

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

Michael T. Seipp,^{1*} David Pattison,¹ Jacob D. Durtschi,¹ Mohamed Jama,¹ Karl V. Voelkerding,^{1,3} and Carl T. Wittwer^{1,2,3}

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.

© 2008 American Association for Clinical Chemistry

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

¹ ARUP Institute for Clinical and Experimental Pathology, ² ARUP Laboratories, 500 Chipeta Way, Salt Lake City, Utah 84108, and ³ Department of Pathology, University of Utah Medical School, 50 N Medical Drive, Salt Lake City, Utah 84132

* Address correspondence to this author at: ARUP Institute for Clinical and

Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108, Phone: (801) 583-2787 x2679, Fax: (801) 584-5114, email: seippmt@aruplab.com
Received August 31, 2007; accepted October 11, 2007

Previously published online at DOI: 10.1373/clinchem.2007.097121

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- μ L volumes containing 1 \times LightCycler FastStart DNA Master HybProbe solution (Roche Diagnostics), 0.6 μ mol/L of each *F5* primer, 0.15 μ mol/L of each of the *MTHFR* 1298 and 677 primers, 0.17 μ mol/L of each *F2* primer, 0.08 μ mol/L of the 50-bp high- and low-temperature correction controls, 3.5 mmol/L MgCl₂ (including 1 mmol/L MgCl₂ contributed by the LightCycler Master solution), 0.01 units heat-labile uracil-DNA glycosylase (Roche Diagnostics) per reaction, 1 \times 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 μ L BigDye Terminator Ready Reaction Mix v. 1.1 (Applied Biosystems), 4 μ L primer (0.8 pmol/ μ L), and 3 μ L 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.

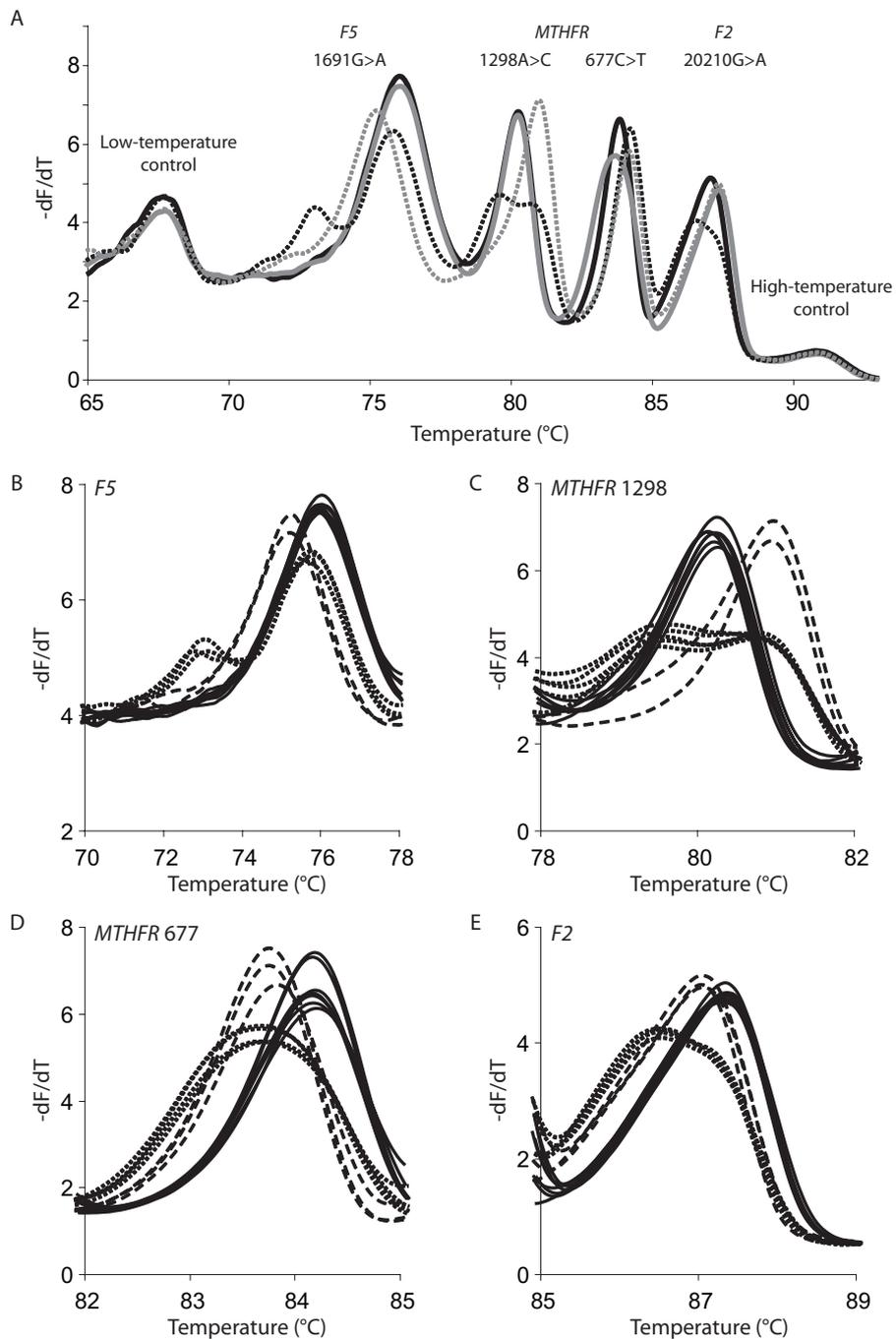


Fig. 2. Derivative melting plots for the multiplex thrombophilia melting assay.

(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* 2021AA), a solid gray line (*F5* 1691GG, *MTHFR* 1298AA and 677CT, and *F2* 2021GG), a dotted black line (*F5* 1691GA, *MTHFR* 1298AC and 677CC, and *F2* 2021GA), and a dotted gray line (*F5* 1691AA, *MTHFR* 1298CC and 677CC, and *F2* 2021GG). 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* 2021 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
<i>F5</i>	75.95	0.067	75.12	0.083
<i>MTHFR 1298</i>	80.14	0.053	80.89	0.095
<i>MTHFR 677</i>	84.19	0.054	83.81	0.027
<i>F2</i>	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 fluores-

cently labeled probes, however, and the number of colors available for multiplexing is limited (19, 20).

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

Table 2. Genotype distribution in the blinded study.

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

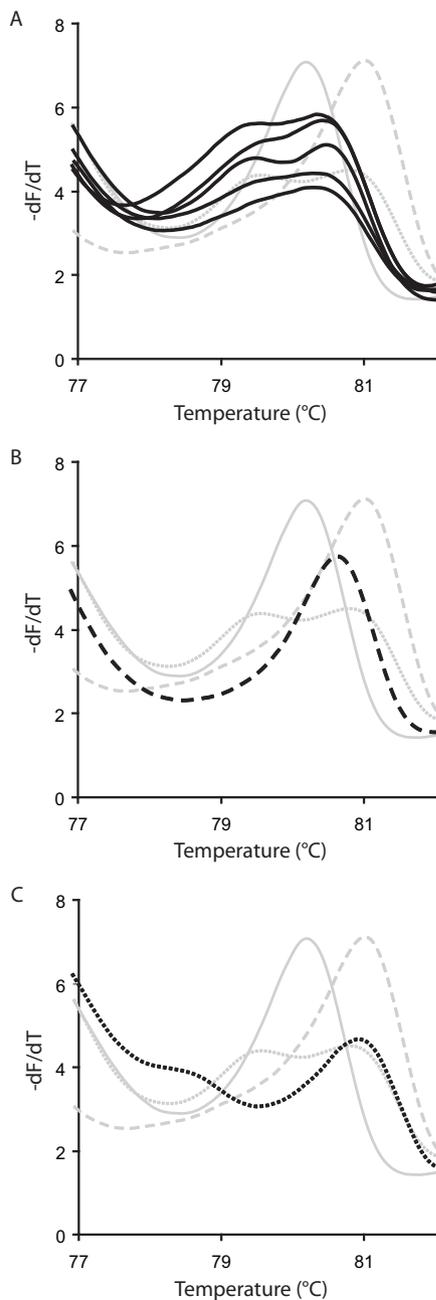


Fig. 3. Atypical derivative melting plots from the *MTHFR* 1298A>C amplicon.

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-resolu-

tion 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.

Financial disclosures: Aspects of the high-resolution melting and rapid-cycle PCR methods are licensed by the University of Utah to Idaho Technology and from Idaho Technology to Roche Applied Systems. One of the authors, Carl T. Wittwer, holds equity interest in Idaho Technology.

References

- Dahlback B. Blood coagulation. *Lancet* 2000;355: 1627–32.
- Key NS, McGlennen RC. Hyperhomocyst(e)inemia and Thrombophilia. *Arch Pathol Lab Med* 2002; 126:1367–75.
- Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, Wittwer C. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem* 2004;50:1156–64.
- Liew M, Seipp M, Durtschi J, Margraf RL, Dames S, Erali M, et al. Closed-tube SNP genotyping without labeled probes/a comparison between unlabeled probe and amplicon melting. *Am J Clin Pathol* 2007;127:341–8.
- Margraf RL, Mao R, Highsmith WE, Holtegaard LM, Wittwer CT. RET proto-oncogene genotyping using unlabeled probes, the masking technique, and amplicon high-resolution melting analysis. *J Mol Diagn* 2007;9:184–96.
- Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* 2007; 8:597–608.
- Vandersteen JG, Bayrak-Toydemir P, Palais RA, Wittwer CT. Identifying common genetic variants by high-resolution melting. *Clin Chem* 2007;53: 1191–8.
- Zhou L, Myers AN, Vandersteen JG, Wang L, Wittwer CT. Closed-tube genotyping with unlabeled oligonucleotide probes and a saturating DNA dye. *Clin Chem* 2004;50:1328–35.
- Dujols V, Kusukawa N, McKinney J, Dobrowolski S, Wittwer C. High-Resolution Melting Analysis for Scanning and Genotyping. In: Dorak M, ed. *Real-Time PCR*, Vol. New York: Garland Science 2006;157–71.
- Erali M, Palais R, Wittwer CT. SNP Genotyping by Unlabeled Probe Melting Analysis. In: Seitz O, Marx A, eds. *Methods in Molecular Biology*, Vol. Totowa, New Jersey: Humana Press 2006.
- Montgomery J, Wittwer CT, Palais R, Zhou L. Simultaneous mutation scanning and genotyping by high-resolution DNA melting analysis. *Nat Protoc* 2007;2:59–66.
- Chou LS, Meadows C, Wittwer CT, Lyon E. Unlabeled oligonucleotide probes modified with locked nucleic acids for improved mismatch discrimination in genotyping by melting analysis. *Biotechniques* 2005;39:644, 6, 8 passim.
- Palais RA, Liew MA, Wittwer CT. Quantitative heteroduplex analysis for single nucleotide polymorphism genotyping. *Anal Biochem* 2005;346: 167–75.
- Herrmann MG, Durtschi JD, Bromley LK, Wittwer

- CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin Chem* 2006;52:494–503.
15. Seipp MT, Durtschi JD, Liew MA, Williams J, Damjanovich K, Pont-Kingdon G, et al. Unlabeled oligonucleotides as internal temperature controls for genotyping by amplicon melting. *J Mol Diagn* 2007;9:284–9.
 16. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clin Chem* 2003;49:396–406.
 17. Wittwer C, Kuskawa N. *Nucleic Acid Techniques*. In: Burtis C, Ashwood E, Bruns D, eds. *Textbook of Clinical Chemistry and Molecular Diagnostics*, Vol. 4th ed. New York: Elsevier 2005;1407–49.
 18. Xu B, Tubbs RR, Kottke-Marchant K. Molecular genetic testing of polymorphisms associated with venous thrombosis: a review of molecular technologies. *Diagn Mol Pathol* 2005;14:193–202.
 19. Lay MJ, Wittwer CT. Real-time fluorescence genotyping of factor V Leiden during rapid-cycle PCR. *Clin Chem* 1997;43:2262–7.
 20. Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KS. Real-time multiplex PCR assays. *Methods* 2001;25:430–42.
 21. Wang J, Chuang K, Ahluwalia M, Patel S, Umblas N, Mirel D, et al. High-throughput SNP genotyping by single-tube PCR with Tm-shift primers. *Biotechniques* 2005;39:885–93.
 22. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003;49:853–60.
 23. Graham R, Liew M, Meadows C, Lyon E, Wittwer CT. Distinguishing different DNA heterozygotes by high-resolution melting. *Clin Chem* 2005;51:1295–8.
 24. Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem* 2004;50:1748–54.
 25. Hill CE, Duncan A, Wirth D, Nolte FS. Detection and identification of cytochrome P-450 2C9 alleles *1, *2, and *3 by high-resolution melting curve analysis of PCR amplicons. *Am J Clin Pathol* 2006;125:584–91.
 26. Dobrowolski SF, McKinney JT, Amat di San Filippo C, Giak Sim K, Wilcken B, Longo N. Validation of dye-binding/high-resolution thermal denaturation for the identification of mutations in the SLC22A5 gene. *Hum Mutat* 2005;25:306–13.
 27. Montgomery J, Wittwer CT, Kent JO, Zhou L. Scanning the cystic fibrosis transmembrane conductance regulator gene using high-resolution DNA melting analysis. *Clin Chem* 2007; Sep 21 [epub ahead of print]
 28. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Instrument comparison for heterozygote scanning of single and double heterozygotes: a correction and extension of Herrmann et al., *Clin Chem* 2006;52:494–503. *Clin Chem* 2007;53:150–2.
 29. Herrmann MG, Durtschi JD, Wittwer CT, Voelkerding KV. Expanded instrument comparison of amplicon DNA melting analysis for mutation scanning and genotyping. *Clin Chem* 2007;53:1544–8.
 30. Bernard PS, Wittwer CT. Homogeneous amplification and variant detection by fluorescent hybridization probes. *Clin Chem* 2000;46:147–8.
 31. Liew M, Nelson L, Margraf R, Mitchell S, Erali M, Mao R, et al. Genotyping of human platelet antigens 1 to 6 and 15 by high-resolution amplicon melting and conventional hybridization probes. *J Mol Diagn* 2006;8:97–104.
 32. Pegoraro RJ, Chikosi A, Rom L, Roberts C, Moodley J. Methylene tetrahydrofolate reductase gene polymorphisms in black South Africans and the association with preeclampsia. *Acta Obstet Gynecol Scand* 2004;83:449–54.
 33. Allen RA, Gatalica Z, Knezetic J, Hatcher L, Vogel JS, Dunn ST. A common 1317TC polymorphism in MTHFR can lead to erroneous 1298AC genotyping by PCR-RE and TaqMan probe assays. *Genet Test* 2007;11:167–73.
 34. Jeffrey GP, Chakrabarti S, Hegele RA, Adams PC. Polymorphism in intron 4 of HFE may cause overestimation of C282Y homozygote prevalence in haemochromatosis. *Nat Genet* 1999;22:325–6.
 35. Teupser D, Rupprecht W, Lohse P, Thiery J. Fluorescence-based detection of the CETP TaqIB polymorphism: false positives with the TaqMan-based exonuclease assay attributable to a previously unknown gene variant. *Clin Chem* 2001;47:852–7.