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High-Resolution DNA Melting Analysis for Simultaneous Mutation Scanning and Genotyping in Solution

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Background: **High-resolution DNA melting analysis with saturation dyes for either mutation scanning of PCR products or genotyping with unlabeled probes has been reported. However, simultaneous PCR product scanning and probe genotyping in the same reaction has not been described.**

Methods: **Asymmetric PCR was performed in the presence of unlabeled oligonucleotide probes and a saturating fluorescent DNA dye. High-resolution melting curves for samples in either capillaries (0.3 °C/s) or microtiter format (0.1 °C/s) were generated in the same containers used for amplification. Melting curves of the factor V Leiden single-nucleotide polymorphism (SNP) and several mutations in exons 10 and 11 of the cystic fibrosis transconductance regulator gene were analyzed for both PCR product and probe melting transitions.**

Results: **Independent verification of genotype for simple SNPs was achieved by either PCR product or probe melting transitions. Two unlabeled probes in one reaction could genotype many sequence variants with simultaneous scanning of the entire PCR product. For example, analysis of both product and probe melting transitions genotyped ΔF508, ΔI507, Q493X, I506V, and F508C variants in exon 10 and G551D, G542X, and R553X variants in exon 11. Unbiased hierarchal clustering of the melting transitions identified the specific sequence variants.**

Conclusions: **When DNA melting is performed rapidly and observed at high resolution with saturating DNA dyes, it is possible to scan for mutations and genotype at the same time within a few minutes after amplification. The method is no more complex than PCR and may reduce the need for resequencing.**

Melting of the DNA double helix into separate random coils is classically monitored by ultraviolet absorbance during slow heating and requires large amounts of DNA. Recently, methods that monitor DNA melting by fluorescence have become popular in conjunction with real-time PCR *(1)*. Because PCR produces enough DNA for fluorescent melting analysis, both amplification and analysis can be performed in the same tube, providing a homogeneous, closed-tube system that requires no processing or separation steps. Dyes that stain double-stranded DNA are commonly used to identify products by their melting temperature (T_m)[1] *(2)*. Alternatively, hybridization probes allow genotyping by melting of product/probe duplexes *(3–6)*.

The power of DNA melting analysis depends on its resolution. Studies with ultraviolet absorbance often required hours to collect high-resolution data at rates of 0.1–1.0 °C/min to ensure equilibrium. In contrast, fluorescent melting analysis is usually acquired at 0.1–1.0 °C/s, equilibrium is not achieved, and resolution is limited to 2–4 points/°C *(1, 7, 8)*. With recent advances in electronics (e.g., 24-bit A-to-D converters), high-resolution melting can be performed rapidly with 10–100 times the data density (50–100 points/°C) of conventional real-time PCR instruments, as recently demonstrated for probe *(6)* and PCR product *(9)* melting. Furthermore, saturating DNA dyes that maximize detection of mismatched duplexes (heteroduplexes) are now available *(10)*. These developments have dramatically increased the power of fluorescence-based DNA melting for robust identification of single-base changes within PCR products.

High-resolution melting analysis for gene scanning

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[1] Nonstandard abbreviations: T_m, melting temperature; SNP, single-nucleotide polymorphism; CFTR, cystic fibrosis transconductance regulator; and LED, light-emitting diode.

relies primarily on the shape of the melting transition of the PCR products, and very little on T_m. Screening for heterozygous single-nucleotide polymorphisms (SNPs) within products up to 1000 bp has a sensitivity and specificity of 97% and 99%, respectively *(11)*. In many cases, high-resolution analysis of the melting transition also allows genotyping without probes *(9–12)*. Even greater specificity for variant discrimination over a smaller region can be obtained by use of unlabeled probes *(6)*. Specific genotypes are inferred by correlating sequence alterations under the probe to changes in the probe T_m. We now report that high-resolution gene scanning and genotyping with unlabeled probes can be done simultaneously in the same reaction. Both PCR product and probe melting transitions are observed in the presence of a saturating DNA dye. In addition to screening for any sequence variant between the primers in the PCR product, common polymorphisms and mutations can be genotyped. Furthermore, unbiased, hierarchal clustering can accurately group the melting curves into genotypes. One, two, or more unlabeled probes can be used in a single PCR.

Materials and Methods

DNA SAMPLES

Human genomic DNA of known factor V Leiden genotype *(3)* was kindly deidentified and provided by Associated Regional and University Pathologists. Heterozygous genomic DNA samples with selected cystic fibrosis mutations (see Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue10/) were from the Coriell Institute for Medical Research. In addition, a G542X homozygote was also obtained from Coriell.

PRIMERS AND PROBES

Primer and probe sequences and the concentrations used in PCR are listed in Table 2 of the online Data Supplement and were synthesized by the University of Utah core synthesis facility. Predicted probe T_ms *(13, 14)* were lower than observed T_ms, perhaps because of dye stabilization and the nonequilibrium conditions/rates used for melting (0.1–0.3 °C/s). The T_ms of different probe/allele duplexes were adjusted by probe length, mismatch position, and probe dU vs dT content *(15, 16)*. Extension of unlabeled probes during PCR was prevented by incorporating a 3′-phosphate during synthesis. When a 5′-exonuclease–negative polymerase is used, probes should be designed to melt lower than the PCR extension temperature. Otherwise, variable displacement of the probe during rapid cycling may favor amplification (and detection) of certain alleles over others. Alternatively, use of an exonuclease-positive polymerase allows a greater range of probe T_ms without bias.

ASYMMETRIC PCR

Primer asymmetry ratios of 1:5 to 1:10 produced sufficient double-stranded product for amplicon melting and enough single-stranded product for probe annealing. PCR for factor V was performed in 384-well format with 5-μL volumes and included 20 ng of genomic DNA in 50 mM Tris (pH 8.3) with 3 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 500 μg/mL bovine serum albumin, 1× LCGreen® PLUS (Idaho Technology), 0.2 U of KlenTaq1™ (AB Peptides), and 70 ng of TaqStart™ antibody (Clontech). PCR was performed in a 9700 thermal cycler (ABI) with an initial denaturation at 94 °C for 10 s, followed by 50 cycles of 94 °C for 5 s, 57 °C for 2 s, and 72 °C for 2 s. After PCR, the samples were heated to 94 °C for 1 s and then cooled to 10 °C before melting.

PCR for amplification of cystic fibrosis transconductance regulator (*CFTR*) exons 10 and 11 was performed in 10-μL volumes and included 50 ng of genomic DNA in 50 mM Tris (pH 8.3) with 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 500 μg/mL bovine serum albumin,, 1× LCGreen I (Idaho Technology), and 0.4 U of Taq polymerase (Roche). The PCR was performed in capillaries on a LightCycler (Roche) with an initial denaturation of 95 °C for 10 s followed by 45 cycles of 95 °C for 1 s, 54 °C for 0 s, and 72 °C for 10 s. After amplification, the samples were heated to 95 °C for 0 s and rapidly cooled to 40 °C before melting.

MELTING ACQUISITION

When samples were amplified in 384-well plates, melting acquisition was performed on a prototype version of the LightScanner™ (Idaho Technology). This instrument is similar to the LightTyper® *(17)*, but is modified for high-resolution melting of LCGreen dyes. The standard 470 nm light-emitting diodes (LEDs) were replaced with 450 nm LEDs (Bright-LED Optoelectronics). In addition, the optical filters were changed to 425–475 nm excitation and 485 nm long-pass emission filters (Omega Optical). Temperature homogeneity across the plate, temperature precision and control, and fluorescence precision and frequency of acquisition were all increased over the LightTyper. The plate was heated from 55 °C to 88 °C at 0.1 °C/s with a 300-ms frame interval, 15-ms exposure, and 100% LED power, giving ~25 points/°C.

Melting of *CFTR* exons was performed on the HR-1 high-resolution melting instrument (Idaho Technology) with 24-bit acquisition of temperature and fluorescence. After PCR, each capillary was transferred to the HR-1 and melted from 50 °C to 90 °C with a slope of 0.3 °C/s, giving 65 points/°C.

MELTING ANALYSIS

Melting curves were analyzed on custom software written in LabVIEW (National Instruments). Normalization and background subtraction were first performed by fitting an exponential to the background surrounding the melting transitions of interest. Derivative plots of probe melting transitions were obtained by Salvitsky–Golay polynomial estimation as described previously *(1)*. Melting curves of PCR products were compared on difference plots of temperature-overlaid, normalized melting curves *(9)*. The

normalized melting curves were temperature-overlaid (to eliminate slight temperature errors between wells or runs) by selecting a fluorescence range (low fluorescence/high temperature, typically 5%–10% fluorescence) and shifting each curve along the *x* axis to best overlay a standard sample within this range. Difference plots of temperature-overlaid, normalized curves were obtained by taking the fluorescence difference of each curve from the average wild-type curve at all temperature points. These analytical methods have been applied previously to mutation scanning *(18–20)* and HLA matching *(21)*.

Agglomerative, unbiased hierarchical clustering of melting curve data was performed by standard methods *(22)*, custom programmed in LabVIEW. The distance between curves was taken as the average absolute value of the fluorescence difference between curves over all temperature acquisitions. The number of groups was automatically identified by selecting the largest ratio of distances between consecutive cluster levels.

Results

Simultaneous SNP genotyping of the human factor V Leiden mutation by both unlabeled probe and PCR product melting is shown in Fig. 1. Different genotypes were patterned in a 384-well plate, amplified by asymmetric PCR in the presence of an unlabeled probe and LCGreen PLUS, and melted at 0.1 °C/s with high-resolution acquisition of fluorescence and temperature data. The entire melting profile, showing the melting region of both the unlabeled probe and the PCR product, is shown in Fig. 1A. Two transitions are apparent. Depending on genotype, the probe melted at 58–68 °C, whereas the PCR product melted at 78–82 °C. Both PCR product and unlabeled probe melting transitions independently identified the genotype. When only the probe melting region was analyzed as a negative derivative plot (Fig. 1B), wild-type samples matched to the probe were most stable, homozygous mutant samples were destabilized by ~6 °C, and heterozygous samples showed both transitions. When only the PCR product region was considered, the wild-type samples were more stable than the homozygous mutants by ~2 °C (Fig. 1A). The heterozygous samples were best distinguished by the skewed shape of the curve and the broad overall transition. The low-temperature shoulder was produced by heteroduplexes and enabled PCR product scanning *(11)*. The heterozygotes were most easily identified by overlaying the plots at high temperature and plotting the difference of each curve against the average wild-type sample (Fig. 1C). When unbiased hierarchal clustering was applied to either the PCR product or the unlabeled probe melting data, identical 3-cluster assignments resulted (Fig. 1D).

To demonstrate simultaneous scanning and genotyping of multiple variants, we chose to analyze exons of the *CFTR* gene. Three SNPs in 2 regions of exon 11 of the *CFTR* gene were analyzed with 2 unlabeled probes (Fig. 2). Two of the mutations were only 6 bases apart, allowing one of the probes to cover both mutations (Fig. 2A). Five replicates of each genotype were amplified and analyzed. The normalized melting curves (Fig. 2B) showed regions of probe melting (56–74 °C) and PCR product melting (80–83 °C). On casual observation, it is not clear from the normalized melting curve what information can be extracted. However, when the probe region is displayed as a derivative plot (Fig. 2C), the melting transitions of all common alleles under both probes are apparent. Both unlabeled probes were matched to the wild-type sequence, but one of the probes was made shorter and contained dU instead of dT to decrease its T_m *(15, 16)*. The more stable probe covered a single SNP giving 2 alleles being separated by T_m, both being more stable than all alleles of the less stable probe. The less stable probe covered 2 SNPs, giving 3 peaks for common genotypes *(23)*. The specific mismatch and its position within the probe affect duplex stability, allowing probe design that distinguishes multiple alleles *(24)*. A difference plot of the PCR product melting transition is shown in Fig. 2D. The heterozygous, wild-type, and homozygous mutant samples are clearly different. However, it is difficult to distinguish between different heterozygotes by PCR product melting alone. Unbiased hierarchal clustering grouped all heterozygotes together (data not shown). The 3 heterozygotes are all in the same SNP class *(12)*, giving the same heteroduplex mismatches (C:A and T:G) and homoduplex matches (C:G and A:T). Although the stabilities of all 3 heterozygotes predicted by nearest-neighbor thermodynamics *(13, 14)* were not identical, definitive genotyping required the use of probes. The strength of product melting is to easily identify the presence of heterozygotes, whereas unlabeled probes further discriminate between heterozygotes and more easily identify homozygous variants.

Three SNPs and 2 deletions within exon 10 of the *CFTR* gene were also analyzed with 2 unlabeled probes (Fig. 3). The probe with the higher T_m covered a single SNP, whereas the probe with the lower T_m covered 2 SNPs and 2 deletions (Fig. 3A). The normalized melting curves (Fig. 3B) showed regions of low temperature probe (56–67 °C), high temperature probe (67–75 °C), and PCR product (80–83 °C) melting. When the probe regions are displayed as a derivative plot (Fig. 3C), all 5 heterozygous genotypes follow unique paths that distinguish them from the wild type and each other. Four of the heterozygotes had resolved peaks, whereas one (the red trace in Fig. 3C) can be identified by a broad peak resulting from a relatively stable mismatch (an A:G mismatch near one end of the probe in an AT-rich region). Allele discrimination does not require a unique T_m for each allele, only that the curves are different in some region of the melting transition. A difference plot of the PCR product melting transition is shown in Fig. 3D. The double heterozygote shows the greatest deviation from wild type because 2 mismatches are present within the PCR product. The 4 single heterozygotes are all easily distinguishable from wild type. In contrast to exon 11, all heterozygotes studied

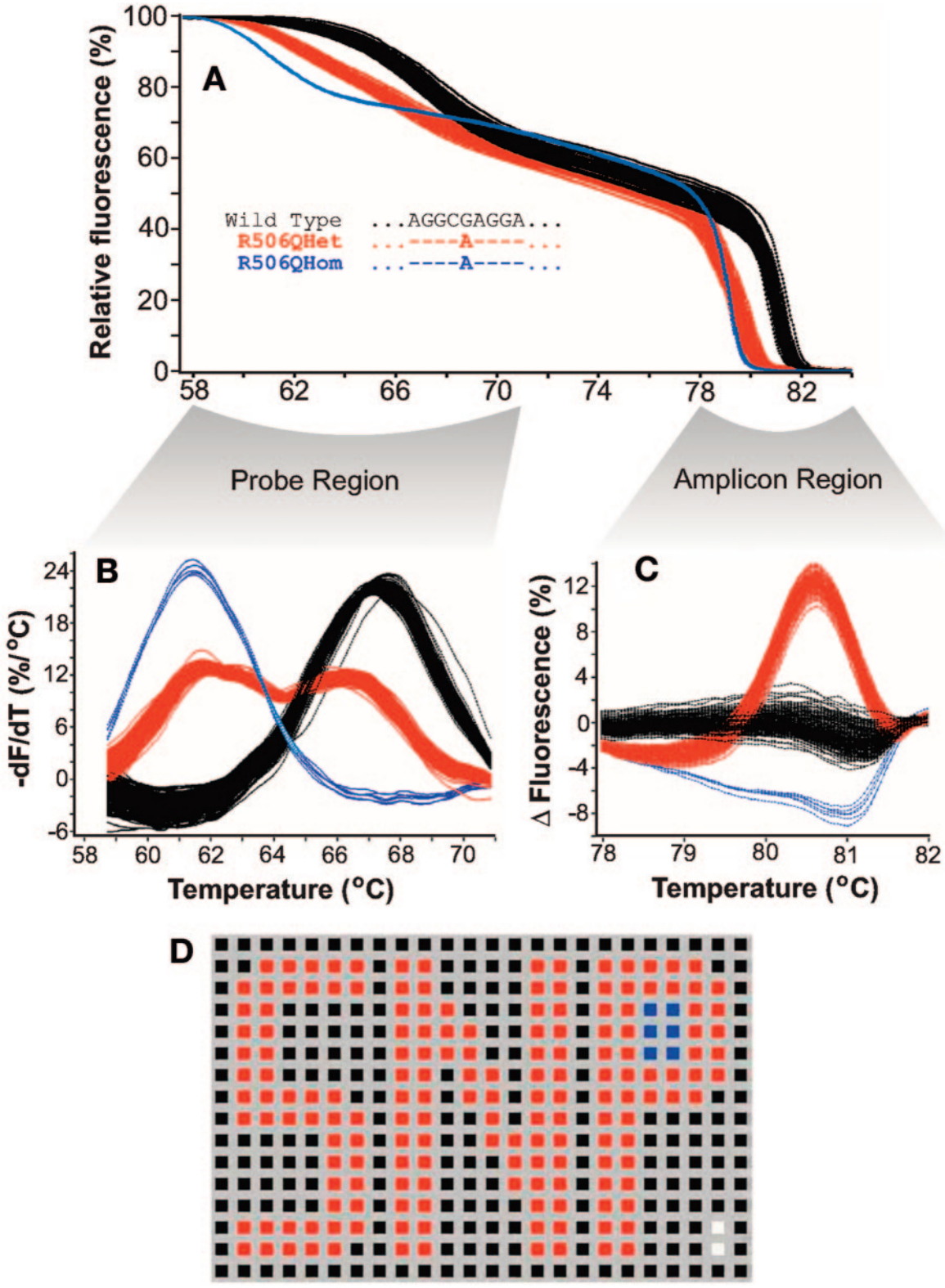

Fig. 1. High-resolution melting analysis showing both PCR product and unlabeled probe melting.

Three genotypes of the factor V Leiden SNP (wild type, R506QHet, and R506QHom) were patterned in a 384-well plate. Asymmetric PCR was performed in the presence of the saturating DNA dye LCGreen PLUS and an unlabeled probe covering the SNP. Without any processing after PCR, the plate was heated in a prototype LightScanner™ at 0.1 °C/s and 25 readings acquired every 1 °C. The no-template control samples (2 wells at *bottom right*) did not show a melting transition. After normalization, both PCR product and probe melting transitions are apparent (*A*). Derivative plots of just the probe region (*B*) visually cluster by genotype, as do difference plots of the PCR product melting transition (*C*). Unbiased hierarchal clustering recovered the previously invisible SNP pattern on the plate through correct genotyping (*D*). The same pattern was obtained after analysis of just the probe region (*B*), the product region (*C*), or both.

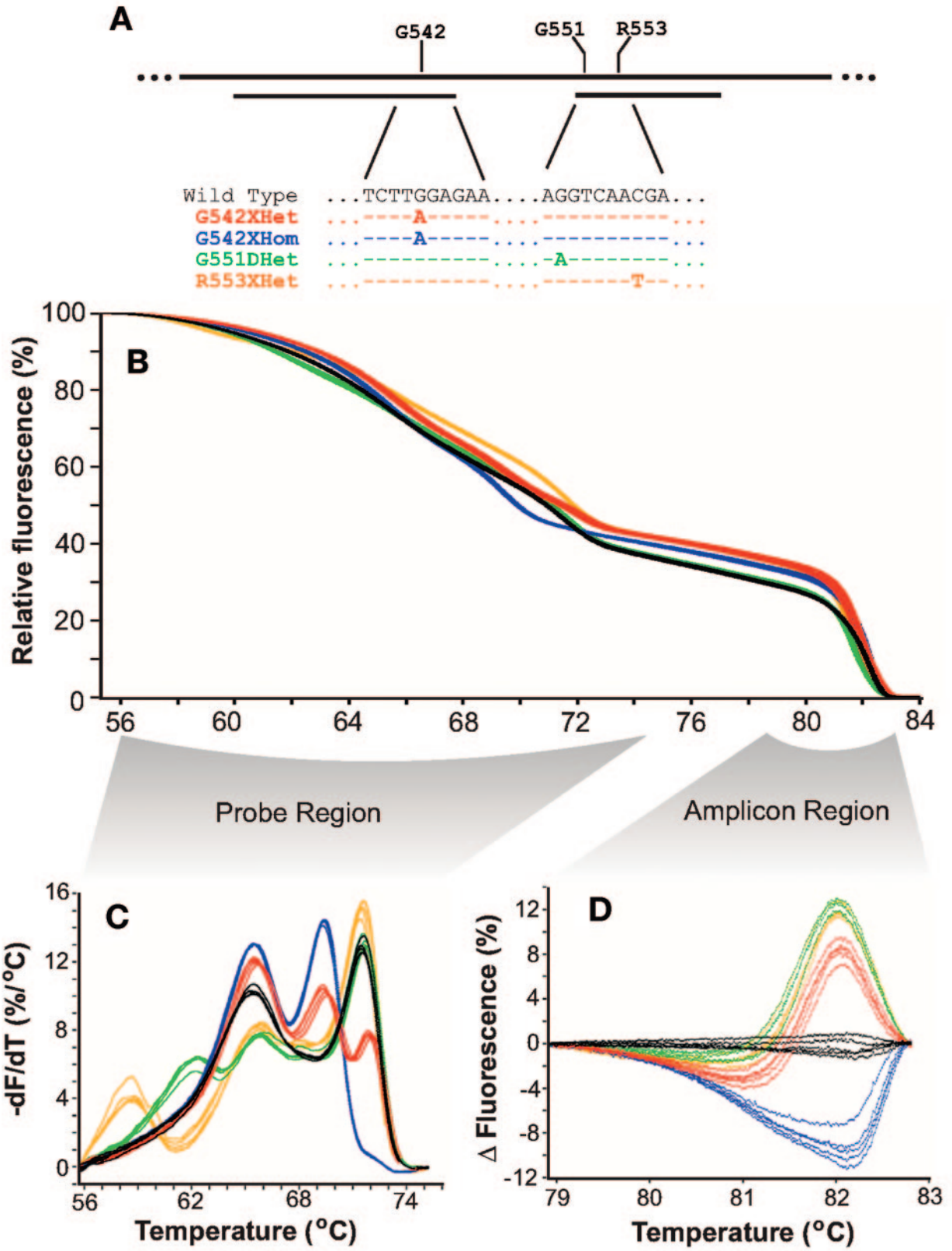

Fig. 2. High-resolution melting analysis of exon 11 of the *CFTR* gene by use of 2 unlabeled probes.

One probe covers 2 SNPs (G551D and R553X) and the other covers G542X (*A*). Melting transitions were observed with the saturating DNA dye LCGreen I on the HR-1 instrument at 0.3 °C/s with 65 readings every 1 °C. Normalized composite melting curves (*B*), probe derivative plots (*C*), and product difference plots (*D*) are shown. Five independent amplifications and melting curves are shown for each genotype.

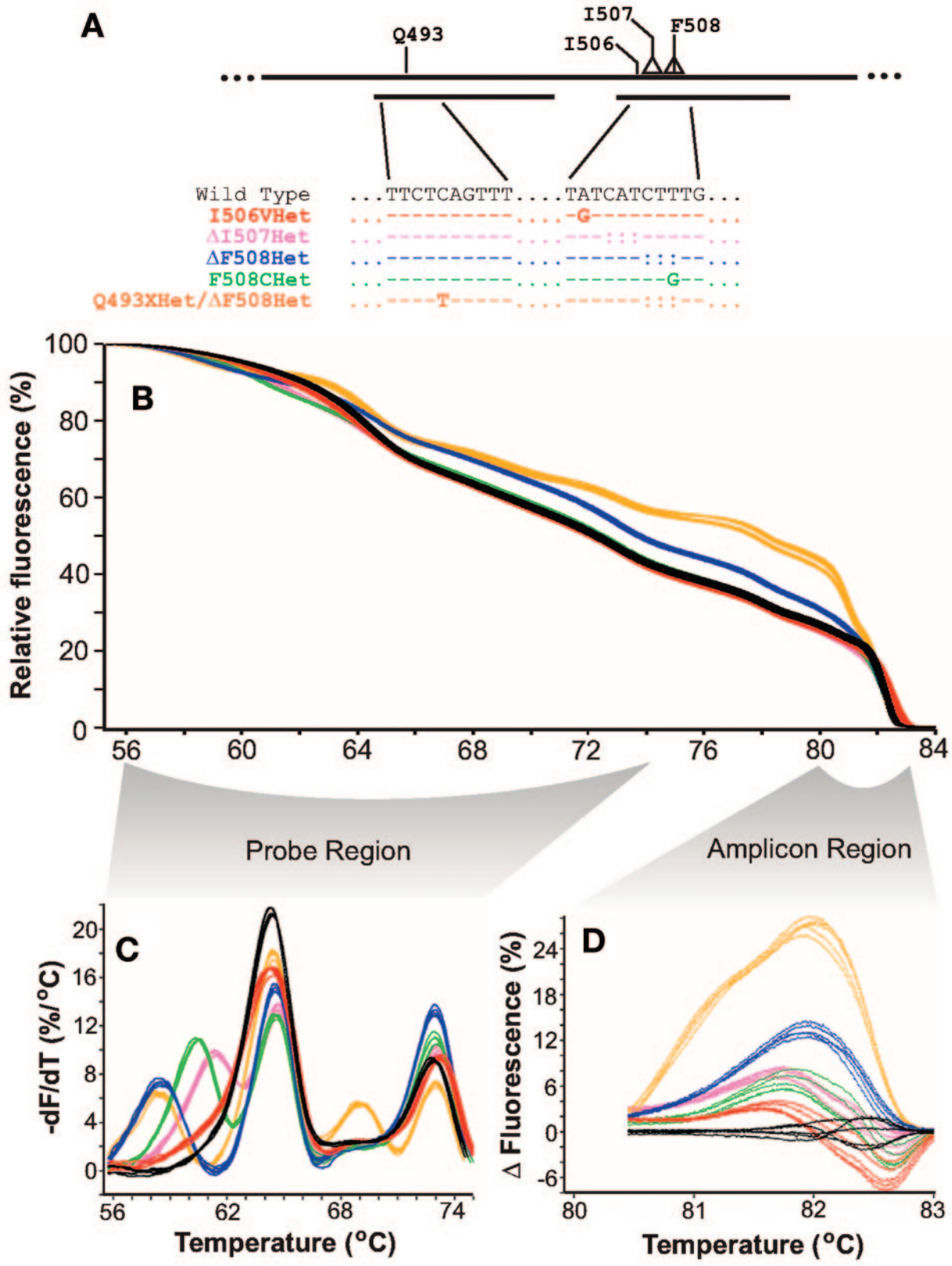

Fig. 3. High-resolution melting analysis of exon 10 of the *CFTR* gene by use of 2 unlabeled probes.

One probe covers 2 SNPs (I506V and F508C) and 2 deletions (I507del and F508del), whereas the other covers Q493X (*A*). Melting conditions are described in the legend for Fig. 2. Normalized composite melting curves (*B*), probe derivative plots (*C*), and product difference plots (*D*) are shown. Five independent amplifications and melting curves are shown for each genotype.

could be genotyped by either PCR product or probe melting. Consideration of both regions often provides independent confirmation of genotype.

Discussion

Genetic analysis has historically been divided into mutation scanning techniques and specific genotyping. Conventional scanning methods require a separation step after PCR and detect sequence differences between amplified fragments of DNA. These methods include single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, heteroduplex analysis, denaturing HPLC, temperature gradient capillary electrophoresis, and chemical or enzymatic mismatch detection methods *(25)*. Scanning methods screen for sequence differences, but specific genotyping or sequencing is required to identify the variation.

Recently, a closed-tube method of mutation scanning by high-resolution melting analysis was developed with high sensitivity and specificity *(11)*. A genotyping method, also based on melting in the presence of the same saturating DNA dyes, also was reported *(6)*. These methods are attractive because (*a*) there are no processing or separation steps after the initiation of PCR, (*b*) only standard PCR reagents, unlabeled oligonucleotides, and a saturating DNA dye are required, and (*c*) analysis is rapid (1–10 min after PCR). Even more attractive is simultaneous scanning and genotyping in the same reaction, as reported here. A composite melting curve generated after asymmetric amplification allows both PCR product (scanning) and probe (genotyping) analysis. Asymmetric PCR produces both full-length duplex product and excess single-stranded product complementary to the probes. When the mixture is heated, melting transitions for both the full-length product and the product/probe duplexes occur at different temperatures.

Product melting analysis detects sequence variants anywhere between 2 primers, whereas probe melting analysis identifies variants under a probe. If a sequence variant is between the primers and under a probe, both the presence of a variant and its genotype are obtained. If product melting indicates a variant but the probe does not, then the variation occurs between the primers but not under the probe, and further analysis for genotyping is necessary. Probes can be placed at sites of common sequence variation so that in most cases, if product scanning is positive, the probes will identify the sequence variants, greatly reducing the need for sequencing. With 1 probe, the genotype of a SNP can be established by both PCR product and probe melting. With 2 probes, 2 separate regions of the sequence can be interrogated for genotype and the rest of the PCR product scanned for rare sequence variants. Multiple probes can be used if they differ in T_m and if each allele presents a unique pattern of probe and/or product melting.

Consider population screening for cystic fibrosis mutations. Because only 3.8% of Caucasians are cystic fibrosis carriers *(26)*, at least 96.2% of randomly screened individuals would be negative by complete (exon and splice site) sequencing. With 27 exons, the percentage of sequencing runs expected to be positive is <3.8%/27, or 0.14%; i.e., only ~1 in 1000 sequencing runs would be useful. This is why sequencing is not recommended for cystic fibrosis screening. Instead, a selected mutation panel is usually performed that detects 83.7% of cystic fibrosis alleles *(26)*.

Consider as an alternative simultaneous scanning and genotyping for cystic fibrosis by high-resolution melting. If the amplicon length is kept under 400 bp, the sensitivity of high-resolution scanning approaches 100.0% *(11)*. If common mutations and polymorphisms are analyzed with unlabeled probes in the same reaction, then ~80% of mutations will also be genotyped. This further reduces the need for sequencing exons from 0.14% to <0.03%. Although this analysis ignores the complication of rare polymorphisms, it is clear that the need for sequencing can be vastly reduced by simultaneous scanning and genotyping.

Closed-tube genotyping methods that use melting analysis have the capacity to scan for unexpected variants (Table 1). Melting methods also use less complex and require fewer probes than allele specific methods, which require 1 probe for each allele analyzed. Allele discrimination by T_m *(7)* or curve shape *(9)* is an interesting option to fluorescent color. Dyes that generically stain double-stranded DNA are attractive for their simplicity and cost *(2, 9)*. Although the reliability of genotyping by amplicon melting is controversial, a recent study found that 21 of 21 heteroduplex pairs tested were distinguishable by high-resolution melting of small amplicons *(27)*.

Although common sequence variants can usually be genotyped with 1 or 2 unlabeled probes in the same reaction, more than 2 probes and/or sequential reactions can also be used. For example, multiple overlapped probes can locate unexpected rare variants to within the region covered by 1 probe *(28)*. Additional probes can be designed to identify the exact position and sequence of the variation. However, DNA sequencing is a more direct approach for identifying new, previously unknown variations, particularly when the amplified region is highly variable. Nevertheless, in the vast majority of genetic

Table 1. Comparison of techniques for real-time SNP genotyping/scanning.

Technique [Ref(s).]	Probes/SNP genotype	Modifications/ probe	Scanning region
Amplicon melting *(9, 11, 12, 27)*	0	0	Between primers
Unlabeled probe *(6)*	1	0	Between primers and within probe
Single Hybprobe *(5)*	1	1	Within probe
Dual HybProbe *(4)*	2	1	Within probes
TaqMan *(29)*	2	2	None
Beacons *(30)*	2	2	None
Scorpions *(31)*	2	3	None
MGB TaqMan *(32)*	2	3	None

analyses, the amplified wild-type sequence is known and potential common variants are limited. In these cases, scanning and genotyping can be performed in 1 step by DNA melting with simple oligonucleotides. No fluorescent probes or separations are required, and both amplification (15 min) and melting analysis (1–2 min) can be rapid *(1)*. Previously, the options for complete gene analysis were direct sequencing, scanning followed by sequencing, and scanning followed by genotyping. The ability to simultaneously scan and genotype with a process no more complex than PCR is a new attractive option.

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