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Homogeneous Detection of Specific DNA Sequences by Fluorescence Quenching and Energy Transfer

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The use of fluorescence quenching and energy transfer in DNA hybridization assays is reviewed. Placement of DNA probe labels within interacting distances by hybridization of DNA probes to target DNA or to one another allows rapid homogeneous analysis of specific DNA sequences. Due to the inherently lower sensitivity relative to heterogeneous assays, the fluorescence assays have been coupled with DNA amplification methods such as PCR to provide highly sensitive, clinically relevant homogeneous assays which can be performed in closed systems.

KEY WORDS: Fluorescence energy transfer; fluorescence quenching; DNA hybridization; homogeneous assays; polymerase chain reaction.

INTRODUCTION

This article reviews homogeneous DNA hybridization assays for the detection of specific DNA sequences which may be important in the detection and identification of infectious organisms or in the characterization of genetic abnormalities. DNA hybridization assays are a member of the broad category of biological binding assays, which use the affinity between antibodies and haptens, complementary nucleic acid strands, receptors and their ligands, enzymes and their substrates or cofactors, and other specifically interacting biological species to measure the presence of a wide variety of biologically important analytes. Homogeneous binding assays can form the basis for rapid and easily automated analyses. As defined here, homogeneous assays are comprised of only a solution phase, in contrast to heterogeneous assays, which also employ a solid support phase as a means to separate bound from unbound analytes and reagents. Homogeneous assays are then free of problems such as nonspecific adsorption of reagents and analytes to the solid phase, slower solution–surface binding kinetics, and the need for physical separation of the phases and subsequent washing steps associated with heterogeneous assays. This is not without consequence, however, as homogeneous assays are generally less sensitive than heterogeneous assays.

Homogeneous assays are conceptually more difficult to design, since a detectable quality must be altered as a result of the binding between two binding partners. Homogeneous immunoassays were developed in the 1970s, well after the heterogeneous radioimmunoassays. Fluorescence quenching and energy transfer, sensitive to the distance separating fluorescent labels from quenching or energy accepting labels, were used to detect binding of several labeled antibody reagents to a multiepitope antigen or binding between labeled antibody and labeled homologous reagents [1]. In the 1980s these concepts were extended to DNA hybridization assays. Two of the early DNA assay formats are shown in Figs. 1 and 2. In the "adjacent probe" format (Fig.1), hybridization of two labeled DNA reagents (DNA probes) to contiguous regions on a single-stranded target DNA strand bring labels attached to the probes into close proximity to one another [2–4]. In fact, a label placed on the 5′-terminus of the 3′-most hybridizing probe can be brought into contact distance to a label placed on the 5-terminus of

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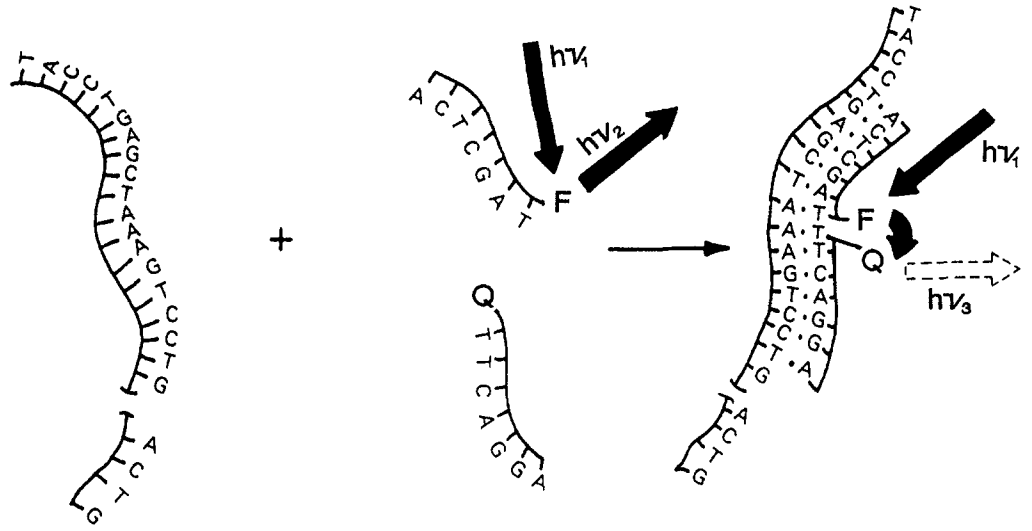

Fig. 1. "Adjacent probe" format for detecting the presence of specific DNA sequences via energy transfer and fluorescence quenching. "*F*" designates the primary fluorescent label excited by light of energy $h\nu_1$, received from an external excitation lamp and emitting light of energy $h\nu_2$. When positioned adjacent to the quencher or energy accepting label, "Q," F and Q interact to reduce the fluorescence intensity of F(quenching). If energy transfer occurs and Q is fluorescent, the transferred energy can be emitted from Q as fluorescence of energy $h\nu_3$.

the 5′-most hybridizing probe. The ability to place labels in highly interacting positions on DNA probes was an advantage DNA assay design had over immunoassay design, since DNA chemistries could be selectively applied to the terminal positions. The "complementary probe" format (Fig. 2) also takes advantage of selective 3′- and 5′-labeling of DNA [5–7]. In this format the single-stranded complementary probes compete for hybridization with each other and with single-stranded analyte DNA of the same sequence, providing for label interactions in the absence of target analyte, where probes are predominantly hybridized to each other, and preventing label interactions at high analyte concentrations, where probes are predominantly hybridized to the unlabeled target. A third format investigated (not shown) used a single labeled probe together with an intercalator or

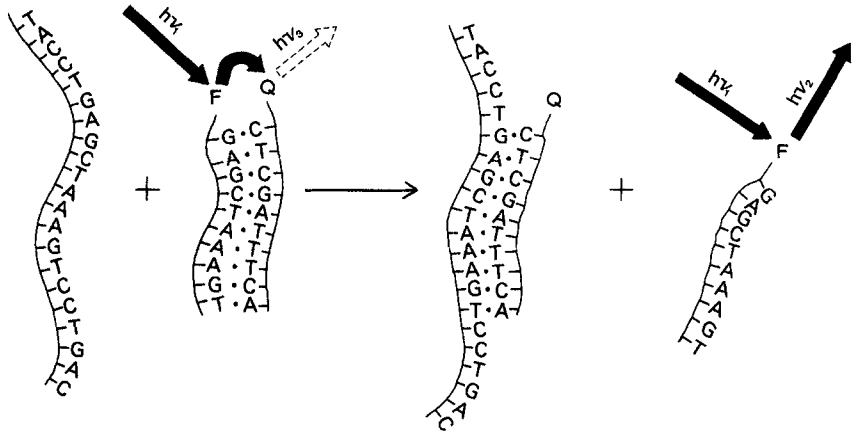

Fig. 2. "Complementary probe" format for detecting the presence of specific DNA sequences via energy transfer and fluorescence quenching. "F" designates the primary fluorescent label excited by light of energy $h\nu_1$ received from an external excitation lamp and emitting light of energy $h\nu_2$. When positioned adjacent to the quencher or energy accepting label, "Q," F and Q interact to reduce the fluorescence intensity of F (quenching). If energy transfer occurs and Q is fluorescent, the transferred energy can be emitted from Q as fluorescence of energy $h\nu_3$.

helix binding dye, such as ethidium bromide [3,6]. The dye preference for double stranded DNA must be very high, however, since dye binding to the free probe strand contributes to background fluorescence. Similarly the residual fluorescence of unbound dye must be low.

LABEL INTERACTIONS

Dual-Label Interactions. Fundamental to the energy transfer and fluorescence quenching assays is the distance-dependent interaction between a luminescent label and a second label which can affect the luminescent label's emission. When the two labels are attached to different probes that are freely dispersed in solution, the average separation distance between the labels is too large to permit detectable amounts of interaction. Binding of the two probes to each other or to a common molecule greatly decreases the average separation distance and interactions become detectable. The separation distances which allow interactions depend upon the mechanisms of deexcitation involved. Fluorescence is one path by which a molecule in an electronically excited state can lose energy and return to its ground state. Nonradiative paths also exist, including internal conversion through vibrational levels with release of heat, intersystem crossing from an excited singlet to a triplet state, and collision with other molecules (for general discussions see Refs. 8 and 9). Intersystem crossing from a fluorescent singlet state usually results in degradation of the excitation energy to heat because the triplet states typically are longer-lived and are therefore more susceptible to internal conversion and collisional energy loss. Collisional quenching is a bimolecular process, as opposed to the other processes mentioned so far, and requires contact between molecules, such as the two probe labels. Collision between the two labels can distort the energy levels of the excited molecule causing the nonradiative paths to dominate the fluorescence path, resulting in a loss of fluorescence emission, otherwise known as fluorescence quenching. If two probes are bound together such that their labels can physically contact one another, fluorescence quenching can be very efficient due to the high collisional rates resulting from their confinement.

An example of two probe labels interacting via collisional quenching is shown in Fig. 3, in which two complementary DNA oligomers, one labeled with fluorescein on its 5′-terminus and the other labeled with pyrenebutyrate on its 3′-terminus, are slowly heated and cooled through the "melting transition" to produce the DNA "melting curve," in this experiment monitored by the fluorescence intensity of the fluorescein label excited with an external lamp near the fluorescein absorbance maximum [7]. At low temperatures the complementary probes hybridize and the labels interact, while at high temperatures the double-stranded structure is not thermodynamically stable and the labels are separated in solution.

Two labels do not have to collide physically to affect one another. Electronic energy can transfer from the excited label to another label in a nonradiative process that requires matching energy transitions in each molecule

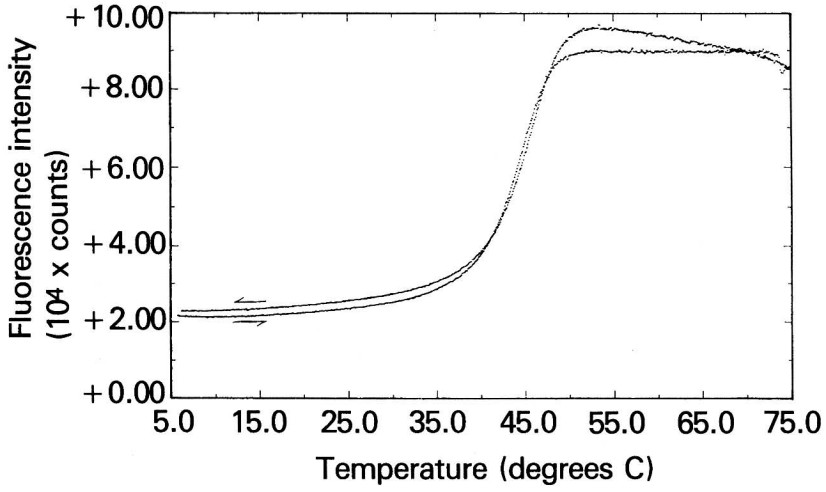

Fig. 3. "DNA melting curve" recorded by monitoring the fluorescein emission intensity of a solution containing 5′-fluorescein–$(dA)_{20}$ mixed in equimolar amounts with 3′-pyrenebutyrate–$(dT)_{20}$ as the solution temperature was slowly increased from the low-temperature extreme to the high-temperature extreme and then slowly returned to the low-temperature extreme.

[10]. This type of energy transfer can occur over long separation distances, 100 Å, for example, and is referred to variously as dipole-coupled energy transfer, long-range energy transfer, and fluorescence resonance energy transfer (FRET). The relationship derived by T. Förster predicts that the first-order rate constant for energy transfer (k_{et}) is proportional to the inverse sixth power of the distance separating two molecules (R; cm):

$$k_{et} = 8.79 \times 10^{-25}\kappa^2\Phi_D k_D \eta^{-4} R^{-6} \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$$

where κ is an orientation factor (equal to 2/3 for randomly oriented donor and acceptor molecules in solution), Φ_D is the donor fluorescence quantum yield, k_D is the first-order rate constant for deexcitation of the donor in the absence of energy transfer, η is the refractive index of the medium, $F_D(\lambda)$ is the normalized emission spectrum of the energy donor (integrated intensity = 1), and $\varepsilon_A(\lambda)$ is the spectrum of the acceptor's molar extinction coefficients for absorption, both expressed as functions of the wavelength, λ.

The integral term provides for the required presence of absorption transitions in the energy accepting molecule that match the deexcitation transitions in the energy donor molecule. If the label that accepts the energy is also fluorescent, then an increase in the acceptor label fluorescence accompanies the quenching of the donor label fluorescence.

An example of energy transfer between two probe labels is provided by the DNA "melting curves" shown in Fig. 4 [12]. Fluorescence from a 5′-pyrenesulfonate label on one DNA strand and a 3′-fluorescein label on a complementary strand was measured simultaneously and plotted, and shows high pyrenesulfonate fluorescence and low fluorescein emission at high temperatures compared to lower pyrenesulfonate fluorescence and higher fluorescein emission at low temperatures. In this experiment the excitation lamp was filtered to provide light near the maximum of pyrenesulfonate absorption. Fluorescein does have absorption transitions overlapping the pyrene emission transitions and a change in the fluorescence of both labels is consistent with an energy transfer mechanism for quenching of the pyrenesulfonate fluorescence at low temperatures, where the labels are brought together by probe-to-probe hybridization.

In contrast to the example shown in Fig. 4, the label pair used in the example in Fig. 3 exhibited no energy transfer, regardless of the label excited with the excitation lamp. This turns out to be the more common case when 3′- and 5′-terminal labeling is used, and the energy transfer example in Fig. 4 turns out to be the exception. In fact, when the labels on the DNA oligomers in the Fig. 4 example were switched (3′-fluorescein and 5′-pyrenesulfonate), the predominant interaction was quenching of the fluorescein emission by pyrenesulfonate. This indicates that the molecular interactions are highly dependent

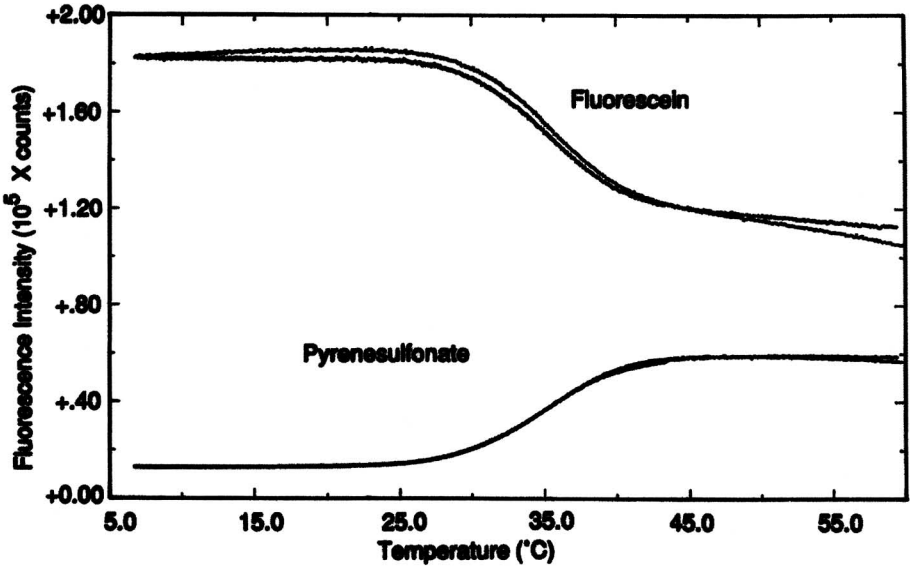

Fig. 4. "DNA melting curve" recorded by monitoring both the fluorescein and the pyrenesulfonate emission intensities of a solution containing 5′-pyrenesulfonate–$(dA)_{20}$ mixed in equimolar amounts with 3′-fluorescein–$(dT)_{20}$ as the solution temperature was slowly increased from the low-temperature extreme to the high-temperature extreme and then slowly returned to the low-temperature extreme.

not only upon the two molecules involved but also upon the manner in which they are linked to the DNA strands. Predicting the type of behavior a priori may not be possible. In general, it appears that if the labels are positioned with linkers that allow contact of the two labels, then collisional quenching will predominate [7], while energy transfer becomes favored if the labels are positioned such that contact is prohibited [11].

Single-Label Interactions. Only one label would be required for a homogeneous hybridization assay if the fluorescence of that label were strongly affected by the hybridization process. One such label is pyrenebutyrate which is quenched to varying degrees by the proximity of different nucleotides. Data for the fluorescence quantum yield of pyrenebutyrate attached to the 5′-terminal position of several different DNA oligomers are listed in Table I [12]. The first oligomer listed is composed of all adenine nucleotides and the pyrenebutyrate fluorescence efficiency is high. Hybridization to the complementary oligomer causes very strong quenching (99%). This would appear to be due at least in part to the approach of thymidine nucleotides in the complementary strand. This effect can be seen by placing thymidine nucleotides in different positions on the labeled strand, as shown in the next three oligomers. Hybridization to a complementary oligomer, which brings more thymidine nucleotides near the pyrenebutyrate, causes additional strong quenching for all of the oligomers listed. This would indicate that probes with a high adenine and low thymidine content near the pyrenebutyrate attachment position should be selected. Further limitations are shown in the last four oligomers. Considerable quenching is observed in the single-labeled strand which is greater for the longer oligomers. Pyrenebutyrate-labeled probes, therefore, also need to be as short as possible to maintain reasonable fluorescence efficiency. Quenching in these examples may be due to electron transfer reactions between the different nucleotides and the excited pyrene label. The limitations placed on the probe, however, would severely limit its use in hybridization assays, although hybridization measurements using pyrene quenching have been reported [13,14].

Table I. Quantum Yields of 5′-Pyrenebutyrate-Labeled Oligomers and Percentage Quenching upon Hybridization to Complementary Oligomers

Probe	Quantum yield of single-stranded probe	% quenching in hybridized probe
Py-AAAAAAAAAAAA	0.52	99
Py-ATAAAAAAAAAA	0.092	91
Py-AATAAAAAAAAA	0.15	91
Py-AAATAAAAAAAA	0.27	90
Py-AAGACCTATGTTAGTC	0.057	91
Py-AAGTTAAGACCTATGT	0.035	81
Py-AAGACCTATG	0.14	95

ASSAY CHARACTERISTICS

The homogeneous hybridization assays have different characteristics depending upon whether the probes bind competitively or noncompetitively to the target nucleic acid. In the competitive assay format (Fig. 2), probes and target are rendered single-stranded, and four hybridization equilibria then compete: binding of probe strands to each other, binding of target strands to each other, and binding of each probe strand to the complementary target strand. If the association equilibria are all large and strand displacement is disregarded, the fraction of probe hybridized to target equals the ratio of the target DNA concentration to the sum of the probe and target concentrations [7]. The theoretical response of the probe fluorescence to the target concentration is plotted in Fig. 5 for two hypothetical probe concentrations, a 10 p*M* probe and a 100 p*M* probe. From these plots it can be seen that the response is half-maximal when the target concentration equals that of the probe and that 80% of the response occurs when the target concentration is between 0.1 and 10 times the probe concentration. The assay sensitivity, therefore, can be improved by lowering the probe concentration. Fluorescence measurements in a model probe/target system were found to follow the theoretical relationship closely [7].

The ideal response of the noncompetitive adjacent probe format (Fig. 1) is also plotted in Fig. 5 for hypothetical 10 and 100 p*M* probe concentrations. If the association constants are large, the probe response is linear with target concentration when probe is in excess, reaching the maximum value when target and probe are equal. At higher target concentrations, hybridization of probe to the excess target serves to separate the two probes, and an increasing target concentration reduces the probability that the adjacent probes will hybridize to the same target strand. As in the complementary probe format, a higher sensitivity is achieved by lowering the probe concentration, however, it its necessary to assure probe excess in the adjacent probe format or take measurements at more than one probe concentration or sample dilution to know on which side of the response maximum the actual target concentration lies.

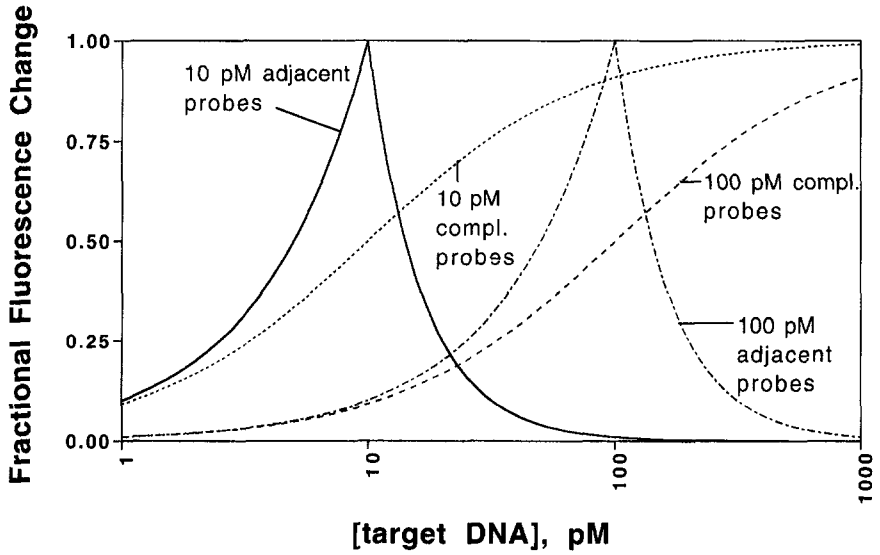

Fig. 5. Theoretical response of complementary and adjacent probe formats to target concentration. The fractional fluorescence change is proportional to the fraction of probe strands hybridized to target strands in the complementary probe format. In a fluorescence quenching assay of this type, the response is proportional to the measured fluorescence. The fractional fluorescence change is proportional to the fraction of probe strands hybridized to adjacent positions on the same target strand in the adjacent probe format. In a fluorescence quenching assay of this format type, a high response corresponds to high quenching and low fluorescence.

In comparing the assay formats, both are essentially linear when the probe is at a 10-fold or greater excess to the target. At higher target concentrations the complementary probe format becomes nonlinear, however, the complementary probe response continually increases with target concentration and responds to the target over a wider range of target concentrations. The fact that the same response level can correspond to two target concentrations is a severe drawback to the adjacent probe format if probe excess is not guaranteed.

Compared to heterogeneous assays, homogeneous assays are generally less sensitive because unhybridized probe is not physically separated from probe hybridized to target before the measurements are made. The homogeneous assay relies upon fluorescence differences between interacting and noninteracting labels, and background probe fluorescence results if the energy transfer or fluorescence quenching is not 100% when the labels interact. For example, in the complementary probe format, if quenching is 99% when the probes are hybridized to each other, then complete absence of target DNA still generates a background fluorescence intensity equivalent to 10 p*M* target DNA when the probes are present at a 1 n*M* concentration. The detection level can be lowered by reducing the probe concentration, however, at some concentration other sources of fluorescence background become a problem. More importantly, the hybridization kinetics become slower as the probe concentration is reduced, causing assay times to go from several minutes at a 1 n*M* probe concentration to hours at a 20 p*M* probe concentration [7,9]. Since unhybridized probe can be physically removed following hybridization in heterogeneous assays, large probe excess can be used to increase hybridization rates. This strategy is successful, however, only if the separation step can remove the increased nonspecific binding of probe to the solid support.

DETECTION OF AMPLIFIED POLYNUCLEOTIDES

The limitations on the homogeneous hybridization assay predict detection limits on the order of femtomoles of target sequence, for conventional assay volumes of 0.1 to 1 ml and assay times of 1 h to several hours. While these are respectable detection limits for many types of assays, including immunoassays, they are not sufficient for the clinical detection of DNA, such as for the identification of infectious diseases, where attomole and lower detection requirements are common. Direct detection would be feasible if specimen DNA could be concentrated with a high efficiency to nano- or picoliter volumes and detected with laser excitation. Alternatively, the energy transfer and fluorescence quenching hybridization assays

could be used to detect amplified polynucleotides, such as products of the polymerase chain reaction (PCR).

Detection of Amplified Sequences. PCR is itself a homogeneous method of amplifying polynucleotides, and identification of the amplification products is still predominantly performed using time- and labor-consuming heterogeneous detection methods such as gel electrophoresis and filter blotting followed by hybridization to radioactive probes. A homogeneous detection system would provide for a completely homogeneous assay of minute quantities of biologically important polynucleotides. This concept was first demonstrated by performing PCR amplification followed by competitive hybridization of the amplification mixture with labeled complementary probes matching the amplified sequence [7]. Subattomole detection of target was achieved in 25 cycles of PCR.

The idea of detecting amplified polynucleotides with homogeneous hybridization assays has gained popularity in the 1990s. Figure 6 shows several formats which have been used to detect the amplified sequences lying between the two PCR primer positions. These include the complementary probe pair in Fig. 6A [7], the adjacent probe pair in Fig. 6B [15], and a variation on the complementary probe pair shown in Fig. 6D, in which the termini at one end of the probes are connected to form a hairpin [16–18]. Related to the adjacent probe pair format is the format shown in Fig. 6C, in which a labeled primer is incorporated in one amplified strand and subsequently hybridizes to a labeled probe complementary to the amplified sequence adjacent to the primer position, placing the labels within interaction distance [19].

The enzymatic activities of DNA polymerases used in the PCR amplifications have been combined with hybridization probes either to separate or to bring together fluorescent and quencher labels. In Fig. 6E, DNA polymerase 5′-to-3′ exonuclease activity cleaves a single probe strand containing both fluorescent and quencher labels, thereby separating the labels and breaking their interaction during the amplification process [20, 15]. This was the first assay in which the hybridization probe was added to the PCR mixture prior to the start of amplification, thereby providing a sealed system in which the reaction solution did not need to be opened following amplification, reducing the possibility of contaminating other PCR reactions with the amplification products [20]. Adding probes prior to the start of PCR also permits monitoring of amplified DNA production during each PCR cycle, by either moving the tube to a fluorometer between cycles or, preferably, using a PCR thermocycling machine with an integral fluorometer. Hybridization formats which do not rely on enzymatic digestion have also been shown to work when added prior to the start of the PCR amplification [15–19].

A multistep PCR detection method is also shown in Fig. 6F, where, following amplification, labeled primer, labeled dideoxynucleosidetriphosphates, and DNA polymerase are added to the reaction mixture. Labeled dideoxynucleotide becomes incorporated to indicate presence of a particular target nucleotide at that location, thereby placing a second label within interaction distance with the first label on the primer strand [21].

Detection of Primer Incorporation. Amplified DNA has also been detected by measuring fluorescence quenching or energy transfer associated with PCR primer incorporation. Two related fluorescence quenching primers are shown in Figs. 7A and B. Figure 7A shows a 5′-labeled PCR primer strand to which a smaller 3′-labeled oligonucleotide is hybridized [22]. After hybridization to the target strand, the primer is extended by the polymerase (Fig. 7C), followed by priming of that strand with the

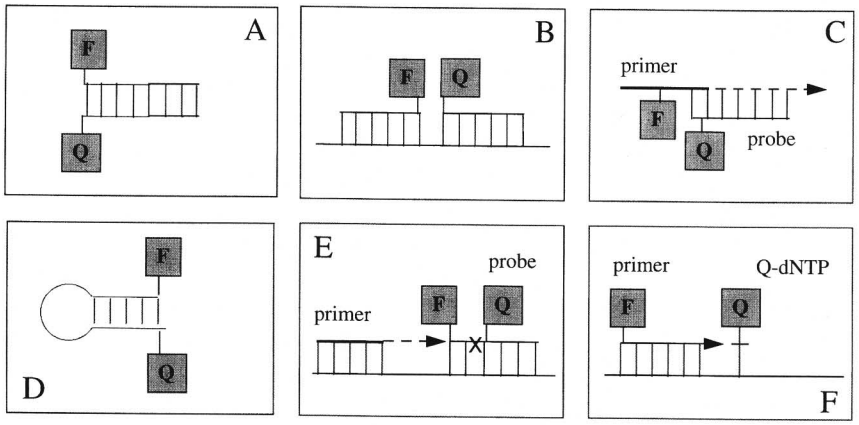

Fig. 6. Formats for detecting PCR-amplified DNA sequences via interaction of two probe labels. Refer to the text for a description of each format.

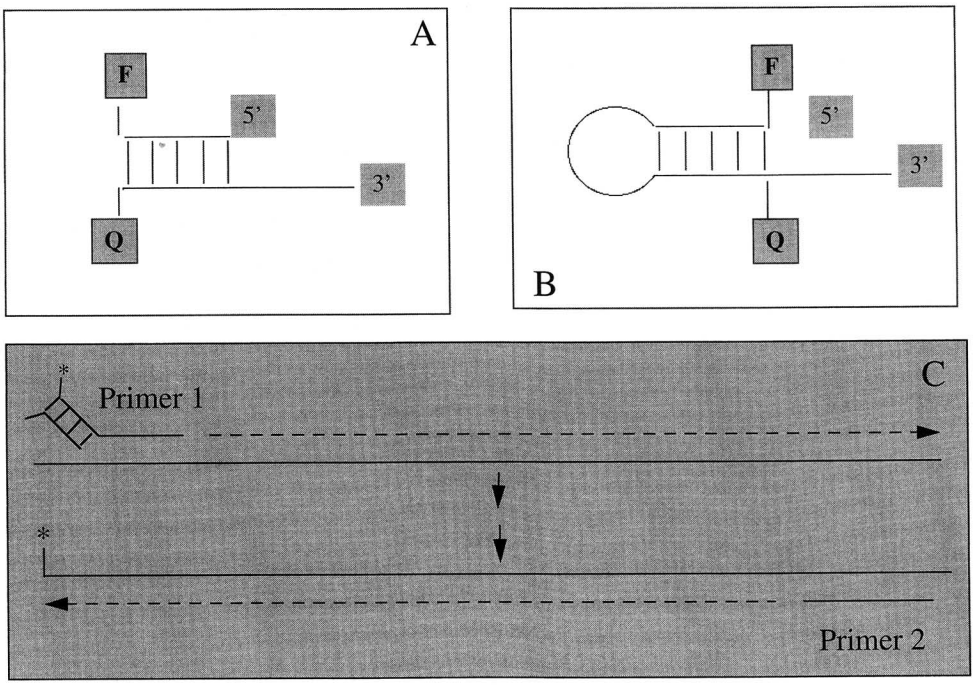

Fig. 7. Formats for detecting PCR primer incorporation via interaction of two probe labels. Refer to the text for a description of each format.

second unlabeled primer in the next PCR cycle. Extension of the second primer serves to displace (or digest) the smaller labeled oligonucleotide, disrupting the label interaction and increasing the fluorescence of the label attached to the first primer. A similar approach is shown in Fig. 7B, where the first primer and labeled oligonucleotide have been connected to form a hairpin, with labels occupying adjacent positions in the closed form [23].

Detection of Amplified Sequences Versus Primer Incorporation. Although fluorescence quenching and energy transfer can be used to detect amplified DNA through both incorporation of primer into the amplified DNA and hybridization to the amplified internal sequence, there are fundamental differences between the two approaches. Both approaches can detect amplification, however, measurement of primer incorporation may have a higher false positive rate since it indicates only that incorporation of primer into a product has occurred. Partial hybridization of primer to nontarget sequences in a specimen, or to other primers, can lead to extension of the primers and separation of the label pairs, falsely indicating the presence of target DNA. Hybridization to the sequence amplified between the primers measures exactly what DNA has been amplified. Measurement of primer incorporation may be useful in very well-characterized research systems where nonspecific priming may be ruled out or in screening situations where a higher level of false positives is acceptable.

A second disadvantage of using labeled primers is that the primer concentration cannot be reduced to achieve lower detection limits since the primer must be present at a high concentration to promote rapid priming during each PCR cycle. The concentration of probes to internal amplified sequences can be changed independently of the primer concentration. One advantage of the labeled primers is that only the primers need to be optimized for a particular target DNA, while both primer pairs and probes must be optimized when a separate set of probes is used to identify the internal sequence.

Linear Versus Hairpin Probes. There has been much recent interest in the hairpin hybridization probes for detecting PCR-amplified DNA [16–18]. As shown in Fig. 6D, the hairpin probe is like the complementary probe pair (Fig. 6A) except that the pair has been connected together to form a single probe strand. Connecting the probe pair together creates a structure with a single-stranded region (the loop) of sequence complementary to the target, which will rapidly self-associate to form a hairpin in the absence of target, thereby bringing the labels into neighboring and interacting positions. At first inspection, the rapid rate of closing would not appear to offer an advantage over the linear probe pair in the PCR

setting. This is because the hairpin probes are used at a relatively high concentration, about 0.3 μM, where linear probe-to-probe hybridization is also very fast. For example, a 20-nucleotide-long oligomer should have a bimolecular association rate constant near $2 \times 10^7\ M^{-1}\ s^{-1}$ at the 37°C hybridization temperature used in PCR and an even higher rate at the 72°C primer extension temperature (calculated from kinetic constants in Ref. 24). At 0.3 μM the rate of conversion of single-stranded oligomer to double-stranded oligomer would be about 2 μM oligomer per s, providing essentially complete hybridization within several seconds. Hairpin closure time would have a significant advantage over probe pair hybridization at a lower concentration, but at a low probe concentration the probe-to-target hybridization time would also be slow, and this rate would be similar for both hairpin and paired probes.

The rapid hairpin closure time, however, may be important in differentiating point mutations. Comparison of linear probe pairs and hairpin probes showed similar melting temperatures for both types of probes hybridized to perfectly matched targets but lower melting temperature for hairpin probes hybridized to target with one base mismatch compared to linear probes hybridized to the same mismatched target [18].

MEASUREMENT OF ACCEPTOR LABEL FLUORESCENCE

Fluorescence quenching and energy transfer can be measured by the emission of only one of the two labels, the quenched or energy donor label. When energy transfer occurs and the energy accepting label is also fluorescent, the acceptor label fluorescence can also be measured. Measuring acceptor fluorescence in addition to donor fluorescence assures that the intended label interaction is occurring, and not an unintended quenching of the donor label by some sample impurity resulting in a false-positive or false-negative result. If energy transfer occurs due to the two probe labels being brought together, then a reduction in donor fluorescence will be accompanied by a gain in acceptor emission. Unfortunately, acceptor fluorescence is rarely measured, in large part because acceptor fluorescence arising from energy transfer is accompanied by acceptor fluorescence due to absorption of the excitation lamp's emission. This background fluorescence can dwarf the amplitude of the fluorescence from energy transfer. An example of this can be seen in Fig. 4, where the emission of the energy acceptor label, fluorescein, has a much higher background component than the donor label, pyrenesulfonate.

One way to eliminate the acceptor background fluorescence problem is to use a chemiluminescent donor label, which eliminates the need for an excitation lamp [3]. However, there is a considerably smaller selection of chemiluminescent compounds available relative to fluorophores, and fluorescence measurements can be repeated on the same sample and are easier to control (light excitation versus chemical excitation).

A second method of removing acceptor background emission has been demonstrated in an immunoassay system and makes use of a long-lifetime donor label and a short-lifetime acceptor label combined with time-resolved detection of the acceptor emission [25]. When a short pulse of excitation light is used to excite the donor label, light absorbed by donor and acceptor labels is emitted as fluorescence with their characteristic emissive rate constants, k_D and k_A, respectively, as described in the following equation, the acceptor being selected to decay faster:

$$A^* = A_0^* e^{-(k_A)t}; \qquad D^* = D_0^* e^{-(k_D)t}; \qquad k_A > k_D$$

where A^* is the number of excited acceptor labels at time t after the excitation pulse, D^* is the number of excited donor labels at time t after the excitation pulse, A_0^* is the initial number of excited acceptor labels at $t = 0$, and D_0^* is the initial number of excited donor labels at $t = 0$. However, if light is transferred to the acceptor from the donor label, then the rate of acceptor label fluorescence decay also depends upon the lifetime of the donor label (k_T = energy transfer rate constant):

$$A^* = k_T D_0^* \left[e^{-(k_D + k_T)t} - e^{-(k_A t)}\right]/(k_A - k_D - k_T) + A_0^* e^{-(k_A t)}$$

and the observed lifetime of the acceptor fluorescence due to energy transfer (first term) can exceed the lifetime of acceptor fluorescence due to direct absorption of light (last term). Use of a time-gated detector (or phase sensitive detection combined with sinusoidally modulated excitation light) can then attenuate the background acceptor emission relative to emission due to energy transfer. Fifteen-fold improvements in energy transfer-to-background ratios were demonstrated, and experimental improvements could theoretically increase this ratio another one to two orders of magnitude.

PHYSICAL STUDIES OF DNA HYBRIDIZATION

Homogeneous detection of DNA hybridization also lends itself to physical studies of DNA. Interaction of

labels attached to terminal positions of complementary oligomers has been used to measure the rates of DNA association and dissociation and measure hybridization equilibrium constants [24,26,27]. Advantages provided over conventional methods included the ability to measure DNA at lower concentrations, which in turn provided more accurate thermodynamic measurements of longer DNA strands and slowed hybridization rates to time scales that allowed kinetic measurements to be performed with inexpensive mixing and monitoring equipment. Energy transfer and quenching measurements have also been applied to assessing the structure of hybridized DNA, particularly in measuring the helical geometry of DNA [4,26,28,29].

CONCLUSIONS

From work dating to the early 1980s, energy transfer and fluorescence quenching have been shown to provide effective routes to homogeneous DNA assays. With terminal labeling of DNA strands, probe labels can be placed in highly interacting positions, providing efficient deexcitation of a fluorescent label by an energy quenching or energy accepting label. Labels can be brought together via hybridization of adjacent probes to a common target polynucleotide and by hybridization of complementary probes which compete with hybridization to target polynucleotides. With the variety of automated DNA chemistries available today, labels can be placed virtually anywhere within synthetic oligonucleotides to optimize label interactions. In general, homogeneous assays provide rapid and simple means to detect specific DNA sequences, however, homogeneous assays have the disadvantage of being less sensitive than heterogeneous assays. While the sensitivity level is adequate for many immunoassays, it is inadequate for most DNA assays of clinical importance. The combination of homogeneous DNA assays with DNA amplification methods (e.g., PCR) provides the route for clinically relevant assays, and in recent years a number of fluorescence quenching and energy transfer-based assay formats have been tested in combination with PCR reactions. As such, these assays are suitable for high throughput screening. Methods such as lifetime-resolved detection of energy transfer may allow further improvements in the detection of energy transfer, providing further confidence in hybridization assay results. In addition to hybridization assays of clinical relevance, energy transfer and fluorescence quenching are providing improved methods for physical studies of DNA hybridization, including kinetic, thermodynamic, and structural studies.

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