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Continuous Fluorescence Monitoring of Rapid Cycle DNA Amplification

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ABSTRACT

Rapid cycle DNA amplification was continuously monitored by three different fluorescence techniques. Fluorescence was monitored by (i) *the double-strand-specific dye SYBR® Green I,* (ii) *a decrease in fluorescein quenching by rhodamine after exonuclease cleavage of a dual-labeled hydrolysis probe and* (iii) *resonance energy transfer of fluorescein to Cy5Ô by adjacent hybridization probes. Fluorescence data acquired once per cycle provides rapid absolute quantification of initial template copy number. The sensitivity of SYBR Green I detection is limited by nonspecific product formation. Use of a single exonuclease hydrolysis probe or two adjacent hybridization probes offers increasing levels of specificity. In contrast to fluorescence measurement once per cycle, continuous monitoring throughout each cycle monitors the temperature dependence of fluorescence. The cumulative, irreversible signal of hydrolysis probes can be distinguished easily from the temperature-dependent, reversible signal of hybridization probes. By using SYBR Green I, product denaturation, annealing and extension can be followed within each cycle. Substantial product-to-product annealing occurs during later amplification cycles, suggesting that product annealing is a major cause of the plateau effect. Continuous within-cycle monitoring allows rapid optimization of amplification conditions and should be particularly useful in developing new, standardized clinical assays.*

INTRODUCTION

Fluorescent probes can be used to detect and monitor in vitro DNA amplification (14). Useful probes include double-stranded DNA (dsDNA)-specific dyes and sequence-specific probes. With the intercalater ethidium bromide and ultraviolet illumination, red fluorescence increases after amplification in microcentrifuge tubes (3) or capillaries (22). Sequence-specific fluorescence detection is possible with oligonucleotide probes. For example, dual-labeled fluorescein/rhodamine probes may be cleaved during polymerase extension by 5′-exonuclease activity, separating the fluorophores and increasing the fluorescein/rhodamine fluorescence ratio (5,8,9). Alternately, "molecular beacons" have been described with a fluorogenic conformational change when hybridized to their targets (7).

Fluorescence can be measured after temperature cycling is complete, once per cycle as a monitor of product accumulation or continuously within each cycle. Sequence-specific methods (7–9) have been limited in the past to endpoint analysis. The potential of once-per-cycle monitoring for quantification of initial template copy number was first suggested and developed by Higuchi et al. using ethidium bromide (3,4). Fluorescence is acquired during the extension or combined annealing/extension phase of each cycle and is related to product concentration. A quantitative assay for hepatitis C RNA using the intercalater YO-PRO-1™ has been reported (6). To date, continuous monitoring of fluorescence within each cycle has found little use. Higuchi et al. continuously monitored amplification during 4-min temperature cycles using a 10-s integration time (3). An inverse correlation of ethidium fluorescence to temperature was noted, with product accumulation resulting in increased fluorescence during annealing/extension.

If fluorescence is continuously monitored within each temperature cycle, the hybridization of amplification products and probes can be followed during amplification. With dsDNA dyes, product denaturation and reannealing can be monitored. With probes that change fluorescence upon hybridization, probe melting temperatures can be determined. With rapid, homogeneous control of sample temperature, the kinetics of hybridization can be followed. We have previously used capillaries and forced-air heating for precise temperature control that allows 30 cycles in less than 15 min (18–22). By minimizing denaturation and annealing times, the specificity and yield of such "rapid cycle" amplifications are also improved (2,12,15,16,20–22). In addition to facilitating rapid heat transfer, glass capillaries are optically clear and make natural cuvettes for fluorescence analysis.

Three different fluorescence techniques for following rapid cycle DNA amplification are studied here using instrumentation described elsewhere (23). Instead of ethidium bromide, SYBR® Green I is used as a dsDNA-specific dye. A 5′-exonuclease probe is then compared to SYBR Green I monitoring. Finally, a novel fluorescence scheme based on adjacent hybridization probes with resonance energy transfer from fluorescein to the cyanine dye Cy5™ (11) is demonstrated. Once-per-cycle monitoring of multiple samples is a powerful quantitative tool. Continuous monitoring within the temperature cycles of DNA amplification can reveal the mechanism of probe fluorescence, rapidly optimize tempera-

ture/time conditions and potentially even control temperature cycling parameters. The melting and annealing of products and probes can be followed during amplification.

MATERIALS AND METHODS

DNA amplification was performed in 50 mM Tris-HCl, pH 8.5 (25°C), 3 mM $MgCl_2$, 500 µg/mL bovine serum albumin, 0.5 µM of each primer, 0.2 mM of each deoxyribonucleoside triphosphate and 0.2 U of *Taq* DNA polymerase per 5-µL sample, unless otherwise stated. Human genomic DNA (denatured for 1 min by boiling) or purified amplification product was used as DNA template. Purified amplification product was obtained by phenol /chloroform extraction and ethanol precipitation (17), followed by removal of primers by repeated washing through a Centricon® 30 microconcentrator (Amicon, Beverly, MA, USA). Template concentrations were determined by absorbance at 260 nm. A_{260}/A_{280} ratios of templates were greater than 1.7.

Primers were synthesized by standard phosphoramidite chemistry (Gene Assembler® Plus; Pharmacia Biotech, Piscataway, NJ, USA). SYBR Green I was obtained from Molecular Probes (Eugene, OR, USA). The β-actin primers and fluorescein/rhodamine dual probe were obtained from Perkin-Elmer (Norwalk, CT, USA). The human β-globin primers RS42/KM29 (536 bp) and PC03/PC04 (110 bp) have been described previously (19). The single-labeled probes 5′-CAAACAG-ACACCATGGTGCACCTGACTCC-TGAGGA-fluorescein-3′ and 5′-Cy5-AAGTCTGCCGTTACTGCCCTGTG-GGGCAAG-phosphate-3′ were synthesized using a fluorescein phosphoramidite (Glen Research, Sterling, VA, USA), a Cy5™ phosphoramidite (Pharmacia Biotech) and a chemical phosphorylation reagent (Glen Research). These adjacent probes hybridize internal to the PC03/PC04 β-globin primer pair on the same DNA strand and are separated by one base pair. Probes were purified by reverse-phase C-18 high-pressure liquid chromatography, and homogeneity was checked by polyacrylamide gel electrophoresis and absorbance (A_{260} and the absorbance maximum of the fluorophore). The β-actin hydrolysis probe and the β-globin hybridization probes were used at 0.2 µM each. Figure 1 schematically compares the differences between the three fluorescence monitoring techniques: ds-DNA-specific dyes, hydrolysis probes and hybridization probes.

Amplification samples of 5 µL were loaded into glass capillary tubes (1.02 mm o.d., 0.56 mm i.d.) and sealed. The tubes were cleaned with optical-grade methanol and then loaded into the carousel of a fluorescence temperature cycler described elsewhere (23). For continuous monitoring of a single sample, the carousel was positioned at maximal fluorescence and signals acquired every 200 ms. For multiple tubes, signals were obtained once each cycle by sequentially positioning the carousel at each tube for 100 ms.

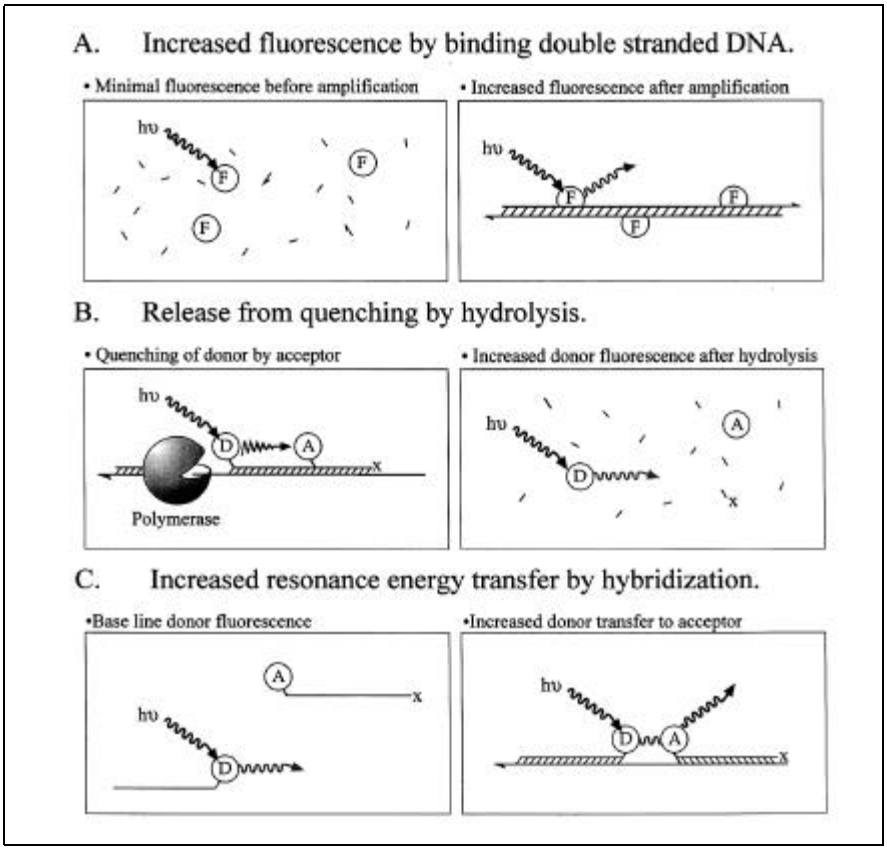

Figure 1. Schematic diagram comparing three different fluorescence-monitoring systems for DNA amplification. System A uses dsDNA-specific dyes (F) such as SYBR Green I, which increase in fluorescence when bound to accumulating amplification product. System B uses dual-labeled probes (8,9) and depends on the 5′-exonuclease activity of the polymerase to separate donor (D) and acceptor (A) by hydrolysis. Donor fluorescence is increased by removing acceptor quenching. System C depends on the independent hybridization of adjacent donor (D) and acceptor (A) probes. Their approximation increases resonance energy transfer from the donor to the acceptor. Other symbols are "hυ" for excitation light and "x" for a 3′-phosphate.

RESULTS

Figure 1 illustrates the three different fluorescence techniques used for continuous monitoring of DNA amplification. Figures 2, 3 and 4 demonstrate the application of these techniques for initial template quantification by fluorescence monitoring once each cycle. In Figure 2, the fluorescence of the ds-DNA-specific dye SYBR Green I is followed. A 10^7–10^8 range of initial template concentration can be discerned. When the data are normalized as the percent maximal fluorescence of each tube, 100 initial copies are clearly separated from 10 copies. However, the difference between 1 and 10 copies is marginal, and no difference is observed

between 0 and 1 average copies per tube. Nonspecific detection of undesired products after many cycles is a limitation of fluorescence monitoring of dsDNA.

Specific fluorescence monitoring can be obtained with sequence-specific fluorescent probes. In Figure 3, amplification is monitored using a dual-labeled hydrolysis probe. The fluorescence signal is expressed as a ratio of fluorescein to rhodamine fluorescence. Signal generation with 5′-exonuclease probes is dependent not only on DNA synthesis, but requires hybridization and hydrolysis between the fluorophores of the dual-labeled probe. Hydrolysis reduces quenching of fluorescein, and the fluorescence ratio of fluorescein-to-rhodamine emission increases. Whereas the fluorescence from dsDNA-specific dyes plateaus with excess cycling, the signal from hydrolysis probes continues to increase after many cycles. Even though no net product is being synthesized, probe hybridization and hydrolysis continue to occur. In contrast to dsDNA dyes, no fluorescence signal is generated in the absence of template.

In Figure 4, amplification is monitored using adjacent hybridization probes and is expressed as a ratio of Cy5 to fluorescein fluorescence. One of the probes is labeled 3′ with fluorescein, and the other probe is labeled 5′ with Cy5. When hybridized to accumulating product, the probes are separated by a 1-bp gap, and the Cy5-to-fluorescein fluorescence ratio increases. The change in fluorescence ratio during hybridization is largely due to an increase in Cy5 fluorescence from resonance energy transfer (data not shown). In contrast to hydrolysis probes, the fluorescence signal of hybridization probes tends to decrease with excessive cycling. No fluorescence signal is generated in the absence of template.

Sequence-specific probes have an even greater dynamic range for template quantification than do dsDNA dyes. As the template copy number decreases below 10^3, signal intensity decreases because specific amplification efficiency decreases, but low copy numbers can still be quantified because the negative control signal is stable (Figures 3 and 4). Although multiple samples would need to be run to confirm Poisson statistics, it appears that these specific techniques can discriminate a single initial template copy from negative controls (compare 0 and 1 average initial template copies in Figures 3 and 4).

With each technique, the fluorescence response is not strictly proportional to the amount of specific product. With SYBR Green I, two factors contribute to this nonlinearity. First,

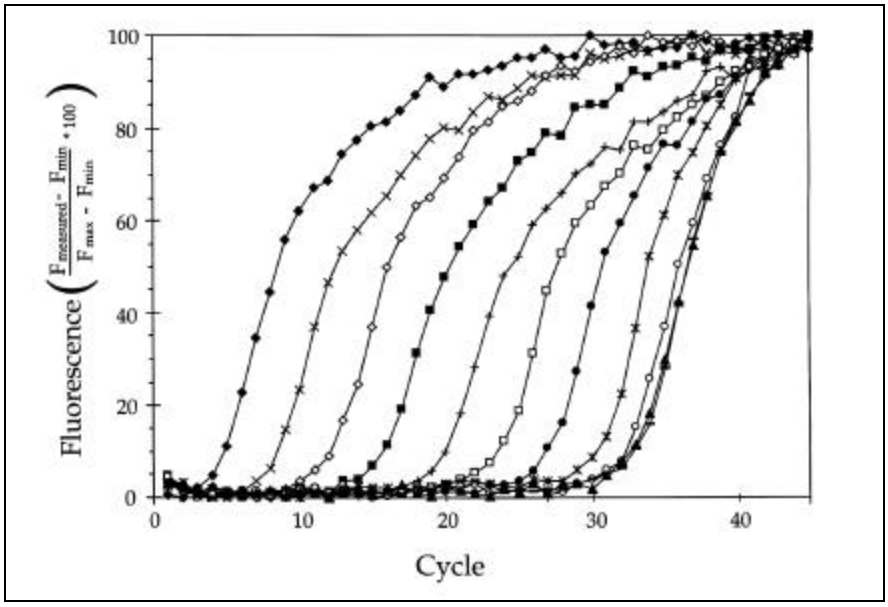

Figure 2. Fluorescence vs. cycle number plot of DNA amplification monitored with the dsDNA-specific dye SYBR Green I. A 536-bp fragment of the human β-globin gene was amplified from 10^9 (◆), 10^8 (×), 10^7 (◇), 10^6 (■), 10^5 (+), 10^4 (□), 10^3 (●), 10^2 (✳), 10 (O), 1 (▲) and 0 (—) average template copies in the presence of a 1:10 000 dilution of SYBR Green I. Each temperature cycle was 28 s long (95°C maximum, 61°C minimum, 15 s at 72°C, average rate between temperatures 5.2°C/s) and 45 cycles were completed in 21 min. All samples were amplified simultaneously and monitored for 100 ms between seconds 5 and 10 of the extension phase. The data are normalized as a percentage of the difference between minimum and maximum values for each tube (y-axis).

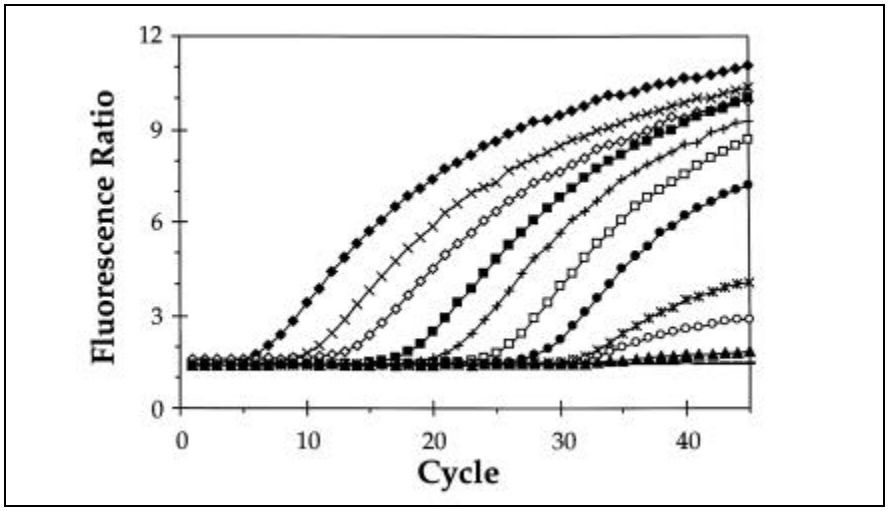

Figure 3. Fluorescence ratio (fluorescein/rhodamine) vs. cycle number plot of DNA amplification monitored with a dual-labeled 5′-exonuclease hydrolysis probe. A 295-bp fragment of the human β-actin gene was amplified from 10^9 (◆), 10^8 (×), 10^7 (◇), 10^6 (■), 10^5 (+), 10^4 (□), 10^3 (●), 10^2 (✳), 10 (O), 1 (▲) and 0 (—) average template copies in the presence of 0.2 μM of the hydrolysis probe. Each temperature cycle was 26 s long (94°C maximum, 60°C for 15 s, average rate between temperatures 6.2°C/s) and 45 cycles were completed in under 20 min. All samples were amplified simultaneously and monitored for 100 ms between seconds 5 and 10 of the annealing/extension phase.

nonspecific amplification of alternative templates results in fluorescence unrelated to specific product, particularly after many cycles (Figure 2). In addition, when the fluorescence of purified DNA standards is measured, the response is only linear to 10–20 ng of DNA per 5-µL reaction under the conditions used (data not shown). Higher concentrations of DNA show proportionally less fluorescence, presumably because the amount of SYBR Green I becomes limiting. Higher concentrations of SYBR Green I than those used here (<1:7000 dilution) inhibit amplification (data not shown). With hydrolysis probes, the fluorescence signal continues to increase after the plateau phase has been reached (Figure 3). With hybridization probes, the fluorescence decreases during the plateau phase (Figure 4). Despite the nonlinearity of these fluorescence techniques, they are very useful for absolute quantification of initial template copy number when fluorescence is measured for each amplification cycle (Figures 2–4).

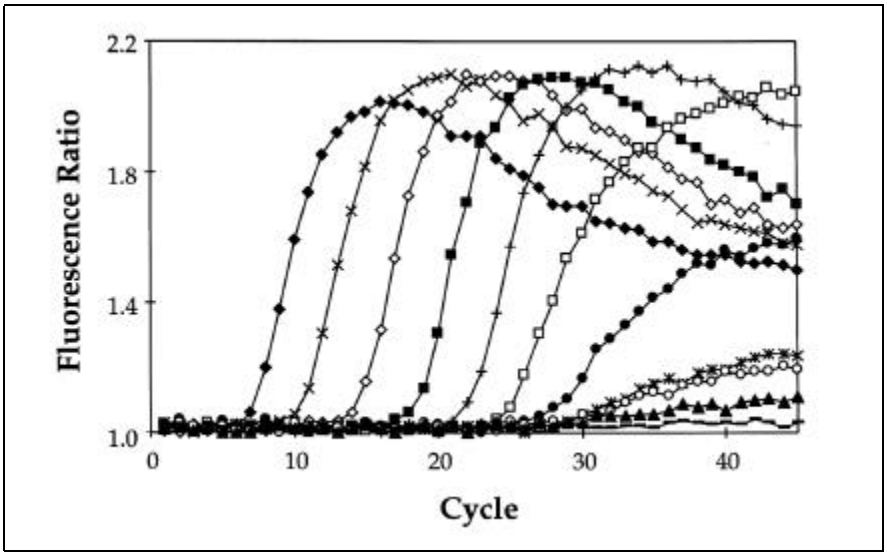

Figure 4. Fluorescence ratio (Cy5/fluorescein) vs. cycle number plot of DNA amplification monitored with 3′-fluorescein and 5′-Cy5 adjacent hybridization probes. A 110-bp fragment of the human β-globin gene was amplified from 10^9 (◆), 10^8 (×), 10^7 (◇), 10^6 (■), 10^5 (+), 10^4 (□), 10^3 (●), 10^2 (✳), 10 (○), 1 (▲) and 0 (—) average template copies in the presence of 0.2 µM of each hybridization probe. Each temperature cycle was 30 s long (94°C maximum, 59°C for 20 s, average rate between temperatures 7.0°C/s) and 45 cycles were completed in under 23 min. All samples were amplified simultaneously and monitored for 100 ms between seconds 12 and 17 of the annealing/extension phase.

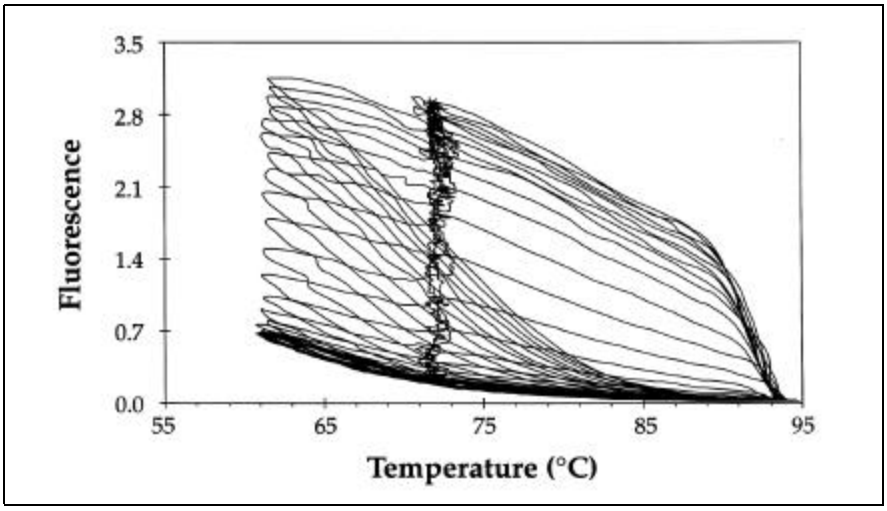

Figure 5. Continuous fluorescence vs. temperature plot of DNA amplification with the dsDNA-specific dye SYBR Green I. A 536-bp fragment of the human β-globin gene was amplified from 25 ng of genomic DNA and a 1:10 000 dilution of SYBR Green I. Temperature cycling conditions are given in Figure 2. Cycles 15–40 are shown. Product denaturation is evident between 90° and 93°C, and significant product-to-product annealing is observed during cooling.

Because fluorescence depends on temperature, fluorescence is usually acquired only once per cycle at a constant temperature to monitor product yield. This eliminates any confounding effect of temperature on fluorescence. However, interesting and useful information about hybridization can be obtained by monitoring fluorescence continuously throughout each temperature cycle. For example, fluorescence vs. temperature plots of amplification with SYBR Green I show the temperature dependence of strand status during DNA amplification (Figure 5). Early cycles appear identical, with a nonlinear increase in fluorescence at lower temperatures. As amplification proceeds, later cycles appear as rising loops between annealing and denaturation temperatures that show significant hysteresis. By hysteresis, we mean that the observed fluorescence during heating is greater than that during cooling. As the sample is heated, fluorescence is high until denaturation occurs (apparent as a sharp drop in fluorescence). As the sample cools from denaturation to annealing temperatures, fluorescence increases rapidly, apparently reflecting product-to-product annealing. Fluorescence also increases during extension while the temperature is held constant.

Fluorescence vs. temperature plots of 5′-exonuclease probes confirm that probe hydrolysis (not hybridization) is the mechanism of signal generation. In Figure 6, a fluorescence vs. temperature plot is shown for amplification with the β-actin exonuclease probe. During each cycle, the fluorescence ratio varies linearly with temperature and there is little, if any, hysteresis. Although the fluorescence of both fluorescein and rhodamine decreases with increasing temperature (data not shown), the rate of change is greater for rhodamine, resulting in an increasing ratio with increasing temperature. The fluorescence ratio increases during the annealing/extension phase at a constant temperature when probe hydrolysis is

presumed to occur. In contrast, with adjacent hybridization probes, the fluorescence signal is dependent only on hybridization, not hydrolysis. Fluorescence vs. temperature plots of amplification with hybridization probes show obvious hysteresis (Figure 7). During heating to product denaturation temperatures, the probes appear to dissociate between 65° and 75°C, returning the fluorescence ratio to background levels. These temperature-dependent hybridization effects are not apparent with the 5′-exonuclease probe (Figure 6).

DISCUSSION

Three different chemistries for fluorescent monitoring of DNA amplification have been studied: a dsDNA-specific dye, a dual-labeled exonuclease hydrolysis probe and adjacent hybridization probes. The hydrolysis and hybridization probes are sequence specific. Hydrolysis probes require one hybridization event for signal generation, whereas hybridization probes require two independent hybridizations. Figure 2 schematically compares and contrasts the three fluorescence monitoring systems.

dsDNA-specific dyes such as ethidium bromide (3,4,22) or SYBR Green I can be used as generic indicators of amplification. We used SYBR Green I instead of ethidium bromide because it has an excitation maximum near fluorescein and, in our hands, gave a stronger signal with DNA than excitation of ethidium bromide with visible light. These dyes depend on the specificity inherent in the amplification primers. Currently, nonspecific amplification after many cycles limits detection sensitivity to about 100 initial template copies (Figure 2, see also Reference 4). Improvements in amplification specificity could remove this limitation.

When low-copy-number detection and quantification are needed, additional specificity can be provided by sequence-specific fluorescent probes. Hydrolysis of a dual-labeled exonuclease probe is sequence specific (8,9). However, the design, synthesis and purification of dual-labeled hydrolysis probes require care. Hybridization is a necessary but not sufficient condition for hydrolysis; all probes are not cleaved efficiently. Synthesis of the dual-labeled probes involves manual addition of the rhodamine label, and at least one stage of high-pressure liquid chromatography is required for purification. In addition, the signal generated by exonuclease probes is cumulative and only indirectly related to product concentration. Hence, the fluorescence signal continues to increase even after the amount of product has reached a plateau (Figures 3 and 6).

Instead of depending on hydrolysis

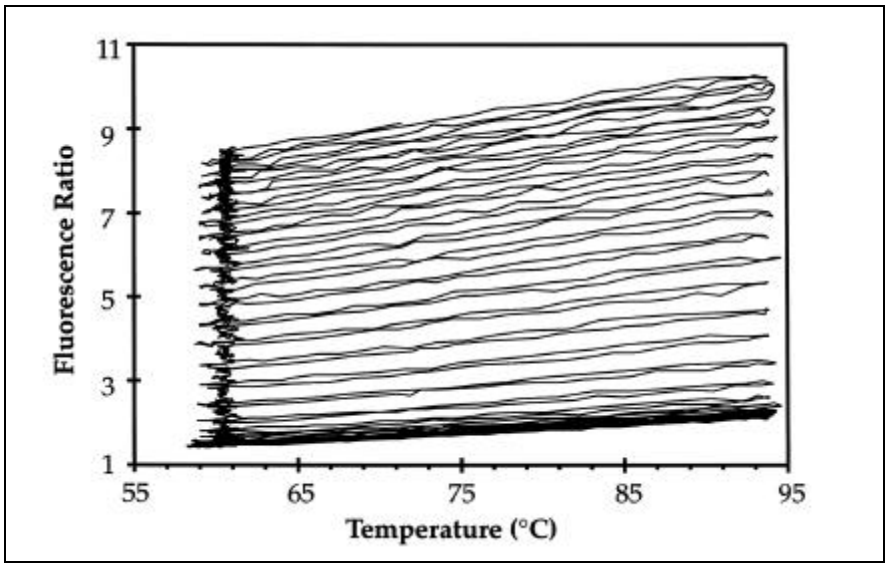

Figure 6. Continuous fluorescence (fluorescein/rhodamine) vs. temperature plot of DNA amplification monitored with a dual-labeled 5′-exonuclease hydrolysis probe. A 295-bp fragment of the human β-actin gene was amplified from 25 ng of genomic DNA. Temperature cycling conditions are given in Figure 3. Cycles 20–45 are shown. The dependence of fluorescence on temperature is linear each cycle, and no hybridization effect is apparent.

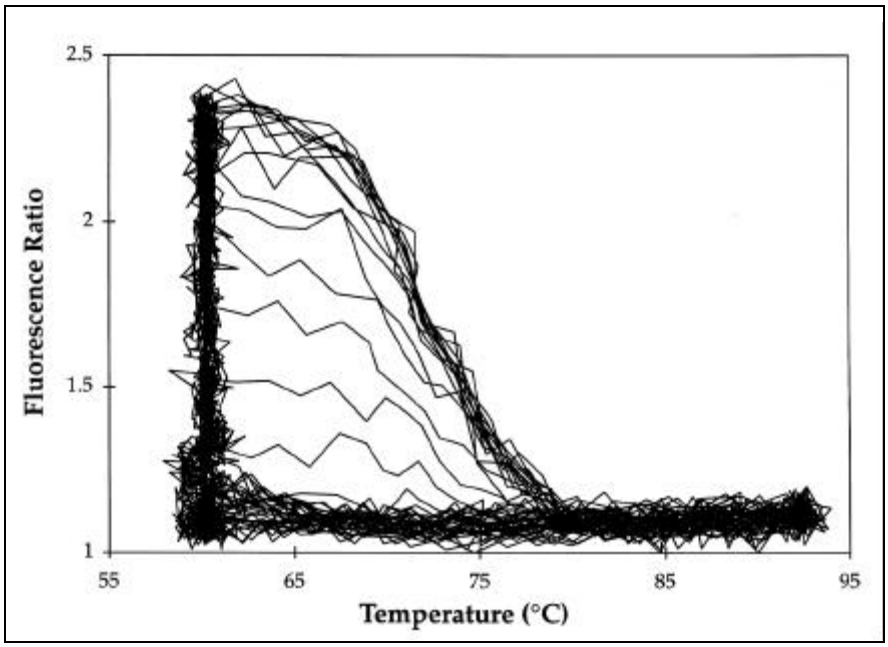

Figure 7. Continuous fluorescence (Cy5/fluorescein) vs. temperature plot of DNA amplification monitored with adjacent hybridization probes. A 110-bp fragment of the human β-globin gene was amplified from 25 ng of genomic DNA. Temperature cycling conditions are given in Figure 4. Cycles 20–40 are shown. The hybridization probes appear to melt off the target DNA sequence each cycle between 65° and 75°C.

of a dual-labeled probe, hybridization can be detected directly through resonance energy transfer as outlined by Morrison (10). By using two adjacent probes labeled separately with fluorescein and Cy5, energy transfer to Cy5 increases with product accumulation (Figures 4 and 7). In contrast to exonuclease probes, probe synthesis is relatively simple because amidites for both fluorescein and Cy5 are available for direct incorporation during automated synthesis. Signal generation requires two independent hybridization events to occur each cycle and is more directly related to product concentration than cumulative hydrolysis probes. However, after many cycles, the fluorescence from hybridization probes decreases (Figure 4), possibly because of probe consumption by exonuclease hydrolysis.

We are not aware of prior reports using fluorescein and Cy5 as a resonance energy transfer pair, although phycoerythrin and Cy7™ (Amersham International, Little Chalfont, Bucks, England, UK) (with similar spectral separation) have been used as a bright tandem dye in immunofluorescence (13). With fluorescein and Cy5, the spectral overlap is small, but the molar absorption coefficient and absorption wavelengths of Cy5 are high. All three factors (overlap, absorptivity and wavelength) contribute to the overlap integral that determines energy transfer rates (24). Cy5 also has low absorbance at fluorescein excitation wavelengths (11), reducing direct excitation of the acceptor.

Many aspects of hydrolysis and hybridization probes remain to be studied. The effects of probe length, melting temperature and concentration, distance between hybridization probes, distance to primers, temperature profiles, acquisition point within a cycle and type of polymerase have not been systematically optimized.

DNA amplification is extensively used but not rigorously understood. Continuous fluorescence monitoring provides an instantaneous window into the amplification process. For example, product denaturation occurs in less than 1 s (20,22), yet most protocols call for 10 s to 1 min of denaturation. By monitoring with dsDNA-specific dyes, product denaturation can be observed during each amplification cycle (Figure 5), a convincing demonstration that most denaturation protocols are excessive. To give another example, many causes of the "plateau effect" have been proposed, but little data are available to distinguish between alternatives. Figure 5 shows that product-to-product annealing is very rapid. In fact, during later cycles of amplification, a majority of product anneals to itself each cycle during cooling before the primer annealing temperature has been reached. This rapid reannealing is observed with cooling rates of 5°–10°C/s, characteristic of rapid cycling. Product reannealing with slower, conventional temperature cyclers would be greater. Product-to-product annealing appears to be a major, and perhaps the sole, cause of the "plateau effect".

As previously suggested (22), continuous fluorescence monitoring within each temperature cycle can be used to control temperature cycling parameters. With dsDNA-specific dyes, amplification can be stopped after a certain amount of product is synthesized, thus avoiding overamplification of alternative templates. The extension phase of each cycle needs to be continued only as long as fluorescence increases. Product denaturation can be assured each cycle by increasing the temperature until the fluorescence reaches baseline. This kind of fluorescence feedback should allow very rapid optimization of new assays. Limiting the time that product is exposed to denaturation temperatures may also be useful for the amplification of long products (1,2).

Additional uses of continuous monitoring with fluorescent dyes can be envisioned. For example, with fine temperature control and dsDNA-specific dyes, product purity could be estimated by melting curves. With rapid temperature control, absolute product concentration could be determined by product-to-product annealing kinetics. The only requirements are fluorescence monitoring, the ability to change temperatures rapidly and strict intra-sample temperature homogeneity. Aspects of instrument design are discussed elsewhere (23).

Conventional end-point analysis of DNA amplification by gel electro-

phoresis identifies product size and estimates purity. However, because amplification is at first stochastic, then exponential, and finally stagnant, the utility of end-point analysis is limited for quantification. Fluorescence monitoring every cycle during DNA amplification is an extraordinarily powerful technique for quantification. With simple instrumentation and fluorescent monitoring each cycle, sequence-specific detection and quantification can be achieved in 5–20 min after temperature cycling has begun. Although the final fluorescence signal is decreased when low copy numbers are amplified, quantification between 0 and 1000 initial template copies appears possible (Figures 3 and 4). These techniques should be particularly useful in assays where rapid quantification is desired, such as in the amplification of clinical serum viruses.

ACKNOWLEDGMENTS

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