

## Multiplex Amplicon Genotyping by High-Resolution Melting

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High-resolution amplicon melting is a simple method for genotyping that uses only generic PCR primers and a saturating DNA dye. Multiplex amplicon genotyping has previously been reported in a single color, but two instruments were required: a carousel-based rapid cyclers and a high-resolution melting instrument for capillaries. Manual transfer of capillaries between instruments and sequential melting of each capillary at 0.1°C/s seriously limited the throughput. In this report, a single instrument that combines rapid-cycle real-time PCR with high-resolution melting [LightScanner-32 (LS-32), Idaho Technology, Salt Lake City, UT] was used for multiplex amplicon genotyping. The four most common mutations associated with thrombophilia, *F5* (factor V Leiden 1691G>A), *F2* (prothrombin 20210G>A), and *methylenetetrahydrofolate reductase* (*MTHFR*; 1298A>C and 677C>T) were genotyped in a single homogeneous assay with internal controls to adjust for minor chemistry and instrument variation. Forty temperature cycles required 9.2 min, and each capillary required 2.2 min by melting at 0.3°C/s, 3× the prior rate. Sample volume was reduced from 20 µl to 10 µl. In a blinded study of 109 samples (436 genotypes), complete concordance with standard assays was obtained. In addition, the rare variant *MTHFR* 1317T>C was genotyped correctly when present. The LS-32 simplifies more complex high-resolution melting assays by reducing hands-on manipulation, total time of analysis, and reagent cost while maintaining the resolution necessary for multiplex amplicon genotyping.

**KEY WORDS:** Thrombophilia, *F5*, *F2*, *MTHFR*, PCR, SNP, LightScanner-32

### INTRODUCTION

Genotyping by high-resolution amplicon melting uses only two PCR primers/locus and a generic, saturating DNA dye that detects heteroduplexes as well as homoduplexes. Heterozygous genotypes have a characteristic melting curve shape and a broader width than homozygous genotypes, which are assigned based on small melting temperature ( $T_m$ ) differences that are usually around 1.0°C but can be less depending on the base change and the length of the amplicon.<sup>1,2</sup> Genotyping accuracy depends on the resolution of the melting instrument and appropriate software for analysis.<sup>3–5</sup> The discrimination of different homozygous genotypes by  $T_m$  is affected by chemistry and instrument variance and can be corrected by the inclusion of temperature correction controls within each PCR.<sup>6,7</sup>

Multiplex genotyping by amplicon melting is also possible. Multiple amplicons targeting different loci may be

separated naturally in  $T_m$  based on their guanine-cytosine content and sequence. If the  $T_m$ s of different loci are not separated naturally, adequate separation can often be achieved by modifying the length of the amplicons or by selectively adding  $T_m$ -shifting primer tails.<sup>8,9</sup> The ability to genotype up to four loci in the same reaction using amplicon melting has been reported using rapid-cycle PCR for amplification in one instrument and high-resolution melting in separate instruments.<sup>9</sup> However, individual capillary samples had to be transferred manually between instruments, and the melting rates were slow (0.1°C/s), resulting in limited throughput.

Here, we report use of a new instrument that combines rapid-cycle PCR with high-resolution melting for multiplex amplicon genotyping to eliminate manual sample handling. Associated advantages include a three-fold increase in melting rate to 0.3°C/s, which triples throughput, and a twofold reduction in reaction volume that halves reagent costs. Genotyping accuracy is demonstrated using a four-plex thrombophilia amplicon melting assay for the *F5* (factor V Leiden, 1691G>A), *F2* (prothrombin 20210G>A), and *methylenetetrahydrofolate reductase* (*MTHFR*; 1298A>C and 677C>T) mutations and also discriminates *MTHFR* 1317T>C successfully.

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## MATERIALS AND METHODS

### LightScanner-32 (LS-32)

LS-32 (Idaho Technology, Salt Lake City, UT) is a new instrument that combines LightCycler® (Roche Applied Systems, Indianapolis, IN) and HR-1™ (Idaho Technology) technologies (Fig. 1). The LS-32 eliminates the manual transfer of individual capillaries from the LightCycler® to the HR-1™. This transfer is replaced by the automatic rotation of the LS-32 carousel into position over the high-resolution melting ingot (Fig. 1B). The ingot is elevated to enclose each capillary in turn to perform the high-resolution melting analysis. When one tube is finished, the ingot drops, and the carousel rotates the next sample into position. This greatly reduces the hands-on time associated with the original method. The LS-32 can perform rapid-cycle PCR, acquire real-time fluorescence, and provide low- and high-resolution melting analysis on 10 µl samples.

### Study Samples and DNA Extraction

Whole blood samples were submitted to ARUP (Salt Lake City, UT) for *F5* (1691G>A), *MTHFR* (1298A>C and 677C>T), or *F2* (20210G>A) genotyping. DNA was extracted with the Roche Applied Systems MagNA Pure LC system (Roche Applied Systems), resulting in concentrations of 10–67 ng/µl by absorbance at 260 nm, which were diluted to a uniform concentration of 10 ng/µl prior to four-plex amplification. Samples were genotyped at all loci using HybProbe (*MTHFR*)<sup>7</sup> or unlabeled probe (*F5* and *F2*) assays.<sup>10</sup> A total of 109 samples was selected to enrich rare genotypes at each locus. The genotypes of these samples were 89 wild-type, 10 heterozygous, and 10 homozygous at *F5* Leiden; 70 wild-type, 24 heterozygous, and 15 homozygous at *MTHFR* 1298; 59 wild-type, 33 heterozygous, and 17 homozygous at *MTHFR* 677; and 90 wild-type, 15 heterozygous, and 4 homozygous at *F2* 20210. At *MTHFR* 1317, 103 of these samples were wild-type, five heterozygous, and one homozygous. The samples were de-identified according to a global ARUP protocol (IRB #7275) after blinding and analyzed by the thrombophilia multiplex amplicon melting assay.

### Thrombophilia Multiplex High-Resolution Amplicon Melting Assay

Oligonucleotide sequences for the primers as well as the internal controls have been published previously.<sup>9</sup> PCR was performed in 10 µl vol with 1× LightCycler® FastStart DNA Master HybProbe (Roche Applied Systems), 0.5 µM each *F5* primers, 0.15 µM each *MTHFR* 1298 and 677 primers, 0.16 µM each *F2* primers, 0.06 µM low-temperature correction control and 0.08 µM high-temperature correction control, 3.5 mM MgCl<sub>2</sub> (including 1 mM MgCl<sub>2</sub> contributed by the

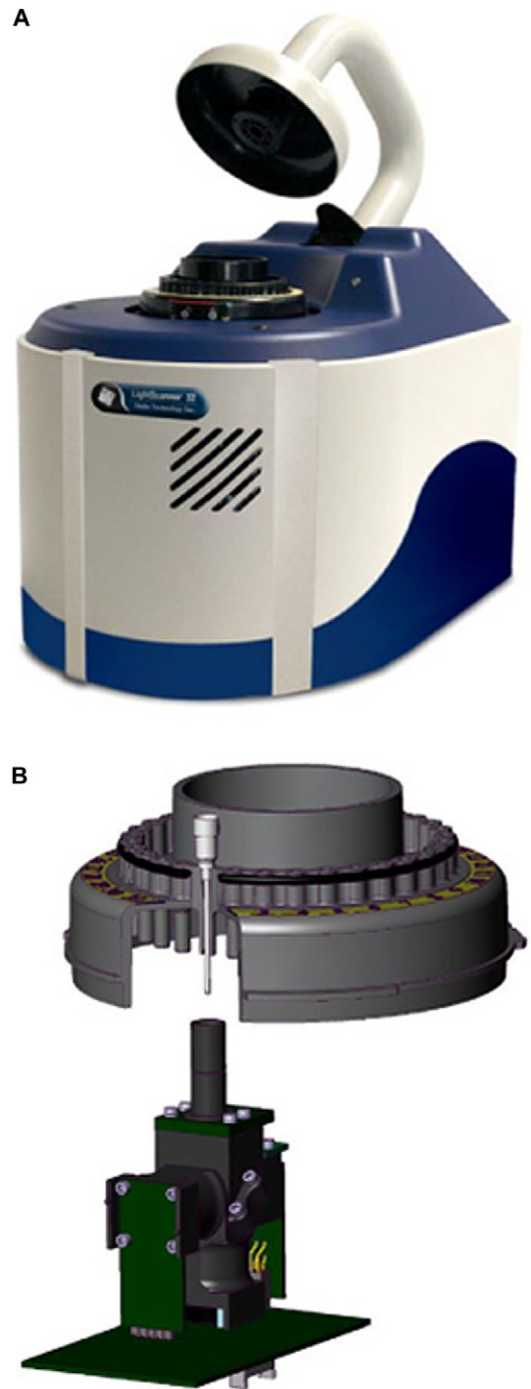


FIGURE 1

Photo (A) and schematic (B) of LS-32. (A) The instrument. (B) The internal design of LS-32 showing the 32-sample carousel and the high-resolution melting ingot with light source/fluorescence-detection housing.

LightCycler® FastStart DNA Master HybProbe solution), 0.01 U/reaction heat-labile uracil-DNA glycosylase (Roche Applied Systems), 1× LCGreen® Plus (Idaho Technology), and 20 ng template DNA. All oligonucleotides were mixed together and stored as a 20× stock solution.

PCR and high-resolution melting were done on the LS-32 (Idaho Technology). PCR included 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. During amplification, no fluorescence acquisition was performed to avoid prolonging the temperature cycles. All heating and cooling steps during PCR were done with ramp rates programmed at 20°C/s. After PCR, samples were cooled (10°C/s) from 95°C to 40°C and melting curves generated with continuous fluorescence acquisition from 55°C to 95°C at 0.3°C/s. Data processing included normalization of fluorescence, exponential background removal,<sup>11</sup> and display of derivative melting curves, which were adjusted by identifying the maxima of the temperature correction control peaks and aligning curves by shifting and linear-scaling using custom software.<sup>7</sup> Heterozygotes were identified by melting-peak width and shape. Homozygotes were assigned genotypes by visual inspection based on  $T_m$  (melting-peak maxima). Predicted  $T_m$ s based on nearest-neighbor parameters were calculated as described previously.<sup>1,2</sup>

## RESULTS

Representative multiplex genotyping results are shown in Figure 2A as derivative melting curves after normalization,

exponential background subtraction, and correction with internal controls. The melting transitions for genotyping are spread over an 18°C temperature range with the controls bracketing this range by an additional 5°C. For each locus, heterozygous genotypes result in an altered melting-curve shape with a wider transition, and homozygous genotypes can be distinguished by  $T_m$ .

All 109 blinded *F5*, *MTHFR* 677C>T, *MTHFR* 1298A>C, and *F2* variants were genotyped correctly. Furthermore, variation at the 1317T>C locus that is internal to the *MTHFR* 1298 amplicon was also detected and genotyped correctly in six samples, which included four 1298AA, 1317TC heterozygotes, one 1298AC, 1317TC double heterozygote, and one 1298AA, 1317CC homozygote. The 1298AA, 1317CC homozygote had a  $T_m$  of 81.6°C between the 1298AA, 1317TT (81.1°C) and 1298CC, 1317TT (81.9°C) homozygotes (Fig. 2B). Nearest-neighbor modeling predicted the same order of homozygote stabilities with separations of 0.4°C between 1298AA, 1317TT and 1298AA, 1317CC and 0.3°C between 1298AA, 1317CC and 1298CC, 1317TT. Four 1298AA, 1317TC heterozygotes followed a melting-curve cluster distinctly different from 1298AC, 1317TT heterozygotes (Fig. 2C). The double heterozygote (1298 AC and 1317TC) cor-

**FIGURE 2**

Derivative melting plots of the multiplex thrombophilia melting assay. (A) Four representative melting profiles are shown, which in combination, contain examples of all genotypes at each locus. The melting plots are shown as a solid black line (*F5* 1691GA, *MTHFR* 1298AC and 677CT, and *F2* 20210GG), solid gray line (*F5* 1691GG, *MTHFR* 1298CC and 677CC, and *F2* 20210AA), dotted black line (*F5* 1691GG, *MTHFR* 1298CC and 677CC, and *F2* 20210GA), and dotted gray line (*F5* 1691AA, *MTHFR* 1298AA and 677TT, and *F2* 20210GG). The derivative melting plot includes all four thrombophilia loci and high and low 50 bp complimentary oligonucleotide temperature correction controls. (B and C) Atypical derivative melting plots from the *MTHFR* 1298A>C amplicon. The gray lines represent characteristic *MTHFR* 1298 melting curves for AA (solid line), CC (dotted line), and AC (dashed line) genotypes. (B) Derivative melting curves of a *MTHFR* 1298 AA sample that was homozygous *MTHFR* 1317T>C (solid black). (C) A representative derivative melting curve of a *MTHFR* 1298 AA sample that was heterozygous *MTHFR* 1317T>C (solid black). The dotted black line represents the derivative melting curve of a sample heterozygous for *MTHFR* 1298A>C and 1317T>C.

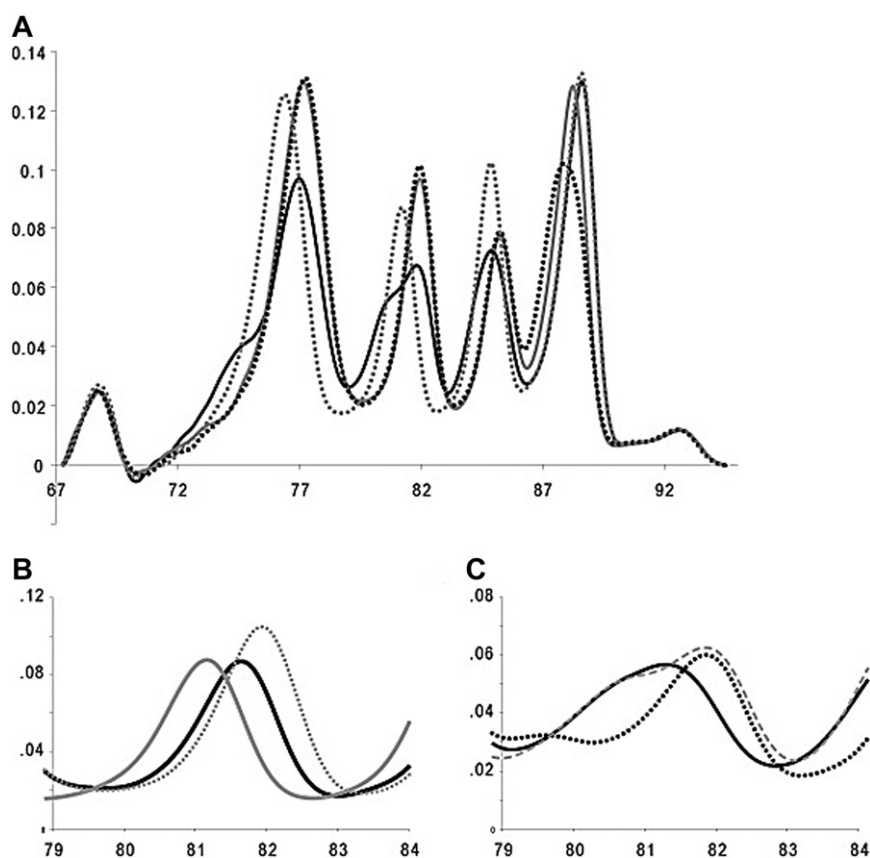


TABLE 1.

	T <sub>m</sub> s and SDs of Homozygous Genotypes							
	Wild-type <sup>a</sup>		Wild-type <sup>b</sup>		Variant <sup>a</sup>		Variant <sup>b</sup>	
	T <sub>m</sub>	SD	T <sub>m</sub>	SD	T <sub>m</sub>	SD	T <sub>m</sub>	SD
<i>F5</i>	77.20	0.121	77.27	0.052	76.60	0.067	76.47	0.048
<i>MTHFR</i> 1298	81.09	0.121	81.16	0.050	81.85	0.155	81.89	0.026
<i>MTHFR</i> 677	85.03	0.133	85.10	0.018	84.64	0.123	84.70	0.039
<i>F2</i>	88.28	0.146	88.33	0.047	88.18	0.150	88.03	0.050

<sup>a</sup>Data without internal controls; <sup>b</sup>Internal control temperature adjusted data.

related with a distinct heteroduplex peak between 79°C and 80°C (Fig. 2C).

The T<sub>m</sub>s and SDs of all homozygotes, with and without correction by internal controls, are shown in Table 1. The T<sub>m</sub>s of alternative homozygotes were separated by 0.60°C (*F5*), 0.76°C (*MTHFR* 1298), 0.39°C (*MTHFR* 677), and 0.10°C (*F2*) without correction and 0.80°C (*F5*), 0.73°C (*MTHFR* 1298), 0.40°C (*MTHFR* 677), and 0.30°C (*F2*) after correction. Internal controls decreased the T<sub>m</sub> SD by an average of 67%. Internal controls decreased the average homozygous T<sub>m</sub> SD from 0.13°C to 0.04°C, a variation low enough to allow accurate genotyping of all homozygotes. Without internal control correction, 14 of the possible 436 genotypes were assigned incorrectly. Six of the 14 incorrectly assigned genotypes were *MTHFR* 1298 samples confounded by the presence of the 1317 variant. The other samples included one *F5* 1691GG assigned as 1691AA, one *F2* 20210GG and two 20210AA called 20210AA and GG, respectively, and one *MTHFR* 677CC assigned as 677TT.

## DISCUSSION

Deep venous thrombosis is dependent on many factors including heredity and acquired lifestyle risk factors.<sup>12</sup> Mutations at several well-defined loci in genes coding for proteins involved in coagulation, fibrinolysis, and homocysteine metabolism contribute to the development of deep venous thrombosis.<sup>13</sup> The potential cooperative interaction of these mutations in the development of thrombophilia suggests multiplex genotyping. High-resolution melting of amplicons is an attractive method for genotyping because of its simplicity and has been reviewed recently.<sup>14–16</sup>

Up to four different single-base variants were amplified and genotyped previously by high-resolution melting in one reaction using a single fluorescence color.<sup>9</sup> However, two different instruments, one for rapid-cycle PCR and the other for high-resolution melting, were required.<sup>9</sup> Furthermore, manual transfer of individual sample capillaries was necessary. Each 20 µl sample was melted at 0.1°C/s requir-

ing 6.7 min on an instrument that only melted one sample at a time. Although the data quality was excellent, the throughput was low and required significant hands-on time. Internal controls decreased the SD within a genotype by 38% to an average weighted SD of 0.06°C. In this prior study, internal controls were not necessary for correct genotyping.

With minimal adjustments of oligonucleotide concentrations, the same quadruplex assay was adapted to the LS-32 platform. The LS-32 integrates rapid-cycle PCR and high-resolution melting on one instrument so that no manual transfers are necessary. Temperature cycling for PCR required 9.2 min, similar to the time of 10.1 min for the LightCycler® 1.5. We found that the LS-32 generated high-resolution melting profiles similar to the HR-1™ at 3× the rate (0.3°C/s), lowering the analysis time of each sample to 2.2 min. In addition, the sample volume was reduced from 20 µl to 10 µl. Internal controls were required for correct genotyping. When internal control adjustments were not made, 14 of the possible 336 genotypes were assigned incorrectly. The T<sub>m</sub> SD after internal control correction was decreased by 67% to an average weighted SD of 0.04°C, even lower than similar data reported on the HR-1™.

The *MTHFR* 1298 primers bracket position 1317, where a known variant is observed in <5% of Caucasians and >40% of Blacks.<sup>17</sup> In our prior study, the 1317 variants were unexpected and resulted in atypical *MTHFR* 1298 melting profiles that could not be assigned a genotype. Sequencing confirmed that the variations were correlated with 1317 variants. In the current study, similar variants also occurred, but the patterns were expected, and by prior experience, all melting curves were genotyped correctly at 1298 and 1317. The 1317 variant can confound *MTHFR* 1298 genotyping with hydrolysis probe and restriction fragment-length polymorphism assays.<sup>18</sup> In contrast, all common genotypes with 1317 variants (1298AC/1317TC, 1298AA/1317TC, and 1298AA/1317CC) were clearly distinct by melting analysis (Fig. 2B

and C). Furthermore, we expect the more rare genotypes 1298CC/1317CC, 1298AC/1317CC, and 1298CC/1317TC to be distinct also. For example, by nearest-neighbor predictions, the  $T_m$  of the 1298CC/1317CC homozygote should be 0.4°C higher than the 1298CC/1317TT homozygote and should be easily identified. In addition, different single heterozygotes within the same amplicon are usually distinct.<sup>19,20</sup>

These studies were performed to evaluate the LS-32 as a rapid-cycle real-time PCR instrument with high-resolution melting. The LS-32 required 9.2 min for 40 cycles compared with 10.1 min on a carousel LightCycler®. High-resolution melting on the LS-32 easily discriminated homozygous from heterozygous melting profiles as well as different heterozygous profiles from each other. The  $T_m$  precision of the LS-32 with internal controls appears superior to the HR-1™, previously reported as the instrument with the highest melting precision.<sup>3</sup> The LS-32 is more convenient than the LightCycler®/HR-1™ pair by eliminating manual transfer of capillaries between instruments. The LS-32 should be able to run all carousel LightCycler® assays with the added advantage of high-resolution melting.

Rapid cycling and high-resolution melting appear necessary for multiplex amplicon genotyping.<sup>9</sup> We have not been able to reproduce this work on instruments other than the LS-32 or the carousel LightCycler®/HR-1™ combination, presumably because of limits in cycling speed and/or melting resolution of other instruments. Access to exponential background subtraction methods<sup>21</sup> may be another reason why multiplex amplicon genotyping has only appeared on these platforms. Further work will focus on automatic genotype analysis in software to provide objective results not requiring expert evaluation.

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