Clinical Chemistry 49:6
853–860 (2003)

Molecular Diagnostics
and Genetics

High-Resolution Genotyping by Amplicon Melting Analysis Using LCGreen

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Background: **High-resolution amplicon melting analysis was recently introduced as a closed-tube method for genotyping and mutation scanning (Gundry et al. *Clin Chem* 2003;49:396–406). The technique required a fluorescently labeled primer and was limited to the detection of mutations residing in the melting domain of the labeled primer. Our aim was to develop a closed-tube system for genotyping and mutation scanning that did not require labeled oligonucleotides.**

Methods: **We studied polymorphisms in the hydroxytryptamine receptor 2A (HTR2A) gene (T102C), β-globin (hemoglobins S and C) gene, and cystic fibrosis (F508del, F508C, I507del) gene. PCR was performed in the presence of the double-stranded DNA dye LCGreen, and high-resolution amplicon melting curves were obtained. After fluorescence normalization, temperature adjustment, and/or difference analysis, sequence alterations were distinguished by curve shape and/or position. Heterozygous DNA was identified by the low-temperature melting of heteroduplexes not observed with other dyes commonly used in real-time PCR.**

Results: **The six common β-globin genotypes (AA, AS, AC, SS, CC, and SC) were all distinguished in a 110-bp amplicon. The HTR2A single-nucleotide polymorphism was genotyped in a 544-bp fragment that split into two melting domains. Because melting curve acquisition required only 1–2 min, amplification and analysis were achieved in 10–20 min with rapid cycling conditions.**

Conclusions: **High-resolution melting analysis of PCR products amplified in the presence of LCGreen can identify both heterozygous and homozygous sequence variants. The technique requires only the usual unlabeled primers and a generic double-stranded DNA dye added before PCR for amplicon genotyping, and is a promising method for mutation screening.**

Melting curve analysis in conjunction with real-time PCR was introduced in 1997 *(1, 2)* and is a natural extension of continuously monitoring PCR within each cycle *(3, 4)*. The double-stranded DNA (dsDNA)[1] dye SYBR Green I was used for amplicon melting analysis and provided a rough characterization of what was amplified *(1)*. Subsequently, hybridization probes were used to interrogate a limited region for sequence alterations by melting curve analysis *(2, 5)*. The primary advantage of melting curve analysis is that, rather than just determining the presence or absence of a specific allele, the method can interrogate the entire region under the probe. A book series on methods and applications of real-time PCR and melting curve analysis has recently been published *(6–8)*.

Two adjacent hybridization probes are most commonly used for melting curve analysis of small sequence variations, but a single hybridization probe can also been used *(9)*. With only a single probe, the fluorescence change observed with hybridization may be small, first suggesting the need for high-resolution melting analysis.

If PCR is performed with a 5′-labeled primer, high-resolution melting analysis of the amplicon can distinguish different homozygotes and heterozygotes *(10)*. The ability to genotype single-nucleotide polymorphisms within amplicons up to 304 bp in length suggests that scanning for unknown polymorphisms may also be successful. Disadvantages of the method include the need for a labeled oligonucleotide and a requirement that the sequence alterations must be within the melting domain that includes the labeled primer.

Limitations in the use of SYBR Green I for detecting small sequence variations are detailed elsewhere *(10)*.

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Received January 27, 2003; accepted March 11, 2003

[1] Nonstandard abbreviations: dsDNA, double-stranded DNA; Hb, hemoglobin; HTR2A, hydroxytryptamine receptor 2A; and T_m, melting temperature.

Briefly, detection of heterozygotes by SYBR Green I has been possible only when extra steps are added between amplification and analysis, such as amplicon purification and addition of high amounts of dye *(11)* or urea *(12)*. A completely closed-tube method using a generic dsDNA dye for effective genotyping and scanning has been elusive. After screening several dsDNA dyes, we found one dye that did detect heterozygotes and at the same time did not inhibit or adversely affect PCR. In this report, we demonstrate the usefulness of LCGreen in genotyping applications that involve high-resolution melting curve analysis of PCR amplicons. We also introduce melting curve subtraction (fluorescence difference) as a useful method of differentiating genotypes that allows better visual grouping of genotypes than the more common first-derivative method. The potential of using LCGreen for unknown variant detection (so-called mutation scanning) is also considered.

Materials and Methods

PCR PROTOCOL

Oligonucleotide primers were synthesized by standard phosphoramidite chemistry by our local core facility (University of Utah). PCR was performed in 10-μL volumes in a LightCycler (Roche Applied Systems) with programmed transitions of 20 °C/s unless otherwise indicated. The amplification mixture included 50 ng of genomic DNA as template, 200 μM each deoxynucleotide triphosphate (dNTP), 0.4 U of KlenTaq1 polymerase (AB Peptides), 88 ng of TaqStart antibody (ClonTech), 3 mM $MgCl_2$, 50 mM Tris (pH 8.3), 500 ng/μL bovine serum albumin, and 0.5 μM primers unless indicated otherwise. Genotyped human genomic DNA was obtained from previous studies *(13, 14)* or from Coriell Cell Repositories. The DNA used for β-globin genotyping was extracted from dried blood spots and was not quantified *(15)*. SYBR Green I was obtained from Molecular Probes and used at a 1:10 000 dilution. LCGreen was obtained from Idaho Technology and used at 1–10 μM as indicated. Ethidium bromide (Sigma), SYBR Gold, PicoGreen, TOTO-1, and YOYO-1 (all from Molecular Probes) were used at final concentrations of 16 μM, a 1:40 960 dilution, a 1:640 dilution, 0.4 μM, and 0.8 μM, respectively. These dye concentrations are near the maximum PCR-compatible concentrations, as determined by real-time PCR analysis using the second-derivative maximum method *(3, 16)*.

MELTING CURVE ACQUISITION

Melting analysis was performed on the LightCycler or on a high-resolution melting instrument (HR-1; Idaho Technology), as described previously for labeled primers *(10)*. After amplification, the samples were heated momentarily in the LightCycler to 94 °C and cooled to 40 °C unless stated otherwise. The LightCycler capillary was then transferred to the high-resolution melting instrument and heated at 0.3 °C/s unless otherwise stated. The HR-1 is a single-sample instrument that surrounds one LightCycler capillary with an aluminum cylinder that is heated by a coil wound around the outside. Sample temperature and fluorescence signals are converted to 16-bit digital signals, ideally providing resolution down to 0.002 °C and 0.002% of normalized fluorescence. Approximately 50 data points are acquired for every 1 °C.

MELTING CURVE ANALYSIS

LightCycler and high-resolution melting data were analyzed with custom software written in LabView (National Instruments). Fluorescence intensity values were usually normalized between 0% and 100% by first defining linear baselines before and after the melting transition of each sample. Within each sample, the fluorescence of each acquisition was calculated as the percentage of fluorescence between the top and bottom baselines at each acquisition temperature.

In some cases after normalization, the temperature axis of each curve was adjusted to superimpose the curves over a certain fluorescence interval. One sample was chosen arbitrarily, and the points within the fluorescence interval were fit to a quadratic. For each remaining curve, the required temperature shift for translation of each point within the fluorescence interval onto the quadratic was calculated. Each curve was then translated by the average shift to allow superimposition of the curves within the selected fluorescence interval.

Different genotypes were most easily discerned by plotting the fluorescence difference between normalized (and optionally, temperature-shifted) melting curves. One melting curve was chosen as a reference, and the difference between each curve and the reference was plotted against temperature to give a "fluorescence difference" plot. The original reference curve became a horizontal line at zero, and different genotypes clustered along different paths for easy visual discrimination.

Derivative melting curve plots were calculated from the Savitsky–Golay polynomials at each point *(17)*. Savitsky–Golay analysis used a second-degree polynomial and a data window including all points within a 1 °C interval.

MELTING CURVE GENOTYPING OF CYSTIC FIBROSIS MUTATIONS WITH SYBR GREEN I OR LCGreen

A 41- to 44-bp fragment was amplified with the primers 5′-GGCACCATTAAAGAAAATAT-3′ and 5′-TCATCATAGGAAACACCA-3′ (GenBank accession no. M55115; positions 413–456R) in the presence of SYBR Green I or 10 μM LCGreen. The primers flanked the mutational hot spot containing the F508del, I507del, and F508C variants. PCR was performed through 40 cycles of 85 and 58 °C (0 s holds). A final melting cycle was performed on the LightCycler by heating to 95 °C, cooling to 55 °C, and collecting fluorescence continuously at a ramping rate of 0.2 °C/s. Five samples were monitored during melting curve acquisition.

MELTING CURVE ANALYSIS OF MULTIPLE PRODUCTS WITH SYBR GREEN I OR LCGreen

A low-mass DNA ladder (Invitrogen), usually used as a length marker for gel electrophoresis, was melted in the presence of SYBR Green I or LCGreen. The DNA ladder (235 ng) was mixed with a 1:10 000 dilution of SYBR Green I or 4 μM LCGreen in the presence of 50 mM Tris (pH 8.3), 3 mM $MgCl_2$, and 500 ng/μL bovine serum albumin. These dye concentrations each produced 9% of the maximum fluorescence observed when excess dye was present. Each sample was melted at 0.1 °C/s in the high-resolution melting instrument. Derivative plots were obtained as described above.

β-GLOBIN MUTATIONS (HbC AND HbS)

A 110-bp fragment of the β-globin gene was amplified with primers 5′-ACACAACTGTGTTCACTAGC-3′ and 5′-CAACTTCATCCACGTTCACC-3′ (GenBank accession no. GI:455025; positions 62150–62259R). The single-nucleotide polymorphisms hemoglobin (Hb)C (G16A) and HbS (A17T) are near the middle of this amplicon. DNA was extracted from dried blood spots of two different individuals with each of the common genotypes. The genotypes included three homozygous (AA, SS, and CC) and three heterozygous (AS, AC, and SC) types. Each reaction contained 10 μM LCGreen. After an initial denaturation for 5 s at 95 °C, the samples were cycled 35 times with the following protocol: 94 °C with no hold, 50 °C with a 2-s hold, and a 1 °C/s ramp to 72 °C with a 2-s hold. After PCR amplification, denaturation, and cooling, fluorescence was acquired while heating from 70 to 93 °C at a rate of 0.3 °C/s with the high-resolution melting instrument.

HTR2A SINGLE-NUCLEOTIDE POLYMORPHISM

A 544-bp genomic fragment was amplified from the human 5-hydroxytryptamine receptor 2A (HTR2A) gene (GenBank accession no. NM_000621.1) with primers 5′-CCAGCTCCGGGAGA-3′ and 5′-CATACAGGATGGTTAACATGG-3′ (positions 14–557R). We used 1 μM LCGreen and 2 mM $MgCl_2$. PCR cycling conditions were as follows: 40 cycles of 92 °C for 0 s, 60 °C for 2 s, and 74 °C for 25 s. Duplicates of one patient sample of each genotype (CC, TC, and TT) were amplified and analyzed. After PCR amplification, denaturation, and cooling, fluorescence was acquired while heating from 70 to 93 °C at a rate of 0.3 °C/s in the high-resolution melting instrument. A melting temperature (T_m) map of the homozygous wild-type sequence was obtained using Poland *(18)* and Fixman–Freire *(19)* algorithms with the MELT94 software (available at http://web.mit.edu/osp/www/melt.html).

Results

INVESTIGATION OF DIFFERENT DNA DYES FOR HETERODUPLEX DETECTION

Several dsDNA dyes were tested for their ability to detect heteroduplexes with use of the short cystic fibrosis amplicon described previously *(10)*. A diagram of the 44-bp amplicon, showing the sequence alterations, is shown in Fig. 1A. Two homozygous and three heterozygous genotypes were analyzed.

Heteroduplexes were not detected with ethidium bromide, SYBR Green I, SYBR Gold, Pico Green, TOTO-1, and

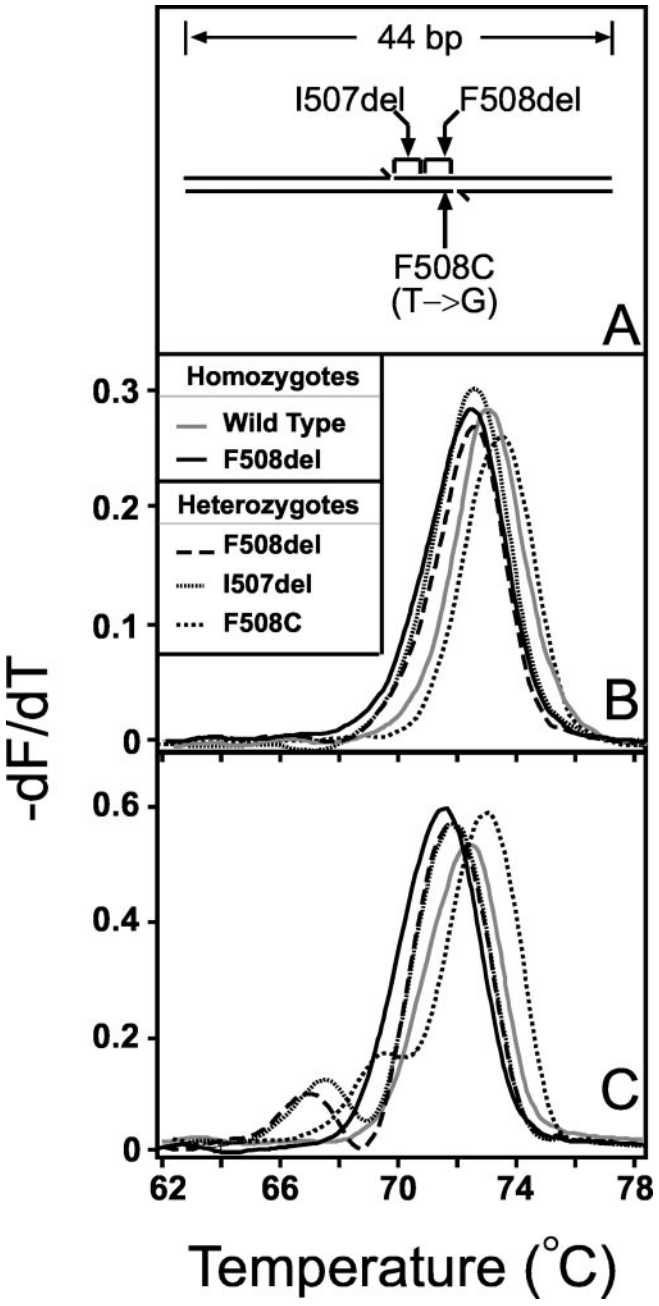

Fig. 1. Genotyping the I507/F508 region of the cystic fibrosis gene with use of either SYBR Green I or LCGreen.

(*A*), scaled drawing of the 44-bp amplicon. When SYBR Green I was used (*B*), the derivative melting curves showed closely spaced amplicon T_ms. No separate heteroduplex products were apparent. In contrast, when LCGreen was used (*C*), clearly separated heteroduplex products were observed that melted at lower temperatures than the homoduplex products. In both cases, two homozygotes and three heterozygotes were studied. The LightCycler instrument was used for melting curve acquisition.

YOYO-1 dyes. The derivative melting curves for SYBR Green I are shown in Fig. 1B. All genotypes showed symmetric derivative curves with T_ms within 1 °C of each other. The T_m of the F508C heterozygote was slightly higher than that of the wild type, reflecting the greater stability of a GC vs an AT base pair *(13)*. The three genotypes containing heterozygous or homozygous deletions melted at a slightly lower temperature than the wild type and were difficult to distinguish from each another.

In contrast, amplification and melting of heterozygotes in the presence of a newly available dye, LCGreen, produced two clearly defined peaks (Figs. 1C). Similar to the results obtained with labeled primers *(10)*, the lower-temperature peak was always smaller than the higher-temperature peak, indicating the melting transition of a heteroduplex product. As might be expected, the heterozygotes with 3 bp deleted (F508del and I507del) produced heteroduplex products that were more destabilized than heteroduplex products from a single base change (F508C). With LCGreen, the major melting transition was spread over 2 °C, approximately twice the range observed with SYBR Green I. This greater range, in addition to the presence of heteroduplex peaks, allowed complete differentiation of all five genotypes. When the concentration of LCGreen was varied from 1 to 60 μM, there was little difference in the percentage area of the heterozygous peak (data not shown). The mean (SD) area for the heterozygote was 12.7 (2.7)% (n = 7 different concentrations).

DYE REDISTRIBUTION DURING MELTING

One reason that LCGreen identifies low-melting transitions better than SYBR Green I is illustrated in Fig. 2. When several DNA fragments of increasing thermal stability are present, the low-temperature peaks are very small with SYBR Green I compared with LCGreen. During melting, SYBR Green I may be released from low-temperature duplexes only to attach to duplexes that melt at higher temperatures. This causes each successive peak to be higher than the last. In contrast, LCGreen shows clear peaks for all duplexes, and the peak height does not always increase with T_m. Compared with SYBR Green I, LCGreen does not appear to redistribute during melting.

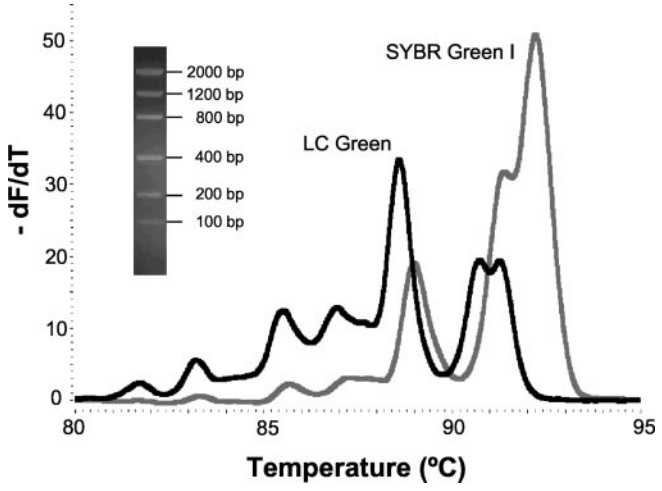

Fig. 2. Derivative melting curve plots of a molecular size ladder (*inset*) using either SYBR Green I (*gray line*) or LCGreen (*black line*).

The DNA ladder consisted of six DNA fragments (100 bp to 2 kb). All six fragments were clearly observed when LCGreen was used (the highest melting product appears to split into two melting domains). With SYBR Green I, the low-temperature peaks were minimized, with the peaks increasing in size with temperature.

The area of each peak in Fig. 2 was determined and divided by the known amount of each DNA species, providing an assessment of the relative sensitivity of each dye for each band (data not shown). LCGreen displayed a tendency for higher sensitivity with low-temperature melting species. In contrast, SYBR Green I strongly favored species that melted at higher temperatures.

ANALYSIS METHODS FOR HETERODUPLEX AND HOMODUPLEX DIFFERENTIATION

Melting curve analysis of six common genotypes of β-globin in a 110-bp amplicon is detailed in Fig. 3. The original high-resolution data are shown in Fig. 3A. Note that the magnitude of the fluorescence was variable between different samples because of differences in the starting template and/or capillary optics. It is difficult to see any consistent difference between genotypes. The derivative plot of this data also did not clearly distinguish genotypes, although the presence of heterozygotes could be identified (data not shown).

The magnitude differences between samples in the original data could be normalized. Two linear regions were selected, one before and one after the major transition. These regions defined two lines for each curve, an upper, 100% fluorescence line and a lower, 0% fluorescence line. The percentage of fluorescence within the transition (between the two regions) was calculated at each temperature as the distance to the experimental data compared with the distance between the extrapolated upper and lower lines. The normalized result for the β-globin data is shown in Fig. 3B. Each genotype now grouped together, most apparent between 84 and 85 °C. Different homozygotes had similar shapes but different T_ms, differing by 0.2–0.4 °C. Although there was some variation within each genotype (0.1–0.2 °C), all homozygotes were clearly differentiated when the entire curve was considered. Heterozygotes have a different shape than homozygotes in the low melting region of the transition.

When the purpose of analysis is to scan for heterozygotes, temperature offsets between samples can be eliminated by shifting the temperature axis of each curve so that the curves are superimposed over a given fluorescence interval. Amplification of a heterozygote produces both low-temperature heteroduplex transitions and high-temperature homoduplex transitions. When the curves were shifted to superimpose their high-temperature, homoduplex regions (low percentage of fluorescence), heteroduplexes could be identified by their early decrease in

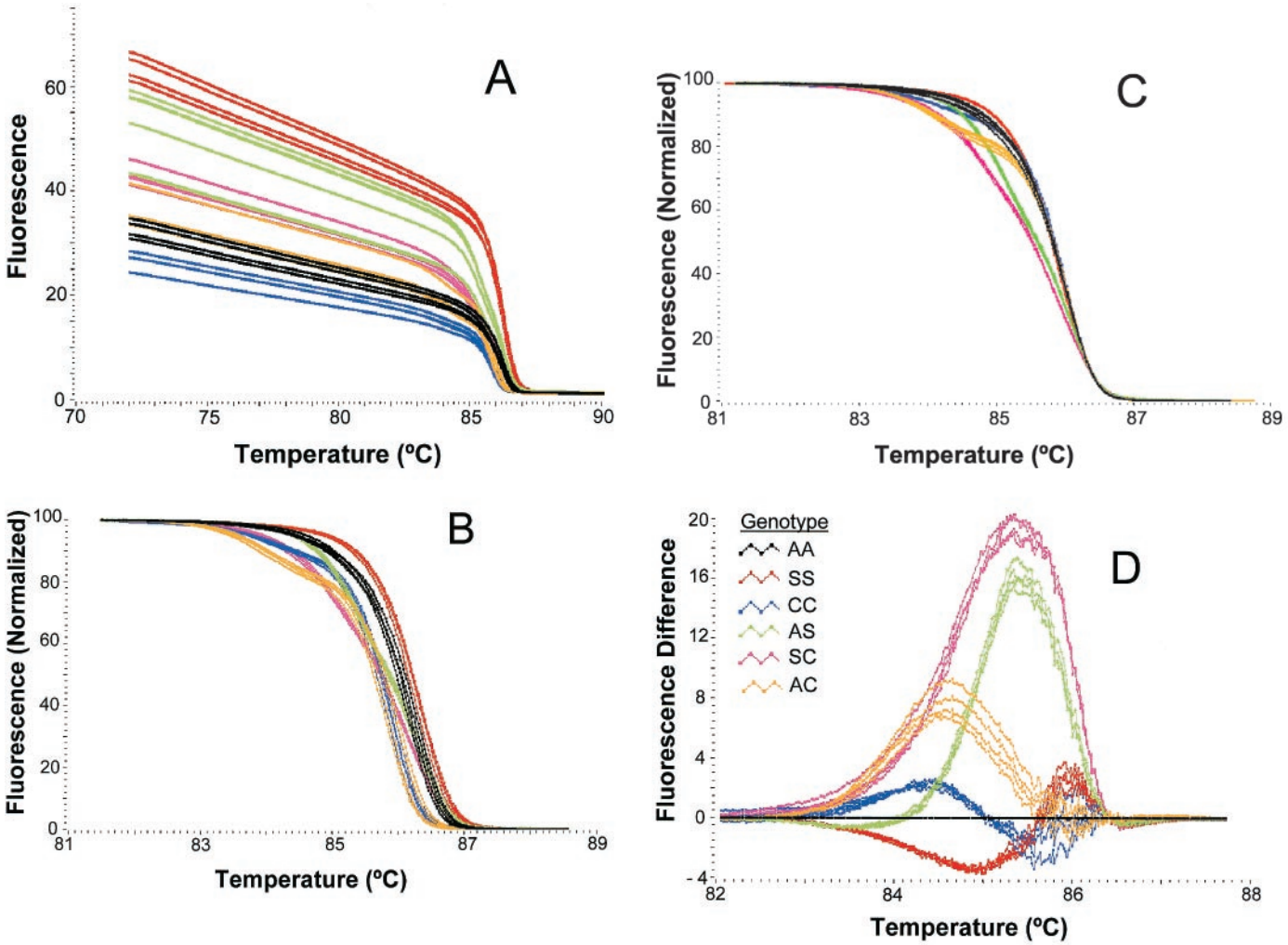

Fig. 3. Genotyping of two adjacent single-nucleotide polymorphisms (HbS and HbC) within a 110-bp β-globin fragment with use of high-resolution amplicon melting curve analysis.

The original high-resolution melting curves (*A*) include two different individuals for each of the six different genotypes, each individual run in duplicate. (*B*), the same curves as in *panel A* after normalization. (*C*), normalized curves after temperature-shifting of each curve to overlay them on one of the AA curves between 5% and 10% fluorescence. (*D*), fluorescence difference curves obtained by subtracting each curve in *panel C* from one wild-type (*AA*) curve (for clarity, the other AA samples are not shown). Because of the shift in temperature of the curves in *panels C* and *D*, the temperature axis no longer reflects absolute temperatures but rather reflects temperature differences relative to the superimposed segments of the curves.

fluorescence at lower temperatures, as shown in Fig. 3C. However, this temperature shift would reduce or possibly eliminate any difference between homozygotes. That is, temperature-shifting alters the apparent T_m (by up to 0.4 °C in Fig. 3) and should not be used for calculating T_ms.

Different genotypes were most easily distinguished by plotting the fluorescence difference between normalized (and optionally, temperature-shifted) melting curves. A reference genotype was first selected (AA in Fig. 3). The difference between all other genotype curves and the reference was then plotted against temperature, as shown in Fig. 3D. The reference sample (subtracted from itself) became zero across all temperatures. Other genotypes traced unique paths that could be identified by visual pattern matching. Each individual (two for each genotype) was run in duplicate to get some indication of the variation between individuals and between genotypes as well as the experimental variation from amplification and melting. Because the duplicates of each individual did not appear to cluster, individual variation does not appear to interfere with the difference between genotypes.

SINGLE-NUCLEOTIDE POLYMORPHISM TYPING IN LONG PCR AMPLICONS WITH MULTIPLE DOMAINS

High-resolution melting analysis of a single-nucleotide polymorphism within a 544-bp fragment of the human HTR2A gene is shown in Fig. 4. Duplicate samples of each genotype (CC, TC, and TT) were amplified and analyzed. The data were normalized and temperature-shifted as described for β-globin genotyping with curve superimposition between 10% and 20% fluorescence. The inset in

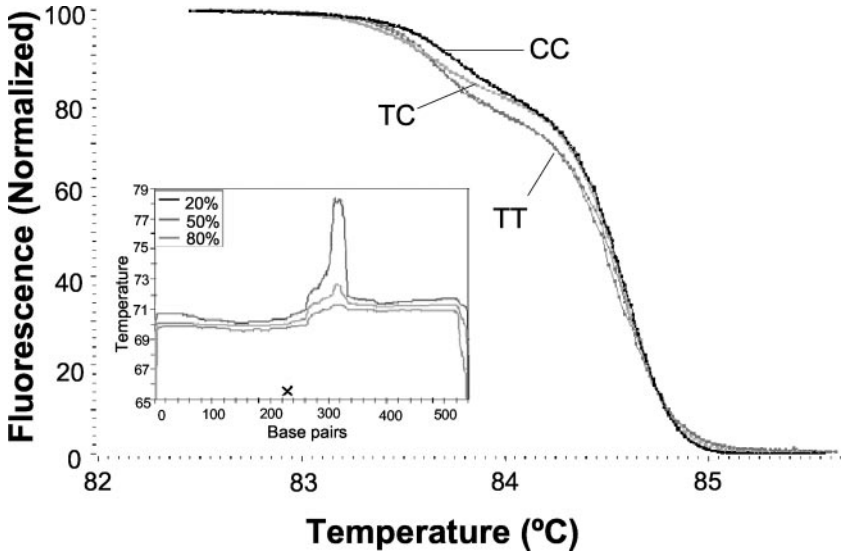

Fig. 4. High-resolution melting curve genotyping of a single-nucleotide polymorphism within a 544-bp fragment of the HTR2A gene.

Duplicate samples of each genotype (CC, TC, and TT) are shown. The data were normalized and temperature-shifted to superimpose the curves between 10% and 20% fluorescence. The *inset* shows a predicted melting map of the homoduplex and the position of the polymorphism in the lower melting domain (marked as *X*). Experimental melting curves also showed two apparent melting domains. All genotypes were similar in the higher melting temperature domain but differed in the lower melting domain.

Fig. 4 shows a predicted melting map of the homoduplex and the position of the polymorphism in the lower melting domain. The experimental data also show two apparent melting domains. All genotypes were similar in the higher melting domain. Genotypes differed in the lower melting domain, and the heterozygote pattern resembled that observed with labeled primers *(10)*.

Discussion

PCR amplification with a labeled primer followed by high-resolution melting analysis of the products has been reported previously *(10)*. Single-nucleotide polymorphisms were correctly genotyped in products as long as 304 bp in a closed-tube system. Although the method may be useful for identifying unknown mutations (scanning), it is limited by the requirement that the sequence variants need to be in the same melting domain as the labeled primer. Another disadvantage of the technique is that a labeled oligonucleotide is required. A closed-tube system for genotyping and mutation scanning that did not require labeled oligonucleotides would be ideal.

In this report, we describe a closed-tube method for genotyping entire amplicons that uses the dsDNA dye LCGreen in combination with high-resolution melting curve analysis. In a short cystic fibrosis model system *(10)*, most of the dsDNA dyes commonly used in real-time PCR did not differentiate heterozygotes from homozygotes. The dyes SYBR Green I (Fig. 1B), ethidium bromide, SYBR Gold, Pico Green, TOTO-1, and YOYO-1 did not reveal heteroduplexes when tested at noninhibitory concentrations.

LCGreen is a dsDNA dye that detects heteroduplexes (Fig. 1C), accurately genotypes amplicons (Figs. 3 and 4), and could be useful for closed-tube mutation scanning. Not only are heteroduplexes detected, the major melting transitions observed with LCGreen are expanded between genotypes compared with SYBR Green I (Fig. 1). This expansion is particularly useful in differentiating different homozygotes. Concentrations between 1 and 10 μmol/L are most useful. At concentrations <1 μmol/L, the signal-to-noise ratio decreases, and at concentrations >10 μmol/L, the melting transition broadens and genotyping is not as reproducible (data not shown).

The reason that LCGreen detects heteroduplexes whereas SYBR Green I does not is not entirely clear. Saturation curves can be obtained by measuring the fluorescence of different dye dilutions with the same amount of DNA (data not shown). If 100 ng/10 μL is chosen as the amount of DNA present at the PCR plateau, LCGreen can be used at >90% saturation, whereas SYBR Green I completely inhibits PCR at >50% saturation. Dye redistribution during acquisition of the melting curve may be one reason that heteroduplexes are not observed with SYBR Green I *(10)*. However, even when the dyes were used at the same low saturation concentration (Fig. 2), melting results obtained with LCGreen were much more representative of the products present than results obtained with SYBR Green I, which is strongly biased against low-melting products.

Heteroduplexes resulting from the amplification of heterozygotes are easier to detect when the PCR product is short. With the 44-bp cystic fibrosis amplicon, the heteroduplex transition was clearly observed on derivative plots (Fig. 1). When we *(20)* used engineered plasmid templates from a DNA mutation toolkit (Cambrex Bio Science Rockland, Inc.), the LightCycler could detect all possible heterozygotes with short amplicons (data not shown). However, as amplicon size increases, it becomes increasingly difficult to identify heterozygotes. The heteroduplex effects on melting curves are still present and could be used for genotyping and scanning if high-resolution methods are used. The requirements of such an approach include not only high-resolution instrumentation, but also adequate analysis techniques. For example,

conventional derivative plots limit the display of high-resolution data because taking a derivative necessarily smoothes the data and the effect of heteroduplexes may be obscured in the rising shoulder of the major transition.

High-resolution techniques may also be needed to distinguish different homozygotes from each other. A similar situation occurs when the DNA analyzed is not diploid, such as with most genes in bacteria or viruses. For example, the T_m difference between some homoduplexes (single base change of A:T to T:A or G:C to C:G) challenges even high-resolution methods. One way to detect homozygous variants with nearly identical T_ms is to mix the variants together. If the samples are different, heteroduplexes will be easily identified.

On first glance, original melting curve data do not appear to contain enough information for genotyping (Fig. 3A). Conventional derivative plots are also not very informative (not shown). However, simple normalization of the fluorescence before and after the melting transitions allows visual clustering of different genotypes (Fig. 3B). If the purpose of analysis is to scan for heterozygotes, temperature-shifting each sample by superimposing the normalized curves at low fluorescence (high temperature) is helpful. Heterozygotes are identifiable by an early decrease in fluorescence before the major melting transition (Fig. 3C). Different heterozygotes trace different paths defined by the unique heteroduplexes and homoduplexes present. The differences between genotypes become even more apparent when displayed as fluorescence difference plots (Fig. 3D). These difference plots should not be confused with conventional derivative plots. Surprisingly, different homozygotes appear distinguishable after normalization, temperature-shifting, and difference analysis. This implies that the shape of the melting curve, as well as the overall T_m, may vary among different homozygous genotypes. Although automatic clustering algorithms could be devised for genotyping, simple visual clustering on difference plots appears simple and accurate.

Although short amplicons produce greater genotyping differences, larger amplicons can also be genotyped. DNA melting domains are usually ~50 to 300 bp in length *(21)*, and larger amplicons, e.g., 100–800 bp, may have multiple melting domains. A sequence alteration in one domain may not affect the melting of other domains. The 544-bp HTR2A amplicon contains two melting domains, both by calculation [Fig. 4, inset, and Refs. *(18, 19)*] and experiment (Fig. 4). The polymorphism is in the lower melting domain, and the melting curves of the different genotypes follow the expected patterns observed for single-nucleotide polymorphisms *(10)*.

Because the melting of one domain appears to be independent of the melting of other domains, an invariant domain may be used as an internal control to adjust the temperature axis to correct instrument and/or sample run variation. Alternatively, temperature calibration of each curve could be provided by control DNA added to each sample before amplification or melting. Because multiple melting domains are present in larger amplicons, the variation in shape may occur in any portion of the curve, and it should be possible to identify the heterozygous domain. Because amplicon melting splits into domains, there may not be an upper limit on the amplicon size for genotyping and mutation scanning. Similar to mass spectrometry, amplicon melting analysis can become a more powerful tool with higher instrument resolution. Recently, we used this technique to distinguish six different genotypes within a 612-bp amplicon of the cystic fibrosis gene (data not shown). Our results suggest that when sufficient resolution is available, most sequence alterations of most amplicons can be genotyped in a single tube. However, potential confounding effects of DNA concentration and salt concentration (particularly Mg^{2+}) should be considered and have been discussed previously *(10)*. When labeled primers are used, heteroduplex formation is favored by lower Mg^{2+} concentrations. With LCGreen, the concentration of Mg^{2+} may also affect dye redistribution during melting.

Heteroduplex detection by high-resolution melting is particularly promising for identifying unknown sequence variants. However, the detection sensitivity remains to be established and will likely improve with advances in instrumentation and analysis methods. Compared with well-established techniques such as denaturing HPLC, high-resolution melting analysis for genotyping and mutation scanning has just begun.

This work was funded by grants from the NIH (GM58983), Idaho Technology, and the University of Utah. We thank Idaho Technology for providing the HR-1 instrument and Steve Dobrowolski for providing the different hemoglobin genotypes as blood spots on filter paper. The LightCycler instrument and related techniques are licensed by the University of Utah to Idaho Technology and from Idaho Technology to Roche Applied Systems. C.T.W. holds equity interest in Idaho Technology.

References

1. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 1997;245:154–60.
2. Lay MJ, Wittwer CT. Real-time fluorescence genotyping of factor V Leiden during rapid cycle PCR. Clin Chem 1997;43:2262–7.
3. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997;22:130–1,134–8.
4. Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The LightCycler: a microvolume, multisample fluorimeter with rapid temperature control. Biotechniques 1997;22:176–81.
5. Wittwer CT, Herrmann MG, Gundry CN, Elenitoba-Johnson KSJ. Real-time multiplex PCR assays. Methods 2001;25:430–42.
6. Meuer S, Wittwer C, Nakaguwara K, eds. Rapid cycle real-time PCR: methods and applications. Berlin: Springer-Verlag, 2001: 408pp.
7. Reischl U, Wittwer C, Cockerill F, eds. Rapid cycle real-time PCR:

methods and applications—microbiology and food analysis. Berlin: Springer-Verlag, 2002:258pp.
8. Dietmaier W, Wittwer C, Sivasubramanian N, eds. Rapid cycle real-time PCR: methods and applications—genetics and oncology. Berlin: Springer-Verlag, 2002:180pp.
9. Crockett AO, Wittwer CT. Fluorescein-labeled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides. Anal Biochem 2001;290:89–97.
10. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clin Chem 2003;49:396–406.
11. Lipsky RH, Mazzanti CM, Rudolph JG, Xu K, Vyas G, Bozak D, et al. DNA melting analysis for detection of single nucleotide polymorphisms. Clin Chem 2001;47:635–44.
12. Elenitoba-Johnson KSJ, Bohling SD. Solution-based scanning for single-base alterations using a double-stranded DNA binding dye and fluorescence-melting profiles. Am J Pathol 2001;159:845–53.
13. Gundry CN, Bernard PS, Herrmann MG, Reed GH, Wittwer CT. Rapid F508del and F508C assay using fluorescent hybridization probes. Genet Test 1999;3:365–70.
14. Herrmann M, Dobrowolski S, Wittwer CT. β-Globin genotyping by multiplexing probe melting temperature and color. Clin Chem 2000;46:425–8.
15. Heath E, O'Brien D, Banas R, Dobrowolski SF. Optimization of an automated protocol to isolate DNA from Guthrie cards. Arch Pathol Lab Med 1999;123:1154–60.
16. Wittwer CT, Kusukawa N. Real-time PCR. In: Persing D, Tenover F, Relman D, White T, Tang Y, Versalovic J, Unger B, eds. Diagnostic molecular microbiology: principles and applications. Washington: ASM Press, 2003:in press.
17. Press WH, Teukolsky SA, Vetterling WT, Flannery BP, eds. Numerical recipes in C, 2nd ed. New York: Cambridge University Press, 1992:650–5.
18. Poland D. Recursion relation generation of probability profiles for specific-sequence macromolecules with long-range correlations. Biopolymers 1974;13:1859–71.
19. Fixman M, Freire JJ. Theory of DNA melting curves. Biopolymers 1977;16:2693–704.
20. Highsmith WE, Jin Q, Nataraj AJ, O'Connor JM, Burland VD, Baubonis WR, et al. Use of a DNA toolbox for the characterization of mutation scanning methods. I: construction of the toolbox and evaluation of heteroduplex analysis. Electrophoresis 1999;20:1186–94.
21. Myers RM, Maniatis T, Lerman, LS. Detection and localization of single base changes by denaturing gradient electrophoresis. Methods Enzymol 1987;155:501–27.