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Review

Real-time assays with molecular beacons and other fluorescent nucleic acid hybridization probes

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Abstract

Background: A number of formats for nucleic acid hybridization have been developed to identify DNA and RNA sequences that are involved in cellular processes and that aid in the diagnosis of genetic and infectious diseases.
Methods: The introduction of hybridization probes with interactive fluorophore pairs has enabled the development of homogeneous hybridization assays for the direct identification of nucleic acids. A change in the fluorescence of these probes indicates the presence of a target nucleic acid, and there is no need to separate unbound probes from hybridized probes.
Conclusions: The advantages of homogeneous hybridization assays are their speed and simplicity. In addition, homogeneous assays can be combined with nucleic acid amplification, enabling the detection of rare target nucleic acids. These assays can be followed in real time, providing quantitative determination of target nucleic acids over a broad range of concentrations.

Keywords: Nucleic acid hybridization; Real-time gene amplification assays; Fluorescence energy transfer; Molecular beacons; Fluorescent nucleic acid hybridization probes

Contents

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1. Introduction

Nucleic acid hybridization is a process whereby a DNA or RNA strand forms an ordered series of hydrogen bonds with its complement, creating a duplex structure. A nucleic acid can identify its complement in a large population of unrelated nucleic acid sequences. The hybrids that are formed are the strongest and most specific macromolecular complexes known. By selecting nucleic acid sequences that are complementary to a target sequence, nucleic acid probes can be designed for the detection of any gene. The applications range from the estimation of similarity between species to the detection of single nucleotide polymorphisms. Nucleic acid hybridization is used to explore complex cellular pathways, for the diagnosis of genetic and infectious diseases and to provide information on the storage, transfer and expression of genetic information in living cells. Aided by the sequencing of the human genome, the development of novel nucleic acid probes has focused on speed, reliability and accuracy in the identification of nucleic acids. In addition, advances in research on pathogenic infections and the characterization of microbes have resulted in a rise in the demand for molecular diagnostic assays.

2. Early nucleic acid hybridization formats

In 1961, in order to answer the question: "Is the RNA synthesized after infection with T2 bacteriophage complementary to T2 DNA?", Spiegelman and Hall developed the basis of a technique now known as nucleic acid hybridization. In their study, DNA isolated from single-stranded T2 bacteriophage was mixed with RNA from T2-infected *Escherichia coli*, and the hybrids were isolated by equilibrium-density gradient centrifugation [1]. The next year, Bolton and McCarthy developed the first simple solid-phase hybridization method [2]. In their method, denatured DNA was immobilized in agar and hybridized to radioactively labeled RNA. In the subsequent washing step, RNA not hybridized to other nucleic acids was removed. The bound RNA was then eluted from the agar by lowering the salt concentration of the elution solution. The radioactivity of the eluent was then shown to be proportional to the fraction of the RNA bound to complements. This method was optimized further by using nitrocellulose membranes as supports on which the hybrids were immobilized [3–5]. The first applications of nucleic acid hybridization were limited to the detection of abundant target nucleic acids. In addition, the preparation of large amount of nucleic acid probes, which is necessary to carry out the hybridization experiments, was difficult and time consuming. In 1975, after the discovery of restriction endonucleases [6] and molecular cloning [7], it became possible to measure both the quantity and the size of specific hybridized DNA and RNA sequences. DNA samples incubated with restriction enzymes were fractionated by gel electrophoresis and then transferred to a nitrocellulose membrane. DNA restriction fragments, containing a specific nucleic acid sequence, were identified by hybridization with labeled nucleic acid probes. This technique, called "Southern blotting", was further developed by and named after Ed Southern [8]. An analogous filter hybridization technique, where RNA is separated by gel electrophoresis, transferred to a membrane and identified by hybridization with a labeled nucleic acid probe, is termed "Northern blotting" [9]. The introduction of sequencing technologies enabled the analysis of the exact composition of the nucleic acids used, increasing the specificity of nucleic acid hybridization [10,11]. Besides being useful in filter hybridization, nucleic acid probes are useful for the detection of nucleic acids within cells and tissues. In these methods, referred to as in situ hybridization, the nucleic acid probe is labeled with a reporter molecule and the sites of hybridization are visualized by microscopy. As with filter hybridization, the earliest phase of in situ hybridization relied on autoradiographic detection of abundant sequences [12,13]. Autoradiographic detection, however, was limited by poor resolution and also lacked the ability to distinguish more than one nucleic acid target simultaneously. In addition, the technique is often time consuming, requiring several weeks before results can be obtained. To overcome these limitations, non-autoradiographic detection methods were developed. The earliest non-autoradiographic techniques used antibodies to recognize RNA–DNA hybrids or used avidin to detect bound biotin-labeled nucleic acid probes [14–16]. Later, methods for the chemical modification of nucleotides and for the recognition of these modified nucleotides by fluorescent molecules, gold particles or enzymatic reporter molecules were developed, increasing assay sensitivity [17]. The introduction of fluorescent dyes as labels for nucleic acid probes, such as derivatives of fluorescein, rhodamine, or Texas red, provided high resolution with the light microscope. One of the biggest advantages of fluorescent probes is that they enable multicolor fluorescence in situ hybridization to detect multiple nucleotide sequences simultaneously [18].

3. Amplification of targets

In clinical diagnostic applications, the total amount of target nucleic acid in a sample is often very low, and therefore the signal generated by the hybridization of specific probes is often not detectable. For example, since only a few copies of the viral pathogen HIV-1 in blood can cause AIDS, it is desirable to have the ability to detect them soon after infection. In order to overcome the limitation in sensitivity of nucleic acid probes, schemes for target amplification have been developed.

The most widely used target amplification technique is the polymerase chain reaction (PCR). This technique, which was first described by Mullis et al. [20] and Saiki et al. in 1985 [19], has made it possible to detect and quantitate rare target nucleic acid sequences isolated from cell, tissue or blood samples. The basis of this technique is the ability of DNA polymerase to extend an oligodeoxyribonucleotide primer that is specifically hybridized to a single-stranded DNA template. PCR consists of three thermally separated steps: denaturation at 95 °C to ensure complete separation of DNA duplexes into single-stranded molecules; annealing at a temperature below 65 °C to allow the primers to hybridize to the DNA template; and extension at 72 °C to allow the synthesis of complementary DNA strands by DNA polymerase. Repeating this cycle of three steps allows the newly formed DNA molecules, as well as the existing DNA molecules to serve as templates for a new round of primer hybridization and extension. Multiple rounds of thermal cycling result in an exponential accumulation of PCR products. In 30 thermal cycles, a 10^8-fold amplification of the target sequence can be achieved. There is a quantitative relationship between the number of target nucleic acid strands initially present in a sample and the number of product strands synthesized at any given thermal cycle. Alternatively, an initial reverse transcription reaction can be used to generate cDNA from a target mRNA, and the resulting cDNA can then be amplified in a polymerase chain reaction (RT-PCR).

Strand displacement amplification (SDA) is an alternative technique for the amplification of DNA molecules. Unlike PCR, SDA is an isothermal process in which the thermal separation of newly synthesized strands from their templates is replaced by an isothermal enzymatic step [21]. The primers used in SDA contain a restriction endonuclease recognition site, and nicks are produced in hybridized primers by a restriction enzyme. In SDA, newly synthesized strands are phosphorothiolated, due to the presence of alpha-thiolated nucleotide triphosphates in the reaction mixture, and single-stranded nicks are produced in the primers, rather than in the template strands, by the restriction enzyme. These single-stranded nicks serve as priming sites for a 5′-nuclease-deficient DNA polymerase. As the DNA polymerase proceeds along the template, it displaces the nicked strand, generating an intact molecule that can serve as template for further amplification.

Another isothermal method for amplifying rare target RNA sequences is nucleic acid sequence based amplification (NASBA), or the very similar transcription-mediated amplification (TMA). The basis of this technique, first described by Guatelli et al. [22], is the incorporation of a T7-promotor sequence during cDNA synthesis with the help of a reverse transcription reaction. This step is followed by transcription of the cDNA product with T7 RNA polymerase, generating 10 to 1000 RNA copies of each cDNA product. Product accumulation is exponential, since the newly synthesized cDNAs and RNAs serve as templates for a continuous series of reverse transcription and direct transcription reactions. This method is able to generate in 90 min as many as 10^9 copies of each RNA target molecule that was initially present in the reaction [23].

The ligase chain reaction (LCR) is another DNA amplification method. In this method, two oligonucleotides are hybridized next to each other on a DNA target. DNA ligase only joins the 3′ and 5′ ends of the two oligonucleotides if their ends are perfectly complementary to the template strands. Ligated oligonucleotides are then amplified by thermal cycling [24]. LCR has better allelic specificity than PCR because of the greater discriminatory properties of ligation compared to primer extension [25].

4. Detection of amplified targets

The classic method for analyzing the products of target amplification involves separating the amplification products by gel electrophoresis and then visualizing the products by either filter hybridization with a nucleic acid probe, direct incorporation of a radioisotope into the amplification product, or the addition of an intercalating agent that becomes fluorescent upon binding to double-stranded nucleic acids. Although these methods can provide useful information on the size, identity and quantity of the amplification products, there are limitations. The main disadvantage of using these methods is the risk of contaminating untested samples, since the amplification products have to be transferred from the reaction solution to a gel matrix for electrophoretic analysis [26]. In addition, the synthesis of non-specific amplification products can be significant when the targets are rare or absent and the sample contains an abundant and diverse nucleic acid population. In these situations, there is an increased chance that amplification will be initiated from unintended sequences, resulting in non-specific amplification products being the primary reaction products.

5. Molecular beacon probes

In order to simplify the detection of amplified products and to perform the detection in sealed tubes in real time, Sanjay Tyagi and Fred Russell Kramer have developed hairpin-shaped nucleic acid hybridization probes that undergo a conformational reorganization when they bind to their target that causes them to fluoresce brightly. These probes are called “molecular beacons” [27] (Fig. 1A). Molecular beacons are single-stranded oligonucleotides possessing a probe sequence that is usually 15 to 30 nucleotides in length. The probe sequence is chosen to be complementary to a specific target sequence that is present in the nucleic acid to be detected. The probe sequence is imbedded within “arm” sequences at either end of the

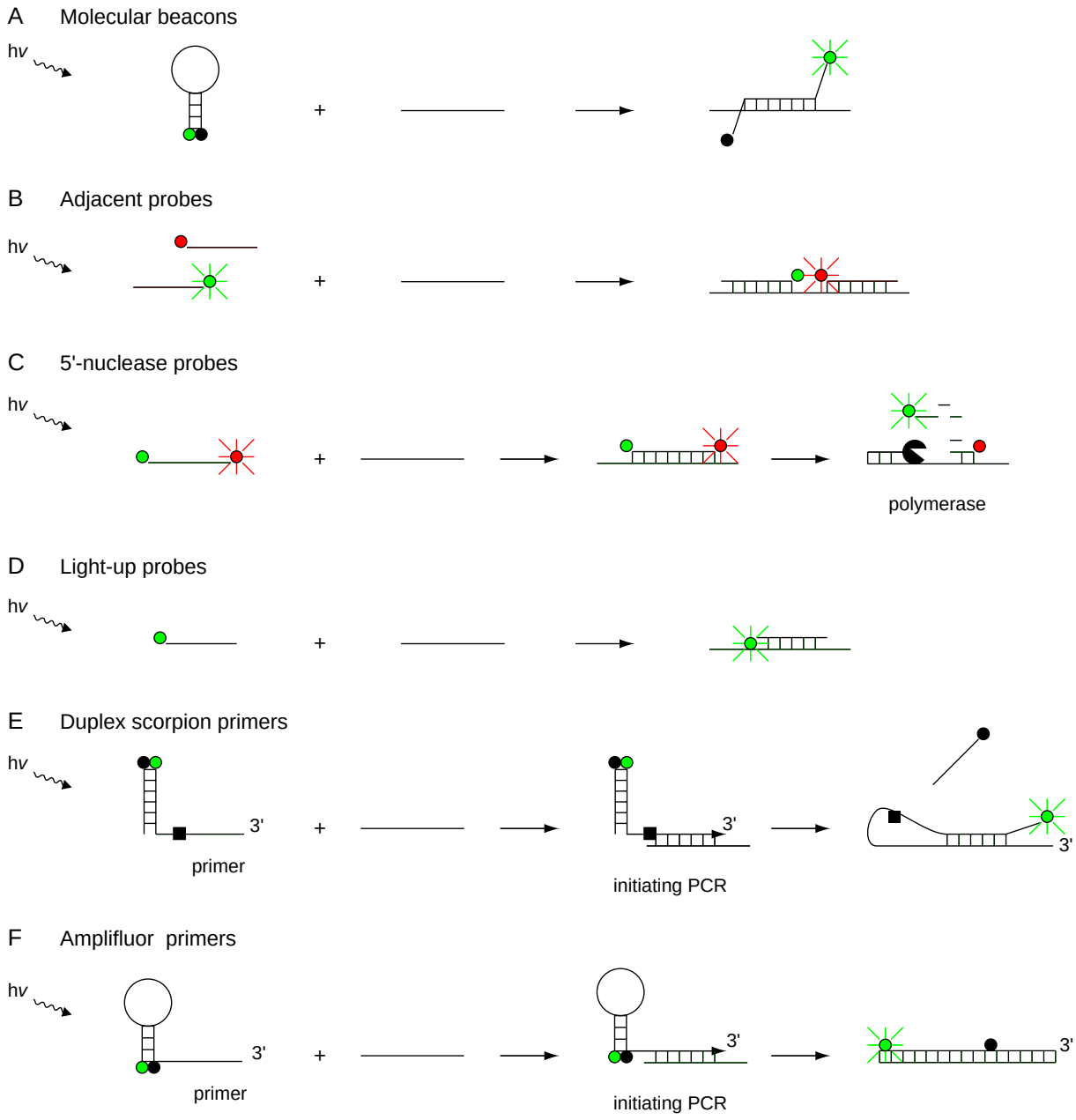

Fig. 1. Schematic representation of fluorescence signal generation with different nucleic acid hybridization probes.

oligonucleotide. The arm sequences are usually 5 or 6 nucleotides long. There is no relationship between the probe sequence and the two arm sequences. However, the sequences of the arms are chosen so that they are complementary to one another and are composed mostly of guanosines and cytidines. Consequently, under assay conditions, molecular beacons can form a hairpin structure, in which the arm sequences are bound to each other forming a hairpin stem and the probe sequence is located in the hairpin loop. A fluorophore is covalently linked to one end of the oligonucleotide and a non-fluorescent quencher moiety is covalently linked to the other end of the oligonucleotide. The stem hybrid brings the fluorophore and quencher into close proximity, allowing energy from the fluorophore to be transferred directly to the quencher through static quenching. In static quenching, the donor and acceptor molecules can interact by proton-coupled electron transfer through the formation of hydrogen bonds. When this complex absorbs light, the excited state immediately returns to the ground state without emission of a photon. When a molecular beacon encounters a target molecule, it forms a relatively rigid probe–target hybrid that is longer and more stable than the stem hybrid. The rigidity of the probe–target hybrid prevents the simultaneous existence of the stem hybrid. The molecular beacon therefore undergoes a spontaneous conformational reorganization that forces the stem hybrid to

dissociate and the fluorophore and the quencher to move away from each other, restoring fluorescence.

In practice, the length of the probe sequence is chosen so that it will form a stable hybrid with its target sequence at assay temperatures, whereas the arm sequences are chosen so that they will form a stable stem hybrid when there is no target present. The kinetics of molecular beacon hybridization are second order. In an assay in which there are more probes available than target molecules, molecular beacon fluorescence is proportional to the amount of target present. The amount of fluorescent light emitted by a molecular beacon when it is bound to its target is typically 100 times as bright as the amount of light given off when it is free in solution. This extraordinary increase is due to the extremely low background signal emitted by molecular beacons, rather than to their fluorescence being especially bright.

Molecular beacons can possess differently colored fluorophores, enabling assays to be carried out that simultaneously detect different targets in the same reaction [28]. Moreover, all differently colored molecular beacons can possess the same nonfluorescent quencher [29]. Recently, new non-fluorescent quenchers have been introduced [30,31]. Fig. 2 is a photograph showing 12 test tubes that all contain the same molecular beacon at the same concentration. The only difference between the tubes is that the molecular beacons in each pair of tubes possessed differently colored fluorophores at their 5′ ends. All of the molecular beacons possessed dabcyl at their 3′ ends, which serves as a universal quencher. When an excess of complementary target oligonucleotides was added to one tube of each pair, the molecular beacons in that tube fluoresced brightly in the characteristic color of their attached fluorophore. Tubes without targets remained dark. Thus, irrespective of the identity of their fluorophore, molecular beacons are dark in the absence of target and fluoresce brightly in the characteristic fluorescent color of their fluorophore when bound to target.

Similar to other fluorescent hybridization probes, molecular beacons enhance the overall specificity of PCR assays. Molecular beacons are added to PCR assays prior to the initiation of synthesis, at the same time the primers are added, and at approximately the same concentration as the primers (Fig. 3). Their target sequence is located at an internal site within one of the two complementary strands of the intended amplicon, away from the terminal sequences where the primers bind. The assay tube is then permanently sealed, and repetitive temperature cycling is begun. At the high temperature setting in each PCR cycle (approximately 95 °C), when the complementary amplicon strands are melted apart, the stem hybrids of the molecular beacons also melt apart, and all of the molecular beacons become fluorescent. As the temperature is lowered (<65 °C) to permit annealing of the primers to the separated amplicon strands, two different things happen to the molecular beacons. The first event, which occurs quickly is the reformation of the stem hybrids, resulting in the return of the molecular beacons to a darkened state, occurs quickly once the temperature has cooled below the melting temperature of the stem. The second event, which occurs over a period of seconds once the annealing temperature is reached, is the hybridization of the molecular beacons to any target amplicon strands that are present, resulting in conformational reorganization of the bound molecular beacons, and the generation of fluorescence. Since the molecular beacons (like the primers) are present at a higher concentration than the amplicons, fluorescence intensity at the end of the annealing stage of each amplification cycle is directly proportional to the number of amplicons present. During the last stage of each PCR cycle, when the temperature is raised (approximately 72 °C) to promote DNA synthesis, the temperature is too high for the molecular beacons to remain on their target sequences, so the molecular beacons fall off of their targets and do not interfere with polymerization. Spectrofluorometric thermal cyclers enable many different PCR assays containing molecular beacons to be carried out simultaneously in sealed tubes. The fluorescence intensity of the molecular beacons in each reaction tube is automatically measured at the end of the annealing stage of each thermal cycle.

Fig. 4 shows the results of a series of PCR reactions, each reaction was initiated with a different number of target strands, and each reaction was monitored in real time with molecular beacons. A control reaction that lacked target strands did not develop fluorescence. The results illustrate how quantitative measurements are made in real-time PCR assays: the fewer the number of target strands initially present in a sample, the more thermal cycles are needed before there are sufficient amplicons present to generate a significant fluorescence signal. The number of thermal cycles needed to generate a significant signal (the "threshold cycle") is inversely proportional to the logarithm of the number of target strands initially present in the sample.

To demonstrate the use of molecular beacons in extremely sensitive, high-throughput, clinical tests, an assay was developed for the detection of retroviral nucleic acids in blood samples and in tissues for transplantation [32].

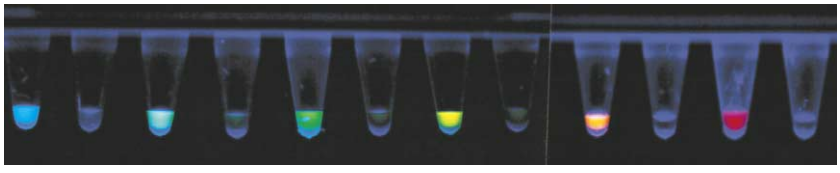

Fig. 2. Differently labeled molecular beacons.

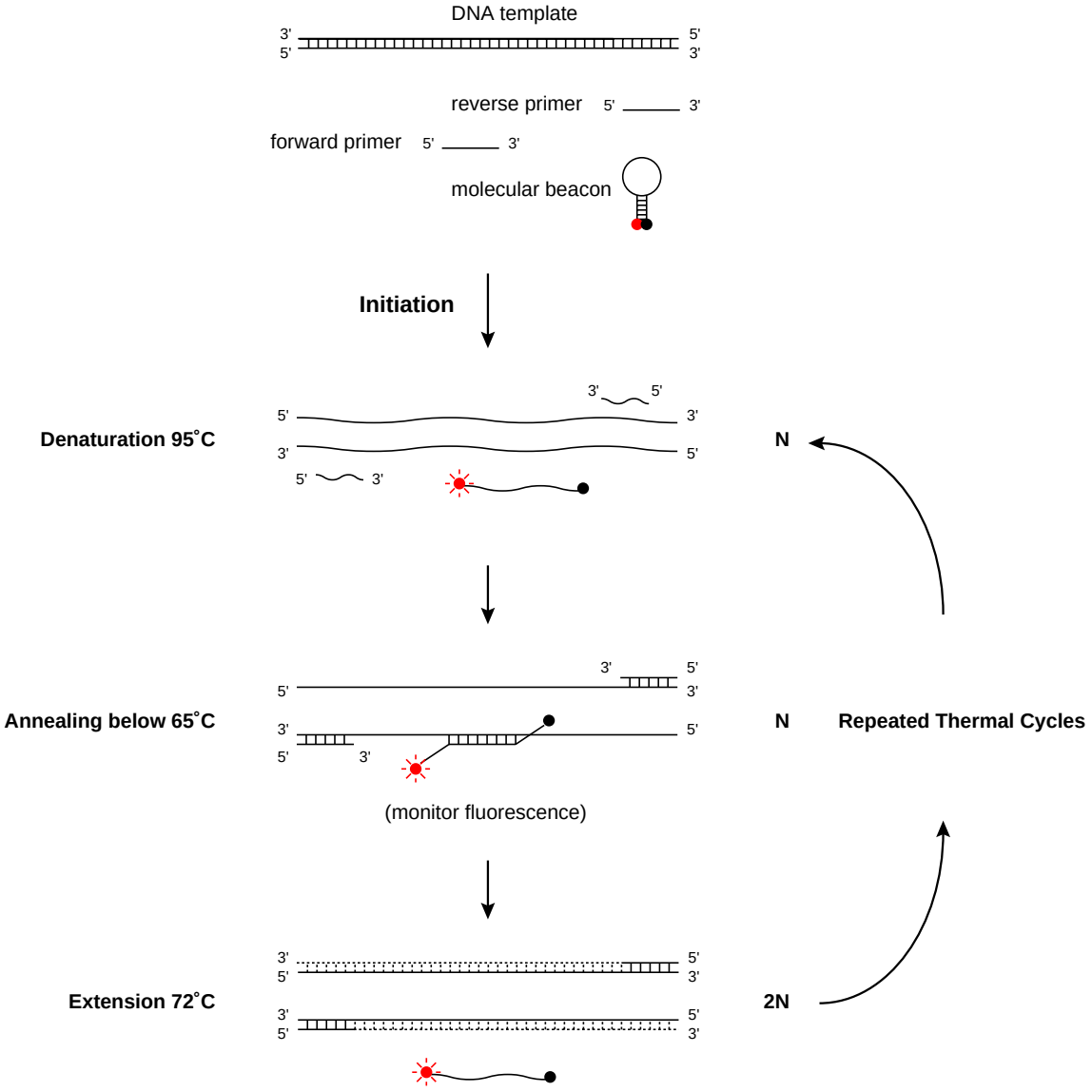

Fig. 3. Schematic representation of the hybridization of molecular beacons to amplification products during polymerase chain reactions.

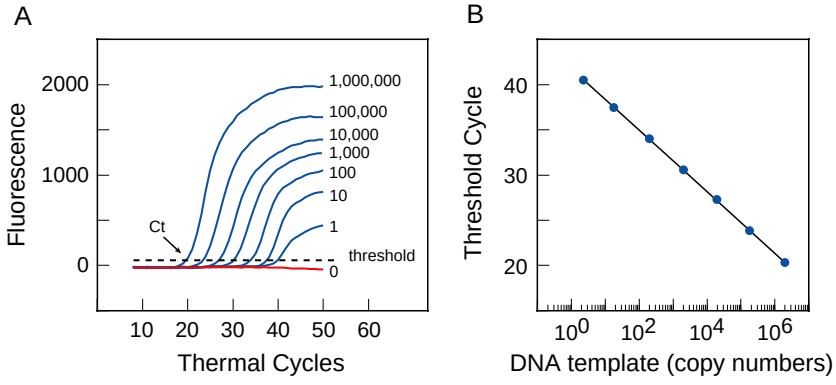

Fig. 4. Real-time measurement of amplification products during polymerase chain reactions using molecular beacons. Seven reactions, each initiated with a different number of template molecules, were incubated simultaneously in a spectrofluorometric thermal cycler. The concentration of the amplicons present after each cycle of amplification was determined by measuring fluorescence during the last few seconds of the annealing step. The number of thermal cycles needed to generate a significant signal (the "threshold cycle") is inversely proportional to the logarithm of the number of target strands initially present in the sample.

A multiplex PCR assay was developed that uses four differently colored, mismatch-tolerant, molecular beacons for the simultaneous detection of amplicons generated from unique sequences found in four different pathogenic retroviruses. The assay contained four compatible sets of PCR primers that are specific for the *gag* gene of HIV-1, the *env* gene of HIV-2, the *tax* gene of HTLV-I, and the *pol* gene of HTLV-II. Each of the four molecular beacons was designed to hybridize to a more or less conserved sequence within one of the four amplicons. To allow the molecular beacons to form stable hybrids, even if there are polymorphisms in the target sequences, the probe sequence in each molecular beacon was increased in length. The HIV-1, fluorescein-labeled, molecular beacon probe was designed to detect HIV-1 subtypes A, B, C, D, F, and G. The HIV-2, tetrachlorofluorescein-labeled, molecular beacon probe was designed to detect HIV-2 subtypes A, D, and SD. The HTLV-I, tetramethylrhodamine-labeled, molecular beacon probe was designed to detect all HTLV-I subtypes; and the HTLV-II, rhodamine-labeled, molecular probe was designed to detect HTLV-II subtypes A and B. Fig. 5 shows that the individual retroviruses could be distinguished from one another in a multiplex format. Four reactions carried out in parallel were initiated with 100,000 molecules of one of the four retroviral DNAs. Each reaction contained all four molecular beacons and all four primer pairs (one pair for each retrovirus). The only significant fluorescence that appeared in the course of the amplification reactions carried out in each assay tube was fluorescence from the molecular beacon that was complementary to the sequence of the retroviral DNA that was originally added to the assay mixture. No significant fluorescence developed in a control assay that did not contain any template DNA. These results demonstrated that each molecular beacon is specific for its intended target amplicon.

Molecular beacons can be designed that are so specific that they only form hybrids with target sequences that are perfectly complementary to the probe sequence. The presence of even a single nucleotide substitution in the target prevents hybridization. Consequently, these molecular beacons can discriminate genetic alleles and can identify the presence of single nucleotide polymorphisms. The key to designing allele-discriminating molecular beacons is to utilize a short embedded probe sequence that is just long enough to form a perfectly complementary hybrid, yet is too short to form a stable hybrid containing a mismatched basepair. Because of their hairpin structure, allele-discriminating molecular beacons are significantly more specific than corresponding conventional linear probes [33]. The rules for designing allele-discriminating molecular beacons for PCR assays are very simple [34]. The length and sequence of the probe (without the arm sequences) should be chosen to be long enough (and strong enough) to form perfectly complementary probe–target hybrids at the PCR annealing temperature, yet be short enough (and weak enough) for the probe–target hybrids to dissociate at a temperature that is 7–8 °C higher than the annealing temperature. Similarly, the length and sequence of the arms should be chosen to be long enough (and strong enough) to form hairpin stems (in the absence of targets) at the annealing temperature, yet be short enough (and weak enough) for the hairpin stems to dissociate at a temperature that is 7–8 °C higher than the annealing temperature. When molecular beacons are designed in this manner and are observed at the PCR annealing temperature, unbound probes are dark, perfectly complementary probes form brightly fluorescent probe–target hybrids, and mismatched probes remain unbound and dark.

The following experiment illustrates the extraordinary specificity of allele-discriminating molecular beacons [35]. Four template DNAs were prepared that had identical nucleotide sequences, except for one position, which was either an adenosine, a cytidine, a guanosine, or a thymidine. Four molecular beacons were prepared, each perfectly complementary to one of the four template DNAs in the region where the variable nucleotide was located, and each labeled with a differently colored fluorophore. The length of the probe sequence within each of these molecular beacons was chosen to be as short as possible. Four PCR assays were then prepared, each of which contained all four molecular beacons, and each of which contained only one of the four template DNAs. The results obtained from these assays are

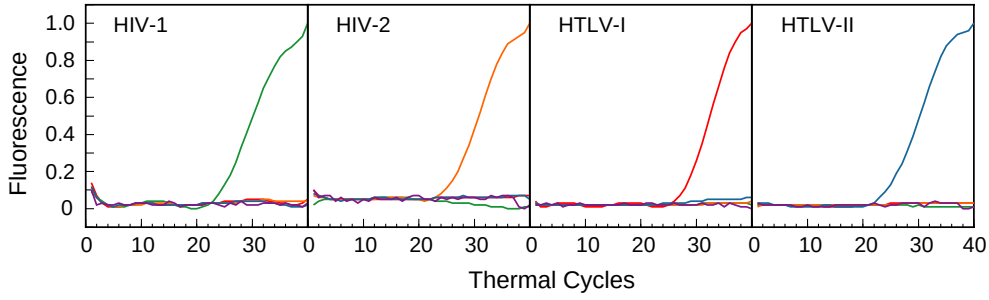

Fig. 5. Real-time detection of retroviral DNAs in a multiplex format. Fluorescence from HIV-1-specific molecular beacons is plotted in green; fluorescence from HIV-2-specific molecular beacons is plotted in orange; fluorescence from HTLV-I-specific molecular beacons is plotted in red; and fluorescence from HTLV-II-specific molecular beacons is plotted in blue.

shown in Fig. 6. In each case, only the perfectly complementary molecular beacon bound to the amplicons. The other three molecular beacons in each tube did not bind to the amplicons and did not fluoresce. Thus, the color of the fluorescence signal reliably identified which nucleotide was present at the variable position in the target sequence. These results demonstrated that molecular beacons can distinguish sequence differences as small as a single nucleotide substitution. Fig. 7 shows the results of an experiment that illustrates how well-designed, allele-discriminating molecular beacons behave. The fluorescence of a solution containing one of the molecular beacons used in the experiment shown in Fig. 6 was measured as a function of temperature in a spectrofluorometer. In the absence of targets, at low temperatures, the molecular beacons were dark. However, as the temperature was slowly raised, the hairpin stems of more and more of the molecular beacons dissociated, separating their fluorophore from their quencher, resulting in an increase in fluorescence. The stems of half of the molecular beacons were dissociated by 65 °C. In the presence of an excess of a perfectly complementary target at low temperatures, the molecular beacons formed probe–target hybrids and fluoresced brightly. However, as the temperature was raised, the probe–target hybrids became less and less stable, and eventually dissociated, resulting in the return of the molecular beacons to their darkened state. Half of the perfectly complementary probe–target hybrids were dissociated by 55 °C. Similarly, in the presence of an excess of targets possessing a single nucleotide substitutions, the molecular beacons formed brightly fluorescent probe–target hybrids. However, as the temperature was raised, the mismatched probe–target hybrids dissociated at a significantly lower temperature (approximately 45 °C) than the perfectly complementary probe–target hybrids. Consequently, at the annealing temperature of the PCR assay for which this molecular beacon was designed (50 °C) unbound probes are dark, perfectly complementary probes form brightly fluorescent probe–target hybrids, and mismatched probes remain unbound and dark.

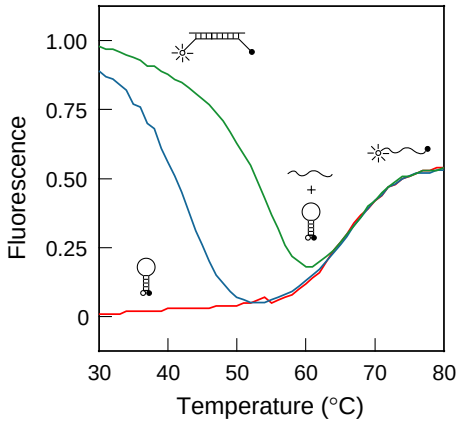

Fig. 7. Characterization of an allele-specific molecular beacon in the presence of either wild-type target (green line), mutant target (blue line), or no target (red line). The state of the molecular beacons at different temperatures indicated by diagrams over the thermal denaturation profiles. The melting temperature of the mismatched hybrid was 10 °C below the melting temperature of the perfectly matched hybrid. Optimal discrimination was achieved at the center of this temperature range. Therefore, in this example 50 °C was chosen to be the annealing temperature of the PCR assay.

Simple, robust and high-throughput molecular beacon-based real-time PCR assays for the detection of infectious agents [36,37], for genetic screening [38], and for mRNA quantitation [39] have been developed. Molecular beacons are able to monitor the progress of any amplification reaction where either single-stranded or double-stranded nucleic acids are formed. They do not interfere with the activity of the DNA or RNA polymerases used. Real-time monitoring of the synthesis of DNA or RNA sequences has also been reported for NASBA [40], rolling circle amplification [41], and isothermal ramification amplification [42]. Another area in which the darkness of unbound molecular beacons is particularly useful is for the observation of specific mRNAs in living cells and in developing embryos, where removal of unbound probes cannot be accomplished without killing the cells [43,44]. Other studies use molecular beacons to measure enzymatic activities [45–49], duplex and triplex

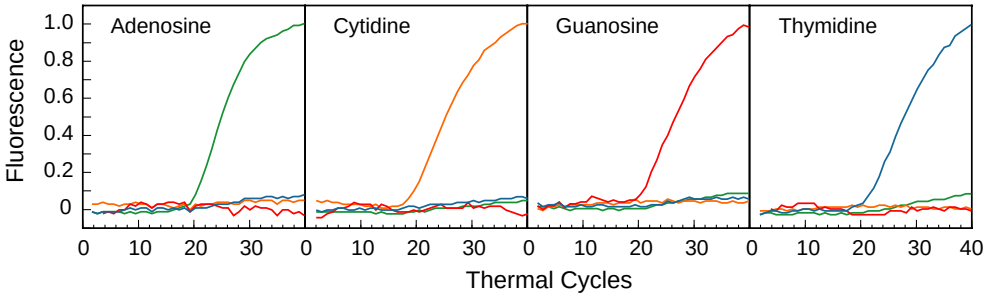

Fig. 6. Multiplex detection of single nucleotide variations in real-time PCR assays. Four differently colored, allele-discriminating molecular beacons were present in each reaction. Fluorescence from the molecular beacons that were perfectly complementary to the target containing an adenosine in the variable position is plotted in green; fluorescence from the corresponding "cytidine-specific" probes is plotted in orange; fluorescence from the "guanosine-specific" probes is plotted in red; and fluorescence from the "thymidine-specific" probes is plotted in blue. The color of the fluorescence that developed in each reaction identified the variant nucleotide that was present in the template DNA. No fluorescence developed in a control reaction that did contain template DNA.

formation in nucleic acids [50–52], and interactions between proteins and nucleic acids [53–56]. Recently, aptamer molecular beacons have been developed for the specific detection of proteins [57,58]. Molecular beacons are also being explored for use as immobilized probes on self-reporting DNA arrays and DNA biosensors [59–62].

6. Other detection methods

There are several other methods that have been developed to detect amplified targets in sealed tubes in real time. Fig. 1 presents a schematic overview of these methods.

6.1. Adjacent probes

Based on the earlier work of Cardullo et al., "adjacent probe" assays utilize two oligonucleotides that bind to neighboring sites on a target [63] (Fig. 1B). One probe is labeled with a fluorescent donor moiety at its 3′ end, and the other probe is labeled with a fluorescent acceptor moiety at its 5′ end. The distance between the two probes, once they are hybridized, is chosen such that efficient fluorescence resonance energy transfer (FRET) can take place from the donor to the acceptor. In this non-radiative process, a photon from one molecule, the "donor", raises the energy state of an electron in another molecule, the "acceptor", to higher vibrational levels of the excited singlet state. As a result, the energy level of the donor molecule returns to the ground state without emitting fluorescence and the acceptor fluorophore will emit fluorescence in its own specific color. This mechanism is dependent on the dipole orientations of the molecules and is limited by the distance between the donor and the acceptor molecule. Typical effective distances between the donor and acceptor molecules are in the 10 to 100 Å range [64]. This is roughly the distance between 3 and 30 nucleotides in the double helix of a DNA molecule. Another requirement is that the fluorescence emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Therefore, no energy transfer should occur when the two probes are apart from each other and are free in solution. Upon hybridization of the oligonucleotides, energy transfer is measured by the decrease in donor fluorescence or the increase in acceptor fluorescence. Wittwer et al. showed that the use of adjacent probes could be combined with rapid-cycle DNA amplification [65]. The use of adjacent probes has also been demonstrated for the detection of specific mRNAs in living cells [66]. A benefit of this approach is higher specificity, as two probes, rather than one, must bind to the target in order to generate a change in the fluorescence signal.

6.2. 5′-Nuclease probes

Utilizing the inherent 5′ to 3′ nuclease activity of *Taq* DNA polymerase, Pamela Holland and her colleagues described a method in which linear DNA probes, called "5′-nuclease probes" (also known as "TaqMan probes"), are cleaved upon specific hybridization to accumulating PCR products [67]. In order to utilize these probes for the real-time monitoring of amplification reactions, Kenneth Livak and his colleagues labeled the probes with a donor fluorophore on one end and an acceptor fluorophore on the other end that interact with each other by FRET [68] (Fig. 1C). Probes that are free in solution transfer energy from the donor fluorophore to the acceptor fluorophore, resulting in a low fluorescence signal from the donor fluorophore. The probe is designed to hybridize at the same time as the PCR primer to one strand of a DNA molecule. When *Taq* DNA polymerase extends the primer, the polymerase encounters the probe, and as a result of its 5′-nuclease activity, it cleaves the probe. Cleavage of the probe results in the separation of the donor and acceptor from each other, leading to an increase in the intensity of the fluorescence signal from the donor fluorophore. With each cycle of amplification, additional fluorophores are cleaved from their probes, and the increasing fluorescence intensity is monitored during the extension step of each PCR cycle. 5′-Nuclease assays are designed to amplify relatively short amplification products (75 to 150 basepairs); the primer melting temperature (T_m) is chosen to be between 58 and 60 °C; and the probe–target T_m is chosen to be between 65 and 67 °C. Using these guidelines, Livak showed that 2 allelic variants can be detected using the 5′-nuclease assay [69]. 5′-Nuclease probes have been widely employed for high-throughput amplification assays, utilizing a 96-well or 384-well thermal cycler that monitors fluorescence in real time. One of the first quantitative real-time RT-PCR assays to detect small copies of HCV RNA used 5′-nuclease probes [70]. Recently, in order to increase accuracy in single nucleotide polymorphism detection, a modified 5′-nuclease probe containing a minor groove binder was introduced to increase the difference in stability between perfectly complementary probe–target hybrids and mismatched probe–target hybrids [71].

6.3. Light-up probes

"Light-up probes" are short peptide nucleic acids (PNA) to which the cyanine dye thiazole orange is linked [72]. Thiazole orange fluorescence increases almost 50-fold upon hybridization to a complementary nucleic acid (Fig. 1D). This increase in fluorescence occurs when the rotation around the bond between the aromatic system of thiazole orange is restricted, which eliminates a way for non-radiative decay to occur [73]. A PNA molecule is a chemically modified nucleic acid in which the entire sugar-phosphate backbone is replaced by a polyamide structure [74]. The resulting polyamide contains all four natural bases, enabling it to hybridize to complementary oligonucleotide targets. PNA was used for light-up probes because of its good hybridization properties and because the binding of thiazole

orange to its linked nucleic acid sequence is minimal in PNA, reducing background fluorescence. Light-up probes, which are not fluorescent when they are free in solution, are useful for monitoring amplification reactions, as reported for real-time PCRs [75]. In addition, they can be used for the identification of specific mRNAs in living cells [76].

6.4. Duplex scorpion primers

In order to increase the speed of hybridization in amplification reactions, David Whitcombe et al. introduced "scorpion primers" [77], which later were modified and named "duplex scorpion primers" [78]. Duplex scorpion primers consist of a primer linked to a probe sequence, which has a fluorophore attached at its 5′ end. The probe sequence is annealed to a complementary oligonucleotide sequence with a quencher at its 3′ end. The probe sequence is designed to hybridize to a target sequence in the extension product of the primer. In a solution in which no target sequence is present, the fluorophore in the probe sequence and the quencher in the complementary sequence are in close proximity, resulting in static quenching of the fluorophore, and fluorescence is low. When the probe sequence hybridizes to its target sequence, a more stable probe–target hybrid is formed, forcing the probe sequence and the complementary sequence to unfold, separating the fluorophore from the quencher, thus restoring fluorescence (Fig. 1E). The primer sequence and the probe sequence are linked through a moiety that prevents the probe sequence from being copied by the DNA polymerase. Extension would lead to displacement of the complementary oligonucleotide possessing the quencher, resulting in an increase in fluorescence (for example in cases where unintended priming occurs, such as primer-dimer formation.) Since duplex scorpion primers depend on the extension of an oligonucleotide primer to generate a signal, they are most suited for monitoring real-time PCR assays [79].

6.5. Amplifluor primers

Nazarenko et al. introduced a method for the direct detection of amplification products. They describe labeled hairpin-primers, called "amplifluor primers," that are directly incorporated into amplification products [80] (Fig. 1F). In this method, a hairpin structure is present at the 5′ end of one of the oligonucleotide primers. The hairpin structure contains a fluorophore and quencher moiety in the hairpin stem. Hairpin-primers that are not incorporated into an amplification product do not generate a fluorescence signal, because the hairpin structure keeps the reporter and quencher in close proximity. During amplification, hairpin-primers are incorporated into the double-stranded amplification products. These primers generate a fluorescence signal because they are linearized by further amplification. When the primers are linearized, the fluorophore and quencher are no longer in close proximity, and fluorescence is restored. A disadvantage of this method is that hairpin-primers can generate "false" amplicons or primer-dimers, resulting in false-positive fluorescence signals. Recently, a multiplex real-time PCR assay, utilizing amplifluor primers, was reported [81]. Another report describes an end-point assay in which amplifluor primers were incorporated during rolling circle amplification [82]. Modified amplifluor primers are used to monitor the accumulation of products in a strand displacement assay [83].

6.6. Alternative fluorescent hybridization probe formats

In addition to probes described earlier, new types of hybridization probes are being introduced continuously. At this moment, they are not as widely used as the probes discussed above. Most of these nucleic acid probes are modifications of previously developed hybridization probes and are now being explored for their specific use in nucleic acid detection. For example, "HyBeacons" are single-stranded oligonucleotides that lack significant secondary structure, in which a fluorophore is quenched by an internal nucleotide. When the probe binds to a nucleic acid, its conformation changes, and as a result, the fluorescence intensity of the fluorophore increases [84]. Another method utilizes "Yin-yang probes," which are probes consisting of two complementary oligodeoxyribonucleotides of different lengths, one labeled with a fluorophore, and the other labeled with a quencher [85]. When hybridized to each other, the fluorophore and quencher are in close proximity, resulting in low fluorescence emission. In the presence of target nucleic acid, one of the probes hybridizes to the target nucleic acid, separating the two strands of the probe complex. As a consequence of this displacement, the fluorophore is separated from the quencher and fluorescence increases. Pseudocyclic oligodeoxyribonucleotides, or "Cyclicons", have been described as hybridization-based fluorescent probes [86]. Cyclicons consist of two oligonucleotides, linked at either their 5′ or their 3′ ends. One of the oligonucleotides is the probe sequence and the other oligonucleotide is complementary to one end of the probe sequence, forming a cyclic structure. A fluorophore is attached to one of the oligonucleotides and a quencher is attached to the other oligonucleotide. Free in solution, the cyclic structure keeps the fluorophore and quencher in close proximity. Once hybridized to a target nucleic acid, the fluorophore and quencher are separated, resulting in an increase of fluorescence. The probe sequence can also be designed to act as a primer to initiate an amplification reaction.

7. Future trends

With the development of automated methods for synthesizing nucleic acid probes, it has become much easier and more efficient to obtain large quantities of hybridization probes. Together with new, simple, and relatively inexpen-

sive methods for labeling nucleic acid probes with non-radioactive labels, doors have been opened that enable nucleic acid probes to be used for research and development, as well as for clinical diagnostic applications. The introduction of fluorescence-based hybridization probes, in particular, enables the development of homogeneous hybridization assays. The advantages of homogenous hybridization are its speed and simplicity. The kinetics of hybridization are faster in homogenous solutions than on solid surfaces, and homogenous hybridization formats are less labor intensive, as changes in the fluorescence of the probe indicate the presence of the target, and there is no need to separate unbound probes from hybridized probes. This enables hybridization to be followed in real time, providing both qualitative results and an immediate quantitative determination of the amount of target nucleic acid that was initially present in a sample over a broad range of concentrations.

As a result of the efforts of the human genome project and the rise in demand for molecular diagnostic assays, the development and optimization of novel hybridization probes has focused on speed, reliability, and accuracy in the identification of nucleic acids. High sensitivity is required to provide early diagnosis of pathogenic infections in clinical samples. This can be achieved by either direct detection of nucleic acids, in living cells or by in situ hybridization, or by detection of nucleic acids during amplification of target DNA and RNA sequences. In order to develop high-throughput assays, it is desirable to perform multiplex assays, where more than one target nucleic acid can be identified in the same solution. The development of highly specific hybridization probes is required for the identification of single nucleotide polymorphisms (SNPs). Some SNP alleles are the actual DNA sequence variants that cause differences in gene function or regulation that directly contribute to disease processes. Most SNP alleles, however, are not directly related to diseases, but are useful as genetic markers. These genetic markers are valuable research tools in understanding genetic differences. For example, the number of SNPs that have been identified in the human genome is approaching 3 million, and this is more than sufficient to enable linkage and association studies to be carried out across the entire genome.

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