon donor for the de novo synthesis of methionine (Fig. 1). The common germ-line variants of MTHFR studied here (677T and 1298C) are less active (10, 11), and this can lead to higher levels of homocysteine and a deficiency of methyl group donors. The same is likely to be true for the MTR 2756G allele, but so far no one has been able to express human MTR in active form at sufficient levels to evaluate the biochemical effects of this polymorphism (12, 13). DNMT3b uses SAM to transfer methyl groups to DNA and is both responsible for de novo and maintenance DNA methylation. Overactivity of this gene has been linked to methylation of tumor suppressor genes and...
cancer (14, 15). Folate is an important player in these pathways as well, as it is required for the synthesis of methionine and SAM (Fig. 1). It has been shown that a deficiency of this nutrient results in DNA hypomethylation. The role of folate and the above germ-line variants in relation to cancer has been reviewed (16, 17).

MTHFR also plays a role in the synthesis of dTMP from dUMP as MTHFR and the enzyme catalyzing this reaction (thymidylate synthase) both utilize 5,10-methylenetetrahydrofolate (Fig. 1). Reduced MTHFR activity leads to elevated levels of 5,10-methylenetetrahydrofolate, which in turn leads to lower levels of dUMP and higher levels of dTMP. This can help prevent cancer because deficiency in dTMP increases the rate of misincorporation of dUMP into DNA, which has been shown to increase the rate of DNA strand breaks and other chromosomal damage (18). Thus, reduced MTHFR activity has been shown to correlate with both increased and decreased risk of getting different forms of malignancies (8, 9).

The DNMT3b promoter C46359T single nucleotide polymorphism (SNP) is located 149 bp upstream from the transcription start site. The C-to-T change significantly increases the promoter activity of the DNMT3b gene (19). This up-regulates the expression of the gene and may in turn lead to aberrant methylation of CpG islands in some tumor suppressor genes. Nevertheless, both the C and the T alleles have been associated with increased risk of getting different malignancies (7). For this reason, further investigations are needed to clarify the function of this SNP in relation to cancer.

The DNMT3b C31721T SNP is intronic, but when the T allele is present the sequence motif MATWAAAT (where M is A or C and where W is A or T) is missing (20). This motif is recognized by the transcription factor N-Oct-3 encoded by the POU3F2 gene. This gene has been shown to regulate the tumorigenic potential of human melanoma cells (21). Strong evidence for an association between this SNP and breast cancer risk has been reported (20).

High-resolution melting (HRM) analysis is a recently developed methodology made possible by the use of a new generation of fluorescent dyes, which do not inhibit PCR when they intercalate into double-stranded DNA at saturating levels (22). As the temperature increases, and the DNA “melts,” the dye is released and stops fluorescing. This change in fluorescence is sequence specific and can be monitored by appropriately designed instrumentation. Heterozygotes are particularly easy to identify because the heteroduplexes formed before the melting step have a characteristic melting profile. Homozygotes are more difficult to differentiate because their melting curves are usually very similar with often marginal differences in their melting temperature ($T_m$).

We used HRM to develop reliable and low-cost SNP genotyping assays that did not require additional probes.

Table 1. Primers and amplicon information (University of California-Santa Cruz Genome Browser, September 2007)

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Direction</th>
<th>Sequence 1</th>
<th>Amplicon size (bp)</th>
<th>Region spanned</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR C677T</td>
<td>Forward</td>
<td>5'-GCACCTTGAGAAGAGGTTGTCTG</td>
<td>50</td>
<td>11778944-11778994 of chromosome 1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGCTGCCGATGTAAGAAATCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFR A1298C</td>
<td>Forward</td>
<td>5'-GGGGAGGAGCCTGAGCGTGA</td>
<td>54</td>
<td>11777032-11777085 of chromosome 1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GAGGTAAGAAGCAAGGACTCAGACAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTR A2756G</td>
<td>Forward</td>
<td>5'-TGTCCCAGCTGTAGATGAAAATC</td>
<td>129</td>
<td>235115044-235115172 of chromosome 1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AAACAGCAAATCAAGCTGTTCACACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTR A2756G</td>
<td>Forward</td>
<td>5'-ATCTATGGAAGATGATGAGATTAGACAGG</td>
<td>57</td>
<td>235115092-235115148 of chromosome 1</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACTTACCTTGAAGACCTGATATAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNMT3b C46359T</td>
<td>Forward</td>
<td>5'-TTCTGGCCCCGGCCGAGACAG</td>
<td>58</td>
<td>30837901-30837958 of chromosome 20</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GAGAAGGACCTCACTGGGCCCTT</td>
<td></td>
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<tr>
<td>DNMT3b C31721T</td>
<td>Forward</td>
<td>5'-ATGGAGAGCTGGCATTAGTGAAAAATGAGAC</td>
<td>70</td>
<td>30863274-30863343 of chromosome 20</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-CATTCAGCTCTCTTGATTCAAGCAC</td>
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<tr>
<td>DNMT3b C31721T</td>
<td>Forward</td>
<td>5'-CCTGAGCAGGCTAAATACATTCAACA</td>
<td>100</td>
<td>30863248-30863347 of chromosome 20</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGTGCATCCGTCCTCTTATG</td>
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NOTE: The position of the interfering SNP is shown in bold.
or spiking with control DNA. This involved developing short amplicons that had $T_m$ differences above 0.4°C to 0.5°C for the homozygotes. These assays were tested on the Rotor-gene 6000 (Corbett Research) and on the LC480 (Roche). Although we did not test the stand-alone HR-1 and LightScanner HRM machines (Idaho), the assays we developed should translate well to those machines (23).

Materials and Methods

Samples and DNA Extraction. The investigations were done after approval by the Peter MacCallum Cancer Centre Ethics of Human Research Committee (Project 02/70). We obtained 94 peripheral blood samples from the Melbourne Branch of the Red Cross Blood Bank. DNA was extracted from theuffy-coat fraction of blood using the X-tractor Gene (Corbett Research) according to the manufacturer’s protocol. We did the incubation step after loading the Liquid Sample Digest Buffer for 20 min at 37°C instead of 10 min at room temperature. The DNA concentrations of the samples were measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies).

Primer Design, PCR, and HRM Conditions. One set of primers was designed for each of the $MTHFR$ and $DNMT3b$ (C46359T) assays and two for each of the $MTR$ and $DNMT3b$ (C31721T) assays. Primers were designed to have an optimal annealing temperature of 60°C using Primer Express 1.5 (Applied Biosystems). All amplicons were chosen so that they contained a single melting domain using the Poland program (http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html). The primer sequences and amplicon information can be found in Table 1. PCR cycling and HRM analysis were done on the Rotor-gene 6000 (Corbett Research) or on the LC480 (Roche).

For the Rotor-gene 6000, the intercalating dye used was SYTO 9 (Invitrogen), and the reaction mixtures were made up using HotStarTaq (Qiagen) and consisted of 2 to 20 ng genomic DNA, 1 × PCR buffer, 2.5 mmol/L MgCl₂ total, 200 nmol/L of each primer [400 nmol/L were used

Figure 2. $MTR$ A2756G. A. Rotor-gene 6000 HRM normalized graph. B. LC480 normalized graph. A and B, red, heterozygous (AG); blue, homozygous for the A allele (AA); green, homozygous for the G allele (GG). Y axis, normalized fluorescence; X axis, temperature (°C). C. Amplification (raw channel). Uneven amplification was observed when using shorter PCR steps. D. HRM normalized graph of the samples shown in C. Black, homozygous for the A allele. Right-shifted melting curves for these samples were observed.
in the DNMT3b (C46359T) assay], 200 μmol/L deoxy-
nucleotide triphosphates, 5 μmol/L SYTO 9, 0.5 units HotStarTaq polymerase, and PCR-grade water in a total
volume of 20 μL. For the LC480, the LC480 HRM
Scanning Master (Roche) was used with the same
concentrations of primers, MgCl₂, and genomic DNA
in the same total volume as the HotStarTaq/SYTO 9-containing MasterMix. The cycling protocol when

Figure 3. MTHFR C677T. A. Rotor-gene 6000 HRM normalized graph. B. LC480 normalized graph. Genotypes were readily
distinguished and are indicated by arrows for both.

Figure 4. MTHFR A1298C. A. Rotor-gene 6000 HRM normalized graph. B. LC480 normalized graph. Genotypes were readily
distinguished and are indicated by arrows for both. C. Amplification (raw channel). Two samples stayed in the exponential phase,
denoted as outliers. D. HRM normalized graph of the samples shown in C. Right-shifted melting curves of the two samples staying in
the exponential phase were observed. These are denoted as outliers.
using HotStarTaq started with one cycle of 95°C for 15 min, whereas the cycling protocol when using the LC480 HRM Scanning Master started with one cycle of 95°C for 10 min as less time is needed to activate the enzyme used in the latter MasterMix.

The cycling protocol for both machines was 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s, 1 cycle of 97°C for 1 min, and a melt from 65°C to 90°C for all assays, except for the DNMT3b C46359T assay. In this assay, 50 cycles were used, and the annealing step was done as a touchdown PCR with 6 cycles decreasing 1°C/ cycle from 70°C to 64°C. For the melt on the Rotor-gene 6000, the temperature was increased at the rate of 0.2°C/ s, and for the LC480, the temperature was increased at the rate of 1°C/s with the acquisitions set at 30 per °C. All reactions were done in duplicate.

The MTHFR C677T and the DNMT3b C31721T assays also worked well when using a more rapid cycling protocol of 45 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 10 s. This was not the case for the MTR A2756G assay as the melting curves of all the replicates showed considerable variation with these conditions (see Results and Fig. 2C and D).

HRM Analysis. The analysis was done with the software supplied with each machine. For the LC480, there is the possibility of temperature shifting the melting curves. This software feature makes it easier to distinguish different samples giving rise to differently shaped melting curves. However, because the two different homozygotes have identically shaped melting curves, temperature shifting was not done in this study.

Results

The samples are from individuals of a variety of ethnicities. Because of this, the actual genotyping results may not be comparable with results derived from a single population. However, the data are shown as it has been proven useful as a quality-control measure to ensure that expected proportions of all the genotypes were observed. In all cases, heterozygotes were readily distinguished by their characteristic biphasic melting patterns. However, in some cases, the two homozygote genotypes were less readily distinguished from each other.

Figure 5. DNMT3b C46359T and C31721T. A. DNMT3b C46359T SNP: Rotor gene 6000 HRM normalized graph. B. DNMT3b C46359T SNP: LC480 normalized graph. C. DNMT3b C31721T SNP: Rotor gene 6000 HRM normalized graph. D. C31721T SNP: LC480 HRM normalized graph. Genotypes were readily distinguished and are indicated by arrows for all.
**MTHFR** C677T (rs1801133). The homozygotes had $T_m$ values that differed by $\pm 0.6^\circ C$ independent of the MasterMix used. These were easily distinguished by HRM analysis on both machines when the data were normalized for the fluorescence before and after the melting transitions (Fig. 3A and B). We typed 94 samples and found 35 CC, 48 CT, and 11 TT.

**MTHFR** A1298C (rs1801131). It was difficult to design primers in this region because of the proximity of other SNPs. In particular, the position of the rs4846051 SNP is close to the MTHFR A1298C SNP. To avoid having two SNPs in between the primers, we designed the reverse primer to span the interfering SNP (see Table 1). The SNP is a C/T SNP, [listed in the SNP database (dbSNP BUILD 126) as an A/G SNP]. The reverse primer was designed to match neither the C nor the T expected in the antisense strand of this SNP by having a C at that position.

The size of the amplicon in this assay resulted in homozygous samples that differed by $\pm 0.8^\circ C$ in $T_m$ when using the SYTO 9 MasterMix and by $\pm 1.2^\circ C$ when using the LC480 HRM Scanning MasterMix. These were readily distinguished by HRM analysis on both machines when the data were normalized for the fluorescence before and after the melting transitions (Fig. 4A and B). We typed 94 samples and found 9 CC, 46 AC, and 39 AA.

As with other studies, we found partial linkage disequilibrium with the 667C>T polymorphism; in the 19 samples that were homozygous for the C allele, 18 were found to be homozygous for the C allele at the MTHFR C677T SNP as well, and all samples that were homozygous for the C allele at the MTHFR C677T SNP were homozygous for the A allele at the MTHFR A1298C SNP.

In one run on the Rotor-gene 6000, one replicate of two of the samples remained in the exponential phase, whereas all the other sample replicates went into the plateau phase (Fig. 4C). This resulted in a melting curve that was shifted to the right for both (Fig. 4D). One of these two samples is actually homozygous for the A allele and the other for the C allele. This could have resulted in an AA sample being interpreted as a CC sample if samples had not been run in duplicate and if normal PCR had been used instead of real-time PCR before the melting analysis.

**DNMT3b** C46359T (rs2424913). The homozygotes had $T_m$ values that differed by $\pm 0.7^\circ C$. These were readily distinguished by HRM analysis on both machines when the data were normalized for the fluorescence before and after the melting transitions (see Fig. 5A and B). We typed 94 samples and found 33 CC, 38 CT, and 23 TT.

**DNMT3b** C31721T (rs406193). For the large amplicon, the homozygotes had $T_m$ values that differed by $\pm 0.4^\circ C$ (Fig. 5C). The size of the amplicon in this assay resulted in poor separation of the homozygotes on the LC480 (data not shown). The smaller amplicon resulted in homozygotes that differed by $\pm 0.7^\circ C$ in $T_m$ values (Fig. 5D). All genotypes could then easily be determined on both machines. We typed 94 samples and found 6 GG, 24 AG, and 64 AA.

In one run on the Rotor-gene 6000 using shorter denaturation, annealing, and elongation steps (see Materials and Methods), we found samples that amplified much later than the others (Fig. 2C). This resulted in melting curves that were shifted to the right (Fig. 2D). These samples are actually homozygous for the A allele not the C allele. Again, this could have resulted in AA samples being interpreted as CC samples if samples had not been run in duplicate and if normal PCR was used instead of real-time PCR. The melting curves of the replicates varied in Fig. 2D. This is caused by uneven amplification of the samples in general under these conditions (Fig. 2C).

**Discussion**

Many different methods for detecting SNPs have been developed. All have their inherent strengths and weaknesses (24). All of these techniques, with the exception of fluorescent probe-based SNP genotyping, require removal of the PCR product for further analysis, making them more laborious and prone to human error as well as causing potential problems with PCR contamination.

Closed-tube assays, in which the genomic DNA and all the reagents required for amplification and genotyping are added at the same time, have become increasingly more attractive due to the continuous development of this approach. Originally, labeled primers or TaqMan-type probes were required. However, due to the introduction of saturating fluorescent DNA dyes, closed-tube SNP genotyping by amplicon melting has become simple, rapid, and inexpensive. These dyes, such as LCGreen, SYTO 9, and the dye in the LC480 HRM Scanning Master (this dye has no commercial name yet), bind to double-stranded DNA and fluoresce. Because the dyes do not inhibit PCR, it is possible to use them at saturating concentrations and thus to accurately monitor the melting of double-stranded DNA. Again, this has eliminated the need for any fluorescently labeled oligonucleotides and the produced amplicons can be directly analyzed after PCR. Generally, the method is called closed-tube SNP genotyping by amplicon melting (25).

The assays developed here are based on this method. We used the Rotor-gene 6000 and the LC480 because they are HRM-enabled real-time machines. The real-time capability allows us to record the amplification profiles and then to readily identify which aberrant melting patterns may have been due to irregular amplification. Real-time data are convenient for assay optimization as well.

Additional probes (26, 27) or control DNA spiking (28) have been used previously to separate homozygotes, but genotyping of nearly all SNPs can be done without the use of probes, and only G/C and A/T SNPs may require spiking with control DNA if the nearest neighboring bases are complementary (29). We obtained identical results for all 94 samples when using two different dyes on the two different HRM instruments. Furthermore, two different primer pairs for the MTR and DNMT3b SNPs gave identical results for all samples.
as well. Thus, SNP genotyping by HRM is a very reliable method that does not require confirmation by sequencing or other methods.

Homozygotes for all amplicons were readily distinguished by the Rotor-gene 6000 although their $T_m$ values differed by only 0.3°C and 0.4°C in the $MTR\ A2756G$ and $DNMT3b\ C31721T$ assays respectively. The smaller size of the amplicon in the $MTHFR\ A1298C$, $MTHFR\ C46359T$, $DNMT3b\ A1298C$, and $DNMT3b\ C46359T\ assays$ resulted in the two different homozygotes having $T_m$ values that differed by 0.6°C, 0.8°C/1.2°C (dependent on the MasterMix; see Results), and 0.7°C respectively.

We found a bigger spread of the melting curves for each genotype when using the LC480. This is not surprising because a plate-based system has intrinsically more temperature variation than a rotor-based system. This did not matter as long as the amplicon size was kept small. However, we could not reliably type some larger amplicons, e.g., the small. However, we could not reliably type some larger DNA fragments, e.g., the $MTR\ A2756G$ and $DNMT3b\ C31721T$ assays respectively. The smaller size of the amplicon in the $MTHFR\ A1298C$, $MTHFR\ C46359T$, $DNMT3b\ A1298C$, and $DNMT3b\ C46359T\ assays$ resulted in the two different homozygotes having $T_m$ values that differed by 0.6°C, 0.8°C/1.2°C (dependent on the MasterMix; see Results), and 0.7°C respectively.

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We recommend designing SNP assays so that the $T_m$ difference is greater than 0.4°C for the LC480. In general, this means that the amplicon size at least should be less than 100 bp, but the $T_m$ difference is sequence dependent as well. However, amplicon size seems to be the most important factor in determining $T_m$ differences. The $MTHFR\ A1298C$ assay showed that $T_m$ differences can be reagent and/or equipment dependent as well. It has been shown previously that ionic strength significantly affects $T_m$ (30). The advantage of optimizing the reaction for the LC480 is that higher throughput is possible compared to the rotor-based system of the Rotor-gene 6000.

Efficient SNP genotyping requires the presence of only one SNP per amplicon. The primers should not overlie a SNP as this may affect the differential amplification. The $MTHFR\ C1298A$ assay showed that SNPs in primers cannot always be avoided but that the problem can be worked around by introducing a mismatch at the SNP position in the primer. In spite of the mismatch in our primer, we were able to get good amplification. This might not have been the case if the position of the mismatch were closer to the 3' end, because this end needs to be stable for the polymerase to extend.

It has been considered that real-time amplification data is unnecessary for SNP genotyping by HRM analysis (27, 29). However, when we occasionally found outliers, these were almost always the result of individual reactions remaining in the exponential phase or amplifying very late. It has been noted previously by us that late amplifying samples should be treated with caution (31). For this reason, observation of real-time amplification data should be included in HRM result interpretations, and we recommend not scoring samples that amplified particularly late.

The results for the $MTHFR\ A1298C$ and $MTR\ A2756G$ SNPs showed that individual reactions staying in the exponential phase or amplifying markedly later than the majority of reactions can show right-shifted melting curves without alteration of shapes. This can lead to misinterpretation of results if certain precautions are not taken. Standardizing for DNA input helps prevent these problems, but even if samples are standardized for DNA input, some replicates occasionally amplify later than the majority. We were able to genotype samples that differed by ~10-fold in input DNA and found no direct association between outliers and DNA concentration within this window. However, when trying to genotype samples that differed in the range from 100-fold to 1,000-fold, we had major problems and found many more outliers (data not shown). Running samples in duplicate or triplicate is preferable for this reason as it can identify outliers. We have found that choosing longer denaturation, annealing, and elongation steps can in some cases also facilitate even amplification of samples. This was the case for the $MTR\ A2756G$ assay, in which the uneven amplification (Fig. 2C) was due to shorter denaturation, annealing, and elongation steps. Thus, the rare event that samples amplify markedly late in spite of standardized DNA concentrations can sometimes be explained by less optimized PCR conditions. Apart from that, we suggest that random effects in the PCR as well as pipetting errors may also be part of the explanation. The phenomena that individual replicates stay in the exponential phase and thus amplify to much higher levels are rarely observed and hard to explain. After optimization of assays, and running samples giving rise to outliers again on both machines, we were able to score all samples for all the SNPs. Thus, a very high percentage of success can be expected, and generally minimal optimization is needed when using the HRM approach.

Compared with TaqMan-based SNP genotyping, the HRM approach is much more cost-effective. Less optimization of assays is needed when no probes are used, and higher percentage of success can be expected. Pyrosequencing is another convenient method for SNP genotyping. However, this technique requires expensive and dedicated equipment and the percentage of success is highly dependent on DNA quality and quantity. The use of SNP chips has also become more widely used, and would be preferred relative to HRM, when conducting studies involving very large numbers of SNPs. However, in studies involving small numbers of SNPs, HRM will be the method of choice.

Germ-line variants in the $MTHFR$, $MTR$, and $DNMT3b$ genes have undergone many association studies in relation to different malignancies (3, 4, 7-9, 32-37). Some of these are contradictory and some have been difficult to replicate. We predict that more correlations between different malignancies and germ-line variants in these genes are to be discovered, and for this reason, reliable, low-cost, and high-throughput methodologies for detecting these variants should be welcomed in many laboratories.

In conclusion, we showed that HRM is one such method by developing genotyping assays specific for the $MTHFR\ C677T$, $MTHFR\ A1298C$, $DNMT3b\ C46359T$, $DNMT3b\ C31721T$, and $MTR\ A2756G$ SNPs. This was done without the use of additional probes or spiking with control DNA. These assays are reliable, of low cost, and are capable of high throughput analysis. Being able to review the real-time PCR amplification is a valuable tool for checking whether aberrant HRM profiles may be due to abnormal amplification.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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