

Real-Time Multiplex PCR Assays

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The ability to multiplex PCR by probe color and melting temperature (T_m) greatly expands the power of real-time analysis. Simple hybridization probes with only a single fluorescent dye can be used for quantification and allele typing. Different probes are labeled with dyes that have unique emission spectra. Spectral data are collected with discrete optics or dispersed onto an array for detection. Spectral overlap between dyes is corrected by using pure dye spectra to deconvolute the experimental data by matrix algebra. Since fluorescence is temperature dependent and depends on the dye, spectral overlap and color compensation constants are also temperature dependent. Single-labeled probes are easier to synthesize and purify than more complex probes with two or more dyes. In addition, the fluorescence of single-labeled probes is reversible and depends only on hybridization of the probe to the target, allowing study of the melting characteristics of the probe. Although melting curves can be obtained during PCR, data are usually acquired at near-equilibrium rates of 0.05–0.2°C/s after PCR is complete. Using rapid-cycle PCR, amplification requires about 20 min followed by a 10-min melting curve, greatly reducing result turnaround time. In addition to dye color, melting temperature can be used for a second dimension of multiplexing. Multiplexing by color and T_m creates a “virtual” two-dimensional multiplexing array without the need for an immobilized matrix of probes. Instead of physical separation along the X and Y axes, amplification products are identified by different fluorescence spectra and melting characteristics. © 2001 Elsevier Science (USA)

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Every PCR product or probe melts at a characteristic temperature. This melting temperature, or T_m , can distinguish between different PCR products. This allows

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PCR multiplexing by T_m , a powerful tool with a multiplexing potential similar to that of fluorescence emission spectra. When both emission spectra (color) and T_m multiplexing are combined, the number of distinguishable products is equal to the number of discernable colors times the number of separable T_m s.

Many different fluorescence techniques are available for real-time PCR. Dyes like SYBR Green I can be used in any amplification and are inexpensive (1). However, they are not sequence-specific and multiplexing is not possible by color.

Color multiplexing is possible with oligonucleotide probes that are labeled with different fluorescent groups. For example, both exonuclease probes (Taq-Man) and hairpin probes (molecular beacons) are sequence specific and can be multiplexed by color. These dual-labeled probes hybridize internal to the primers and include one fluorescent reporter and one quencher (2, 3). The fluorescence from exonuclease probes results from probe cleavage by 5'-exonuclease activity. This fluorescence change is not reversible with temperature and T_m analysis is not possible. With hairpin probes, melting curves are complicated by two melting transitions: probe hybridization to target and internal hairpin melting (4). A better design for combined color and T_m multiplexing is the simple hybridization probe.

Simple hybridization probes have only a single fluorescent label, in contrast to other probe designs with two (2, 3) or more (5, 6) added groups. The observed change in fluorescence depends only on hybridization to target and is reversible. This change in fluorescence occurs either by resonance energy transfer (1, 7) or from deoxyguanosine quenching (8, 9). Because these probes require only a single functional group, they are easier to synthesize and purify than more complex designs. They are also ideal for combined color and T_m multiplexing. Simple hybridization probes and SYBR Green

I in real-time PCR were first introduced in 1997 as part of the LightCycler system (1, 10). This system arose out of prior work with rapid temperature cycling for PCR (11, 12). In what follows, basic methods for rapid-cycle PCR are presented first, followed by modifications for real-time monitoring, color multiplexing, T_m multiplexing, and combined color/ T_m multiplexing. Many specific applications of this technology have been published and cannot be reviewed in detail here. A methods and applications book on rapid-cycle, real-time PCR, including 38 chapters on specific applications, has recently been published (13).

DESCRIPTION OF METHOD

Rapid-Cycle PCR

Rapid-cycle PCR was developed in the early 1990s (11, 12, 14). Amplification yield and product specificity are optimal when denaturation and annealing times are less than 1 s (12, 14). There is no advantage to extended denaturation or annealing times, as long as the PCR solution reaches the target temperatures and genomic DNA is denatured before amplification. Indeed, longer annealing times result in more nonspecific amplification. The time required for 30 cycles of most products less than 500 bp is 10–15 min. Rapid temperature cycling with minimal annealing and denaturation times improves quantitative PCR (15) and increases the discrimination of allele-specific amplification (16).

The reagents used for a standard 10- μ l rapid-cycle PCR are given in Table 1. PCR is robust and can tolerate a wide variety of reagents. Reagents A–E can be combined into a premix without primers and template DNA if desired. The reaction does not include KCl because initial studies suggested higher yields with a low-ionic-strength buffer, possibly because of slower double-stranded product formation during cooling. In addition, other PCR buffers, such as the commonly used 50 mM KCl, 10 mM Tris can be used in rapid-cycle PCR (17).

TABLE 1

Standard Reagent Concentrations for Rapid-Cycle PCR

| Reagent | Concentration |
|---------|---------------------------------------------------------|
| A | 50 mM Tris, pH 8.3 |
| B | 500 μ g/ml bovine serum albumin |
| C | 200 μ M each dNTP |
| D | 1–5 mM MgCl ₂ |
| E | 0.4 U heat-stable polymerase |
| F | 0.5 μ M each primer |
| G | 50 ng genomic DNA (15,000 copies of a single-copy gene) |

Bovine serum albumin or other protein is necessary to coat the glass surface of the capillaries or cuvettes, presumably to avoid surface denaturation of the polymerase on the glass surface. The MgCl₂ concentration is varied to adjust the stringency of the reaction. The polymerase can be any heat-stable polymerase, including standard 5'-exonuclease-positive *Taq* polymerase. Polymerases with higher extension rates will allow faster cycling. Primer concentrations are generally high in rapid-cycle PCR so that annealing rates are fast and minimal annealing times can be used. Finally, genomic DNA at an $A_{260} = 1.0$ is a convenient 10 \times solution of template. The quality of reagents is not critical, except for the absence of target amplicon. If the concentration of template is low, hot start techniques can be used. These include the use of antipolymerase antibodies (18), and Fast-Taq, a rapid heat-activating polymerase (Roche Applied Systems). If analysis will be on agarose gels instead of in real time, gel-loading agents (Ficoll or sucrose and a tracking dye) can be added (12). Additional recipes can be found at <http://www.idahotech.com>.

Suggested temperature/time parameters for rapid cycling are summarized in Table 2. If the template is complex genomic DNA not previously denatured, an initial denaturation step at 94°C for 15 s should be added on the first cycle only. There is no advantage to appending a 7-min final extension to the process. For products with a high-melting GC domain, adding dimethyl sulfoxide (DMSO) or formamide and increasing the denaturation temperature above 94°C should be considered. The extension temperature may need to be lowered below 74°C for products with a low-melting AT domain. The suggested annealing temperature is approximate and will need to be tested at various Mg²⁺ concentrations. Finally, for amplicons of less than 100 bp, a “0”-s extension time is adequate.

Rapid-Cycle Real-Time PCR

Integrating a fluorometer with a rapid temperature cyler led to rapid-cycle, real-time instrumentation (10). SYBR Green I is used to monitor the accumulation

TABLE 2

Suggested Temperature and Time Parameters for Rapid-Cycle PCR

| Step | Temperature (°C) | Time (s) |
|--------------|----------------------|-----------------------|
| Denaturation | 94 | 0 |
| Annealing | 30 + 0.5(primer %GC) | 0 |
| Extension | 74 | 0.03 (product length) |

of double-stranded amplicon and simple hybridization probes are used to monitor sequence-specific product (1).

To monitor the accumulation of double-stranded DNA during PCR, SYBR Green I is added to the standard reagents used in rapid-cycle PCR (Table 1) at a final concentration of 1:20,000. Final dilutions from 1:7000 to 1:40,000 can be used. Stock solutions of SYBR Green I in DMSO are available commercially, although the concentration and molecular structure are trade secrets (Molecular Probes, Eugene OR). Dilutions of the stock should be made with a positive displacement pipet. The dye has limited stability in dilute aqueous solution, particularly to freezing and thawing, but is stable in 10 mM Tris, pH 8.0, 0.1 mM EDTA at 4°C for days as a 10× (1:2000 dilution) or 100× (1:200 dilution) solution. Master solutions of SYBR Green I integrated with other required PCR reagents are available commercially (DNA Master SYBR Green I, RNA Master SYBR Green I, etc., Roche Applied Systems).

Simple Hybridization Probes

Compared with other probes used in real-time PCR (2, 3, 5, 6), simple hybridization probes are easy to design (Fig. 1). Each probe only has a single dye attached. The first format uses two adjacent probes (1). The energy transfer donor is attached to one probe, and a long-wavelength acceptor is placed on the other probe. As product accumulates during PCR, the probes hybridize next to each other during the annealing segment of each cycle. The donor transfers absorbed energy to the acceptor and the acceptor emits at a longer wavelength. The increased emission of the acceptor and/or the decreased emission of the donor are monitored to follow the appearance of target. Resonance energy transfer decreases as the separation between dyes increases. A single base between dyes appears optimal. The signal decreases about 50% at a 5- to 10-base separation but can still be observed at distances of up to 15–25 bases.

The second format uses one labeled primer and one labeled probe (19). The labeled primer is incorporated into the PCR product during amplification. In contrast to the probe/probe system, only a single hybridization each cycle is necessary in the primer/probe design. A primer that has a T positioned 3 to 6 bases from the 3' end is selected. The primer is synthesized with a modified T at this position (e.g., C6-dT, Glen Research) for conjugation to one of the resonance energy transfer dyes. The other dye is placed 3' on a probe that hybridizes to the strand extended by the labeled primer. On hybridization, the dyes are on opposite strands and should be separated by 4–6 bases. This requires either separating the probe and labeled primer by 0–3 bases

or overlapping them by up to 2 bases, depending on the position of the label on the primer.

The third format uses only one label on a single probe (8). Observed fluorescence from the probe may increase or decrease with hybridization. For example, reliable fluorescence quenching occurs during probe hybridization when fluorescein (8) or BODIPY-FL (9) is approximated to deoxyguanosine nucleotides. The probe can be labeled on either the 3' or the 5' end. The probe should hybridize such that a G is present at the first overhang position on the target strand. Additional neighboring G's on the target strand increase quenching incrementally, but the first overhang position is most important.

In all simple hybridization probe formats, any 5'-labeled probe must be blocked at the 3' end with phosphate to prevent polymerase extension during PCR. Amplicons are usually 100–500 bp, although any size can be used. Short amplicons allow more rapid cycling.

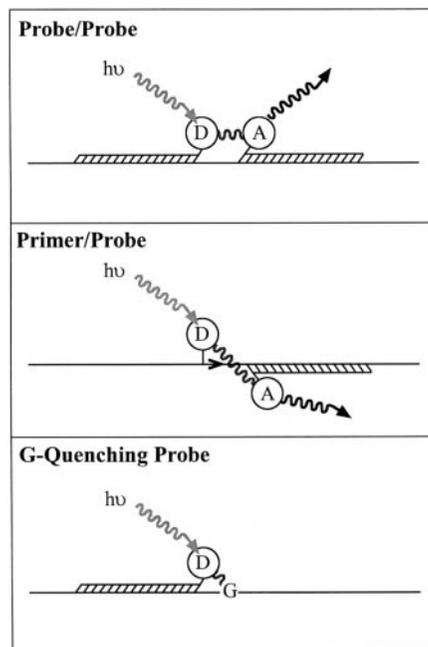


FIG. 1. Three different formats for simple hybridization probes. In the probe/probe format (top), two hybridization probes anneal next to each other on the target strand. The donor dye (D) is excited with light ($h\nu$). If the acceptor dye (A) nears the donor dye because of probe hybridization, resonance energy transfer occurs and the acceptor emits radiation at a longer wavelength. This dual hybridization event can be monitored as an increase in the fluorescence of A, a decrease in the fluorescence of D, or an increase in the ratio A/D. In the primer/probe format (middle), one primer is internally labeled with a dye and extended during PCR. The probe anneals to this extended strand during PCR, generating fluorescence from the resonance energy transfer pair. In the deoxyguanosine-quenching format (bottom), only one probe and one dye are necessary. Hybridization of the probe to target decreases fluorescence of the dye if the dye hybridizes near target G residues.

The probes can be designed to hybridize to either strand. In the primer/probe format, the probe must be near the labeled primer. In all formats, there may be an advantage to placing the probe(s) close to one primer, allowing the polymerase to nearly complete strand extension before hesitating at the probed region. When the probes are displaced, strand extension is quickly completed. Most protocols use standard *Taq* polymerase. Exonuclease-negative polymerases have also been used (20, 21). The concentration of hybridization probes usually used is 0.2 μM (range: 0.1–0.5 μM), and they are added to the other rapid-cycle PCR reagents (Table 1) before PCR.

For quantification, the probe(s) must hybridize each cycle. Therefore, probe T_m should be 5–10°C greater than primer T_m and sufficient time must be allowed for probe annealing. At a probe concentration of 0.2 μM , annealing times of 10–20 s are commonly used. If the amplicon is short, the annealing and extension segments may be combined in a two-temperature cycling protocol. If the amplicon is long, an additional extension segment at a higher temperature (three-temperature cycling) will complete extension more rapidly. In either case, fluorescence is acquired after the probes have hybridized at the end of annealing. A slow temperature ramp (1°C/s) through the probe melting transition may ensure completion of strand extension.

For allele typing, the detection probe is placed over the variable site. For single-nucleotide polymorphisms, the ends and penultimate bases on the probe should be avoided to maximize destabilization to the mismatched allele. Mismatches with shorter probes result in greater destabilization than those with longer probes, although the melting transition is broader. In the probe/probe format, the T_m of the second probe should be 5–10°C above that of the first probe, so that the “anchor” probe remains annealed during the melting transition of the detection probe from all possible alleles. For allele typing, the T_m of the probes can be greater or less than the T_m of the primers. However, fluorescence is observed during PCR only if the detection probe anneals during PCR, that is, if the detection probe T_m is greater than the primer T_m s. Melting curve data are usually acquired during a final modified temperature cycle. After denaturation, the probes are annealed at 5–10°C below the T_m of the least stable allele. The temperature is then increased at a rate of 0.05–0.2°C and fluorescence continuously acquired through all possible melting transitions until 5–10°C above the T_m of the most stable allele. Hybridization (cooling) curves have also been used for allele typing (22). Probes can detect large deletion/insertion polymorphisms or alternative splice sites by placing the detection probe partly into the polymorphic boundary. In addition to choice of strand, probes can

be made complementary to any allele or even mismatched to all alleles. In general, different alleles destabilize to different extents and can be distinguished. However, when the expected T_m of different alleles is calculated, some mismatches result in very nearly the same T_m (23). Although cases of incorrect typing from unexpected alleles have not been reported, the chance of miscalling a random polymorphism as a relevant mutation can be minimized by making the probe complementary to the lower-frequency allele (usually the mutation). In this case, unexpected polymorphisms will destabilize the probe at two positions: wild type will be mismatched at 1 position, and the mutation will be perfectly matched, spreading these possibilities out over the T_m dimension.

Probe T_m Estimation

Over the past few years, there has been significant progress in nearest-neighbor probe T_m estimation. A unified thermodynamic database has been compiled (24), and new parameters for all possible single mismatches (25–29) and dangling ends have been estimated (30). Many software programs and websites are available for *in silico* T_m estimation (e.g., <http://jsl1.chem.wayne.edu>). The effect of certain PCR conditions is still empirical (PCR product concentration, dNTPs, stabilization effect of Mg^{2+} , etc.). Modifications specific to LightCycler conditions have been studied (17, 31), and programs incorporating these changes are available over the web: <http://www.meltcalc.de> and <http://www.idahotech.com>. Predicted T_m s are usually within $\pm 2^\circ\text{C}$ of measured T_m s on the LightCycler and are very useful for designing assays that incorporate melting curves. This becomes even more important when designing multiplex assays that are differentiated by T_m . Each different allele (or target) must melt at a distinguishable melting temperature.

Simple Hybridization Probe Synthesis

One advantage of simple hybridization probes is that they are relatively easy to synthesize and quality control. Many commercial sources of single-labeled fluorescent probes are available. Fluorescein-labeled probes are particularly common and are often used as the donor in resonance energy transfer and deoxyguanosine-quenching formats. It is easiest to incorporate fluorescein at the 3' end by starting with the fluorescein already attached to the synthesis support (e.g., fluorescein-labeled CPG cassette, BioGenix or Glen Research). Fluorescein can also be added to the 5' end during automated synthesis with a fluorescein phosphoramidite (Clontech or Glen Research). Postsynthetic addition at any position is possible if an amino

group has been attached (e.g., with amino modifier C6-dT, Glen Research).

Long-wavelength dyes are available as phosphoramidites (Cy5, LCRed705) or as activated derivatives for postsynthetic addition (LCRed640). Cy5 became available as an amidite in 1995 (Amersham) and was used as the long-wavelength dye in initial development. The current LightCycler, manufactured by Roche Applied Systems, has optics optimized for LCRed640 and LCRed705, dyes specifically manufactured for the LightCycler system. These long-wavelength dyes are usually placed on the 5' end of probes (probe/probe format) or within the sequence of a primer (primer/probe format).

Synthesis details can be found elsewhere (32) and in manufacturers' protocols. Briefly, the oligonucleotide is synthesized trityl-ON when possible to aid in purification. The crude product is HPLC purified on a C₁₈ reversed-phase column with a triethanolamine acetate/acetonitrile gradient, collecting the later peak(s) with the hydrophobic dyes attached. The trityl group is removed if present, any necessary post synthetic steps are performed, and the product is again HPLC purified if necessary. The HPLC eluate is monitored with both an absorbance (A_{260}) and a fluorescence monitor.

Assessing Simple Hybridization Probe Purity

The purity of a labeled oligonucleotide can be difficult to assess. Absorbance measurements are easy to obtain and are a good estimate of purity, particularly with simple hybridization probes. Concentrations of the dye and the oligonucleotide components are calculated from extinction coefficients. The most common reason for failure of simple hybridization probe assays is poor probe purity. The following procedure is strongly recommended.

First, the predicted extinction coefficient of the oligonucleotide at 260 nm ($\epsilon_{260(\text{oligo})}$) is calculated from nearest-neighbor absorbance values. These values have been tabulated (33) and are also used by many software programs (e.g., Oligo 5.0, National BioSciences) to calculate the related value, nmol/ $A_{260(\text{oligo})}$. A T is used for an amino modifier C6-dT or C2-dT (Glen Research). Other amino modifiers and fluorophores should not be considered in the nearest-neighbor calculations. The $\epsilon_{260(\text{oligo})}$ is equal to $10^6/(\text{nmol}/A_{260(\text{oligo})})$.

Next, the concentrations of the oligonucleotide (c_{oligo}) and the fluorophore (c_{fluor}) are calculated. For extinction coefficients of the fluorophores, the measured values (if available) in Table 3 are used:

$$c_{\text{fluor}} = A_{\text{MAX}(\text{fluor})} / \epsilon_{\text{MAX}(\text{fluor})} \quad [1]$$

$$c_{\text{oligo}} = [A_{260} - (A_{\text{MAX}(\text{fluor})} * \epsilon_{260(\text{fluor})} / \epsilon_{\text{MAX}(\text{fluor})})] / \epsilon_{260(\text{oligo})} \quad [2]$$

The concentrations of fluorophore and oligonucleotide should be near equal; that is, the ratio of fluorophore to oligonucleotide should be near one. Acceptable ratios are between 0.8 and 1.2. Ratios less than 0.8 suggest incomplete labeling or destruction of the attached dye. Ratios greater than 1.2 suggest the presence of free dye. A ratio near one is a necessary but not sufficient condition for a pure probe. Coelution of A_{260} and fluorescence on reversed phase HPLC is additional evidence of purity.

Multiplexing Real-Time PCR by Color (Fluorescence Emission Spectra)

Multiplexing by color is widely used in flow cytometry and fluorescence sequencing. Color multiplexing is partly limited by the broad emission spectrum characteristics of common dyes. The emission wavelengths of LCRed640, Cy5, and LCRed705 are nicely separated

TABLE 3

Simple Hybridization Probe Fluorescence Constants

| Dye | Excitation max ^a (nm) | $\epsilon_{\text{MAX}(\text{fluor})}$ (M ⁻¹ cm ⁻¹) | | $\epsilon_{260(\text{fluor})} / \epsilon_{\text{MAX}(\text{fluor})}$ ^d | Emission max ^a (nm) |
|-------------|----------------------------------------|------------------------------------------------------------------------------|--------------------|-----------------------------------------------------------------------------------|--------------------------------------|
| | | Lit. ^b | Meas. ^c | | |
| Fluorescein | 494 | 68,000 | 68,000 | 0.18 | 524 |
| LCRed640 | 622 | 110,000 | 135,000 | 0.28 | 638 |
| Cy5 | 649 | 250,000 | | 0.09 | 670 |
| Cy5.5 | 678 | 250,000 | | 0.20 | 698 |
| LCRed705 | 685 | 170,000 | 257,000 | 0.42 | 712 |

^a Determined by fluorescence measurements in 50 mM Tris, pH 8.3, 3 mM MgCl₂, and 250 μg/ml BSA.

^b Literature values for the maximal extinction coefficients of the free fluorophores, taken from product literature (fluorescein, Cy5, Cy5.5) or personal communications (LCRed640, LCRed705, Roche Molecular Biochemicals).

^c Measured values for the maximal extinction coefficients of the fluorophores attached to oligonucleotides. Using the literature coefficients, the following [dye]/[oligo] ratios (±SD) were calculated from highly purified, labeled probes: fluorescein, 1.00 ± 0.09, $n = 16$; LCRed640, 1.23 ± 0.08, $n = 13$; LCRed705, 1.51 ± 0.3, $n = 17$ (data courtesy of Eric Parkinson, ITBiochem, Salt Lake City, UT). These measurements were used to determine new extinction coefficients for the bound fluorophores.

^d This ratio was experimentally determined as $A_{260(\text{fluor})} / A_{\text{MAX}(\text{fluor})}$. Absorbance was measured in 10 mM Tris, pH 8.3, and 0.1 mM EDTA, except for LCRed640, which was measured in 10% (v/v) isopropanol, 90% 10 mM Tris, pH 8.3/0.1 mM EDTA. Species measured were fluorescein and the NHS esters of LCRed640, Cy5, Cy5.5, and LCRed705.

(Fig. 2). In addition, efficient resonance energy transfer occurs between fluorescein and LCRed640, Cy5, and LCRed705, necessary in the probe/probe and primer/probe formats. Although the spectral overlap is small, the molar absorption coefficient and absorption wavelengths of the acceptors are high. Spectral overlap, acceptor absorptivity, and acceptor wavelength all contribute to the overlap integral that defines resonance energy transfer (34). The spectra in Fig. 2 were taken in PCR buffer (Table 1) which empirically augments the blue end of the absorption spectra of the acceptors. Preliminary calculations for Ro (34) indicate a value of approximately 50 Å for the fluorescein/Cy5 pair.

Color Compensation

Color compensation for spectral overlap of fluorescent dyes was first described in the field of flow cytometry (35). Calibration data for each pure fluorescent dye and autofluorescence control are first obtained with fluorescence values acquired in each channel. Signal crossover constants are calculated from these values and used to convert observed fluorescence (o) to actual signal fluorescence (s) by matrix algebra.

Several modifications to the color compensation algorithms developed for flow cytometry are necessary for use on real-time PCR data, and the technique was first applied to apolipoprotein E genotyping (36). Following the notation given in (35), and eliminating the acquisition of multiple particles (relevant to flow cytometry but not to solution fluorescence), the crossover constants are

$$k(i, j) = \frac{o(i, j) - a(j)}{\sum_{n=1}^N [o(i, n) - a(n)]}, \quad [3]$$

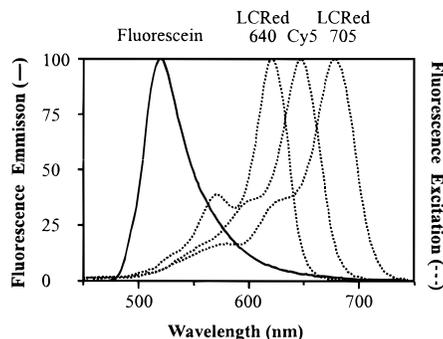


FIG. 2. The normalized emission spectrum of fluorescein is overlapped by the normalized excitation spectra of LCRed640, Cy5, and LCRed705. Spectra were obtained under rapid-cycle PCR conditions (Table 1). These conditions are advantageous because the low-wavelength shoulders of the excitation spectra are augmented and increase resonance energy transfer from fluorescein. LCRed640, Cy5, and LCRed705 are nicely separated spectrally for color multiplexing.

where $k(i, j)$ = crossover constant of dye i in channel j , $o(i, j)$ = observed signal of dye i in channel j , $a(j)$ = autofluorescence in channel j , n = channel index, and N = maximum channel.

The fluorescence of most dyes, and hence the derived crossover constants, is temperature dependent. Therefore, calibration runs are obtained by acquiring fluorescence continuously during a 0.2°C/s temperature ramp from 40 to 95°C. The temperature-versus-fluorescence curves are well approximated by third-degree polynomials. The best-fit third-degree polynomial coefficients for each dye/channel combination and the autofluorescence controls are stored for later interpolation.

To color compensate fluorescence data, the temperature of each acquisition is used to interpolate the temperature-specific fluorescence values from the calibration curves. These values are then adjusted for any change in electronic gain between the calibration and data run as follows:

$$o(i, j) = [w(i, j)T^3 + x(i, j)T^2 + y(i, j)T + z(i, j)][G_D(j)/G_C(j)], \quad [4]$$

$$a(j) = [m(j)T^3 + n(j)T^2 + p(j)T + q(j)][G_D(j)/G_C(j)], \quad [5]$$

where $w(i, j)$, $x(i, j)$, $y(i, j)$, and $z(i, j)$ are the third-degree polynomial coefficients for the temperature-versus-fluorescence curve of dye i in channel j ; $m(j)$, $n(j)$, $p(j)$, and $q(j)$ are the third-degree polynomial coefficients for the temperature-versus-autofluorescence curve in channel j ; T = acquisition temperature; $G_D(j)$ = gain of channel j during the data run; and $G_C(j)$ = gain of channel j during the calibration run. The actual signal fluorescence of each dye is calculated with the matrix equation

$$S = K^{-1}[O - A], \quad [6]$$

where S = actual signal fluorescence of each dye, K = crossover constants of each dye in each channel, O = observed fluorescence in each channel, A = observed autofluorescence in each channel. Equation [6] applies when the number of fluorophores is equal to the number of fluorescence detectors. When the number of detectors is greater than the number of fluorophores, such as with an array detector, the matrix solution is overdetermined and given by

$$S = (K^T K)^{-1} K^T [O - A]. \quad [7]$$

In this case, K^T is the transpose of K and is used to make the crossover constant matrix square so that its

inverse can be easily solved. Mathematically, a best fit to the overdetermined system of equations is found, effectively reducing noise. The required matrix functions are available as routines in many programming languages, including the language we used for development (LabView, National Instruments).

Temperature Correction

If temperature correction is desired as well as color compensation, the calibration data are color compensated by the above procedure, the temperature-versus-signal fluorescence curves are fit to third-degree polynomials, and the coefficients are stored for temperature interpolation. The data run is color compensated and

the fluorescence of each dye during each acquisition is temperature corrected as

$$s_{TC} = s[s_{C(Ts)}/s_{C(TD)}], \quad [8]$$

where s_{TC} = temperature-corrected signal fluorescence, s = signal fluorescence, $s_{C(Ts)}$ = interpolated signal fluorescence from the calibration run at a standard temperature (selected by the user), and $s_{C(TD)}$ = interpolated signal fluorescence from the calibration run at the data acquisition temperature.

Discrete Optics versus Spectral Dispersion

The current commercial LightCycler is a three-color system with discrete optics and detectors. Figure 3

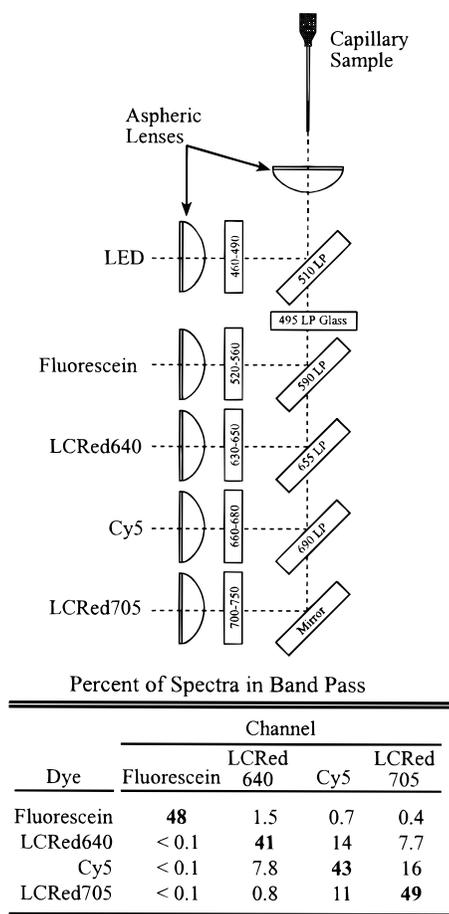


FIG. 3. The optical design of a four-color, rapid-cycle real-time PCR instrument. Bandpass filters are shown vertically, dichroics diagonally, and absorbance glass horizontally. Bandpass and long-pass (LP) cutoffs are shown in nanometers. The percentage of emitted light that passes through ideal filters is indicated for fluorescein, LCRed640, Cy5, and LCRed705. Four photodiode sensors are used for detection.

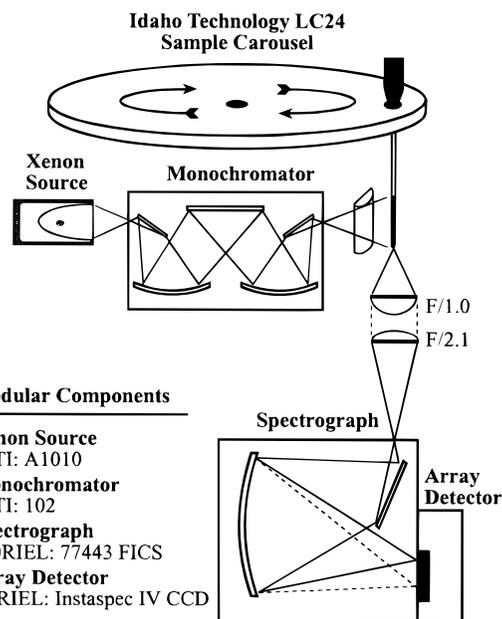


FIG. 4. Real-time PCR prototype with variable excitation, spectral dispersion of emission, and array detection. A thermal cycler capable of amplification in 10–20 min (10) was coupled to optics modules for excitation and emission. A 75-W xenon arc source with an elliptical reflector is attached to a conventional monochromator to provide the excitation light. The monochromator has slit widths for bandpasses up to 20 nm. A cylindrical filter is placed at the exit slit of the monochromator to focus the expanding beam along the vertical length of the capillary. The intensity of emitted light at the 1-mm capillary tip is increased by total internal reflection. Light from the capillary tip is collected through an aspheric lens ($f/1.0$). A longer lens ($f/2.1$) is used to match the numerical aperture to the emission spectrograph. The spectrograph has a concave holographic grating ruled at 1200 lines/mm with a blaze at 500 nm, giving 25–35% efficiency between 300 and 800 nm. The CCD is cooled to between 0 and -70°C and has a 1024×128 array of elements. The light from the 1-mm capillary tip is magnified by the lenses to cover the vertical detector area. The detector slit width can be limited to obtain the desired spectral resolution. The detector has a 16-bit A-to-D converter with vertical binning.

shows similar optics in a four-color instrument that detects fluorescein, LCR640, Cy5, and LCR640. The dye fluorescence that is directed to the appropriate channel (assuming ideal filter performance) is 40–50%. To go beyond four colors, the optical losses and expense of discrete optics suggest that simultaneous separation of wavelengths onto a linear array detector is a better approach. Such a system is shown in Fig. 4.

TABLE 4

Dependence of Color Compensation Precision on the Number of Detector Channels

| No. of bins | Precision (CV) |
|-------------|----------------|
| 4 | 0.13 |
| 10 | 0.11 |
| 47 | 0.10 |
| 282 | 0.08 |

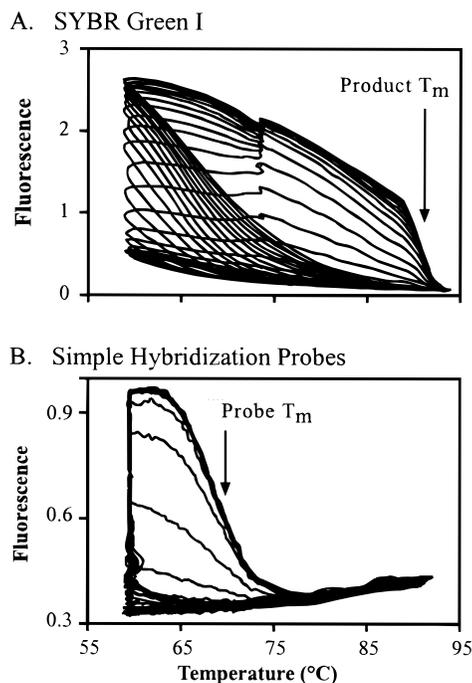


FIG. 5. Continuous monitoring of PCR. (A) SYBR Green I at a 1:20,000 dilution was used to monitor amplification of a 536-bp fragment of human β -globin from genomic DNA (1). Temperature cycling conditions were 94°C for 0 s, 60°C for 0 s, and 74°C for 15 s, and acquisitions were taken every 200 ms. (B) Adjacent hybridization probes at 0.2 μ M were used to monitor amplification of a 110-bp fragment of β -globin from genomic DNA (1). Temperature cycling conditions were 94°C for 0 s and 59°C for 20 s. The approximate melting temperatures of PCR product (A) and hybridization probes (B) are indicated.

With continuous spectral dispersion, all of the collected light is directed to the detector and there are no losses from multiple dichroic filters. The precision of color compensation also improves. Table 4 summarizes precision data collected on the instrument shown in Fig. 4. Color compensation (calibration and analysis) was performed after averaging the data in adjacent channels into different numbers of bins. Not surprisingly, as the number of bins increases, so does the precision of repeated measurements.

High-Order Color Multiplexing

Even with array analysis of emitted light, variable excitation may be needed when more than three or four colors are used. For example, on one commercial platform, only three colors could be distinguished in real time, but up to seven colors were resolved when the excitation wavelength was scanned with an off-line synchronous scanning fluorometer (37). The prototype instrument shown in Fig. 4 has variable excitation. That is, different excitation wavelengths can be selected by adjusting the monochromator. By interrogating at multiple wavelengths, different fluorophores can be optimally excited. This means that two or more resonance energy transfer donors can be excited in the same tube, each transferring to multiple acceptors (probe/probe and primer/probe formats). Alternately, G-quenching probes could be labeled with different fluorophores for multiplexing. Only fluorescein (8) and BODIPY-FL (9)

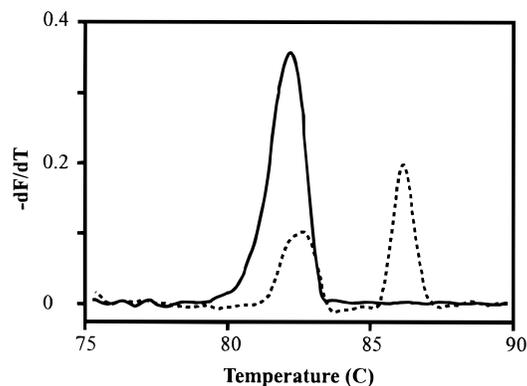


FIG. 6. Derivative melting curves of the Huntington's disease-associated CAG repeat region. The solid line is the derivative melting curve profile of an 80-bp homozygote, corresponding to 16 CAG repeats in a normal individual. The dotted line is the profile of an affected heterozygote with 18 and 49 CAG repeats. The samples were amplified for 40 cycles with denaturation at 97°C for 0 s and annealing/extension at 69°C for 25 s. A final cycle included denaturation followed by melting curve acquisition from 70 to 97°C at 0.05°C/s. Standard reagents were used (Table 1) with 1 mM MgCl₂, 7% DMSO, SYBR Green at 1:30,000, KlenTaq (AB Peptides) at 0.25 U/ μ l, and anti-Taq antibody (ClonTech) at 27.5 ng/ μ l. The primers were CGAGTCCCTCAAGTCTTCCAGCA and GTGGCGGCTGTTGCTGCT.

have been reported as G-quenching dyes, although many others probably exist. For high-order color multiplexing, simple hybridization probes combined with multiple-wavelength excitation is an alternative to single-wavelength excitation of complex dyes with internal resonance energy transfer for wavelength shifting (38, 39).

Multiplexing Real-Time PCR by T_m (Melting Temperature)

Discrimination based on hybridization temperatures is a powerful tool to distinguish different sequences. Multiplexing real-time PCR by T_m is less familiar than multiplexing by color. If fluorescence is monitored continuously as the temperature is changed, melting curves can be obtained. These melting curves are "dynamic dot blots," where the extent of hybridization is monitored at multiple temperatures. In T_m multiplexing, the temperature of hybridization is used as a dimension independent of color for multiplexing. Different duplexes melt at different temperatures. When fluorescence is acquired as the temperature is raised, the change in fluorescence identifies each target by its T_m , making multiplexing possible.

Monitoring Hybridization during PCR

The first application of real-time PCR was template quantification by monitoring fluorescence only once each cycle (40). However, if fluorescence is monitored

continuously within each cycle during temperature transitions (1, 41), the hybridization characteristics of PCR products and probes can be determined. With SYBR Green I, the T_m of the amplicon identifies the product amplified (42). With simple hybridization probes, melting occurs at a characteristic temperature that can be used for product identification and fine sequence analysis (43).

Monitoring hybridization during PCR is best demonstrated with continuous fluorescence-versus-temperature plots (Fig. 5). With SYBR Green I, a sharp drop in fluorescence near the denaturation temperature identifies the product T_m . With simple hybridization probes, the fall in fluorescence with heating follows the melting of the probe and indicates the T_m of the probe/target duplex.

T_m Multiplexing with SYBR Green I

Even though SYBR Green I is not sequence specific, different products can be identified by melting curves (42). For example, primer dimers can be distinguished from the intended products because they melt at a lower temperature. If fluorescence is acquired at a higher temperature where nonspecific products have melted, only the intended product will contribute to fluorescence (44).

One example of using T_m to distinguish different products is in trinucleotide repeat disorders, such as

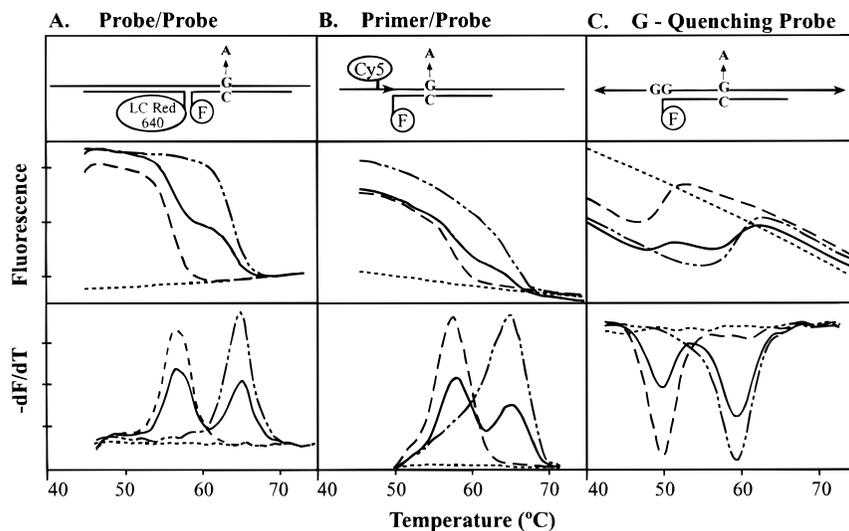


FIG. 7. Homogeneous, real-time factor V Leiden (G1691A) genotyping using three different formats of simple hybridization probes. (A) The probe/probe format of a commercial kit was used (LightCycler - Factor V Leiden Mutation Detection Kit, Roche Applied Systems). (B) The primer/probe format as originally published (32) was used. (C) Genotyping was performed with a single-labeled fluorescein probe and deoxyguanosine quenching as described (8). Melting curve data are presented as fluorescence versus temperature and first-derivative plots. Curves for homozygous wild type (---), homozygous mutant (···), heterozygous (—) and no template control (-·-) are shown. The factor V Leiden mutation results in a C:A mismatch.

Huntington's disease. In this autosomal dominant disorder, an expanded number of repeats results in a long allele and the disease phenotype. Figure 6 shows the derivative melting curve results of a normal subject (with one melting peak) and an affected individual (with two melting peaks). The long allele in the affected individual is identified by its high T_m and is clearly distinguishable from normal, low- T_m alleles.

How small a difference in T_m can be detected? Early work clearly separated mixtures of PCR products differing by 2°C (42). Subsequent work suggests that even smaller differences can be detected, including T_m shifts from heteroduplexes caused by single-base substitutions (45). Homoduplex melting temperatures are largely determined by the fractional GC content (%GC) and the length (L). One commonly used empirical equation (46) includes terms of $0.41(\%GC)$ and $-500/L$. In other words, each 2.4% change in GC content is expected to change the T_m by 1°C if the length remains the same. If the GC content is constant, the difference in T_m between two products of different lengths L_1 and L_2 is $500/L_2 - 500/L_1$. Given one product of length L , the number of base pairs (bp) that need to be subtracted from L to get a T_m change, ΔT_m , is

$$\text{bp} = \Delta T_m * L / (500/L + \Delta T_m). \quad [9]$$

Similarly, the number of base pairs that need to be added to L to get a T_m change, ΔT_m , has a solution when $500/L > T_m$:

$$\text{bp} = \Delta T_m * L / (500/L - \Delta T_m). \quad [10]$$

When these formulas are applied to the data in Fig. 6, the predicted T_m difference between the 16 and 18 repeat alleles is 0.48°C (measured 0.37°C), and between the 18 and 49 repeat alleles, 3.35°C (measured 3.63°C). The resolution that can be obtained with SYBR Green

I for polymorphism detection ultimately depends on the instrumentation, experimental conditions, and method used for analysis.

In addition to heterogeneity analysis within a single amplicon, SYBR Green I multiplexing by T_m can be used to distinguish different products amplified at the same time. For example, a control gene can be simultaneously amplified with a target that may or may not be present (translocations, infectious organisms). Even if the target is absent, amplification of the control gene ensures that amplification was not prevented by inhibitors (47).

T_m Multiplexing with Simple Hybridization Probes

The melting temperature of a probe is dependent on the stability of the probe/target duplex and can be used to identify different PCR products and/or alleles. One common application of simple hybridization probes is to distinguish different alleles under the same probe. Single-nucleotide polymorphisms (SNPs) are easily typed with a single detection probe. Even the most stable G:T mismatch is readily identified (48). With simple hybridization probes, alternate alleles are distinguished by unique T_m s with a single detection probe. In contrast, allele typing with other homogeneous probe designs (2, 3, 5, 6) requires a different probe for each allele uniquely labeled with a different color. Figure 7 demonstrates the use of simple hybridization probes for detection of factor V Leiden. All three hybridization probe formats are shown. Fluorescence decreases with melting in the probe/probe and primer/probe formats, but increases with melting of G-quenching probes. In all formats, all genotypes are readily distinguished. In the G-quenching format, only a single fluorescein-labeled probe is needed.

T_m multiplexing provides an ideal format for competitive PCR. A competitor with a single base change can easily be distinguished from the target by hybridization probe melting temperature. This method has been applied to identifying gene duplications and deletions (49) and quantification of Her-2-*neu* (50).

With biallelic SNPs, only two alleles need to be considered. However, many different alleles can be distinguished under the same probe. For example, a probe over the common three-base deletion delta F508 can identify not only the wild-type and deletion alleles, but also the single-base substitution, F508C (22). Compared with the wild-type sequence, the three-base deletion decreases probe T_m by 10°C , while the single-base mismatch lowers T_m by 6°C . Different single-base mismatches under the same probe can also be distinguished by melting curves. Rare polymorphisms near the factor V Leiden locus occur and interfere with restriction enzyme digestion. In addition to factor V

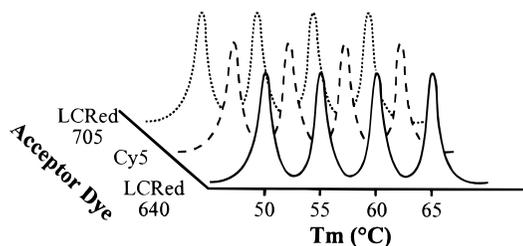


FIG. 8. Idealized multiplex design using both color and melting temperature. Use of three colors and four melting temperatures (T_m s) can differentiate 12 different PCR products. Different colored probes with different emission spectra are distinguished by software color compensation. All melting temperatures must be unique within the same color.

Leiden (G1691A), G1689A and A1692C have been identified (43). The melting peaks of these alleles are all within 1.6°C of each other. Nevertheless, each can be distinguished, including the same mismatch displaced by two bases (G1689A and G1691A). The standard deviations of the T_m estimates were about 0.1°C within a run (43).

T_m multiplexing with simple hybridization probes is not limited to a single amplicon. For example, two different segments of the hemochromatosis gene can be

simultaneously amplified and four alleles all distinguished by T_m (51). Target identification by the T_m of simple hybridization probes is a very powerful tool to identify amplified sequences.

Multiplexing by Color and T_m

Both color and T_m can be used simultaneously for multiplexing, creating a two-dimensional matrix (Fig. 8). Three colors and four T_m s can potentially distinguish

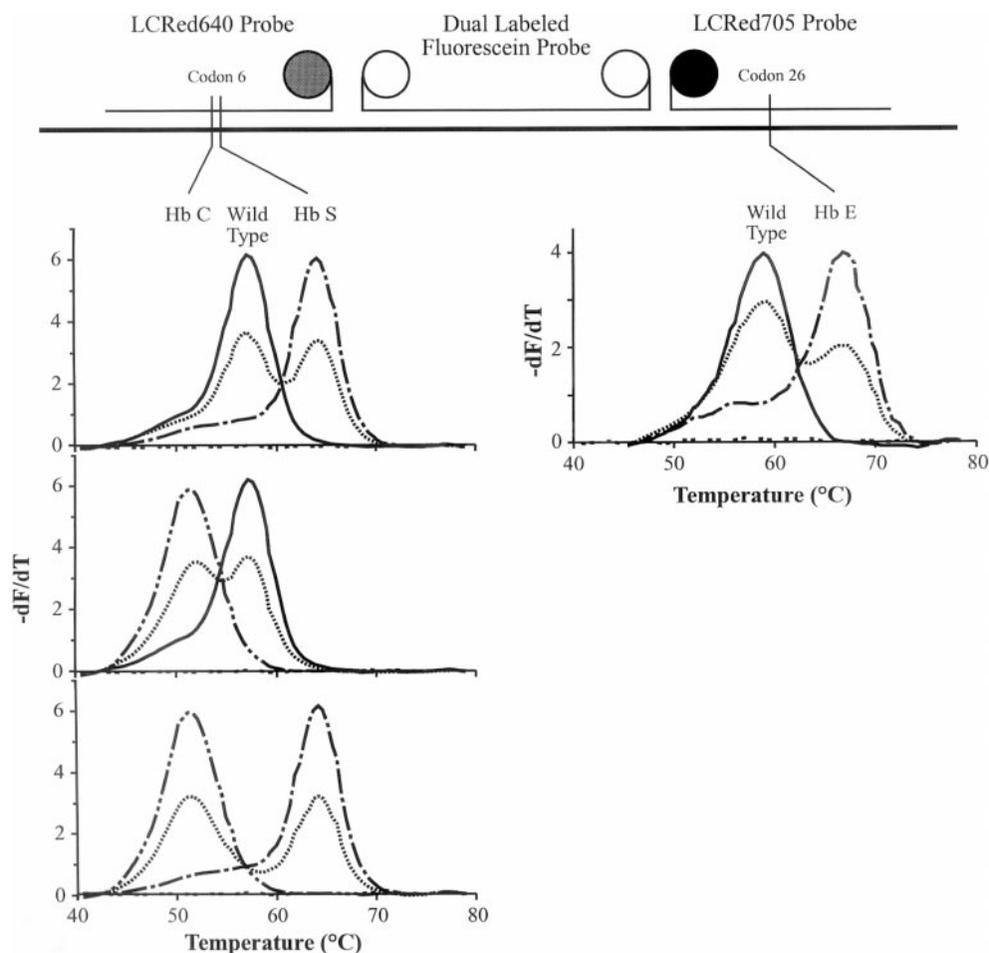


FIG. 9. Multiplex genotyping of Hb S, Hb C, and Hb E by color and T_m . A 214-bp segment of human β -globin, exon 1, was amplified with primers GTCAGGGCAGAGCCATCTA and GTTTCTATTGGTCTCCTTAAACCTG. Because of the proximity of the mutations, a unique combination of probes was designed to detect Hb S, C, and E alleles. Two detection probes were labeled with the acceptor fluorophores, LCRed640 and LCRed705. A third probe was dual-labeled with fluorescein as the donor and served as an anchor probe (F-GTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGA-F), spanning the distance between the detection probes. The codon 6 detection probe (CTCCTGTGGAGAAGTCTGC-LCRed640) completely matched the Hb S allele antisense strand. The codon 26 probe (LCRed705-GTTGGTGGTAAGGCCCTGG-phosphate) completely matched the Hb E allele antisense strand. Standard reagents were used (Table 1) with 3 mM MgCl₂, 0.5 μ M sense primer, 1.0 μ M antisense primer, 0.1 U/ μ l KlenTaq, 0.2 μ M detection probe, and 0.4 μ M anchor probe. Amplification was performed through 40 cycles of 94°C for 0 s, 63°C for 30 s, and a 1°C/s ramp from 63 to 75°C. After a final denaturation (94°C for 5 s) and annealing (1°C/s ramp from 94 to 35°C with a 20 s hold at 35°C), melting curves were acquired at 0.1°C/s. The data shown are color compensated. All possible genotypes were easily distinguished.

12 different products. In general, the degree of multiplexing possible is the product of the number of colors resolved and the number of T_m s distinguished.

To demonstrate combined multiplexing by color and T_m , exon 1 of the human β -globin gene was simultaneously analyzed for hemoglobins S, C, and E (20). The top of Fig. 9 shows the probe format. Hemoglobins S and C are near the beginning of the exon and hemoglobin E is near the end. A long anchor probe with a fluorescein label on both ends hybridizes to the center of the exon. Two additional probes, one labeled with LCRed640 and one with LCRed705, flank the anchor probe. The LCRed640 probe matches the Hb S allele and the LCRed705 probe matches the Hb E allele. After amplification, the temperature is increased and the fluorescence monitored as the probes on each end melt away from the anchor probe.

Derivative melting curves of all genotypes are shown in the bottom of Fig. 9. Color multiplexing followed by color compensation separated the Hb E locus from the Hb C/Hb S locus. At the Hb E locus, wild-type alleles had a T_m 9°C lower than that of the Hb E allele. At the Hb C/Hb S locus, wild-type alleles were destabilized 7°C, while Hb C alleles were destabilized 13°C compared with Hb S alleles. The assay distinguishes the 10 most common hemoglobin genotypes in exon 1 (wild type, Hb S, Hb C, Hb E, S trait, C trait, E trait, and the compound heterozygotes Hb S/E, Hb C/E, and Hb S/C). Complete single-step, homogeneous genotyping required less than 30 min from the start of amplification.

Another example of combined color/ T_m multiplexing is screening for *ras* mutations at codons 12, 13, and 61 (52). An LCRed640 probe was used to report mutations at codons 12 and 13 and an LCRed705 probe at codon 61. All 27 possible mutations at these sites could be distinguished, although in some cases a second analysis with a different probe was required.

SUMMARY

When both color and T_m discrimination are employed in the same reaction, high border multiplexing can be achieved. Simple hybridization probes can easily provide both color and T_m discrimination, a task that appears more difficult to achieve with other real-time probes. Solution multiplexing by color/ T_m virtual arrays will never reach the high densities of solid-phase microarrays. However, PCR itself often restricts solution multiplexing to low to moderate complexity. Color/ T_m multiplexing is a promising alternative in this domain (53).

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