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Journal of Chromatography B, 741 (2000) 3–13

JOURNAL OF CHROMATOGRAPHY B

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Review

Monitoring hybridization during polymerase chain reaction

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Abstract

The polymerase chain reaction (PCR) is usually analyzed by gel electrophoresis for size separation of PCR products. Additional separation techniques, such as single-stranded conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and denaturing high-performance liquid chromatography (DHPLC), can also be used to scan for sequence alterations. These techniques are all based on the effect of PCR product hybridization on mobility. Hybridization can also be monitored with fluorescence during PCR without chromatographic or electrophoretic separation. Continuous monitoring of PCR allows the detection, quantification and sequence specificity of PCR products to be assessed, often without any need for further analysis. In such a closed system, PCR quantification with sensitivity to the single copy level can be achieved using either double-stranded DNA binding dyes or fluorescently labeled allele-specific oligonucleotide (ASO) probes. Melting curve analysis with ASO probes can be used to genotype various alleles, including single base alterations. The integration of rapid cycle PCR and ASO probes in an automated system greatly facilitates research and clinical applications of nucleic acid analysis in genetics, oncology, and infectious disease.

Keywords: Reviews; Hybridization; Polymerase chain reaction; Nucleic acids

Contents

1. Introduction

The polymerase chain reaction (PCR) is a process that can amplify minute amounts of nucleic acid, thus generating ample material for further analysis.

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0378-4347/00/$ – see front matter
PII: S0378-4347(00)00055-4

Typically, PCR and nucleic acid analysis are separate, consecutive processes that can take several hours to perform and interpret. Often, electrophoretic or chromatographic separation techniques are used to distinguish the size or hybridization state of the PCR products. Agarose gel electrophoresis is simple to perform and, when an unknown is compared to DNA size standards, the product length can be estimated. However, there is no information on the sequence of the amplified product.

There are several techniques that can detect sequence alterations in PCR products by their sensitivity to hybridization conformation. For example, different sequences can often be identified by their single-stranded conformation during gel electrophoresis [1,2]. Single-stranded conformational polymorphism (SSCP) is a common screening method for sequence alterations. Similarly, minor alterations in the sequence of low-temperature melting domains or the presence of heteroduplexes effects migration and can be detected by several methods, including denaturing gradient gel electrophoresis (DGGE) [3,4], temperature gradient gel electrophoresis (TGGE) [5,6] and denaturing high-performance liquid chromatography [1,7].

Many methods are also available for probe-based hybridization analysis. Monitoring hybridization of a sequence-specific, labeled oligonucleotide to a complex mixture of immobilized nucleic acid is perhaps the simplest way to detect a sequence of interest [8,9]. This technique is commonly referred to as allele-specific oligonucleotide (ASO) hybridization. ASO hybridization is usually carried out post-amplification. The technique has several limitations, including the difficulty of establishing stringent washing conditions required for discrimination between specific versus non-specific binding of the oligonucleotide probe. In addition, the process can be expensive and very time consuming.

It is also possible to follow ASO hybridization in solution by fluorescence. A new generation of instrumentation has emerged that handles the two processes of amplification and hybridization simultaneously, providing a powerful tool for complex nucleic acid analysis. With such instrumentation, all the hybridization events occurring in a given reaction can be followed in real time. In contrast to hybridization on immobilized supports, there is no need to develop complex washing protocols for discrimination of products. One such instrument, the LightCycler®, a rapid air thermocycler with an integrated fluorometer [10], allows rapid nucleic acid amplification with continuous fluorescence monitoring of hybridization reactions. Amplification reactions occur in 15–30 min with the fluorescent analysis of the product occurring simultaneously.

There are several benefits to combining amplification and hybridization into a single system. It greatly reduces the sample handling requirements, minimizes errors and, if hybridization and amplification are performed simultaneously, reduces dramatically the time taken to perform the analysis.

Fluorescence monitoring of nucleic acid can be achieved by using either a double-strand DNA dye (e.g., ethidium bromide or SYBR® Green I) or fluorescent ASO hybridization probes. The dye or probes are added to the reaction mixture before amplification and the fluorescence followed in real time. Combining fluorescence monitoring with thermocycling allows fluorescence acquisition once every cycle so that the amount of product accumulation can be followed. In addition, continuous monitoring can be performed during each cycle as the temperature is changing to follow ASO probe hybridization. These methods provide rapid, simple and powerful quantification and allele discrimination.

2. Models of PCR

Conventional PCR thermocycling is based on the equilibrium paradigm of PCR (Fig. 1A) that describes the three steps of PCR, denaturation, annealing and extension, as three separate reactions that occur after reaching equilibrium at defined temperatures. This paradigm excludes the possibility of concurrently occurring reactions during the temperature transitions between steps. A single PCR cycle performed in a conventional heat block thermocycler can take between 3 and 6 min due to the long transition times between temperatures and the holds at each temperature.

Although the equilibrium paradigm is a simple description of the steps required for PCR, in reality the reactions occurring in the actual samples do not follow this pattern. In terms of chemistry, long holds

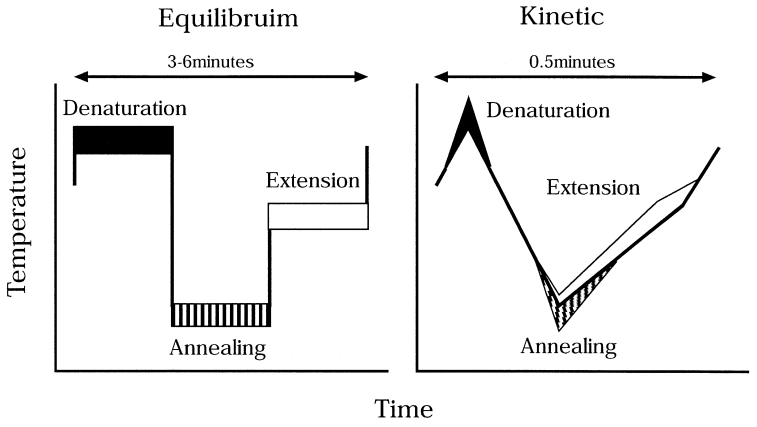

Fig. 1. Equilibrium and kinetic paradigms of PCR. Reproduced from Ref. [42] with permission, Academic Press, 1999.

at denaturation, annealing and extension are unnecessary. An alternate paradigm that can be used to describe PCR is the kinetic paradigm, which takes into account the fact that denaturation, annealing and extension occur over a variety of temperature and can overlap. In fact, much of the reactions occur during the temperature transitions. This paradigm was exploited in the design of a rapid air thermocycler, the RapidCycler® (Idaho Technology, Salt Lake City, UT, USA) [11,12], that allows a single cycle of PCR to be completed in as little as 20–60 s (Fig. 1B) and 30 cycles to be completed in 10–30 min.

The RapidCycler relies on fast, precise sample temperature control, and temperature homogeneity within and between samples. The rates of temperature transitions between PCR cycle segments are included as programmable parameters in rapid cycle instrumentation to better describe the reaction conditions. Amplification yield and product specificity are optimal when denaturation and annealing times are less than 1 s; and for short products, extension can often be completed during a slow transition from annealing to extension. These rapid cycling parameters do not compromise product formation, on the contrary, by limiting denaturation times template degradation is minimized, and decreasing the annealing and extension times increases the specificity of the reaction [13].

Most rapid cycle instruments use capillary tubes as sample containers and control the temperature with circulating air [11,12]. Capillary tubes have the advantage of providing a high surface area to volume ratio for rapid temperature control, since the sample is distributed along a single axis with heat transfer along the remaining two axes. A fan is used to circulate the air within the sample chamber which is heated with a halogen bulb and cooled with ambient air. The circular design of the chamber ensures that accurate temperature homogeneity is achieved in all samples.

3. Design of the LightCycler

By integrating the RapidCycler with the optics used in flow cytometry, continuous monitoring of fluorescence during PCR is possible. The design of the RapidCycler is ideal for such an adaptation since, in addition to facilitating heat transfer, the glass capillaries used in rapid cycling make natural cuvettes for fluorescence analysis. The total internal reflection of a fluorescence signal along the glass–air surface of the capillary tube concentrates the signal at the capillary tip by about an order of magnitude [10].

The design of a commercially available fluorescence air thermocycler, the LightCycler® (Roche Molecular Biochemicals, Mannheim, Germany), is shown in Fig. 2. The temperature within the sample

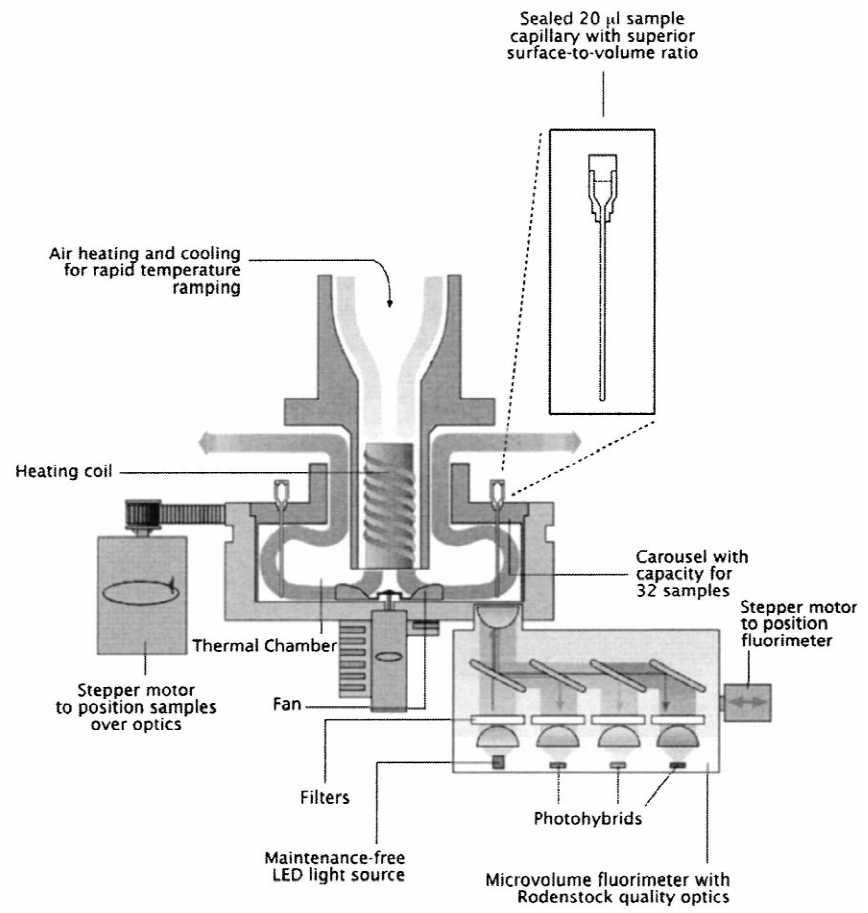

Fig. 2. Cross section of the LightCycler, a rapid air thermocycler with an integrated fluorimeter. The 32-sample chamber is placed above the optics for epi-illumination. Samples are contained in sealed capillary tubes and a stepper motor is used to position the samples above the optics for fluorescence acquisition. A central heating coil and a chamber fan are used to control the temperature in the sample chamber. Reprinted, with permission, from Roche Molecular Biochemicals, Mannheim, Germany.

chamber is controlled with a heating cartridge and a motor that drives a chamber fan. The optics required for epi-illumination of the capillary tips are placed beneath the circular sample chamber, and the samples are arranged in a carousel that is moved by a stepper motor to position each sample over the optics during fluorescence acquisition. A blue light emitting diode provides excitation and silicon photodiodes are used to measure fluorescent emissions. The instrument is interfaced to a personal computer for recording temperature, time and fluorescent signals from one to three reporter dyes.

4. Sample handling

Samples are contained in composite plastic-glass cuvettes that can hold reaction volumes of between 10 and 25 µl. The glass capillaries are 1.5 mm in diameter. A liquid sample is placed in the plastic collar of the cuvette and is spun briefly at low speed to bring the sample to the bottom of the tube. The cuvette is then capped and placed in the carousel. The samples remain closed during amplification and analysis, thus reducing the likelihood of contamination. A closed-tube system also saves time by

eliminating post-amplification sample handling and identification errors.

5. Real-time parameters: time, temperature, and fluorescence

Time, temperature and fluorescence are all parameters that can be used to describe real-time fluorescence monitoring of PCR. Data obtained by continuously acquiring the fluorescence of the double-strand DNA dye, SYBR Green I, during PCR amplification is plotted in three different ways in Fig. 3. Following changes in temperature with time describes the temperature cycling conditions (Fig. 3A). Data obtained by monitoring fluorescence with time is illustrated in Fig. 3B. As the reaction proceeds the fluorescent signal that monitors the amount of product increases. This increase varies during each cycle and is only seen when the DNA is double stranded. At denaturation temperatures, no double-stranded DNA is present and the fluorescence of the double-strand dye falls to zero. The most informative presentation of data from this type of experiment is obtained by displaying the fluorescence signal vs. temperature (Fig. 3C). As each cycle proceeds through annealing and extension, the fluorescence signal increases as new, complementary strands of DNA are formed. During the denaturation phase of each cycle, however, the signal is lost completely as the double-strand product is melted apart. This loss of fluorescence occurs as the two strands of complementary DNA melt apart at a melting temperature (T_m) that is related to the length and GC content of the sequence [14]. The ability to measure the T_m of a product is very useful for product analysis and identification.

6. Double-strand DNA dyes

The use of double-strand DNA dyes for continuous monitoring of PCR was first presented by Higuchi et al. using ethidium bromide [15,16]. These dyes are useful since they are compatible with PCR, inexpensive and can be used to detect any sequences. Commonly used double-strand DNA dyes include

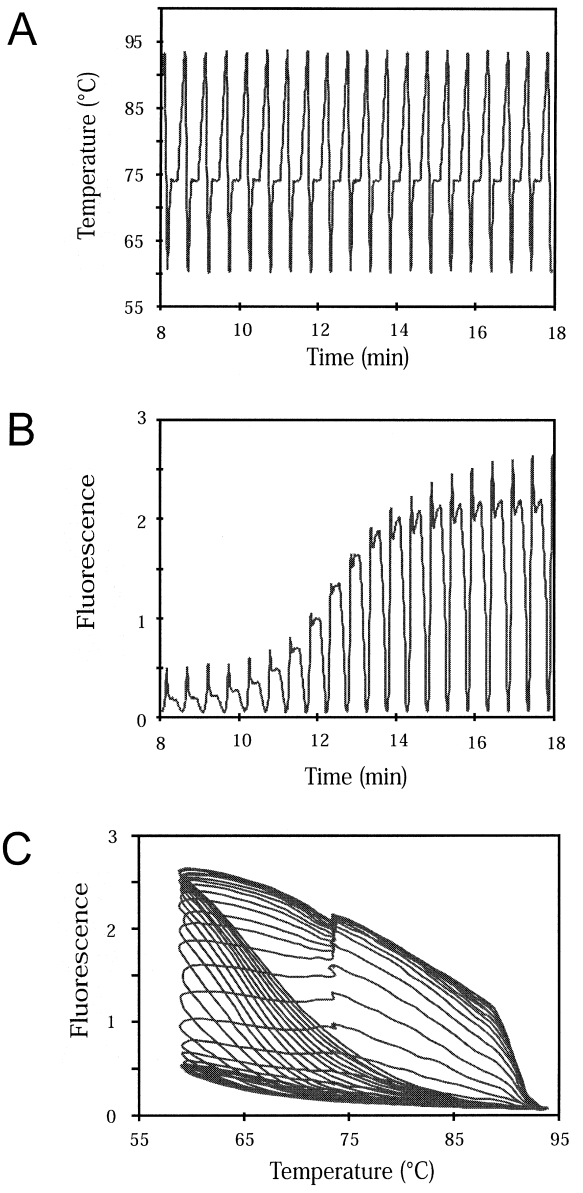

Fig. 3. Continuous monitoring of PCR with SYBR Green I. A 536-bp product of the human β-globin gene was amplified from human genomic DNA in the presence of a 1:20,000 dilution of SYBR Green I (Molecular Probes). Temperature cycling conditions were 94°C for 0 s, 60°C for 0 s, and 74°C for 15 s (A). The fluorescence signal increases over time as more product is formed (B). Continuous monitoring of fluorescence with temperature (C) displays the strand status as the temperature changes. Both high-temperature melting and low-temperature reassociation of product are observed for each cycle. Reproduced, in part, from Ref. [42] with permission.

ethidium bromide, PicoGreen® and SYBR® Green I. The latter is the dye of choice for use with the LightCycler because of greater sensitivity and compatibility of the dye spectra with the instrument optics. These dyes bind almost exclusively to double-strand DNA and can, therefore, be used for both quantification and product identification of any PCR reaction. The major drawback to the dyes is their lack of sequence specificity. Since any double-stranded sequence is recognized by the dye, a fluorescent signal can reflect the presence of desired as well as non-specific products (e.g., primer-dimers).

The acquisition of fluorescence at a given temperature once per cycle generates a sigmoidal amplification curve that can be used for PCR quantification. Using a serial dilution of starting template (Fig. 4) a horizontal displacement of the curves is observed with increasing dilution. This horizontal displacement is inversely related to the log of the initial template concentration [17–20]. The most sophisticated way to quantify the horizontal displacement is to calculate a fractional cycle number where the fluorescence level is increasing at the greatest rate. This is the second derivative maximum of the fluorescence vs. cycle number curve. This calculation can be performed automatically with no user input or assumptions about baseline regions, in contrast to methods that use a fluorescence threshold to identify a cycle number where the fluorescence is significantly above background.

The linear detection range in Fig. 4 is between 10^9 and 10^2 copies of template. The inability to discriminate between 10, 1 and 0 copies is a result of the non-specific nature of the dye. This limitation, however, can be decreased by acquiring the signal at a temperature above the melting temperature of any non-specific products generated during the reaction where the product of interest remains hybridized [18,21]. Non-specific products generated during rapid cycling almost always have T_m less than the desired product because short extension times usual-

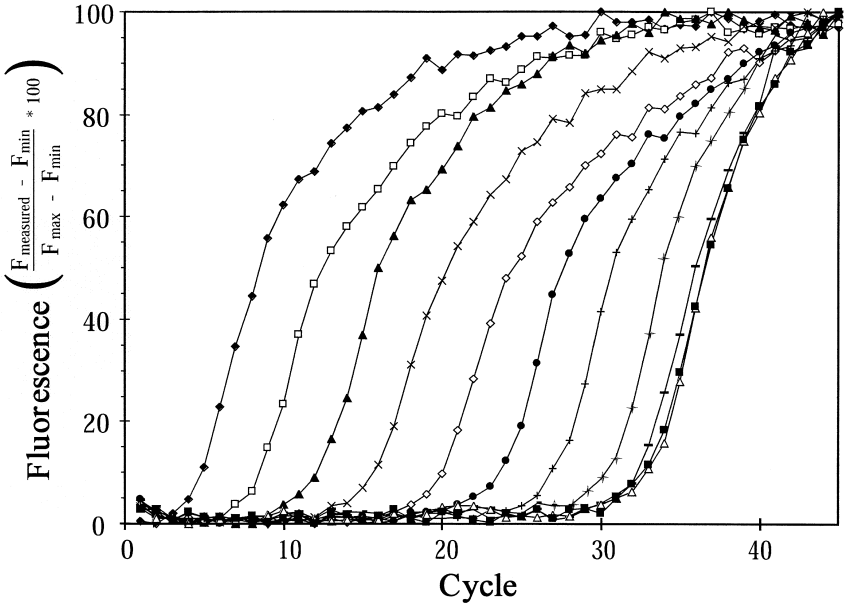

Fig. 4. Fluorescence vs. cycle number plot of DNA amplification monitored with the dsDNA-specific dye SYBR Green I. A 536-bp product was amplified from 10^9 (♦), 10^8 (◇), 10^7 (▲), 10^6 (×), 10^5 (◊), 10^4 (●), 10^3 (+), 10^2 (☆), 10 (–), 1 (△), and 0 (△) average template copies in the presence of a 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). Reproduced, with permission, from Ref. [17].

ly preclude the formation of products longer than the desired target.

Additional information about PCR products can be obtained by continuously following fluorescence in a reaction mixture that is slowly heated immediately post-amplification. As the temperature approaches the melting temperature of a double-strand DNA product there is a marked loss of fluorescence. The melting temperature of a given product is determined by its length, sequence and GC content and can be estimated using simple mathematical equations [14]. Because the T_m of a product is not defined solely by length, melting curve analysis offers a superior alternative to gel electrophoresis for separating products of the same size [22,23]. The temperature dependence of product strand status measured using SYBR Green I is illustrated in Fig. 5A. Two products that differ in length and sequence have very different melting profiles, with a mixture of the two products showing temperature transitions at the expected melting temperatures of both products. A simpler representation of melting curve data is obtained by displaying the first derivative of the data as 'melting peaks', where the midpoint of the peak is the melting temperature of the product (Fig. 5B). Similar methods have been used for DNA analysis with spectrophotometry [24].

Identification of products based on SYBR Green I melting curve analysis has universal application. The system has been used for the detection of the MBR/JH chromosomal translocation [25], the bcl-1 translocation in mantle cell lymphoma [26] and detection of free cancer cells in the peritoneal cavity of patients with gastric and ovarian cancer [27]. Additionally, the ability to identify products based on melting temperature is ideal for multiplex reactions where two products can be differentiated by T_m and the relative amounts of the products can be gauged by the area of the respective peaks. The melting curves can even be generated during the amplification itself, precluding the need for any post-amplification measurements.

PCR products can also be quantified by following the renaturation of products after denaturation. The formation of double-stranded product is monitored by SYBR Green I after rapidly decreasing the sample temperature from above the product T_m to below the product T_m. Product reannealing follows second-order kinetics [28]. Thus, for any given product and temperature a second-order rate constant can be measured. Once the rate constant is known, an unknown DNA concentration can be calculated using experimental reannealing data [19]. Although this method requires initial experiments with pure product for calculation of the rate constant it offers a method for direct, absolute quantification of DNA.

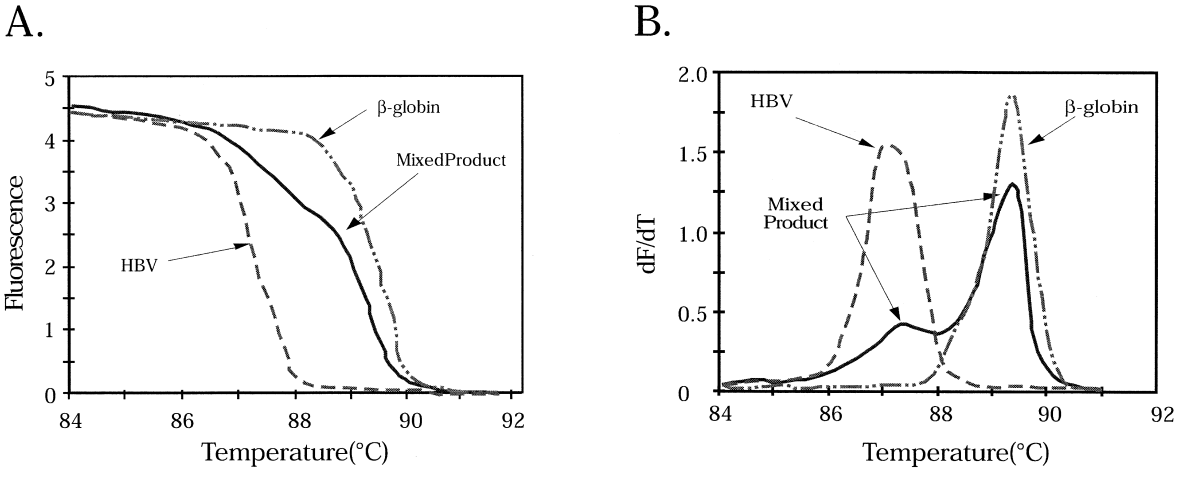

Fig. 5. Melting curves and melting peaks for mixtures of amplification products. Melting curves (A) were acquired on a purified 180-bp fragment from the hepatitis B surface antigen gene (- - -), a 536-bp product from the human β-globin gene (– · · –) and a mixture of the two (———). The melting curves were transformed into first derivative melting peaks (B). Reproduced, with permission, from Ref. [21].

7. Sequence-specific probes

All sequence-specific probes that are currently employed to follow PCR by fluorescence work on the principle of fluorescent resonance energy transfer (FRET). FRET involves a donor and acceptor fluorophore. Excitation of the donor results in energy transfer to the acceptor. In response, the acceptor can either emit light of a longer wavelength or dissipate the energy in the form of heat (i.e. can quench the emission from the donor).

There are several types of probe design that can be adopted for fluorescence monitoring of PCR. Fluorescent hybridization probes are perhaps the simplest illustration of the usefulness of FRET [19,29,30]. These probes are designed as a pair, with each probe labeled with either a donor or acceptor fluor. Ideally, the probes are designed to hybridize next to each other on the template of interest with the dyes lying adjacent. As they hybridize, excitation of the donor and resonance energy transfer results in an emission from the acceptor that can be measured with a fluorometer. As a probe melts off a template sequence, FRET is lost and the fluorescence signal drops sharply making this design ideal for melting curve analysis as well as quantification. The mechanism of action of hybridization probes is illustrated in Fig. 6. Hybridization probes can also be designed as a primer probe pair, where the primer is internally labeled with a dye (either donor or acceptor) and a single, labeled oligonucleotide probe is included in the reaction [29]. The primer generates a fluorescent template stand that can be over represented by the use of asymmetric PCR. The probe is designed to hybridize close to the primer fluorophore thus allowing FRET. This probe design scheme relies only on a single hybridization event.

Other options for probe designs include exonuclease probes [17,31], hairpin probes [32,33] or hairpin primers [34,35]. In the case of exonuclease probes (TaqMan®), a donor and a quencher are attached to a single probe. Hydrolysis of the probe by the 5′ exonuclease activity of Taq polymerase while the probe is hybridized to the target sequence releases donor from quencher and results in a measurable fluorescence signal. In this design, the efficiency of the exonuclease cleavage influences the strength of the signal. Cleavage efficiency varies and is increased as the distance between the fluors is increased. Because the signal from exonuclease probes is irreversible and, therefore, temperature-independent, these probes cannot be used for melting curve analysis. All probe designs can be used to monitor product formation once per cycle for quantification [19,36]. Fluorescence is acquired at the annealing or extension steps, generating a characteristic sigmoidal fluorescence curve. These probes generate a sequence-specific signal allowing accurate quantification of templates down to the single copy level [17].

Hybridization probes have an added advantage over the other probe designs because the signal generated is temperature-dependent and directly related to the formation of target-oligo hybrids. These probes are ideally suited for melting curve analysis. At lower temperatures, where hybridization is favored, the signal is high, however as the temperature is increased, probe melting causes the fluorescence

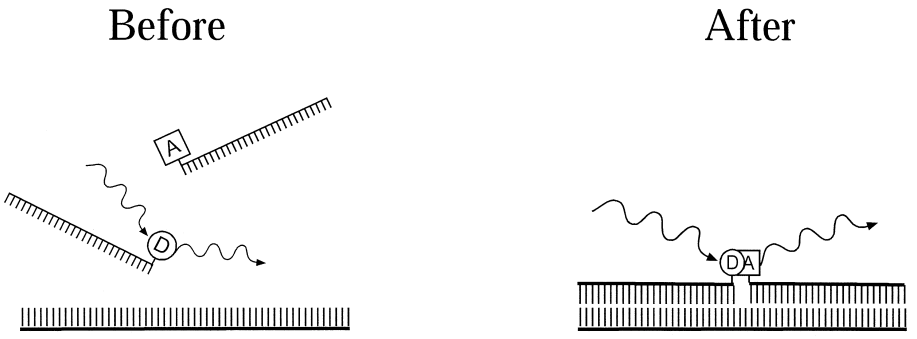

Fig. 6. Fluorescence resonance energy transfer between hybridization probes. A fluorescence signal from the acceptor probe is only produced when both probes are annealed to the target and donor and acceptor fluors are adjacent.

signal to return to baseline levels. This is in essence a 'dynamic dot blot' where ASO hybridization can be followed over an entire range of temperature.

Following oligo-DNA hybrid status can provide information about the target DNA sequence. Any mismatch between probe and template is reflected in a reduction in probe melting temperature due to destabilization of the duplex [29,30]. The shift in T_m is dependent on a variety of factors including the nature of the mismatch and its neighboring base pairs. This is an ideal system for genotyping disease-related point mutations. Factor V Leiden genotyping by melting curve analysis using hybridization probes [29] is illustrated in Fig. 7. By looking at the melting peaks it is easy to distinguish the wild-type sequence that shows a single peak at a high T_m from a homozygous mutant with a single peak at a lower T_m. Heterozygotes are represented by two peaks, one wild-type and one mutant, each at the expected temperature. This method has been applied to a variety of clinically relevant targets including methylenetetrahydofolate reductase (MTHFR) [30], hereditary hemochromatosis [37], prothrombin [38] and human platelet antigen HPA-1 [39]. In the case of MTHFR, the mutation is a G to A transition that is surrounded by GC pairs, creating a relatively stable mismatch between probe and target. Although this mismatch causes only a slight decrease in probe T_m, it is still easily detected using hybridization probes [30].

Since the type and position of any mismatch affects the melting temperature of a probe, it should be possible to discriminate between different mutations by the T_m shift observed. A study of 200 factor V heterozygous or homozygous mutant samples showed that this is indeed the case [40]. A single base alteration two base pairs away from the Leiden mutation may be mistyped as a factor V mutation since it eliminates the same restriction site as the Leiden mutation. With melting curve analysis, this polymorphism was reliably discriminated from both wild-type and the Leiden mutation, even though the T_m differed by less than 1°. Such discrimination was possible because the intra-assay standard deviations of the melting peaks were approximately 0.1°C [40].

The facile differentiation of products based on melting temperature is ideal for T_m multiplexing and can be exploited to increase the number of alleles that can be identified in a single reaction. This was recently demonstrated with the gene for hereditary hemochromatosis [37]. Two point mutations in codons 282 and 63 are commonly associated with disease chromosomes from patients of Northern European ancestry. Currently, screening for these mutations is performed by either ASO hybridization or PCR restriction fragment length analysis, both time-consuming techniques that require post-amplification processing. By multiplexing with two hybridization probe sets, both sites could be typed simultaneously in a closed-tube system in under 1 h [37]. The specificity of the method also allowed the identification of a polymorphism in codon 193.

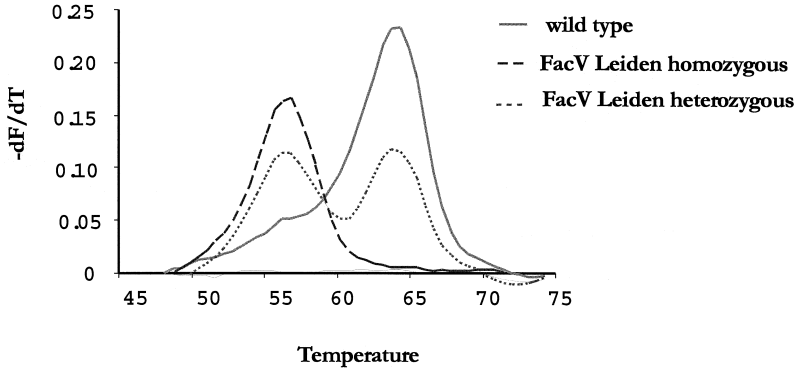

Fig. 7. Factor V Leiden genotyping with melting peaks. Each peak represents the melting temperature of a fluorescein-labeled probe off the template. The melting temperature of the probe off a perfectly matched wild-type template (———) is 64°C. The presence of the Leiden mutation (— — —) causes a 7°C shift in melting temperature to 57°C. Heterozygotes (- - -) show peaks representing both normal and mutant alleles. Reprinted, with permission, from *Biochemica*, Vol. 2 (1998), Roche Molecular Biochemicals, Mannheim, Germany.

By using acceptor fluorophores with different emissions it is possible to multiplex by both T_m and color, increasing the level of complexity available for analysis. Color multiplexing with hybridization probes was recently demonstrated with the apolipoprotein E locus using model oligonucleotide targets [41]. Codons 112 and 158 were analyzed with acceptor probes that emitted at either 640 or 705 nm; both probe sets used fluorescein as the donor fluorophore. Software modified from flow cytometry was used for color compensation of temperature-dependent spectral overlap between the fluorescent dyes [41]. The separation by color in this assay was particularly useful since the probes used to recognize the two codons melted in the same T_m range.

Fluorescence monitoring of PCR is a powerful tool for nucleic acid analysis, without any requirement for chromatographic or electrophoretic separation. The LightCycler provides a rapid, versatile system for amplification and analysis of nucleic acid in a closed system. Either double-strand DNA dyes or fluorescently labeled, sequence-specific oligonucleotide probes can be used for PCR quantification. Melting curve analysis with ASO probes provides additional information about clinically relevant targets. Multiplexing by color and T_m can significantly increase the amount of information that can be obtained from a single reaction. The integration of rapid cycle PCR and ASO probes in an automated system greatly facilitates research and clinical applications of nucleic acid analysis in genetics, oncology, and infectious disease.

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