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Genotyping of Single-Nucleotide Polymorphisms by High-Resolution Melting of Small Amplicons

Michael Liew,[1] Robert Pryor,[2] Robert Palais,[3] Cindy Meadows,[1] Maria Erali,[1] Elaine Lyon,[1,2] and Carl Wittwer[1,2*]

Background: **High-resolution melting of PCR amplicons with the DNA dye LCGreen™ I was recently introduced as a homogeneous, closed-tube method of genotyping that does not require probes or real-time PCR. We adapted this system to genotype single-nucleotide polymorphisms (SNPs) after rapid-cycle PCR (12 min) of small amplicons (≤50 bp).**

Methods: **Engineered plasmids were used to study all possible SNP base changes. In addition, clinical protocols for factor V (Leiden) 1691G>A, prothrombin 20210G>A, methylenetetrahydrofolate reductase (MTHFR) 1298A>C, hemochromatosis (HFE) 187C>G, and β-globin (hemoglobin S) 17A>T were developed. LCGreen I was included in the reaction mixture before PCR, and high-resolution melting was obtained within 2 min after amplification.**

Results: **In all cases, heterozygotes were easily identified because heteroduplexes altered the shape of the melting curves. Approximately 84% of human SNPs involve a base exchange between A::T and G::C base pairs, and the homozygotes are easily genotyped by melting temperatures (T_ms) that differ by 0.8–1.4 °C. However, in ~16% of SNPs, the bases only switch strands and preserve the base pair, producing very small T_m differences between homozygotes (<0.4 °C). Although most of these cases can be genotyped by T_m, one-fourth (4% of total SNPs) show nearest-neighbor symmetry, and, as predicted, the homozygotes cannot be resolved from each other. In these cases, adding 15% of a known homozygous genotype to unknown samples allows melting curve separation of all three genotypes. This approach was used for the HFE 187C>G protocol, but, as predicted from the sequence changes, was not needed for the other four clinical protocols.**

Conclusions: **SNP genotyping by high-resolution melting analysis is simple, rapid, and inexpensive, requiring only PCR, a DNA dye, and melting instrumentation. The method is closed-tube, performed without probes or real-time PCR, and can be completed in less than 2 min after completion of PCR.**

LCGreen™ I is a new fluorescent DNA dye designed to detect heteroduplexes during homogeneous melting curve analysis *(1)*. Genotyping of single-nucleotide polymorphisms (SNPs)[4] by high-resolution melting analysis in products as large as 544 bp has been reported. Unlike SYBR® Green I, LCGreen I saturates the products of PCR without inhibiting amplification and does not redistribute as the amplicon melts. This allows closed-tube, homogeneous genotyping without fluorescently labeled probes *(2–4)*, allele-specific PCR *(5, 6)*, or real-time PCR instruments. Heterozygotes are identified by a change in melting curve shape, and different homozygotes are distinguished by a change in melting temperature (T_m). However, it was not clear whether all SNPs can be genotyped by this method.

SNP genotyping by amplicon melting analysis requires high-resolution methods. The differences among genotypes are easier to see when the amplicons are short *(7)*.

Use of small amplicons of <50 bp also allows for very rapid thermal cycling *(8)*; amplification is complete in <12 min and is followed by high-resolution melting, which requires <2 min.

We studied all possible homozygous and heterozygous genotypes with differences at one base position, using engineered plasmids. In addition, we developed assays to

[1] Institute for Clinical and Experimental Pathology, ARUP, Salt Lake City, UT.
[2] Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT.
[3] Department of Mathematics, University of Utah, Salt Lake City, UT.
*Address correspondence to this author at: Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132. Fax 801-581-4517; e-mail carl.wittwer@path.utah.edu.
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[4] Nonstandard abbreviations: SNP, single-nucleotide polymorphism; T_m, melting temperature; MTHFR; methylenetetrahydrofolate reductase; Hb, hemoglobin; and UNG, uracil N-glycosylase.

genotype the common clinical markers prothrombin 20210G>A *(9)*, factor V (Leiden) 1691G>A *(2)*, methylenetetrahydrofolate reductase (MTHFR) 1298A>C *(10)*, hemochromatosis (HFE) 187C>G *(11)*, and β-globin [hemoglobin S (HbS)] 17A>T *(12)* as examples for each class of SNP.

Materials and Methods

DNA SAMPLES

Most of the samples used in this study were blood specimens submitted to ARUP (Salt Lake City, UT) for routine clinical genotyping of prothrombin, factor V, MTHFR, or HFE mutations. DNA was usually extracted with the MagNa Pure instrument (Roche) according to the manufacturer's instructions. Additional samples genotyped at the β-globin locus for HbS were provided as dried blood spots by Pediatrix Screening Inc. (Pittsburgh, PA) and were extracted as described previously *(13)*. All samples were genotyped at ARUP or Pediatrix Screening by melting curve analysis on the LightCycler® (Roche) using adjacent hybridization probe (HybProbe™) technology and either commercial assays (Roche) or in-house methods *(2, 11, 12)*. At least three different individuals of each genotype for prothrombin 20210G>A, MTHFR 1298A>C, HFE 187C>G, and β-globin 17A>T SNPs were selected. We obtained 104 samples (35 wild type, 35 heterozygous, 34 homozygous mutant) previously genotyped for factor V (Leiden) 1691G>A. We also studied three rare DNA samples with mutations near factor V Leiden (1691G>A); the first sample was heterozygous 1690delC, the second was heterozygous 1690C>T, and the third was compound heterozygous 1696A>G and 1691G>A. These samples were originally identified by aberrant melting profiles during adjacent hybridization probe genotyping and confirmed by sequencing. All samples were deidentified according to a global ARUP protocol under Institutional Review Board no. 7275.

DNA samples obtained with the MagNa Pure or from dried blood spots were not routinely quantified, but contained ~10–50 ng/μL. However, for HFE 187C>G genotyping, DNA was extracted with a QIAamp DNA Blood Kit (QIAGEN, Inc.), concentrated by ethanol precipitation, and quantified based on the absorbance at 260 nm (A_{260}).

Engineered plasmids with an A, C, G, or T at a defined position amid 50% GC content *(14)* were kindly provided by Cambrex BioScience, Inc. (Rockland, ME). Plasmid copy number was quantified by A_{260}.

PRIMER SELECTION AND SYNTHESIS

To maximize the T_m difference between wild-type and homozygous mutant genotypes, the amplicons were made as short as possible. The following process was systematized as a computer program using LabView (National Instruments) and is available for remote use as "SNPWizard", at http:\\DNAWizards.path.utah.edu. After input of sequence information surrounding the SNP, the 3′ end of each primer was placed immediately adjacent to the SNP. The length of each primer was increased in its 5′ direction until its predicted T_m was as close to a user-selectable temperature (usually 55–60 °C) as possible. The primer pair was then checked for the potential to form primer-dimers or alternative amplicons. If the reaction specificity was acceptable, the primers were selected. If alternative products were likely, the 3′ end of one of the primers was shifted one base away from the SNP and the process was repeated until an acceptable pair was found.

Duplex T_ms were calculated with use of nearest-neighbor thermodynamic models described previously *(15–22)*. Best-fit values of 0.2 μM for the amplicon concentration at the end of PCR and the Mg^{2+} equivalence (74-fold that of Na^+) were obtained by use of a data set of 475 duplexes *(23)*. The effective concentration of Mg^{2+} was decreased by the total deoxynucleotide triphosphate concentration, assuming stoichiometric chelation. The effect of $Tris^+$ was assumed equal to Na^+, and the [$Tris^+$] (20 mM) was calculated from the buffer concentration and pH. Oligonucleotides were obtained from Integrated DNA Technologies, IT Biochem, Qiagen Operon, and the University of Utah core facility.

For the in silico calculation of the expected ΔT_m distribution of SNP homozygotes, the six amplicons studied (factor V, prothrombin, MTHFR, HFE, β-globin, and lambda) were considered. For each target, all combinations of the three base pairs centered on the SNP were evaluated. A/A, C/C, G/G, and T/T homozygotes were paired with each other, giving six pairs of homozygotes for ΔT_m calculations. For each pair, 16 possible combinations of neighboring bases were considered. Therefore, $6 \times 6 \times 16$, or 576 ΔT_m values were calculated and plotted as a frequency distribution. Because the bases around SNPs are not completely random *(24)*, the frequency of all three base combinations centered on human SNPs was calculated from the human SNP database (ftp://ftp.ncbi.nih.gov/snp/human, build 120). The analysis included 7 291 660 biallelic SNPs on chromosomes 1–22, X, and Y having no immediately adjacent polymorphisms. The ΔT_m distribution was adjusted for the frequency of each three-base combination for SNPs in the human genome.

PCR

Reaction conditions for the engineered plasmids and the β-globin samples consisted of 50 mM Tris (pH 8.3), 500 μg/mL bovine serum albumin, 3 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 0.4 U of *Taq* polymerase (Roche), 1× LCGreen I (Idaho Technology), and 0.5 μM each primer in 10 μL. The DNA templates were used at 10^6 copies (plasmids) or 20 ng (genomic), and a two-temperature PCR was performed with 35 cycles of 85 °C with no hold and 55 °C for 1 s on either the LightCycler (Roche) or the RapidCycler II (Idaho Technology). PCR was completed within 12 min.

PCR for the prothrombin, factor V, MTHFR, and HFE targets was performed in a LightCycler with reagents

commonly used in clinical laboratories. The 10-μL reaction mixtures consisted of 10–50 ng of genomic DNA, 3 mM $MgCl_2$, 1× LightCycler FastStart DNA Master Hybridization Probes master mixture, 1× LCGreen I, 0.5 μM forward and reverse primers, and 0.01 U/μL *Escherichia coli* uracil N-glycosylase (UNG; Roche). The PCR was initiated with a 10-min hold at 50 °C for contamination control by UNG and a 10-min hold at 95 °C for activation of the polymerase. Rapid thermal cycling was performed between 85 °C and the annealing temperature at a programmed transition rate of 20 °C/s. The primer sequences, amplicon sizes, the number of thermal cycles, and the annealing temperatures for each target are listed in Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue7/.

Differentiating HFE wild-type and mutant homozygotes required adding a known genotype to the samples. The known DNA could be added either before or after PCR. To add the DNA after PCR, equal volumes of known wild-type and unknown PCR homozygous products were mixed. To add before PCR, precisely 50 ng of unknown genomic DNA was used as template, along with an additional 7.5 ng of known wild-type DNA.

MELTING CURVE ACQUISITION AND ANALYSIS

Melting analysis was performed either on the LightCycler immediately after cycling or on a high-resolution melting instrument (HR-1; Idaho Technology). When the LightCycler was used, 20 samples were analyzed at once by first heating to 94 °C, cooling to 40 °C, heating again to 65 °C (all at 20 °C/s), and then melting at 0.05 °C/s with continuous acquisition of fluorescence until 85 °C. LightCycler software was used to calculate the derivative melting curves.

When high-resolution melting was used, amplified samples were heated to 94 °C in the LightCycler and rapidly cooled to 40 °C. The LightCycler capillaries were then transferred to the HR-1 high-resolution instrument and heated at 0.3 °C/s. Samples were analyzed between 65 and 85 °C with a turnaround time of 1–2 min. High-resolution melting data were analyzed with HR-1 software. In most cases, plots of fluorescence vs temperature were normalized as described previously *(1, 7)*. For direct comparison with LightCycler data, derivative plots were used without normalization. All curves were plotted using Microsoft Excel after export of the data.

Results

Melting analysis of short PCR products in the presence of the heteroduplex-detecting dye, LCGreen I, was used to genotype SNPs. Rapid-cycle PCR of short products allowed amplification and genotyping in a closed-tube system in <15 min without probes or allele-specific amplification. The primer locations surrounding the six polymorphic sites analyzed are shown in Fig. 1. The PCR products were 38–50 bp in length, and the distance from

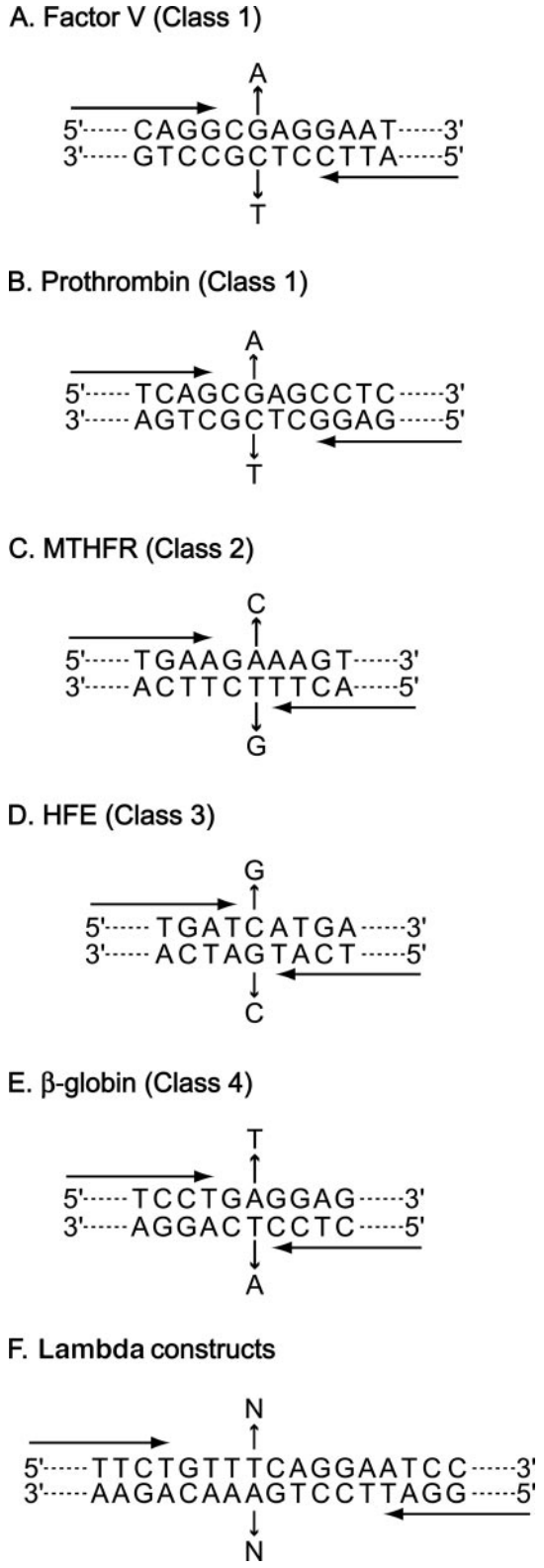

Fig. 1. Details of the SNPs studied, including the primer positions and the SNP class (see Table 1).

Both strands of DNA are shown. The *large arrows above* and *below* the sequences indicate the 3′ position and directions of the primers. The *small vertical arrows* indicate the SNP base change. For the lambda constructs, *N* indicates that all possible changes were studied.

the 3′ end of the primers to the polymorphic site varied from 1 to 6 bases.

The difference between standard and high-resolution melting techniques is shown in Fig. 2, which shows derivative melting curves for different factor V (Leiden) genotypes. Although the heterozygotes can be identified by the presence of a second, low-temperature melting transition even with standard techniques, genotype differentiation is much easier with high-resolution methods. All subsequent studies were done at high resolution.

Engineered lambda constructs *(14)* were used to study all possible SNP base combinations at one position. Four plasmids (identical except for an A, C, G, or T at one position) were either used alone to simulate homozygous genotypes or in binary combinations to construct "heterozygotes". The normalized melting curves of the four homozygotes and six heterozygotes are shown in Fig. 3. All homozygotes melted in a single transition (Fig. 3A), and the order of melting was correctly predicted by nearest-neighbor calculations as A/A < T/T < C/C < G/G *(22)*. Heterozygotes produced more complex melting curves (Fig. 3B), arising from contributions of two homoduplexes and two heteroduplexes *(7)*. Each heterozygote traced a unique melting curve path according to the four duplex T_ms. The order of melting was again according to nearest-neighbor calculations (A/T < A/C < C/T < A/G < G/T < C/G) based on the mean of the two homoduplex T_ms. The six heterozygote curves merged at high temperatures into three traces, predicted

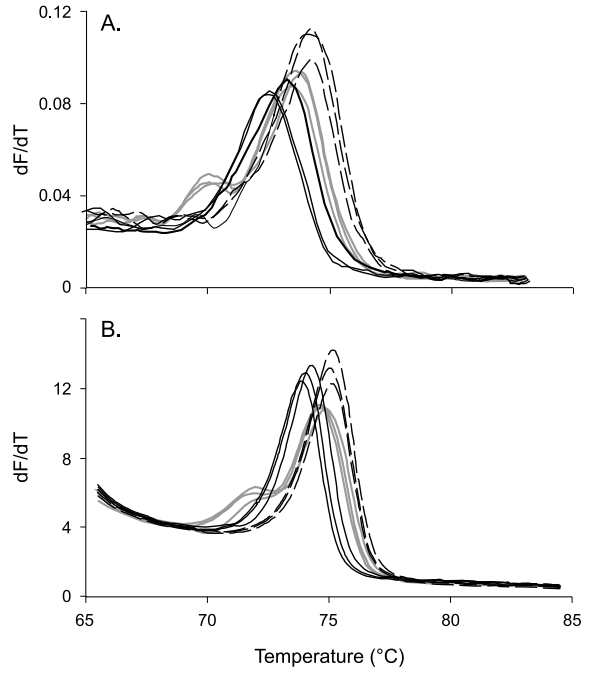

Fig. 2. Derivative melting curves for factor V Leiden genotyping obtained on the LightCycler (*A*) and the HR-1 high-resolution instrument (*B*).

Three individuals of each genotype were analyzed: wild type (*solid black line*), homozygous mutant (*dashed black line*), and heterozygous (*solid gray line*).

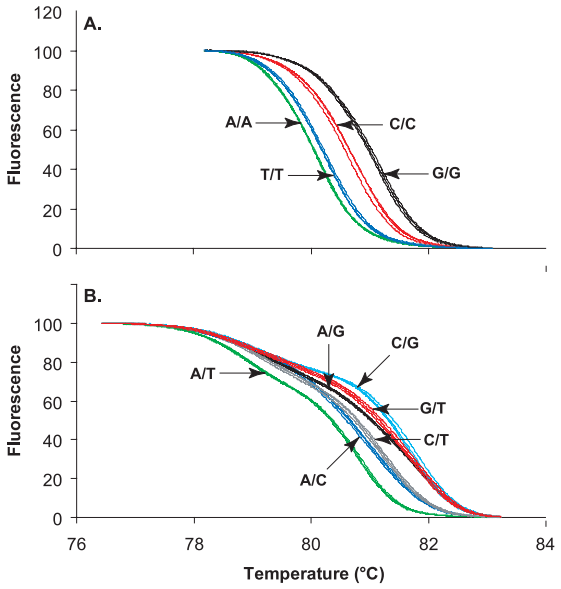

Fig. 3. Normalized, high-resolution melting curves of all possible SNP genotypes at one position using engineered plasmids.

Three samples of each genotype were analyzed and included four homozygotes (*A*) and six heterozygotes (*B*).

by the highest melting homoduplex present (T/T for the A/T heterozygote, C/C for the A/C and C/T heterozygotes, and G/G for the A/G, G/T, and C/G heterozygotes). All genotypes could be distinguished from each other by high-resolution melting analysis.

The genomic SNPs shown in Fig. 1 include all four classes of SNPs. Table 1 lists the four classes of SNPs that resulted from grouping the six different binary combinations of bases by the homoduplex and heteroduplex products that were produced when a heterozygote was amplified. Class 1 SNPs are C/T and G/A transitions that produce C::G and A::T homoduplexes and C::A and T::G heteroduplexes. In contrast, class 2 SNPs (C/A and G/T) are transversions that produce C::T and A::G heteroduplexes. Class 3 SNPs (C/G) produce C::G homoduplexes with C::C and G::G heteroduplexes. Class 4 SNPs (A/T) produce A::T homoduplexes with A::A and T::T heteroduplexes. The clinical SNPs studied were chosen to include two examples (factor V and prothrombin) in the most common SNP class and one example in each of the other three classes.

The melting curves for the five clinical SNP targets are shown in Fig. 4. For all SNP classes, heterozygotes were easily identified by a low and/or broad melting transition. For SNPs in class 1 or 2 (factor V, prothrombin, MTHFR), homozygous wild-type and homozygous mutant samples were easily distinguished from each other by a shift in T_m. However, the T_m difference between homozygous genotypes for SNPs in class 3 or 4 was smaller than in class 1 or 2. Homozygous HbS (17A>T; class 4) could be distinguished from the wild type with a T_m difference of ~0.2 °C, but the HFE homozygous mutant

Table 1. SNP classification according to the homoduplexes and heteroduplexes produced after amplification of a heterozygote and the predicted number of distinct nearest-neighbor thermodynamic duplexes (T_ms).[a]

Class	SNP (frequency)[b]	Homoduplex matches (no. of T_ms)	Heteroduplex mismatches (no. of T_ms)	Examples in Figs.
1	C/T or G/A	C::G and A::T	C::A and T::G	3B, 4A, 4B
	(0.662)	(2)	(2 or 1)[c]	
2	C/A or G/T	C::G and A::T	C::T and A::G	3B, 4C
	(0.176)	(2)	(2 or 1)[c]	
3	C/G	C::G	C::C and G::G	3B, 4D, 5
	(0.088)	(2 or 1)[c]	(2)	
4	T/A	A::T	T::T and A::A	3B, 4E
	(0.074)	(2 or 1)[c]	(2)	

[a] SNPs are specified with the alternative bases separated by a slash, e.g., C/T indicates that one DNA duplex has a C and the other a T at the same position on the equivalent strand. There is no bias for one allele over the other, i.e., C/T is equivalent to T/C. Base pairing (whether matched or mismatched) is indicated by a double colon and is not directional, i.e., C::G indicates a C::G base pair without specifying which base is on which strand.

[b] The human SNP frequencies were calculated from ftp://ftp.ncbi.nih.gov/snp/human dbSNP, build 120, March 18, 2004, based on 7 291 660 biallelic SNPs on chromosomes 1–22, X, and Y having no immediate neighboring polymorphisms.

[c] The number of predicted thermodynamic duplexes depends on the nearest-neighbor symmetry around the base change. One-fourth of the time, nearest-neighbor symmetry is expected, i.e., the position of the base change will be flanked on each side by complementary bases. For example, if a C/G SNP is flanked by an A and a T on the same strand (Fig. 1D), nearest-neighbor symmetry occurs and only one homoduplex T_m is expected (as observed in Fig. 4D).

(187C>G; class 3) could not be distinguished from the wild type.

Complete genotyping of HFE C187G by high-resolution melting analysis was possible by adding a known genotype to the unknown sample. Fig. 5A shows the result of mixing wild-type amplicons with unknown homozygous amplicons after PCR. If the unknown sample is wild type, the melting curve does not change. However, if the unknown sample is homozygous mutant, heteroduplexes are produced, and an additional low-temperature transition appears. An alternative option is to add a known genotype to the unknown sample before PCR. If a small amount of wild-type DNA is added, wild-type samples generate no heteroduplexes, homozygous mutant samples show some heteroduplexes, and heterozygous samples show the greatest amount of heteroduplex formation (Fig. 5B). Table 2 shows that there was complete concordance between the fluorescent hybridization probe (HybProbe™) and high-resolution amplicon melting methods for 167 samples.

Unexpected sequence alterations under primers or very near the mutation of interest can adversely affect genotyping assays. The melting curves for three rare heterozygous sequence changes near factor V Leiden are shown in Fig. 1 of the online Data Supplement. Although only single samples of each rare genotype were available, each curve appeared to have a unique shape, suggesting that genotype differentiation may be possible.

Shown in Fig. 6 is the in silico frequency distribution *(25)* of the calculated ΔT_ms among the homozygous genotypes of SNPs, adjusted for the frequency of each three base combination centered on SNPs in the human genome. All possible ΔT_m combinations (576) were calculated by varying the three bases centered on the natural SNPs of the six amplicons studied here. Class 1 and 2 SNPs form the broad cluster around 0.8–1.4 °C and are easily distinguishable by melting analysis. Class 3 and 4 SNPs include the minor peaks around 0.00 and 0.25 °C. Although most class 3 and 4 SNPs can be fully genotyped by high-resolution melting analysis, one-fourth have identical predicted T_ms, and the homozygotes cannot be differentiated without use of the addition methods described above.

Discussion

There are many ways to genotype SNPs *(26)*. Available techniques that require a separation step include restriction fragment length polymorphism analysis, single-nucleotide extension, oligonucleotide ligation, and sequencing. Additional methods, including pyrosequencing *(27)* and mass spectroscopy *(28)*, are technically complex but can be automated for high-throughput analysis. Some methods require two analyses to obtain a genotype.

Homogeneous, closed-tube methods for SNP genotyping that do not require a separation step are attractive for their simplicity and containment of amplified products. Most of these methods are based on PCR and use fluorescent oligonucleotide probes. Genotyping occurs either by allele-specific fluorescence *(29, 30)* or by melting analysis *(31)*. Melting analysis has the advantage that multiple alleles can be genotyped with one probe *(32)*. Most of these techniques can be performed after amplification is complete, although they are often associated with real-time PCR *(33–37)*.

Some closed-tube fluorescent methods for SNP genotyping do not require probes. Allele-specific PCR can be monitored in real time with SYBR Green I *(5)*. The method requires three primers, two PCR reactions for each SNP, and a real-time PCR instrument that can monitor each cycle of PCR. An alternative method uses allele-specific amplification, SYBR Green I, and melting curve analysis at the end of PCR *(6)*. Monitoring of each cycle is not necessary, and a SNP genotype can be obtained in one reaction. However, a melting instrument and three prim-

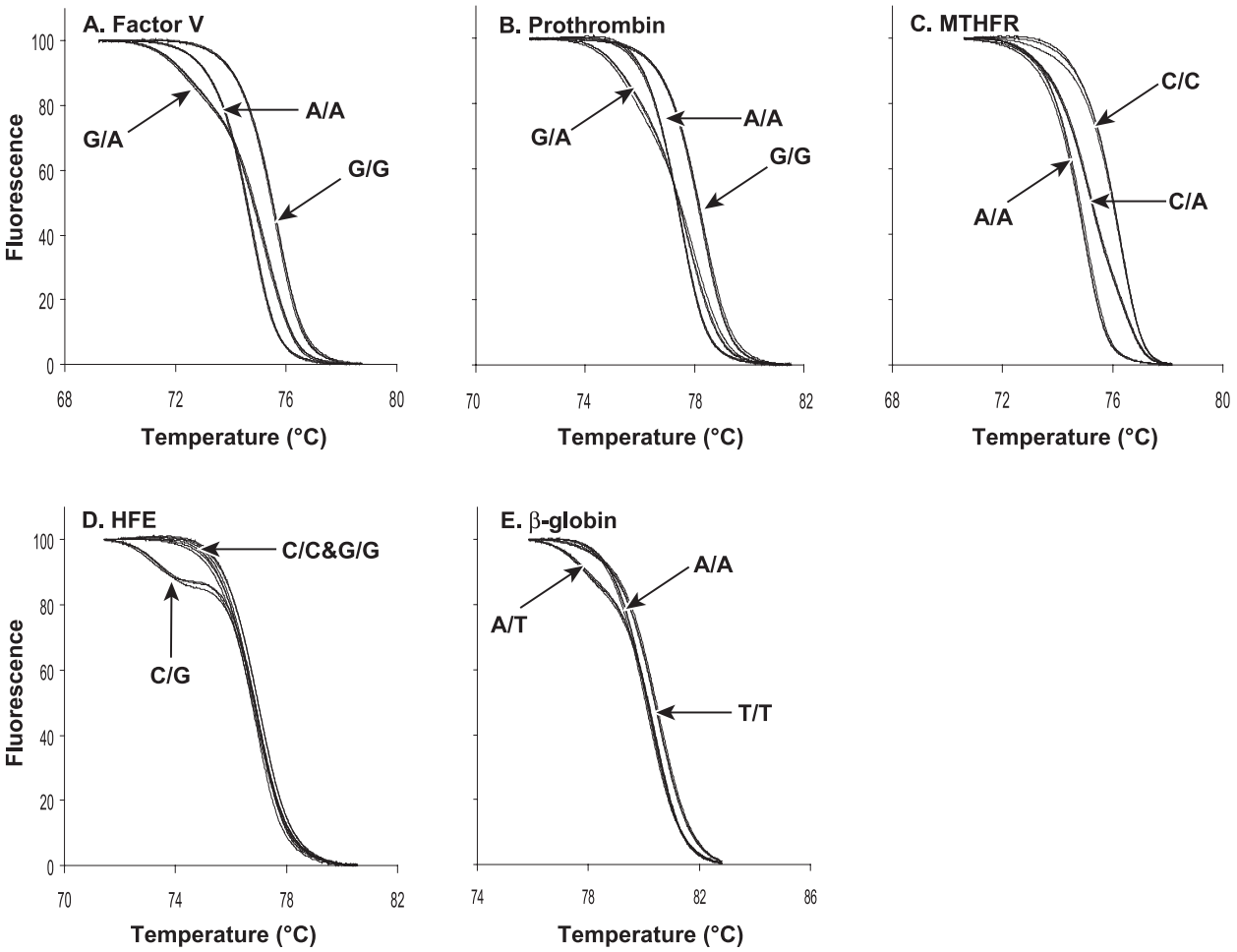

Fig. 4. Normalized, high-resolution melting curves from factor V Leiden 1691G>A (class 1; *A*); prothrombin 20210G>A (class 1; *B*); MTHFR 1298A>C (class 2; *C*); HFE 187C>G (class 3; *D*); and β-globin 17A>T (class 4; *E*) SNPs.
Three individuals of each genotype were analyzed and are displayed for each SNP.

ers are necessary, with one of the primers modified with a GC clamp. Both techniques are based on allele-specific PCR, and each allele-specific primer is designed to recognize only one allele.

SNP genotyping by high-resolution melting with the dye LCGreen I does not require probes, allele-specific PCR, or real-time PCR. Only two primers, one PCR reaction, and a melting instrument are required. Reagent costs for genotyping by amplicon melting are low because only PCR primers and a generic dye are needed. No probes or specialized reagents are required.

Although SNPs have been genotyped within amplicons up to 544 bp long *(1)*, use of a small amplicon for genotyping has numerous advantages. Assay design is simplified because primers are selected as close to the SNP as possible. The T_m differences among genotypes increases as the amplicon size decreases, allowing better differentiation. Cycling times can be minimized because the melting temperatures of the amplicons (74–81 °C in Figs. 3 and 4) allow low denaturation temperatures during cycling that in addition increase specificity. Furthermore, the amplicon length is so small that no temperature holds are necessary for complete polymerase extension. Potential disadvantages of small amplicons include less flexibility in the choice of primers, less effective contamination control with UNG *(38)*, and difficulty distinguishing between primer-dimers and desired amplification products on gels or during real-time analysis.

Small amplicons allow rapid-cycle protocols that complete PCR in 12 min with popular real-time (LightCycler) or inexpensive (RapidCycler II) instruments. Heteroduplex detection in small amplicons is favored by rapid cooling before melting, rapid heating during melting, and low Mg^{2+} concentrations *(7)*. Although conventional real-time instruments can be used for melting (Fig. 2), their resolution is limited. Small T_m differences between homozygotes (see Fig. 4E for an example) are not distinguished on conventional instruments (data not shown).

The effects of ionic strength and product concentration on amplicon T_m have been discussed previously *(7)*.

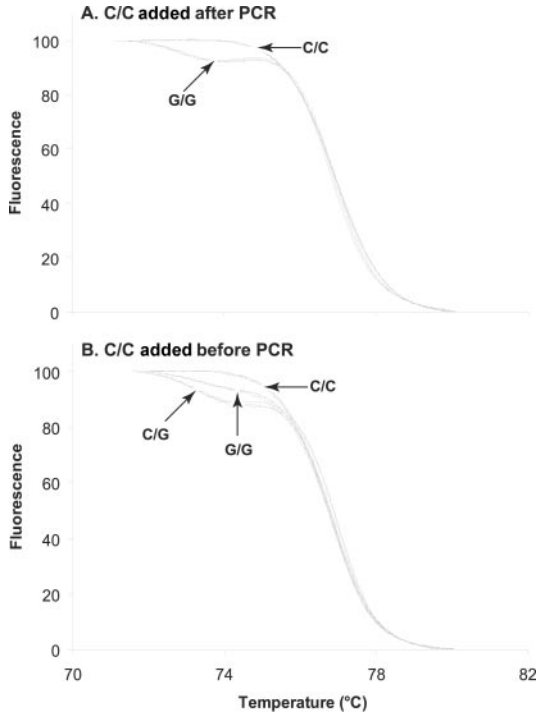

Fig. 5. Genotyping at the HFE 187C>G locus by adding wild-type DNA to each sample.

(*A*), wild-type amplicons were mixed with amplicons from three individuals of each homozygous genotype after PCR. (*B*), 15% wild-type genomic DNA was added to the DNA of three individuals of each genotype before PCR.

Because homozygous SNP genotypes are distinguished only by T_m, these issues are a concern for accurate genotyping. However, the effect of amplicon concentration on T_m is small and usually dwarfed by the temperature (in)accuracy of most melting instruments. Furthermore, amplifying well into the plateau phase usually equalizes by PCR any differences in initial product concentration. Because T_m is strongly dependent on the ionic strength, it is important that all DNA samples are extracted in the same way and end up in the same buffers. We did not find it necessary to quantify the DNA samples before PCR unless addition studies were performed and never attempted to quantify amplicon after PCR but before melting.

Dedicated melting instruments have recently become available (LightTyper and HR-1). The HR-1 provides the highest resolution and is the least expensive. Although only one sample is analyzed at a time, the turnaround time is so fast (1–2 min) that the throughput is reasonable. The LightTyper is an interesting platform for high-throughput melting applications. However, the temperature homogeneity across the plate needs to be improved before homozygotes can be reliably distinguished (data not shown).

Can all SNPs be genotyped by simple high-resolution melting of small amplicons? Studies with engineered plasmids of all possible base combinations at one location initially suggested that the answer was "yes" (Fig. 3).

Table 2. Genotype concordance based on adjacent hybridization probes (HybProbe) and small-amplicon, high-resolution melting analysis (Amplicon melting).

Marker	Genotypes	HybProbe[a]	Amplicon melting[b]
Factor V 1691G>A	Wild type	35	35
	Heterozygous	35	35
	Homozygous mutant	34	34
Prothrombin 20210G>A	Wild type	8	8
	Heterozygous	3	3
	Homozygous mutant	11	11
MTHFR 1298A>C	Wild type	6	6
	Heterozygous	7	7
	Homozygous mutant	7	7
HFE 187C>G	Wild type	4	4[c]
	Heterozygous	4	4
	Homozygous mutant	4	4[c]
β-Globin 17A>T	Wild type	3	3
	Heterozygous	3	3
	Homozygous mutant	3	3

[a] All samples were originally genotyped by ARUP (factor V, prothrombin, MTHFR, and HFE) or Pediatrix Screening (β-globin) as clinical samples with adjacent hybridization probes and melting curve analysis.

[b] Genotyping results for the same samples using LCGreen I, the HR-1 high-resolution melting instrument, and amplicon melting.

[c] Genotyping required addition of homozygous DNA (see text).

Heterozygotes were always easily identified. Whether the different homozygotes were easy to distinguish depended on the class of SNP (Table 1). The six possible binary combinations of bases (C/T, G/A, C/A, G/T, C/G, and T/A) group naturally into four classes based on the homoduplex and heteroduplex base pairings produced when a heterozygote is amplified. SNP homozygotes are easy to distinguish by T_m in the first two classes because one homozygote contains an A::T pair and the other a G::C pair. These short amplicons show homozygote T_m

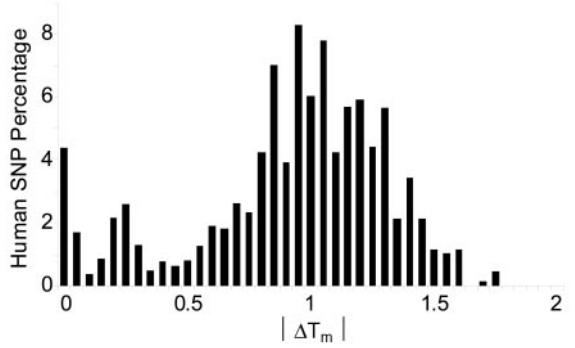

Fig. 6. In silico estimation of the T_m difference among homozygous genotypes of small amplicon SNPs.

The frequency distribution is adjusted for the relative occurrence of each SNP and immediately adjacent bases in the human genome. The mean amplicon length was 43.5 bp. The larger the ΔT_m, the easier it is to differentiate the homozygous genotypes. Approximately 4% of human SNPs have a predicted ΔT_m of 0.00 °C and are expected to require addition of known homozygous DNA for genotyping of the homozygotes.

differences mostly between 0.8–1.4 °C, and these two classes make up >84% of human SNPs *(39)*.

It is more difficult to distinguish the homozygotes of SNPs in classes 3 and 4 (Table 1) because the base pair (A::T or C::G) is simply inverted, that is, the bases switch strands but the base pair remains the same. Differences in amplicon T_m still result from different nearest-neighbor interactions with the bases next to the SNP site, but they are usually <0.4 °C (Fig. 3A and Fig. 4, D and E). Class 3 and 4 SNPs make up ~16% of human SNPs. Genotyping of homozygotes is still possible in most cases with high-resolution analysis.

Clinical SNPs of each class were selected for concordance studies with standard genotyping methods. Factor V (Leiden) 1691G>A and prothrombin 20210G>A were class 1 SNPs, MTHFR 1298A>C was class 2, HFE 187C>G was class 3, and β-globin (HbS) 17A>T was class 4.

The class 3 SNP studied (Fig. 4D) was unique in that we could not differentiate the different homozygotes by T_m using simple melting analysis. Inspection of the bases neighboring the SNP site revealed why (Fig. 1D). In this case, the neighboring bases were complementary, giving nearest-neighbor stability calculations that were identical for the two homozygotes. To the extent that nearest-neighbor theory is correct, the duplex stabilities are predicted to be the same. By chance alone, this nearest-neighbor "symmetry" is expected to occur 25% of the time. When this occurs in class 1 or 2 SNPs, nearest-neighbor calculations indicate that the stabilities of the two heteroduplexes formed are identical. This is not of consequence to SNP typing because all three SNP genotypes still have unique melting curves. However, nearest-neighbor symmetry in class 3 or 4 SNPs predicts that the two homoduplex T_ms (homozygous genotypes) are identical. This will occur in ~4% of human SNPs.

When nearest-neighbor symmetry of class 3 or 4 SNPs predicts that the homozygotes will not be distinguished, complete genotyping is still possible by adding to the reactions a known genotype either before or after PCR. If the amplicon is added after PCR, only the homozygotes need to be tested, but potential amplicon contamination is a disadvantage. Adding before PCR requires either that the DNA concentration of the sample is carefully controlled or that samples are run both with and without added DNA.

Unexpected sequence variants can adversely affect many different genotyping assays, including the amplicon melting assay described here. Variants under a primer can cause allele-specific amplification of heterozygotes, giving apparent homozygous genotypes. Variants very close to the targeted mutation can confound assays based on restriction enzyme digestion or hybridization probes. However, different sequence variants under probes *(40)* or within amplicons (this study) can often be differentiated by melting analysis.

High-resolution amplicon melting with LCGreen I can also be used to scan for sequence differences between two copies of DNA *(1)*. In mutation scanning, the method is similar to other heteroduplex techniques, such as denaturing HPLC *(41)* or temperature gradient capillary electrophoresis *(42)*. However, high-resolution melting is unique in that homozygous sequence changes can often be identified without the use of added DNA. In the case of SNP genotyping with small amplicons, addition is rarely required.

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