Amplicon DNA Melting Analysis for Mutation Scanning and Genotyping: Cross-Platform Comparison of Instruments and Dyes

MARK G. HERRMANN,[1*] JACOB D. DURTSCHI,[1] L. KATHRYN BROMLEY,[1] CARL T. WITTWER,[1,2]
and KARL V. VOELKERDING[1,2]

Background: DNA melting analysis for genotyping and mutation scanning of PCR products by use of high-resolution instruments with special "saturation" dyes has recently been reported. The comparative performance of other instruments and dyes has not been evaluated.

Methods: A 110-bp fragment of the β-globin gene including the sickle cell anemia locus (A17T) was amplified by PCR in the presence of either the saturating DNA dye, LCGreen Plus, or SYBR Green I. Amplicons of 3 different genotypes (wild-type, heterozygous, and homozygous mutants) were melted on 9 different instruments (ABI 7000 and 7900HT, Bio-Rad iCycler, Cepheid SmartCycler, Corbett Rotor-Gene 3000, Idaho Technology HR-1 and LightScanner, and the Roche LightCycler 1.2 and LightCycler 2.0) at a rate of 0.1 °C/s or as recommended by the manufacturer. The ability of each instrument/dye combination to genotype by melting temperature (T_m) and to scan for heterozygotes by curve shape was evaluated.

Results: Resolution varied greatly among instruments with a 15-fold difference in T_m SD (0.018 to 0.274 °C) and a 19-fold (LCGreen Plus) or 33-fold (SYBR Green I) difference in the signal-to-noise ratio. These factors limit the ability of most instruments to accurately genotype single-nucleotide polymorphisms by amplicon melting. Plate instruments (96-well) showed the greatest variance with spatial differences across the plates. Either SYBR Green I or LCGreen Plus could be used for genotyping by T_m, but only LCGreen Plus was useful for heterozygote scanning. However, LCGreen Plus could not be used on instruments with an argon laser because of spectral mismatch. All instruments compatible with LCGreen Plus were able to detect heterozygotes by altered melting curve shape. However, instruments specifically designed for high-resolution melting displayed the least variation, suggesting better scanning sensitivity and specificity.

Conclusion: Different instruments and dyes vary widely in their ability to genotype homozygous variants and scan for heterozygotes by whole-amplicon melting analysis.

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Fluorescent melting analysis of PCR products in conjunction with real-time PCR was introduced on the LightCycler® approximately 9 years ago (*1–3*). Many melting techniques use fluorescently labeled oligonucleotide probes to genotype short segments of the PCR product (*4–7*). Melting curves are usually converted to negative first-derivative melting plots that reveal melting transitions of the probe-target hybrids as peaks. Samples with single peaks indicate a homozygous genotype, and those with 2 peaks indicate a heterozygous genotype. The higher temperature peak corresponds to the allele perfectly matched to the probe, and the lower temperature peak corresponds to the mismatched allele. Although accurate, these methods require expensive fluorescently labeled oligonucleotide probes and provide information only if the sequence variant is under the probe.

In contrast to probe methods, entire PCR products can be melted in the presence of DNA-binding dyes such as SYBR® Green I that differentiate double-stranded from single-stranded DNA by a change in fluorescence intensity. Sequence variants are inferred from changes in the

melting transition of the PCR product. Different PCR products generally have different melting temperatures (T_ms), depending on their GC content, length, and sequence *(3)*. Temperature resolution is limited, however, and small sequence differences, such as single-nucleotide polymorphisms (SNPs), may be difficult to identify. Several reports involving genotyping and scanning for minor sequence variants with SYBR Green I have appeared *(8–13)*, but the reliability of the methods has been questioned *(14, 15)*. Amplicon melting is most often used as a low-resolution tool for confirming the identity of PCR products on real-time PCR instruments.

Recently, techniques for high-resolution amplicon melting have appeared along with the introduction of a new family of LCGreen® dyes *(16, 17)*. These reports claim accurate SNP genotyping and heterozygote scanning in products up to 1 kb in length *(18)*. Most homozygous sequence changes produce a T_m shift compared with the wild type *(19, 20)*. In contrast, heterozygous samples are identified not by product T_m, but by differences in melting curve shape *(21)*. PCR products of heterozygous loci include destabilized heteroduplexes that form by the annealing of one wild-type and one variant strand. The resulting melting curve is a composite of both heteroduplex and homoduplex components. Homozygous samples typically have sharp, symmetric melting transitions, whereas heterozygotes show a more gradual, complex transition resulting from the different homo- and heteroduplex species present. In general, different genotypes show unique transitions that are revealed by high-resolution melting, shape comparison, and difference plots of the melting curves *(16, 21)*.

There is growing interest and use of melting analysis in clinical diagnosis. However, no study has evaluated the melting performance of commercially available instruments. Although melting analysis with labeled probes is well documented on many platforms, a comparative analysis of amplicon genotyping and heterozygote scanning on different platforms is lacking. In the current study, we perform a systematic comparison of melting analysis on 9 instruments from 6 vendors. These instruments vary in sample format from single glass capillary tubes to multiwell plates and use a variety of heat transfer methods. As a model analytical target, the sickle cell mutation was chosen. The sickle cell mutation in the β-globin gene is an A>T transversion in the second nucleotide of codon 6. A 110-bp PCR product including this mutation with a predicted T_m difference *(22)* between homozygotes (AA and TT) of 0.09 °C provides a stringent test for differentiation. Furthermore, the heterozygous AT genotype provides a convenient sample to assess the quality of mutation scanning by melting. Both homozygote resolution (AA vs TT) and heterozygote scanning (AA vs AT) were studied with either SYBR Green I or LCGreen Plus as fluorescent indicators of DNA melting.

Materials and Methods

INSTRUMENTATION

Nine instruments (representing 6 vendors) capable of melting analysis were available for comparison in our laboratory: the Prism® 7000 SDS and 7900HT (Applied Biosystems), iCycler iQ (Bio-Rad), SmartCycler® II (Cepheid), Rotor-Gene™ 3000 (Corbett Research), LightScanner® (Idaho Technology), HR-1™ (Idaho Technology), and the LightCycler 1.2 and LightCycler 2.0 (Roche). All instruments except the HR-1 and the LightScanner also provide temperature cycling for PCR. The sample formats, heating and cooling methods, and excitation and emission wavelengths for each instrument are shown in Table 1.

When LCGreen Plus was monitored in the iCycler, dye-specific excitation (438/24) and emission (485/20) filters (center wavelength/bandpass at half height; Semrock) were used. In this case, a pure dye calibration was performed on the iCycler with 50 pg/μL genomic DNA in 50 mmol/L Tris, pH 8.5 (25 °C) with 0.3× LCGreen Plus. LCGreen Plus was observed in the SYBR Green I Channel on all other instruments.

FLUORESCENCE SPECTRA

Spectra were obtained for both SYBR Green I (Invitrogen) and LCGreen Plus (Idaho Technology) by use of a scanning fluorometer and associated FeliX software (Photon Technology International) in a 60-μL cuvette containing 5 ng/μL human genomic DNA, 50 mmol/L Tris (pH 8.5 at 25 °C), and either a 1:3000 dilution of SYBR Green I or 1× LCGreen Plus. The emission spectrum for SYBR Green I was obtained by exciting at 485 nm and scanning the fluorescence from 490 to 650 nm, whereas LCGreen Plus was excited at 420 nm with scanning from 430 to 530 nm. The excitation spectrum for SYBR Green I was measured from 440 to 520 nm, with fluorescence monitored at 525 nm, whereas LCGreen Plus was measured from 380 to 480 nm with monitoring at 500 nm.

PCR

Exon 1 of the β-globin gene (GenBank accession no. U01317) was amplified by use of primers PCO3 (position 62150) and PCO4 (position 62259) *(23)*. EDTA-anticoagulated whole-blood samples were obtained from residual, deidentified clinical samples that had been submitted for hemoglobin evaluation, and DNA was extracted by use of a QIAamp DNA Blood Mini Kit (Qiagen). Samples were handled according to a global ARUP protocol under Institutional Review Board #7275. These samples were sequenced to identify those that were identical to the β-globin consensus sequence except at the base producing the hemoglobin S mutation. A single patient sample of each genotype was used; wild-type (AA), homozygous mutant (TT), and heterozygous (AT). Nearest-neighbor thermodynamic calculations predicted T_ms of 85.80 °C for the wild type and 85.89 °C for the homozygous mutant. Thermodynamic calculations used values of 0.2 μmol/L

Table 1. Instrument and sample characteristics.

Instrument	Volume, μL	Format	Heating/Cooling	Excitation, nm	Emission, nm	Acquisition mode	Data points/°C	Melting rate,[a] °C/s	Melting time, min
ABI: 7000	25	96-Well plate	Peltier	–[b]	–[b]	Continuous	3.1	0.022	27
ABI: 7900 HT	25	96-Well plate	Peltier	488	–[b]	Continuous	4.2	0.014	42
Bio-Rad: iCycler	25	96-Well plate	Peltier	480–500[c] / 423–447[d]	515–545[c] / 475–495[d]	Step	10	0.009	65
Cepheid: SmartCycler	25	16 Individual cassettes	Thermoelectric/Air	450–490	510–527	Continuous	10	0.1	5.8
Corbett: Rotor-Gene 3000	20	72 Polypropylene tubes	Circulating air	470	510	Step	10	0.008	73
Idaho Technology: HR-1	10	Glass capillary	Thermoelectric	455	490 LP[e]	Continuous	200	0.1	5.8
Idaho Technology: LightScanner	10	96-Well plate	Thermoelectric/air	470	510 LP[e]	Continuous	14	0.1	5.8
Roche: LightCycler 1.2	10	32 Glass capillaries	Circulating air	470	520–540	Step	19	0.023	25
						Continuous	5.2/1.9[f]	0.1	5.9
Roche: LightCycler 2.0	10	32 Glass capillaries	Circulating air	470	520–540	Step	11	0.040	15
						Continuous	4.1/1.9[f]	0.1	6.5

[a] Observed melting rates (programmed at 0.1 °C/s when possible).
[b] Proprietary.
[c] Filter bandpass for SYBR Green I.
[d] Filter bandpass for LCGreen Plus.
[e] LP, longpass filter.
[f] 9 samples/32 samples.

target (40% amplification efficiency), 20 mmol/L Na^+ equivalents from monovalent cations, and 133 Na^+ equivalents from Mg^{2+} (3 mmol/L Mg^{2+} reduced by 1.2 mmol/L deoxynucleoside triphosphates $\times$ 74 for the influence of divalent cations) *(19, 22)*.

A single 96-well plate PCR amplification for each dye was performed on a GeneAmp 9700 (Applied Biosystems) with each well containing 50 μL of 50 mmol/L Tris (pH 8.5 at 25 °C); 3 mmol/L $MgCl_2$; 0.5 μmol/L each primer; 250 mg/L bovine serum albumin; 0.2 mmol/L dATP, dGTP, and dCTP; 0.6 mmol/L dUTP; 1 U of heat-labile Uracil DNA glycosylase; 0.04 U/μL Taq polymerase; and 250 ng of human genomic DNA containing either a 1:30 000 dilution of SYBR Green I or 1× LCGreen Plus. The samples were thermally cycled with maximum temperature ramping under the following conditions: initial holds at 30 °C for 10 min and 95 °C for 10 min, followed by cycling for 35 cycles at 95 °C for 15 s, 65 °C for 20 s, and 72 °C for 1 s. After cycling, a 7-min hold at 72 °C was performed with subsequent rapid cooling to 4 °C. The individual samples were pooled by genotype and briefly centrifuged to remove any bovine serum albumin precipitate.

MELTING ACQUISITION

Samples on each instrument were run at the manufacturer's recommended volume, ranging from 10 to 25 μL (Table 1). Triplicate samples for each of the 3 genotypes for each dye were prepared for each instrument (3 × 3 × 2 × 9) and stored in the dark at 4 °C before melting (<8 h). Samples positions in instruments with 96-well heat blocks were assigned randomly, and the residual wells were filled with water.

In separate runs to more accurately assess the T_m SD and signal-to-noise ratio on each instrument, only the wild-type genotype was analyzed in 96 (ABI 7000, ABI 7900, iCycler, and LightScanner), 72 (Rotor-Gene), or 32 (LightCycler 1.2, LightCycler 2.0, SmartCycler, and HR-1) replicates. The sample capacity of each instrument dictated whether the replicates were intrarun (ABI 7000, ABI 7900, iCycler, LightScanner, and Rotor-Gene), interrun (HR-1), or mixed (SmartCycler: 2 runs of 16 samples).

Unless otherwise specified, after an initial hold at 60 °C for 30 s, the samples were melted by increasing the temperature to 95 °C at a programmed rate of 0.1 °C/s. However, the ABI 7900 had a mandatory premelting cycle of 95 °C for 15 s followed by 60 °C for 15 s. The ABI 7000, LightScanner, and HR-1 had no temperature hold before melting. Furthermore, different instruments had different methods of acquiring fluorescence during melting. One method was "continuous", in which the melting rate is held constant and the fluorescence is acquired as fast as possible. In contrast, some instruments used a "step" mode, in which the instrument acquires fluorescence in fixed temperature steps, ensuring a constant number of data points per 1 °C but at a much slower temperature
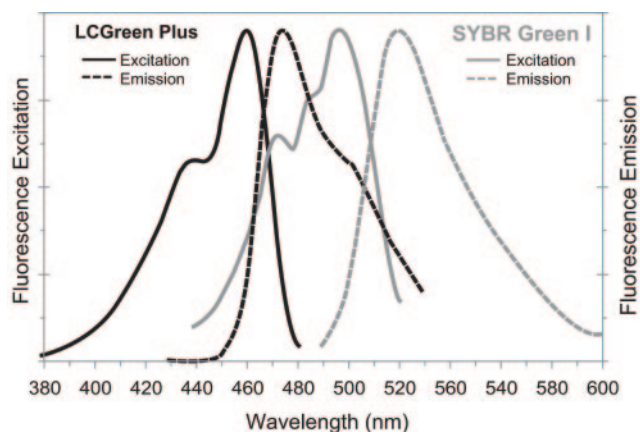
Fig. 1. Fluorescence spectra of LCGreen Plus and SYBR Green I.

The excitation (460 nm) and emission (475 nm) maxima of LCGreen Plus are blue-shifted compared with the excitation (495 nm) and emission (521 nm) maxima of SYBR Green I. Spectra were obtained in the presence of DNA and PCR buffer.

ramp rate. The iCycler and Rotor-Gene both used 10-s holds at each step for temperature equilibration before fluorescence acquisition. On the ABI 7900HT, the melting rate was expressed as "% heating rate", and was run at the company-suggested 2%. On the ABI 7000, only one preset melting protocol was available. On the LightCyclers, both continuous and step modes were available. Preliminary experiments showed that although step mode provided more data points, the continuous mode was preferable because of less noise. The data density, observed melting rates, and times required for melting on all instruments are shown in Table 1.

MELTING ANALYSIS

Original temperature and fluorescence data were extracted from each instrument, arranged in Microsoft Excel, and analyzed by custom software written in LabVIEW, which is designed for high-resolution melting analysis as described previously (16, 24). All operations were strictly arithmetic without smoothing or manipula-

tion of the data. Data from the "component fluorescence" file were used from the 2 ABI instruments. A reference dye was not used on the ABI instruments because all data were normalized between 0% and 100% fluorescence during analysis. Because temperature data are not associated with component fluorescence data on the ABI 7000, temperatures were inferred by dividing the total number of acquisitions (n = 109) by the temperature differential (35 °C), offset to the starting temperature (60 °C).

For genotyping, melting curves were normalized by selecting linear regions before and after the melting transition. These regions defined 2 lines for each curve: an upper (100%) fluorescence line and a lower (0%) baseline. The percentage fluorescence within the transition (between the 2 linear regions) was calculated at each temperature as the distance from the experimental data to the lower line compared with the distance between the extrapolated upper and lower lines. T_ms were interpolated from the normalized data as the temperature at 50% fluorescence. Signal-to-noise ratios were obtained by dividing the signal (the difference between the upper and lower baselines at the T_m) by the noise (the average SD of the points defining the upper baseline about their best-fit second-degree polynomial).

For heteroduplex scanning, melting curve shapes were compared by superimposing the normalized curves at low fluorescence. Typically a region at <5% fluorescence was selected, although lower data quality from some instruments required extending the upper bound of this interval up to 20%. Temperature shifting was not possible on the 96-well run of the ABI 7900 (high noise) or on the 32-sample run of the LightCycler (low data density on continuous mode). Temperature shifting eliminates slight temperature offsets between samples so that heteroduplexes can be easily identified.

Results

The spectral excitation and emission curves for SYBR Green I and LCGreen Plus in the presence of DNA and
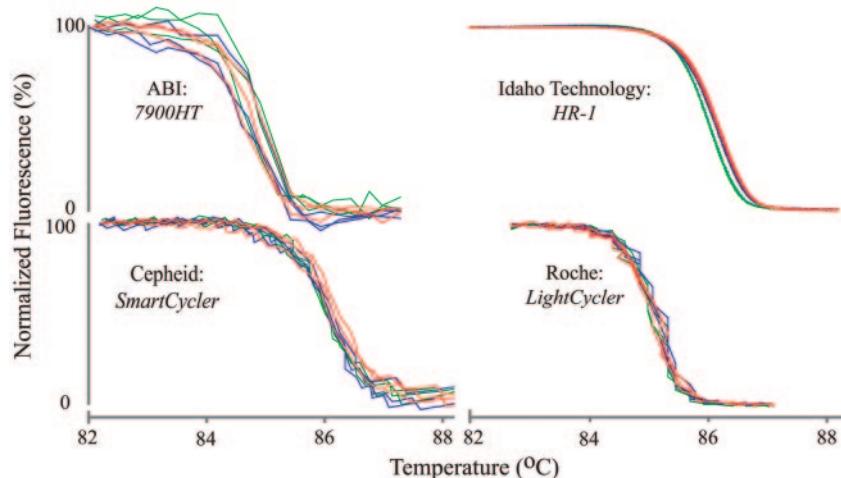

Fig. 2. Melting curves of a 110-bp amplicon including the sickle cell SNP in the presence of SYBR Green I.

Each genotype was melted and displayed in triplicate on 4 representative instruments. Wild-type (AA) samples are shown in *green*, heterozygotes (AT) in *blue*, and the homozygous mutants (TT) in *red*. The heterozygous genotype cannot be distinguished from the homozygous genotypes.
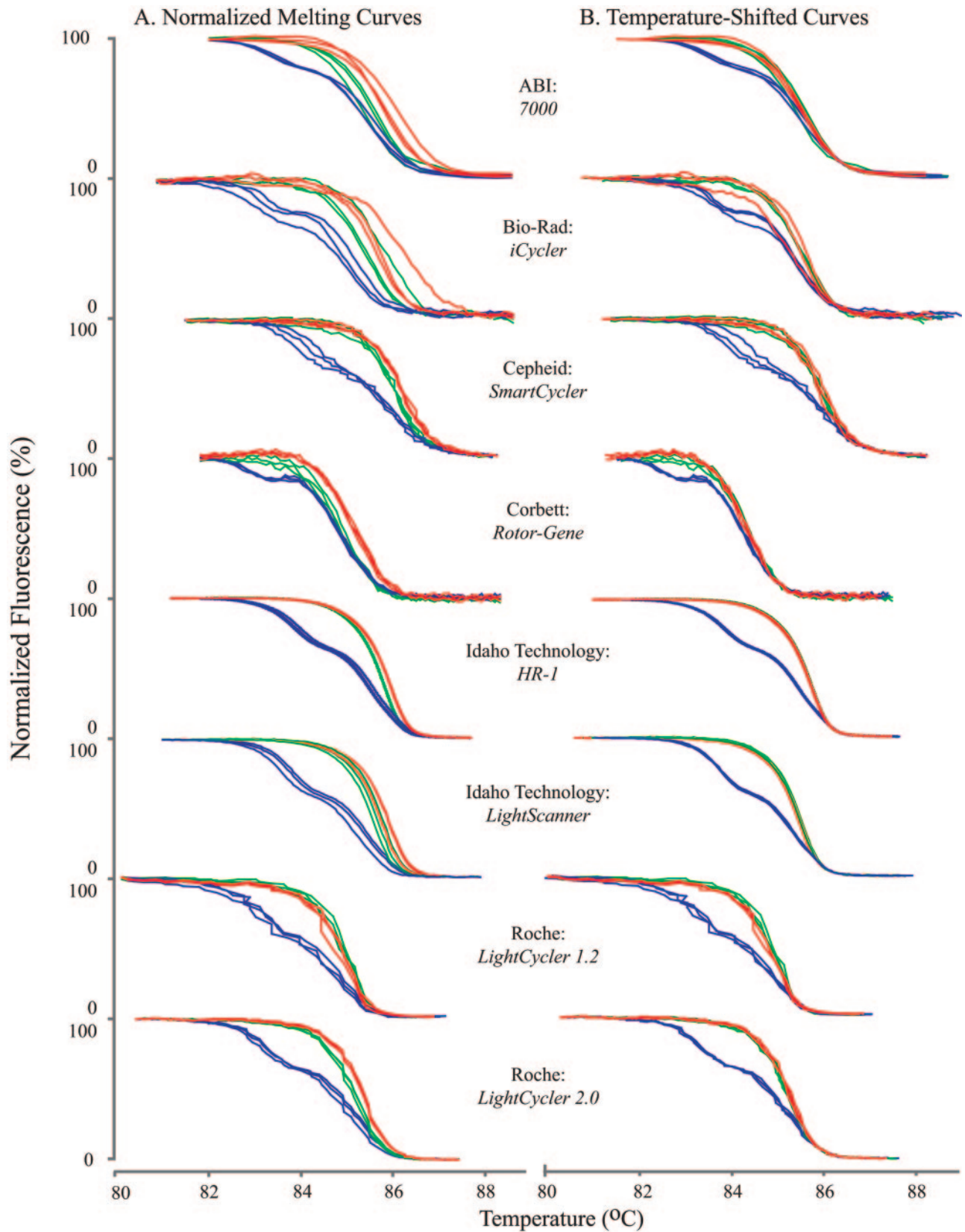
Fig. 3. Melting curves of a 110-bp amplicon including the sickle cell SNP in the presence of LCGreen Plus.

Each genotype was melted and displayed in triplicate on 8 different instruments. Wild-type (AA) samples are shown in *green*, heterozygotes (AT) in *blue*, and the homozygous mutants (TT) in *red*. (*A*), normalized melting curves for genotyping; (*B*), temperature-shifted curves for heterozygote scanning. In contrast to SYBR Green I (Fig. 2), heterozygous samples are easily identified by the presence of a lower temperature heteroduplex transition in addition to a higher temperature homoduplex transition.

Table 2. Temperature variance and signal-to-noise ratios for the melting instruments studied.

Instrument	n	T_m SD, °C		S/N[c]	
		Genotyping[a]	Scanning[b]	LCGreen Plus	SYBR Green I
ABI: 7000	96	0.117	0.065	560	530
ABI: 7900 HT	96	0.274	—[d]	—[e]	180
Bio-Rad: iCycler	96	0.173	0.064	410	170
Cepheid: SmartCycler	32	0.065	0.051	310	120
Corbett: Rotor-Gene 3000	72	0.045	0.051	180	60
Idaho Technology: HR-1	32	0.018	0.012	3500	1600
Idaho Technology: LightScanner	96	0.092	0.013	1800	2000
Roche: LightCycler 1.2	32	0.045	—[d]	260	140
Roche: LightCycler 2.0	32	0.047	—[d]	490	770

[a] SD of the absolute T_m within a genotype (relevant to genotyping homozygotes).

[b] SD of the T_m within a genotype after temperature shifting (relevant to heterozygote scanning).

[c] S/N, signal-to-noise ratio (see *Materials and Methods* for description).

[d] Calculation not possible (see *Materials and Methods*).

[e] No LCGreen Plus signal detected.

PCR buffer are shown in Fig. 1. The maximum absorbance and fluorescence for SYBR Green I were similar to published values at 495 nm and 521 nm, respectively *(25)*. Compared with SYBR Green I, the spectrum of LCGreen Plus was blue-shifted with peak absorbance at 460 nm and peak fluorescence emission at 475 nm.

SYBR Green I was compatible with all instruments. The means (SD) of the average T_ms across instruments were 85.36 (0.43) °C for the AA and 85.42 (0.49) °C for the TT genotypes. These values are, on average, only −0.46 °C off from the nearest-neighbor predicted values. The mean T_m difference between homozygous genotypes (TT − AA) across instruments was 0.06 (0.11) °C. This mean is very close to that predicted from nearest-neighbor parameters, although the magnitude of the SD suggests that genotyping of individual samples may be difficult. This is demonstrated in Fig. 2, in which SYBR Green I melting curves for 4 of the 9 instruments are shown. In general, variations between samples of the same genotype preclude accurate genotyping. An exception may be the HR-1 instrument, for which the AA genotype appeared to the left of the AT and TT genotypes. However, on all instruments, the heterozygous AT genotype was not distinct from the homozygous genotypes when SYBR Green I was used.

In contrast to SYBR Green I, 2 of the instruments as supplied by the manufacturer were not compatible with LCGreen Plus. The standard iCycler filter set for fluorescein/SYBR Green I was too narrow to include LCGreen Plus. Because the iCycler excitation source is broadband and filter sets are exchangeable, a custom filter set matched to LCGreen Plus allowed evaluation. However, the excitation source of the ABI 7900HT is an argon ion laser, and the optics could not be modified to allow evaluation of LCGreen Plus.

The mean (SD) of the average T_m across instruments using LCGreen Plus was 85.40 (0.40) °C for the AA and 85.59 (0.47) °C for the TT genotypes with a T_m difference (TT − AA) of 0.19 (0.14) °C. Very similar T_ms were ob-

tained irrespective of whether the dye was LCGreen Plus or SYBR Green I. Normalized melting curves using LCGreen Plus on all instruments compatible with the dye are shown in Fig. 3A. When the melting curves were temperature-shifted (Fig. 3B), heterozygotes could be identified on all instruments. The amount of noise on different instruments varied widely.

With only 3 samples per genotype, the different homozygotes appeared to separate on some instruments (Fig. 3A). To more accurately assess the ability to differentiate homozygotes for genotyping, we determined the SD of the T_m on each instrument by analyzing 32 to 96 wild-type samples (Table 2). The 9 different instruments varied by >15-fold in the SD of replicate samples. The SDs of the four 96-well plate instruments (0.092 to 0.274 °C) were greater than instruments based on circulating air (0.045–0.047 °C) or individually controlled samples (0.018 and 0.065 °C). The dynamic thermal uniformity of the 96-well instruments based on amplicon T_m is shown in Fig. 4. The LightScanner had the smallest T_m range at 0.35 °C across the plate, with the ABI 7000 at 0.66 °C, the iCycler at 1.10 °C, and the ABI 7900 at 1.24 °C.

Because homozygous melting curves differ predominately in position (T_m) rather than shape, the ability of each instrument to distinguish homozygous genotypes depends only on the SD of the measured T_m (Table 2) and the actual T_m difference between genotypes. The estimated error rates (assuming gaussian distributions) for the 9 instruments at T_m differences of 1.0, 0.5, 0.25, and 0.09 °C are shown in Table 3.

For heterozygote detection, absolute T_m is irrelevant and curve shape is paramount. Resolution can be assessed by curve variation after temperature shifting and by signal-to-noise ratios. Temperature shifting reduces the variation within a genotype (Fig. 3), giving SDs at 50% fluorescence (T_m SD for scanning in Table 2) from 0.012 to 0.065 °C. Temperature shifting was particularly effective in reducing curve shape variance in the iCycler and the
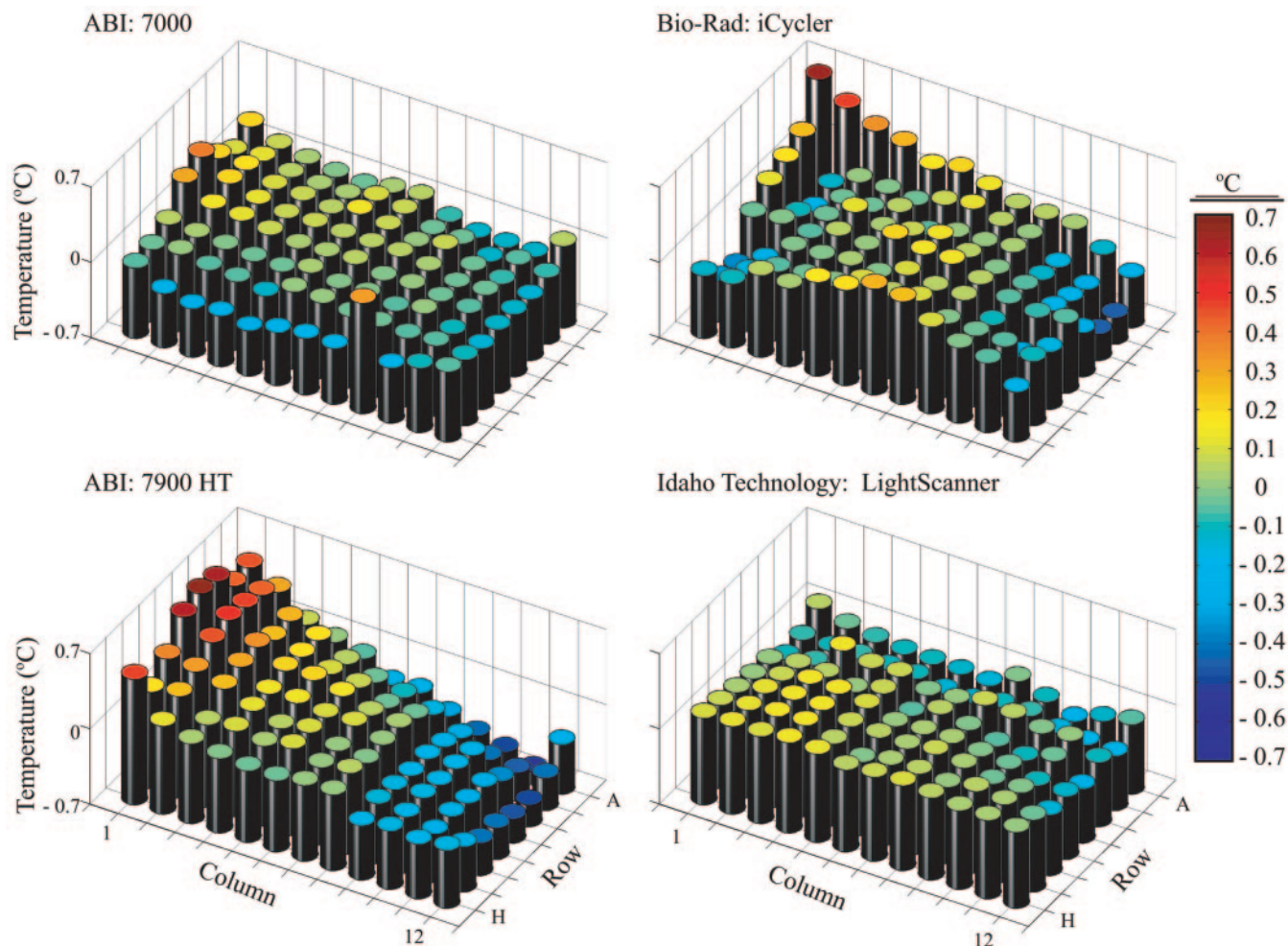
Fig. 4. Dynamic thermal profiles of the four 96-well instruments studied based on the apparent T_m of the wild-type amplicon.
The absolute temperature difference about the mean of each instrument is shown in *false color* as indicated in the key to the *right*.

LightScanner. The signal-to-noise ratios of the different instruments varied >19-fold with LCGreen Plus and 33-fold with SYBR Green I. The dedicated melting instruments had, on average, ~10-fold greater signal-to-noise ratios than conventional real-time instruments.

Discussion

DNA dyes allow for rapid, inexpensive interrogation of PCR products without exposure of amplicon to the environment. Although many DNA binding dyes can monitor PCR in real time, the adequacy of results depends on the dye *(26)*, the instrument used (this study), the specific sequence difference, and whether genotyping or heterozygote scanning is desired. For genotyping assays by T_m (homozygote differentiation), either SYBR Green I or LCGreen Plus can be used. However, only LCGreen Plus appears to detect heteroduplexes and is useful for mutation scanning (Figs. 2 and 3).

SYBR Green I is used routinely on many instruments for real-time product confirmation *(3)* and quantitative PCR assays *(27)*. The product T_m is usually used for amplicon identification, and its SD is the appropriate metric to assess temperature precision for genotyping. Limitations of genotyping small sequence variants by amplicon T_m *(14)* include concerns over T_m resolution and theoretical predictions that some variants will not be detected. Indeed, complete genotyping of the H63D *HFE* mutation by SYBR Green I amplicon melting has been reported *(8)*. However, both on theoretical grounds of nearest-neighbor symmetry *(20)* and empirical results with high-resolution instrumentation *(19)*, the H63D mutant and wild-type homozygotes are not distinguishable by melting. This suggests that genotyping by amplicon melting can indeed be error prone and dependent on instrument resolution.

The first report of PCR product melting in 1997 *(3)* claimed differentiation of PCR products with a T_m separation of 2 °C. Today's instruments do much better; 7 of 9 instruments in this study had a predicted genotyping error rate of <0.01% for products that differed in T_m by only 1.0 °C (Table 3). Most human SNPs (84%) are class 1 or class 2 SNPs *(19)* with a mean T_m difference of 1.0 °C

Table 3. Estimated error rate for genotyping homozygotes as a function of their T_m difference.

Instrument	Error rate[a] (%) at an absolute value of the T_m difference between homozygous genotypes of			
	1.0 °C[b]	0.5 °C[c]	0.25 °C[d]	0.09 °C[e]
ABI: 7000	<0.01	1.6	14	35
ABI: 7900 HT	3.4	18	32	44
Bio-Rad: iCycler	0.19	7.4	24	40
Cepheid: SmartCycler	<0.01	<0.01	2.7	25
Corbett: Rotor-Gene 3000	<0.01	<0.01	0.27	16
Idaho Technology: HR-1	<0.01	<0.01	<0.01	0.59
Idaho Technology: LightScanner	<0.01	0.33	8.7	31
Roche: LightCycler 1.2	<0.01	<0.01	0.27	16
Roche: LightCycler 2.0	<0.01	<0.01	0.45	17

[a] The error rate is estimated from the genotyping SDs calculated in Table 2 and the tail area of gaussian distributions, taken from standard tables (31).

[b] Average T_m difference between homozygotes in small amplicons of class 1 and class 2 SNPs (19), which account for 84% of human SNPs.

[c] Nearly all class 1 and 2 SNPs have a T_m difference >0.5 °C between homozygotes in small amplicons (19).

[d] Approximately 10% of human SNPs have a T_m difference between homozygotes averaging 0.25 °C in small amplicons. These consist of class 3 and 4 SNPs that are not nearest-neighbor symmetric (19).

[e] The T_m difference between homozygotes of the SNP in this study.

between homozygotes in short amplicons. This suggests that many homozygous single-base changes can be genotyped by simple amplicon melting on most instruments. However, the error rate was >1% on 3 of the 9 instruments when the T_m difference was decreased to 0.5 °C, a temperature separation that includes nearly all class 1 and class 2 SNPs (19). The remaining 16% of human SNPs are class 3 or class 4 with T_m differences clustering near either 0.25 °C or 0.0 °C. At a 0.25 °C difference (~10% of human SNPs), the error rate was >8% on 4 instruments, ~3% on 1 instrument, between 0.2% and 0.5% on 3 instruments, and <0.01% on only 1 instrument. Our genotyping study was performed on an SNP having a very small T_m difference (0.09 °C). At such a small T_m difference, only the HR-1 instrument reliably genotyped (<1% error rate), a conclusion supported by Figs. 2 and 3. Approximately 4% of SNPs are class 3 or class 4 with nearest-neighbor symmetry, and the wild type and mutant homozygotes cannot be distinguished by T_m. In these cases, there are 2 options to retain the simplicity of closed-tube genotyping with a dye: (*a*) wild-type DNA can be added to all samples and quantitative heteroduplex analysis performed (20); or (*b*) an unlabeled probe can be included in the PCR to differentiate the homozygotes (28).

Mutation scanning for heterozygote detection (as opposed to complete genotyping) is another application of amplicon melting analysis. Although heterozygote detection with SYBR Green I has been reported (10), the dye was added after PCR at concentrations that inhibited amplification. When added before PCR at noninhibitory concentrations, heteroduplexes could not be detected (Fig. 2). SYBR Green I preferentially detected high T_m products,

which is possibly explained by dye redistribution or product reassociation during melting (16, 29). In Fig. 2B, the melting curve of the heterozygote appears to be superimposed on the highest T_m product.

In contrast to SYBR Green I, LCGreen Plus is a dye that does not inhibit PCR at concentrations that saturate the PCR product produced. LCGreen Plus readily detected heteroduplexes in all instruments tested by a change in the melting curve shape (Fig. 3). This provides a homogeneous scanning method that does not require any processing or separations after PCR, such as those required by denaturing HPLC or temperature gradient capillary electrophoresis. On the HR-1 instrument, the accuracy of heterozygous SNP detection approached 100% for amplicons <400 bp in length. For longer PCR products (400–1000 bp), the sensitivity was 96.1% and the specificity was 99.4% (18). The accuracy of scanning by melting compares favorably with denaturing HPLC (30). The HR-1 instrument had the highest signal-to-noise of all the instruments tested, and the scanning accuracy of other instruments would be lower. This is suggested by the temperature-shifted curves in Fig. 3B, in which significant variation in most of the instruments contrasted to the precise traces of the HR-1 and LightScanner, instruments that were specifically designed for high-resolution scanning. Furthermore, the scanning target evaluated was only 110 bp in length; as the amplicon length increases, the difference between wild-type and heterozygous curves becomes smaller. Any variation introduced by the instrument will lower scanning accuracy, particularly in longer amplicons.

The power of DNA melting analysis depends directly on the resolution of the melting instrument. Melting resolution depends on temperature control, temperature measurement, and fluorescence measurement. Temperature control determines both intra- and intersample variations. Melting rate, sample volume, and sample geometry affect temperature variations within a sample. For example, slower melting rates, smaller sample volumes, and greater surface-to-volume ratios limit temperature gradients within a sample. Intersample variation can be either spatial or temporal. Perhaps surprisingly, spatial variation appears more difficult to control than temporal variation. Fixed, 96-well instruments showed the greatest sample variation, whereas the single-sample instrument had the least, although sequential measurements over time were required. The resolution of temperature and fluorescence measurement depends on sensor and electronic noise, signal intensity, integration time, and the bit depth of analog-to-digital conversion. Overall measurement resolution is also determined by melting rate, extent of data averaging, and the resulting data density.

Although we tried to extract all data before any processing, integration times and data averaging on the instruments were not under our control. Furthermore, only 5 of the 9 instruments could be set to a melting rate of 0.1 °C/s (Table 1), requiring ~6 min for data acquisi-

tion. The other 4 instruments required between 27 and 73 min for melting. These instruments appear to achieve acceptable melting resolution by slow melting rates, long integration times, and/or data averaging. When turn-around time is important, both PCR (26) and melting analysis can be rapid. For example, scanning on the HR-1 is usually performed at 0.3 °C/s, requiring <2 min for melting. When high-throughput mutation scanning is desired, the 96/384-well LightScanner has 5 times the resolution of other plate-based instruments while running at speeds 5–11 times faster than the other instruments.

There are several limitations to the current study. Only those melting instruments available to us at the time were evaluated. Some have since been updated with more recent models. Only 1 instrument of each type was tested and assumed to be representative. Only the HR-1 instrument was designed for genotyping by high-resolution melting, and only the HR-1 and the LightScanner were designed for mutation scanning. Real-time PCR instruments are not primarily intended for melting. In contrast to the static temperature variation considered important in conventional PCR, we studied dynamic temperature measurements (T_ms) during melting. High-resolution melting is a new technique, and the limited genotyping performance of most instruments should not be discouraging. Better differentiation of homozygotes may be possible by inclusion of synthetic duplexes as internal temperature controls, allowing temperature correction between samples. Finally, although all LCGreen Plus–compatible instruments could detect a single-nucleotide heterozygote within the 110-bp amplicon studied, scanning sensitivity and specificity will depend on instrument resolution and amplicon size. Scanning accuracy (with the possible exception of the LightScanner) will not be as good as for the HR-1 (18).

Simple solutions for genotyping (19, 28) and scanning (18) are available that do not require expensive probes, multicolor analysis, real-time PCR, or complex separations. Scanning and genotyping can even be performed simultaneously in the same reaction (17). As the resolution of DNA melting instruments improves, the speed and accuracy of genotyping and scanning by amplicon melting will also improve.

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