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Quadruplex Genotyping of *F5*, *F2*, and *MTHFR* Variants in a Single Closed Tube by High-Resolution Amplicon Melting

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BACKGROUND: Multiplexed amplicon melting is a closed-tube method for genotyping that does not require probes, real-time analysis, asymmetric PCR, or allele-specific PCR; however, correct differentiation of homozygous mutant and wild-type samples by melting temperature (T_m) analysis requires high-resolution melting analysis and controlled reaction conditions.

METHODS: We designed 4 amplicons bracketing the *F5* [coagulation factor V (proaccelerin, labile factor)] 1691G>A, *MTHFR* (NADPH) 1298A>C, *MTHFR* 677C>T, and *F2* [coagulation factor II (thrombin)] 20210G>A gene variants to melt at different temperatures by varying amplicon length and adding GC- or AT-rich 5′ tails to selected primers. We used rapid-cycle PCRs with cycles of 19–23 s in the presence of a saturating DNA dye and temperature-correction controls and then conducted a high-resolution melting analysis. Heterozygotes were identified at each locus by curve shape, and homozygous genotypes were assigned by T_m. We blinded samples previously genotyped by other methods before analysis with the multiplex melting assay (n = 110).

RESULTS: All samples were correctly genotyped with the exception of 7 *MTHFR* 1298 samples with atypical melting profiles that could not be assigned. Sequencing revealed that these 5 heterozygotes and 2 homozygotes contained the unexpected sequence variant *MTHFR* 1317T>C. The use of temperature-correction controls decreased the T_m SD within homozygotes by a mean of 38%.

CONCLUSION: Rapid-cycle PCR with high-resolution melting analysis allows simple and accurate multiplex genotyping to at least a factor of 4.

Deep venous thrombosis has the potential to lead to pulmonary embolism, and its occurrence depends on many factors, including heredity, acquired risk factors, and other contributors *(1)*. The genetic factors include mutations at several well-defined loci in genes that code for proteins involved in coagulation, fibrinolysis, and homocysteine metabolism *(2)*. The existence of these mutations, and their potential to cooperatively interact in the development of thrombophilia, highlights a potential need for multiplexed analytical genotyping methods that are simple, fast, reliable, and cost-effective.

The use of saturating DNA dyes and high-resolution melting analysis provides some attractive solutions for genotyping *(3–8)*. For example, unlabeled probes (simple oligonucleotides) can be used to genotype single-base variants and small insertions or deletions *(3, 5–12)*, and the genotype is assigned by the probe's characteristic melting temperature (T_m)[4]. Even simpler conceptually is high-resolution analysis of amplicon melting, which uses only PCR primers *(3, 4)*. Heterozygous genotypes are easily identified by the shape and width of the melting curve. Homozygous genotypes are assigned on the basis of T_m differences that are usually approximately 1.0 °C, but the T_m difference can be less, depending on the base change and the length of the amplicon *(3, 13)*. High-resolution analysis of amplicon melting is limited by any source of T_m variance, including differences in salt concentrations (arising from evaporation during processing or differences in buffers used for DNA preparation) and variation in instrument temperature *(4, 14–16)*. One method for addressing T_m variance is to include temperature-correction controls within each PCR. For example, temperature-correction controls consisting of unamplified, 3′-blocked, complementary oligonucleotides improved melting precision for genotyping of variants of human platelet antigen genes, *LCT* (lactase), and *MTHFR* (NADPH) *(4, 15)*.

Another concern regarding multiplex single-color melting assays is the limited space along the temperature axis for distinguishing different amplicons or

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[4] Nonstandard abbreviations: T_m; melting temperature.

probes. The feasibility of duplex amplicon genotyping *(3)* with internal temperature calibrators *(15)* has previously been demonstrated. In the present study, we demonstrate the genotyping accuracy of a quadruplex high-resolution amplicon-melting assay for mutations in *F5*[5] [coagulation factor V (proaccelerin, labile factor)] (1691G>A), *F2* [coagulation factor II (thrombin)] (20210G>A), and *MTHFR* (1298A>C and 677C>T) genes.

Materials and Methods

STUDY SAMPLES AND DNA EXTRACTION

Whole-blood samples were submitted to ARUP Laboratories for genotyping of *F5* (1691G>A), *MTHFR* (1298A>C and 677C>T), or *F2* (20210G>A) variants. We extracted DNA with the Roche MagNA Pure LC system (Roche Diagnostics), and our absorbance measurements at 260 nm indicated DNA concentrations of 20–40 mg/L. DNA preparations were diluted to a uniform concentration of 20 mg/L before quadruplex amplification. *F5* and *F2* samples were genotyped in an assay with an unlabeled probe *(12)*. All MTHFR samples were genotyped with a duplex multicolor HybProbe® assay (Roche Diagnostics) *(15)*. For each locus, we chose examples of all 3 genotypes for the enrichment of heterozygous and homozygous mutants and selected 110 samples for analysis. These samples were deidentified according to a global ARUP protocol (IRB #7275), fully genotyped for all 4 loci with HybProbe or unlabeled-probe assays, blinded, and analyzed by means of the quadruplex thrombophilia amplicon-melting assay.

OLIGONUCLEOTIDES

Fig. 1 shows the primer sequences for the 4 genotyping loci and the internal temperature-correction controls. To create 4 different amplicons with nonoverlapping melting curves, we designed primer sets by varying amplicon length and adding GC- or AT-rich tails to selected primers. Synthetic oligonucleotide controls of 50 bases were blocked at the 3′ end with a phosphate group. Low-temperature controls were AT rich, and high-temperature controls were GC rich with added locked nucleic acid bases *(12)*.

QUADRUPLEX HIGH-RESOLUTION AMPLICON-MELTING ASSAY

We adjusted primer concentrations and temperature-correction controls to minimize interferences and to equalize the signal magnitudes of all controls and genotyping loci. PCRs were performed in 20-μL volumes containing 1× LightCycler FastStart DNA Master HybProbe solution (Roche Diagnostics), 0.6 μmol/L of each *F5* primer, 0.15 μmol/L of each of the *MTHFR* 1298 and 677 primers, 0.17 μmol/L of each *F2* primer, 0.08 μmol/L of the 50-bp high- and low-temperature correction controls, 3.5 mmol/L $MgCl_2$ (including 1 mmol/L $MgCl_2$ contributed by the LightCycler Master solution), 0.01 units heat-labile uracil-DNA glycosylase (Roche Diagnostics) per reaction, 1× LCGreen Plus (Idaho Technology), and 40 ng of the DNA template. PCR was carried out on a LightCycler (version 1.5; Roche Diagnostics) with an initial hold of 95 °C for 10 min, followed by 15 cycles of 95 °C for 2 s, 56 °C for 1 s, and 72 °C for 1 s and 25 cycles of 95 °C for 2 s, 58 °C for 1 s, and 72 °C for 4 s. To avoid prolonging the temperature cycles, we did not acquire fluorescence data during amplification. All heating and cooling steps during PCR were carried out with ramp rates of 20 °C/s. After PCR, we prepared samples for melting analysis by rapidly (20 °C/s) cooling them in the LightCycler from 95 °C to 40 °C.

HIGH-RESOLUTION MELTING

High-resolution melting analysis was performed on the HR-1 instrument (Idaho Technology). We generated melting curves by continuously acquiring fluorescence data from 55 °C to 95 °C at a temperature-transition rate of 0.1 °C/s. Data processing included normalization of fluorescence data, exponential background removal *(10)*, and the display of derivative melting curves. We adjusted melting curves by identifying the maxima of the peaks of the temperature-correction controls and then aligning the curves by shifting and linear scaling with the aid of custom software *(15)*. Heterozygotes were identified by melting-peak width and shape. Homozygotes were assigned genotypes by visual inspection of the T_m values (melting curve maxima).

SEQUENCING

We removed excess primers and unincorporated deoxynucleoside triphosphates from the PCR products of selected samples with ExoSAP-IT (USB Corporation) and performed bidirectional DNA sequencing. On a GeneAmp® PCR System 9700 (Applied Biosystems), 5 μL BigDye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems), 4 μL primer (0.8 pmol/μL), and 3 μL purified PCR product underwent 25 temperature cycles (96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min). Unincorporated terminators were removed with Sephadex G-50, and the extension products were sequenced on an ABI Prism 3100 Genetic Analyzer (Ap-

[5] Human genes: *F5*, coagulation factor V (proaccelerin, labile factor); *MTHFR*, 5,10-methylenetetrahydrofolate reductase (NADPH); *F2*, coagulation factor II (thrombin); *LCT*, lactase.

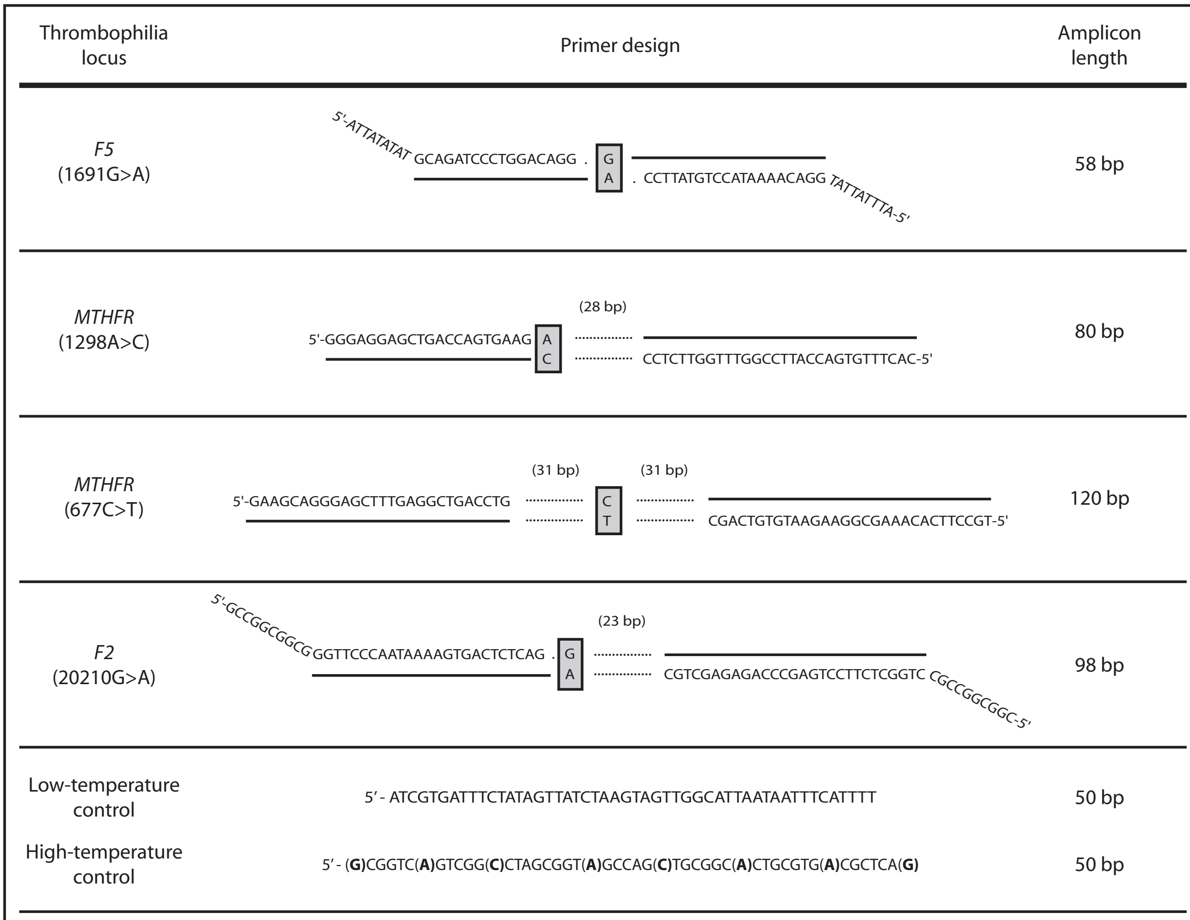

Fig. 1. Primer design and temperature controls for the thrombophilia quadruplex assay.

The common base (upper) and variant (lower) are shown in the gray box for all loci. *F5* primers and *F2* primers are tailed as indicated. The temperature-correction controls are shown without the complementary strand. Bracketed bold letters in the high-temperature control indicate locked nucleic acid bases.

plied Biosystems). Bases were assigned automatically with the instrument's sequence-analysis software.

Results

Representative quadruplex thrombophilia genotyping results are summarized in Fig. 2A as derivative melting curves after temperature adjustment. The melting transitions of the genotyping loci are spread over a temperature range of 15 °C, with the temperature controls positioned an additional 5 °C on either side. All of the controls and genotyping loci are clearly separated in temperature without overlap. Heterozygous genotypes are assignable by melting-curve shape and width at all loci. Table 1 presents the mean T_m values and SDs for all of the homozygotes. The T_m values of alternative homozygotes are separated by 0.82 °C (*F5*), 0.75 °C (*MTHFR* 1298), 0.38 °C (*MTHFR* 677), and 0.26 °C (*F2*).

Because the average SD within a homozygous genotype is only 0.06 °C, homozygotes are also accurately genotyped. Reproducibility within genotypes is shown for each locus in Fig. 2, B–E. Implementation of temperature-correction controls decreased the T_m SD by a mean of 38%.

To assess the accuracy of the quadruplex genotyping assay, we conducted a blinded study of 110 selected samples. The genotype distribution of the selected samples is shown in Table 2. All 110 *F5*, *MTHFR* 677C>T, and *F2* genotypes were correctly assigned, and all but 7 of the *MTHFR* 1298A>C genotypes were assignable. The 7 "indeterminate" samples displayed melting profiles that varied from those predicted for *MTHFR* 1298A>C.

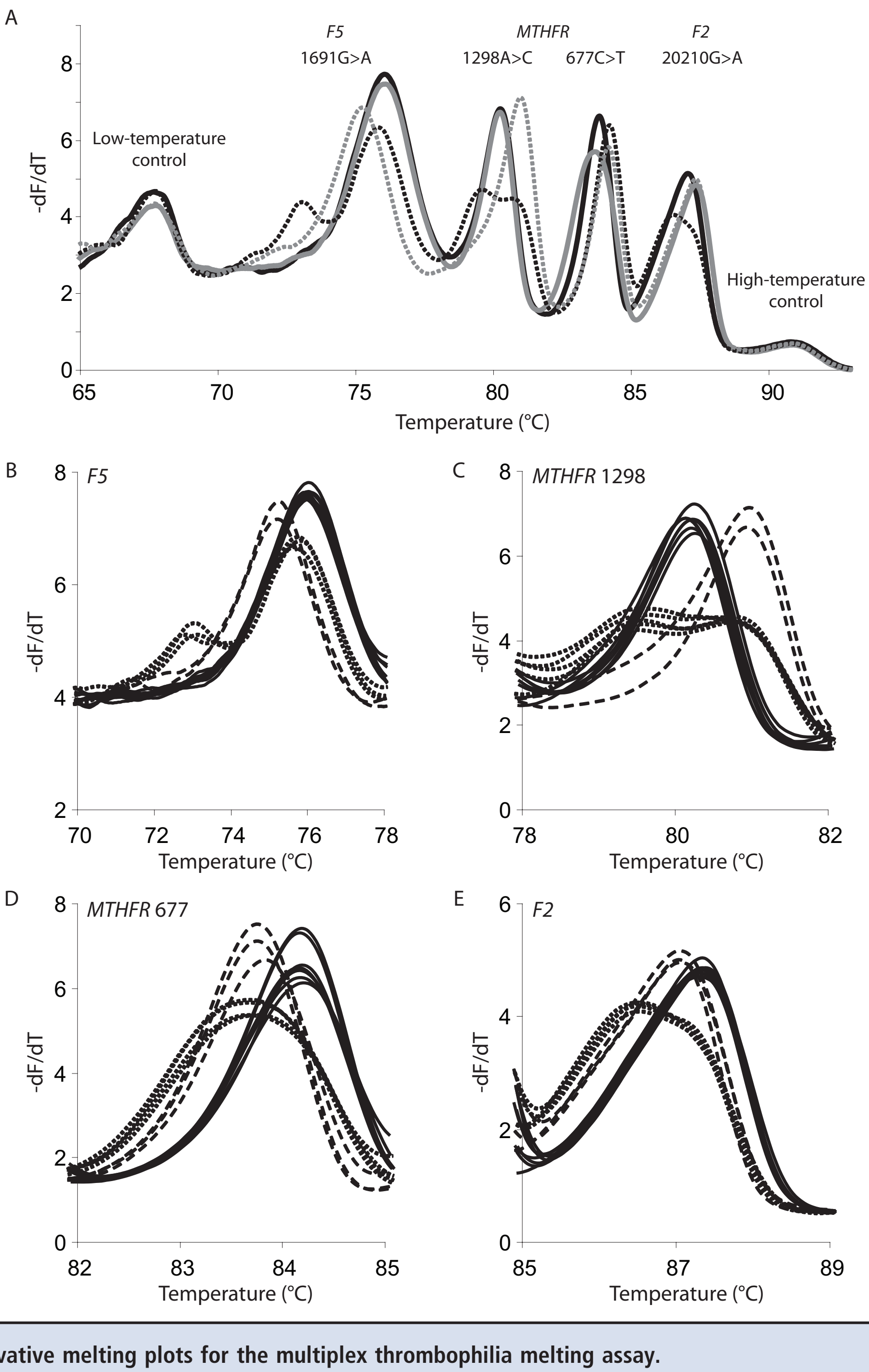

Fig. 2. Derivative melting plots for the multiplex thrombophilia melting assay.

(*A*), 4 representative melting profiles containing examples of all of the genotypes for each locus. Melting plots are shown as a solid black line (*F5* 1691GG, *MTHFR* 1298AA and 677TT, and *F2* 20210AA), a solid gray line (*F5* 1691GG, *MTHFR* 1298AA and 677CT, and *F2* 20210GG), a dotted black line (*F5* 1691GA, *MTHFR* 1298AC and 677CC, and *F2* 20210GA), and a dotted gray line (*F5* 1691AA, *MTHFR* 1298CC and 677CC, and *F2* 20210GG). The derivative melting plot includes all 4 thrombophilia loci and 50-bp complementary oligonucleotide temperature-correction controls for high and low temperature. (*B*–E), representative derivative melting plots for the *F5* 1691, *MTHFR* 1298, *MTHFR* 677, and *F2* 20210 loci, with homozygous wild-type, homozygous variant, and heterozygous genotypes indicated by solid black lines, dashed black lines, and dotted black lines, respectively.

Table 1. Mean T_m values and SDs of homozygous genotypes.

	Wild type[a]		Variant[a]	
	T_m, °C	SD, °C	T_m, °C	SD, °C
F5	75.95	0.067	75.12	0.083
MTHFR 1298	80.14	0.053	80.89	0.095
MTHFR 677	84.19	0.054	83.81	0.027
F2	87.35	0.056	87.08	0.041

[a] The numbers of samples for each category are given in Table 2.

The indeterminate samples with atypical melting characteristics followed 3 patterns (Fig. 3). Five of the samples appeared as heterozygotes that did not match the *MTHFR* 1298A>C pattern (Fig. 3A); these samples sequenced as 1298AA, 1317TC. One indeterminate sample was an apparent homozygote with a T_m between those for 1298AA and 1298CC (Fig. 3B). This sample sequenced as 1298AA, 1317CC. The last indeterminate sample had an unexpectedly low temperature transition and sequenced as the double heterozygote 1298AC, 1317TC (Fig. 3C). Each indeterminate case was due to the presence of an unexpected variant, *MTHFR* 1317T>C (rs4846051).

Discussion

Common genetic variants that predispose to thrombosis can be genotyped by many methods, including RFLP, allele-specific PCR, surface microarrays, microsphere arrays, DNA sequencing, and PCR-independent genotyping *(17, 18)*. Considerations in selecting a genotyping method include reagent and instrument costs, turnaround time, throughput needs, and the complexities of amplicon processing and/or allele separations. Closed-tube methods that require no processing after amplification are widely used in clinical laboratories. These methods usually require fluorescently labeled probes, however, and the number of colors available for multiplexing is limited *(19, 20)*.

Table 2. Genotype distribution in the blinded study.

	Genotype		
Mutation	Wild type, n	Heterozygote, n	Homozygote, n
F5 (1691G>A)	85	16	9
MTHFR (1298A>C)	71	27	12
MTHFR (677C>T)	59	37	14
F2 (20210G>A)	92	11	7

A few closed-tube genotyping methods use DNA dyes instead of labeled probes. These methods usually use melting analysis to identify the different alleles, either with unlabeled probes *(8)* or by tagging allele-specific primers to shift the T_m values of the corresponding amplicons *(21)*. The ultimate in simplicity is directly genotyping amplicons via melting analysis *(22)*. The amplicon-melting approach uses only 2 PCR primers per locus and a generic saturating DNA dye that detects all duplexes, irrespective of their T_m values or whether they are completely matched (as homoduplexes) or mismatched (as heteroduplexes). Primer modifications, probes, and allele-specific or asymmetric PCR are not needed. High-resolution methods for detecting heterozygotes *(23, 24)* and homozygotes *(3, 13)* have been applied to genotyping *(4, 25)* and mutation scanning *(7, 26, 27)*. Genotyping and scanning accuracy depends on the resolution of the melting instrument and the appropriate analytical software *(14, 28, 29)*. Instrument resolution is critical because homozygotes may differ in T_m by <1 °C *(3, 13)*. The identification of heterozygotes requires targeted software that compares the shapes of melting curves, instead of the more familiar T_m analysis that depends only on establishing a specific temperature.

Most single-base changes can be detected directly by analyzing amplicon melting. Single-base variants have been divided into 4 classes according to the homoduplexes and heteroduplexes produced after amplification *(3)*. C/T and G/A variants are class I, which includes the *F5*, *F2*, and *MTHFR* 677 variants, and constitute 66% of human single-base variants. C/A and G/T variants are class II, which includes the *MTHFR* 1298 variant. This class constitutes 18% of human single-base variants. Because a G:C base pair is exchanged with an A:T base pair, the T_m difference between alternative homozygotes is relatively large, averaging approximately 1.0 °C in small amplicons (<50 bp). Class III (G/C) and class IV (A/T) variants occur less frequently and make up the remaining 16% of human single-base variants. In class III and class IV variants, 1 base pair is inverted in the alternative homozygotes, and the GC content does not change; however, in three quarters of class III and class IV variants, nearest-neighbor changes produce a T_m difference between alternative homozygotes with a mode of 0.25 °C in small amplicons. In the remaining one quarter of such variants, the nearest-neighbor base pairs are not changed. Therefore, 4% of human single-base variants may have alternative homozygotes that are very difficult or impossible to distinguish by T_m. Homozygous insertions or deletions can also be very difficult to distinguish from the wild type, as is the case with F508del in cystic

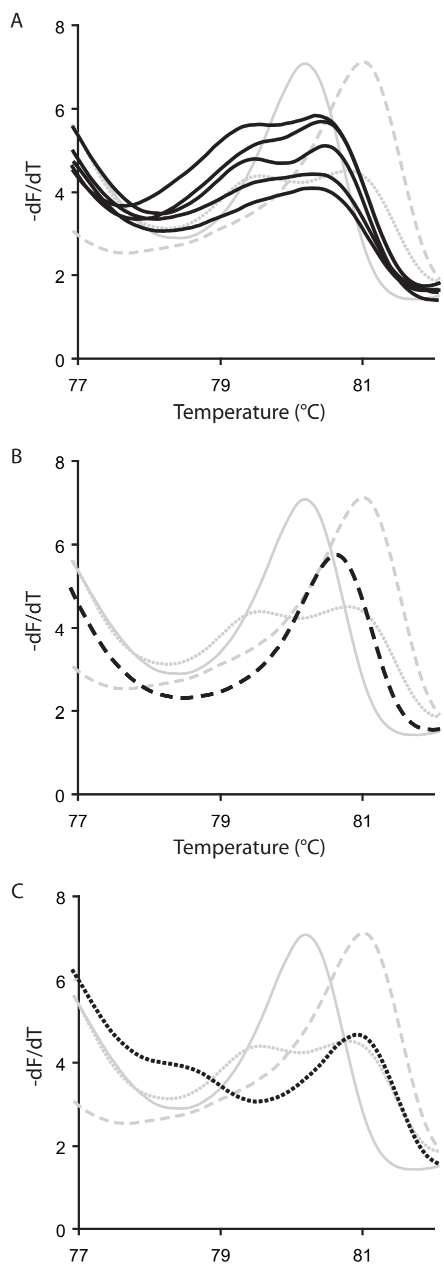

Fig. 3. Atypical derivative melting plots from the *MTHFR* 1298A>C amplicon.

The gray lines represent characteristic *MTHFR* 1298 melting curves for AA (solid line), CC (dashed line), and AC (dotted line) genotypes. (*A*), derivative melting curves for *MTHFR* 1298 AA samples heterozygous for *MTHFR* 1317T>C (solid black lines). (*B*), derivative melting curve of a *MTHFR* 1298 AA sample homozygous for *MTHFR* 1317T>C (dashed black line). (*C*), derivative melting curve of a sample heterozygous for both *MTHFR* 1298A>C and 1317T>C (dotted black line).

fibrosis *(27)*. In cases in which homozygotes are difficult to distinguish, amplicon-melting analysis can still provide complete genotyping information via their mixing with a known homozygote followed by quantitative heteroduplex analysis *(13)*. The variants we have described were all class I or II variants within amplicons of 58–120 bp that produced homozygous T_m differences of 0.3 °C–0.8 °C.

Internal temperature-correction controls can increase the T_m precision of melting assays by allowing the correction of differences in instruments and chemistries *(4, 15)*. The HR-1 instrument we used has the highest precision of all commercially available melting instruments *(14)*. Because of this instrument's precision, our incorporation of temperature-correction controls decreased the T_m SD by a mean of only 38%. Indeed, when temperature correction was not performed on our samples, the same genotyping results were obtained (data not shown). Temperature-correction controls may be critical, however, when instruments of lower resolution are used for melting analyses. For example, temperature correction substantially improves genotyping results with plate-based systems *(4)*.

Cycle times for the LightCycler protocol were only 19 s for each of the first 15 cycles and 23 s for each of the last 25 cycles. Rapid cycling appeared to be critical for the success of the assay. In addition to the short annealing and denaturation times, we turned off fluorescence data acquisition during thermal cycling to minimize the extension time. Because extension times vary slightly with the number of samples on the LightCycler (each acquisition requires approximately 200 ms), short extension times independent of the number of samples were obtained by disabling real-time data acquisition in each cycle.

Melting assays can be multiplexed by exploiting color differences, temperature differences, or both *(30)*. Amplicon melting monitored with a DNA dye uses temperature for multiplexing instead of probe color. Prior multiplex genotyping via amplicon melting has been limited to duplex assays *(15, 31)*. We have demonstrated that the method can be multiplexed to at least 4 amplicons. We achieved this capability by clearly separating each locus in T_m by (*a*) varying amplicon size, (*b*) the selective use of tailed primers to either increase or decrease amplicon T_m, and (*c*) the use of locked nucleic acids to increase the T_m of the high temperature-correction control. The use of locked nucleic acids and deletions to modify the T_m values of temperature-correction controls has previously been reported *(15)*. Once the loci of interest are separated by T_m, the relative fluorescence of each transition is adjusted by varying primer concentrations.

Multiplexing beyond 4 amplicons should be possible. For example, because temperature-correction controls may not be necessary with high-resolution melting analysis, the upper temperature-correction control could be replaced with an additional amplicon, and the lower control region might permit T_m space for 1 or 2 more amplicons. Although separating all of the loci simplifies visual analysis, genotyping may still be possible when loci do overlap in temperature. With separated loci, 3 genotype curves are expected for each biallelic locus. With temperature overlap, 4 unlinked biallelic loci could have 3 (i.e., 81) possible genotype curves. The ability to distinguish many different melting curves depends on instrument resolution and may be enhanced for automated analysis with future software; however, lower-resolution instruments are likely to require temperature-correction controls and may have difficulty distinguishing some genotypes, even with the deployment of appropriate controls *(14, 28, 29)*.

Unexpected sequence variants are often detected with melting assays. With analysis of probe melting, the scanned region is limited to the probe. With amplicon melting, the sequence between the primers is interrogated. In this study, we had 7 indeterminate *MTHFR* 1298 genotypes because their melting profiles did not match any 1298 genotype in shape or in T_m. In each of these 7 cases, sequencing revealed that a nearby 1317T>C synonymous variant was responsible. The 1317T>C variant is common in blacks and has been associated with preeclampsia in black South Africans *(32)*. This variant has caused errors in common restriction endonuclease gel assays and real-time 5′-exonuclease assays that have been used for *MTHFR* 1298 genotyping *(33)*.

Although scanning methods are very good at detecting variants, variant identification usually requires sequencing or specific genotyping *(7)*. High-resolution analysis of amplicon melting is unusual among scanning methods in that many homozygotes *(3)* and approximately 93% of heterozygotes *(27)* can be distinguished, suggesting that direct identification of most variants is possible with melting analysis alone. Nevertheless, specific variants are not identified with certainty, and genotyping accuracy depends on the frequencies and types of variants in the population tested. Of course, the accuracy of all PCR-based genotyping (including sequencing) can be compromised by unexpected variation in the sequences complementary to the primers *(34)*. Methods that rely on restriction enzymes, probes, ligation, or extension from internal oligonucleotides can be further compromised by unexpected variants in such regions *(35)*. The magnitude of the risk due to unexpected variants depends directly on the length of the susceptible sequence. For melting analysis of single-base variants of small amplicons, the size of the region between the primers can be varied and can often be limited to a single base *(3)*.

These studies were performed to assess the multiplexing capability of amplicon-melting analysis under ideal conditions. We have not attempted to transfer the method to platforms with higher throughputs. Two critical components in this study were melting precision/resolution and PCR cycling speed. Use of temperature-correction controls may allow use of melting instruments with lower precision. High-speed cycling appears particularly advantageous for multiplex melting analysis of small amplicons.

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