Advances in Nucleic Acid-Based Detection Methods†

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INTRODUCTION

Clinical microbiologists have traditionally been concerned with the isolation and identification of pathogenic organisms from humans. Conventional methods involve isolating the organism of interest in pure culture and performing predetermined biochemical or immunologic tests to identify it. In many respects, cultures and the adjunct methods used for identification are limited in sensitivity, specificity, or both. To improve test sensitivity, shorten detection times, and identify hard-to-culture microorganisms, immunoassays were developed. These immunoassays allowed both large and small laboratories to expand services to meet diagnostic requirements in a timely fashion. The outlook for even more sensitive, more specific, and more rapid testing is currently being founded in the recent advances in molecular biology methods.

Histologists, cytologists, and cytogeneticists have been concerned with the preparation and staining of various tissue sections for microscopic examination. The traditional stains have allowed differentiation of various cellular components. Over the years, special stains to improve the detection of numerous cellular components and infectious agents of interest have been developed. This area of laboratory practice was one of the first areas to benefit from the advances in molecular biology over the past decade.

Nucleic acid analysis has attracted much attention over the past few years and is now becoming available for clinical laboratory testing on a routine basis (105). The advantages of nucleic acid probes in identifying organisms without special or tedious isolation, detecting nonviable organisms, and detecting genetic mutations or alterations in other cellular material represent the promise of nucleic acid-based technology (105). This review focuses on some methods for detecting nucleic acid molecules and presents some newer and more novel methods that are being developed for possible introduction into the clinical laboratory.

THEORY OF NUCLEIC ACID-BASED DETECTION METHODS

Principles of Nucleic Acid Hybridization

In 1953, Watson and Crick described the structure of DNA and the role of the DNA molecule in holding the information of cell reproduction and function (100). DNA is composed of four repeating nucleotides (sometimes called nucleotide bases or simply bases): adenine, guanine, cytosine, and thymine. DNA is coiled to form a double helix (double-stranded DNA [dsDNA]) composed of two strands held together by hydrogen bonds that can be broken by heat or high pH. The single strands of DNA (ssDNA) are relatively stable, but on removal of the heat source or pH extreme, the DNA molecule will re-form (reanneal) into the double-stranded configuration. When the ssDNAs are from different sources, the reannealing process is called hybridization. The realigning of the dsDNA is possible because nucleotide bases will re-form hydrogen bonds only with specific com-

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plimentary bases; adenine pairs with thymine, and cytosine pairs with guanine. In RNA, the nucleotide base uracil replaces thymine and pairs with adenine. The stability of the hybridization depends on the nucleotide sequences of both strands. A perfect match in the sequence of nucleotides produces a very stable dsDNA, whereas one or more base mismatches impart increasing instability that can lead to weak hybridization of strands. The same basic principles apply to RNAs, the transcriptional products of cell growth and reproduction. RNA-RNA and RNA-DNA hybridizations can occur.

Characteristics of Probe and Target Nucleic Acids

**Probe form.** A probe is normally a short sequence of nucleotide bases that will bind to specific regions of a target sequence of nucleotides. The degree of homology between target and probe results in stable hybridization. In developing a probe, a sequence of nucleotides must be identified, isolated, reproduced in sufficient quantity, and tagged with a compound (label) that can be detected (89). Most often, a DNA sequence is cloned (inserted) into a host organism that will reproduce the sequence that becomes the probe. Another method of producing probe sequences is by manufacturing a synthetic oligonucleotide, which usually consists of a short piece of nucleic acid composed of 15 to 30 nucleotides. The ability to produce demand a synthetic piece of nucleic acid that will function as a probe has allowed the economical commercial development of many probe systems. Although manufactured probes require a detailed knowledge of the target sequence to produce a complementary DNA molecule that will hybridize specifically, their advantage (single strandedness, noncomplementarity) favor their use over cloned probes. It is beyond the scope of this paper to discuss genetics and DNA cloning or synthesis, and reviews by Haas (35), Hilborne and Grody (37), Mulcahy (69), and Tenover (88) are recommended.

An ideal probe is single-stranded nucleic acid that can hybridize to the target of concern. The probe is composed of either DNA or RNA, with DNA probes being more common (38, 99). In general, DNA and RNA probes behave in the same manner; therefore, references to probes in the following discussion include both DNA and RNA probes. The suitability of a probe for microbial detection is based on whether a single strain or a broad family of organisms of interest and whether the probe will detect other organisms that are present (28, 76). Berry and Peter (7) adroitly summarized the basic principles of hybridization and the application of probes: “Hence, the whole nature of an organism is ultimately related to the sequence of nucleotide bases in its DNA. Not only can an organism be identified by its DNA sequence, but even minor differences between similar organisms are detectable if the areas of nucleotide probe sequence responsible for these differences can be observed. This is exactly what a hybridization probe does.”

**Probe size.** Probes can range in size from as short as 10 nucleotide bases (molecular weight of 3,300) to as long as 10,000 bases or more (molecular weight of 3,300,000) (30, 37, 81). The most common size range for most probes is between 141 and 140 bases, whereas 29 is a more statistical number of target nucleic acids. The choice of targets for probes should be of interest to clinical laboratory personnel in evaluating probe kits for possible use in their laboratory. Probes can be designed to detect genera, species, or, sometimes, strains of various organisms, depending on the expected target of hybridization. Another type of probe detects a conserved gene (one that is the same in several genera or
species) or a portion of it. Conserved regions of genes usually include portions of rRNA that represent a broad group or family of organisms. Even if the probe is targeted to a portion of nucleotide sequence that is present in microorganisms other than the one of interest, the probe will still be sufficiently specific to be useful if the cells not of interest are usually absent from the sample. The use of rRNA nucleotide sequences, such as one for members of the family Enterobacteriaceae, would be very useful in screening clinical specimens before genus-specific probes are used to identify the exact pathogen (11). Targets in cellular DNA are the most specific because they contain unique regions. However, targets in RNA are also used because there are many more copies of RNA than DNA per cell, which increases the sensitivity of detection. Although mRNA is a possible target source, it is rarely used owing to the often rapid turnover of the molecule in the cell (101). However, this disadvantage of mRNA targets can be an advantage when it is important to distinguish between dormant and actively growing cells. rRNA is the most useful probe target in a screening assay. The 16S and 23S ribosomal genes are very useful for detecting taxonomic because of the highly conserved nature of the genes (47, 48). rRNA is more highly conserved than mRNA, and many more copies of rRNA than DNA exist in each cell. E. coli, for instance, contains about 10,000 ribosomes per cell compared with only one or at most four copies of genomic DNA during active growth. Thus, the detection of rRNA rather than DNA increases the sensitivity 1,000-fold or more (50). Other targets must be used for viruses, because viruses lack rRNA. Plasmid DNA (DNA that exists as extrachromosomal elements) is an effective target in some microorganisms. The limitation of plasmid DNA, however, is that plasmids are transferred between some organisms and may be present in nontarget strains or lost from the cell altogether. Be it RNA or DNA, the best target is a sequence of nucleotides (e.g., virulence factors, toxin genes) not present in nontarget cells (28).

HYBRIDIZATION STRATEGIES

Like immunoassays, probes can be used in several different formats. These formats are generally classified into those that employ a solid phase and those that use a liquid phase for the hybridization reaction. An easy-to-use format is a large factor in determining the commercial success of various manufacturers` probe systems. This section describes various formats that are available.

Filter Hybridizations

Solid-phase, or filter, hybridization is usually in the form of dot or colony blots (32). The basic procedure for filter hybridization is shown in Fig. 1. This format is the oldest and most often used in the research laboratory, but it is subject to more nonspecific background interference than some of the other hybridization formats. The purpose here is to remove probe that is not specifically bound to the solid phase and reduce background, methods to remove probe that is not specifically bound to the solid phase are being developed. The sandwich hybridization assay (80) employs a three-component system consisting of a solid-phase bound capture probe, the target probe, and a separate signal-generating probe (Fig. 2). The target probe acts as the link to the solid-phase and signal-generating probes. If the target probe is not present, the signal-generating probe does not bind to the solid phase, and on washing, no signal is generated; background interference is minimized.

One variation of the solid-phase format is the strand displacement assay (25, 96, 99). In this format (Fig. 3), the capture probe is attached to a solid phase and has a short signal-bearing nucleic acid strand weakly hybridized to it. On addition of the target sequence, the target, through competition, displaces the single-bearing strand. In one application (96), the signal-bearing strand, which contained a polyadenylated signal, was detected by conversion to ATP, which was then detectable in a bioluminescence assay using luciferase. Although designed as a way of increasing sensitivity by decreasing background noise, this format does have shortcomings. The signal-bearing probe is not always displaced by the target DNA, and thus it can competitively block hybridization of the capture probe with the target. Further, the signal-bearing strands are continuously leached from the capture probe, and this can lead to either false-positive reactions or high background.

Reversible target capture is a solid-phase format that offers a distinct advantage over other formats in that it can be used with crude preparations without the generation of background noise that can lead to false-positive reactions (67, 68, 77). Magnetic microparticles are used to separate the reactants from the cell lysates. The magnetic particles contain an oligonucleotide composed of a single nucleotide such as poly(dA). This nucleotide will bind strongly to its complementary nucleotide, poly(dT). If a probe has a poly(dT) tail, the tail will be captured by the poly(dA) on the magnetic particle. The magnetic particle is removed from solution and washed free of contaminating materials. Chemical or thermal elution of the dA-dT pair and its recapture on fresh microparticles a few times leads to an exceedingly clean solid phase and increased sensitivity (67, 68, 77).

A variation on the reversible target capture format is the dipstick format (46, 77) (Fig. 4). As in reversible target capture, the probe has a long stretch of a single nucleotide and the dipstick has been coated with the complementary nucleotide. Because a second probe that has been labeled with a detectable ligand is used, this format becomes very versatile both in cleaning up the reactants and in allowing detection. In one commercial application, fluorescein is the label on the probe. It is detected colorimetrically with a horseradish peroxidase-labeled antifluorescein antibody and hydrogen peroxide-tetramethyl benzidine substrate-chromogen (46).

Solution or Liquid Hybridizations

Solution hybridization is more rapid (5 to 10 times faster) than hybridization on solid supports, but unless the assay is a homogeneous assay, a separation step is required before final detection (89, 99). In addition, accelerators can be added to increase the rate of hybridization even further; these are removed later to prevent background interference. Several separation steps, including the dipstick assay mentioned above, can be used.

Hydroxyapatite binds only dsDNA. This means that ssDNA must be hybridized either with a probe or with a complementary strand before it can bind to hydroxyapatite. When a solution of DNA is passed through a hydroxyapatite column, only the dsDNA is retained. The hybridization mixture can be washed to remove nonhybridized components (background noise) before being eluted from the column and before the label is activated.

Methidium, a nucleic acid intercalator dye that is coupled
NUCLEIC ACID-BASED DETECTION METHODS

ISOLATE CELLS ON A SOLID SUPPORT

CONVERT dsDNA TO ssDNA AND BIND TO SOLID SUPPORT

HYBRIDIZE PROBE TO TARGET

DISRUPT CELLS TO OBTAIN dsDNA

ADD Labeled PROBE

DETECT PROBE'S SIGNAL

FIG. 1. Basic steps in a DNA probe hybridization assay. The sample containing cells is captured (or blotted) on a solid support such as a nitrocellulose filter disk. The cells are then lysed to release the DNA, which binds to the solid support. Usually the filter is washed, and unreacted sites on the filter are blocked. A labeled probe is then added and allowed to hybridize to the sample DNA. Unbound probe is washed away, and the hybridized probe is detected.

to Sepharose beads, has also been used to separate DNA from solutions (5). After the DNA has been chemically released from the sample, the methidium binds the DNA to the Sepharose beads, and contaminants can be removed by washing. The DNA is then released from the dye with a dilute base and collected.

A homogeneous hybridization assay format that recently became commercially available is based on the protection of a phenol ester of an acridinium ester chemiluminescent probe label (1, 71). In this assay, if the probe is hybridized to the target nucleic acid, the phenyl ester is protected from hydrolysis. However, unhybridized probe does not protect the acridinium ester from hydrolysis, resulting in loss of chemiluminescence when the unhybridized probe label is released. The hybridization protection assay format permits the use of large concentrations of probe without background problems (1). The resulting hybridization reactions are complete within 10 min and have a sensitivity of $10^{-16}$ to $10^{-21}$ mol of nucleic acid (1).

AMPLIFICATION METHODS

Amplification of very minute quantities of nucleic acids before hybridization assays are performed holds great promise for successful commercialization of DNA probes for use in clinical and anatomical laboratories. Most infectious agents or cellular materials of interest are available only in limited quantities unless they are first cultured. Some infec-
Protection signal probes in nucleic acid. To perceive limited amounts of nucleic acids with DNA probes without cell cultivation, some method of enhancing the detection is required. With the rapidly emerging methods of amplifying nucleic acids, the future use of DNA probes in clinical and anatomical laboratories is almost assured.

Probe assays can be amplified by either of two general strategies or a combination of both. The target nucleic acid sequence can be amplified, thus increasing the amount of target nucleic acid available for detection. Alternatively, the probe can be equipped with an amplification system that will allow a very small hybridization reaction to generate a detectable signal.

Polymerase Chain Reaction

In 1983, a conceptually simple process of amplifying, or increasing, the number of specific nucleic acid fragments present in a sample was developed by Mullis (70). In 1989, this process was deemed the "major scientific development" of the year (34). This process is termed polymerase chain reaction (PCR). In just a few years and after some refinements, PCR has become the most frequently used method for amplifying nucleic acids, especially DNA. The PCR technique is based on the reiteration of a three-step process (Fig. 5): denaturing dsDNA into single strands, annealing primers (specific synthetic oligonucleotides) to the ssDNA, and enzymatically extending the primers complementary to the ssDNA templates. After the primers are annealed to the denatured DNA, the ssDNA segment becomes the template for the extension reaction. Nucleotides, present in the solution in excess, are enzymatically joined to form the cDNA sequences. During the second and subsequent cycles, the original DNA segment and the newly generated cDNA strands become templates. Each cycle of PCR therefore doubles the amount of specific DNA present. A typical amplification is 20 to 40 cycles and results in a 10^6-fold amplification of the original DNA (77, 94). The improvement of PCR by the use of a thermostable enzyme, Taq DNA polymerase, has allowed semiautomation and simplification of the process to the point that it has become widely adopted in research laboratories (26, 40).

PCR does have some shortcomings. The system is susceptible to contamination with extraneous DNA fragments that could be amplified along with the sample. This is important in that extraneous DNA can be carried over from previous amplifications (amplicons) or introduced from other sources. To eliminate this problem, adherence to careful laboratory procedures, such as rigid quality control of enzyme preparations and the use of dedicated pipettes and pre aliquoted reagents, is necessary. Various other techniques for reducing extraneous DNA contamination of PCR products have been described (17, 21, 22, 47, 54, 75, 79, 82, 83, 85).
Methods to eliminate or modify the amplicons so that they do not contaminate subsequent PCR amplifications (sterilization) are also being developed (27, 78). In one method (18), the sterilization process is the result of treating the PCR amplicons with 4'-aminomethyl-4,5'-dimethylisopsoralen (4'-AMDMIP), an isopsoralen derivative that reacts with pyrimidine to form a cyclobutane ring when photoactivated with 320- to 400-nm light. The cross-links formed effectively block the polymerase extension reactions. However, the process must be optimized for each reaction on the basis of the length and sequence of the amplicon and on the amount of carryover tolerable. Another method uses the enzyme uracil DNA glycosylate (62). Uracil DNA glycosylate removes the uracil base from the sugar-phosphate backbone of ssDNA or dsDNA. The resulting apyrimidinic site cannot be replicated by polymerase. The result is that either a DNA primer that was synthesized with uracil in place of thymidine or an amplicon that was generated with uracil in place of thymidine can be sterilized by the addition of uracil DNA glycosylate. Normal PCR temperature processing inactivates uracil DNA glycosylate. One of the simplest methods for sterilization of PCR reactions is the use of UV radiation (75, 83). Use of UV light to dimerize extraneous or leftover DNA, together with a dedicated work area and appropriate PCR handling techniques (54), normally reduces the contaminating DNA to a level that can be tolerated. Cimino et al. (17), however, recommend caution in relying on UV radiation to completely solve the contamination problem.

PCR requires much technical skill and some special laboratory equipment. Although PCR has been semiautomated, the technique still requires a significant amount of labor for sample preparation and subsequent detection of DNA. The
Ligase Amplification Reaction

The ligase amplification reaction (104), also called the ligase chain reaction (LCR is the trade name) (4, 56, 94, 103) or the oligonucleotide ligation assay (27, 73), is another technique for detecting or amplifying a target sequence. Unlike PCR (which creates new DNA molecules from individual nucleotides), the ligase reaction uses a thermostable enzyme (2, 4) to join two oligonucleotides that are immediately adjacent to each other (Fig. 6). Like PCR, this reaction is cyclic; denaturation, annealing, and ligation are the basis for the amplification reaction. As in PCR, the ligated oligonucleotide pairs, along with the original sequence, become templates for the next cycle (104). From 20 to 30 cycles of this reaction yield a 10^6-fold increase in the original target. The method is currently able to detect as few as 10 nucleic acid targets (2).

In the ligase reaction, the entire target sequence must be known, because a 1-bp mismatch at the point of ligation can prevent ligation of the oligonucleotides. Although this could be detrimental in many situations, failure to ligate can be used to detect point mutations in the target sequence. Barany used this approach to differentiate normal and sickle β-globulins in blood samples from patients with sickle cell
anemia (4). As with PCR, a thermostable ligase is necessary for the cyclic amplification reaction. However, the ligase reaction appears better suited than PCR for diagnostic work. Whereas PCR generates new DNA or RNA molecules from nucleotides, in many situations the newly generated molecules may still require some form of verification or identification to determine the exact specificity of the DNA. The ligase reaction makes use of known sequences for the sole purpose of detection; the probes must hybridize to adjacent sites in order to be ligated together and detected.

Bond et al. described the use of LCR to detect human papillomavirus (10). In their approach, both a separation step and a signal detection mechanism are incorporated into the oligonucleotide probe.

Another variety of ligase reaction involves the use of both a polymerase and a ligase (8). Two oligonucleotide primers are annealed to the target but are spaced so that polymerase fills a gap between the two primers; the ligase then connects the filled gap and the second primer. This modification, called gapped-LCR (G-LCR is the trade name), has a European patent pending.

In addition to Barany's detection of the mutation that causes sickle cell hemoglobin (4), the ligase amplification reaction has been used to diagnose the mutation responsible for cystic fibrosis (73), for detection of gene segments in the human T-cell receptor β-chain locus (73), and for detection of human papillomavirus (10).

The final development and marketing of ligase reactions may depend on rulings by the U.S. Patent Office and the courts on conflicts between various patent claims (102).

Transcription-Based Amplification Systems

In 1989, Kwoh et al. (53) described another technique for nucleic acid amplification, a transcription-based amplification system (TAS). TAS is a two-cycle scheme based on DNA synthesis and RNA transcription. In this procedure, denatured DNA or a strand of RNA is the target for an oligonucleotide primer that contains a polymerase-binding site. After hybridization of the primer to the target, a reverse transcriptase elongates the primer complementary to the target sequence. After heat denaturation, another oligonucleotide primer is annealed to the new DNA strand. Reverse transcriptase is again added to produce a dsDNA molecule.

FIG. 6. Ligase amplification of a target by use of two oligonucleotide probes. Two probes that hybridize adjacent to each other on a target strand are obtained and ligated with T4 DNA ligase into a longer sequence. The ligated probes can also function as a template for further hybridizations and thereby amplify the original target DNA in a cyclic reaction system. The ligase reaction has also been used to ligate two probes in a solid-phase extraction system to produce cleaner reactions. Modified and reprinted from the Journal of Food Protection (103a) with permission of the publisher.
By the addition of an RNA polymerase, multiple copies (10 to 1,000 per cycle) of RNA are produced. In this method, only four cycles of the reaction are necessary to obtain up to \(10^6\) copies.

Self-sustained sequence replication (3SR is the trade name) RNA transcription is a modification of TAS (31, 33). The 3SR reaction differs from the TAS in that 3SR is isothermal (37 to 42°C) and the RNA target is degraded (Fig. 7). The procedure also can be used to amplify DNA if an initial denaturing step is included. RNase H is used to degrade the RNA-DNA duplexes formed in the TAS reaction and allows for conversion to a dsDNA that has a polymerase-binding site on each end. The dsDNA continues the cycle by acting as template for the synthesis of RNA that reenters the reaction. A \(10^2\)-copy amplification occurs within 15 min; it would take PCR 85 min to amplify that much if the procedure were 100% efficient.

The advantages of 3SR over PCR are that no thermal cycling is required. Also, since the 3SR reaction is based in part on the transcription of RNA, multiple copies of RNA are transcribed in each cycle. This means that fewer cycles are needed for 3SR to generate the same amount of nucleic acid that PCR generates. In addition, 3SR can distinguish DNA and RNA targets. Like the other amplification methods, 3SR is subject to contamination by extraneous nucleic acids. It is also suffers from a low inherent specificity because the stringencies of the reactants are lower than with other amplification methods and because multiple enzymes are used.

Nucleic acid sequence-based amplification (NASBA is the trade name) is another commercial development of the isothermal TAS method (45, 63) and is very similar to 3SR (20, 94). In 1989, a European patent on NASBA was filed (94).
Six cycles of TAS have been shown to be capable of detecting one human immunodeficiency virus (HIV)-infected lymphocyte in a field of 10⁶ uninfected cells (30). Nucleic acid amplification with 3SR has been used to analyze and monitor 3'-azido-3'-deoxythymidine (zidovudine) drug resistance in HIV (29). Compton (20) reported that NASBA detected "as few as three HIV particles" in a 100-μl sample of human plasma, but a later report by Kievits et al. (45) reported that the limit of detection was 10 HIV particles or five infected cells.

Cycling Probe Reaction

The cycling probe reaction is almost the exact opposite of LCR. In this procedure, a nucleic acid probe that incorporates DNA-RNA-DNA sequences is synthesized (Fig. 8). After the probe is allowed to hybridize to the target, RNase H, an enzyme that specifically degrades the middle RNA portion of the probe, is added. The DNA fragments that remain dissociate from the target. The target is now free to hybridize with another probe molecule; therefore, the reaction is inherently cyclic without external manipulations. The free DNA fragments can then be detected or amplified by a secondary amplification system (23). This is a relatively new method, and further development is needed before it can be commercialized.

A similar amplification method is based on the use of restriction enzymes to cleave an oligonucleotide probe. In this technique a labeled oligonucleotide and a restriction enzyme are used to cut the labeled probe into a shorter product. With the use of a second complementary oligonucleotide probe, the reaction can become isothermally cyclic.

Qβ Replicase Amplification

Another method of nucleic acid amplification that is being commercially developed is known as Qβ replicase amplification (16, 52, 59, 94, 99). This amplification method is named after the major enzyme responsible for the amplification, the replicase of the Qβ bacteriophage. The enzyme is an RNA-directed RNA polymerase, meaning that it enzymatically assembles RNA from an RNA template. The Qβ enzyme comprises four subunits, of which one is from the Qβ bacteriophage and three are from the phage host E. coli (14, 60). This amplification system was first reported by Haruna and Spiegelman in 1965 (36). Later, an RNA molecule called midivariant 1 (MDV-1) was isolated. MDV-1 acts as the specific target for the Qβ replicase and allows the replicase to be used in nucleic acid amplifications (42). Following sequencing of the MDV-1 molecule (66), it was learned that a short nucleic acid sequence could be inserted into one of the complementary loops within the MDV-1 molecule without harming replication of the molecule (65). This modified MDV-1 could then be used as a hybridization probe (65).

Although both PCR and Qβ replicase amplify nucleic acids, the amplification of RNA serves as a reporter system for signal detection rather than as a method of increasing the amount of target nucleic acid as in PCR (Fig. 9). The increase in the amount of RNA can readily be detected by using an intercalating fluorescent dye such as ethidium bromide. In less than 15 min, Qβ replicase can amplify an MDV-1 RNA molecule into 10⁶ to 10⁹ RNA molecules (14, 48). One thousand RNA molecules can be amplified to 125 to 200 ng, a 10⁶-fold amplification! Because of the synthesis of multitudinous RNAs, hybridization reactions can be measured by simple colorimetric techniques (59). Quantitation of the reaction is possible because of the kinetics, which allow construction of a standard curve and determination of the initial concentration of bound complex (99).

Another innovation in the use of Qβ replicase amplification came with the development of reversible target capture (14, 68). A poly(dA)-tailed capture probe and magnetic
microparticle beads containing poly(dT) are used to wash the reaction mixture clean of any residual contaminating materials, thereby lowering background noise (14, 59, 61). The use of chaotropic salts also aids Qβ replicase amplification. The salts lyse cells, denature proteins (including nucleases), and liberate and linearize DNA without interfering in hybridization of the MDV-1 probe (61, 90).

Modifications to the use of MDV-1 RNA as a probe include adding a “molecular switch” to a region of the probe or using probes that are not replicable but can synthesize replicable reporter RNA (51, 60). A molecular switch could be used to help clean the reaction system and eliminate unbound probes that might cause a false-positive reaction. For example, the probe sequence could be incorporated into the RNase III-binding site on the MDV-1 molecule (Fig. 10). If the MDV-1 molecule is not hybridized, the RNase-binding site is intact and available for RNase destruction. If the MDV-1 molecule is hybridized, the probe sequence obliterates the complementary loop structure that forms the RNase III binding site. MDV-1 RNA can also be synthesized through the action of two separate probes that each contain a polymerase promoter sequence and a sequence that can serve as a primer for its own extension. In two separate hybridizations and extensions, a dsDNA promoter region that can, with the aid of a polymerase, transcribe a replicatable RNA is made. The replicatable RNA can then be amplified by Qβ replicase.

Although the tests are not commercially available, one company has used the Qβ replicase nucleic acid amplification to detect both HIV and cytomegalovirus (57). In detection of synthetic HIV target RNA, 600 molecules could be detected in approximately 2.5 h (57).

**Strand Displacement Amplification**

Another isothermal amplification method is based on the displacement of one probe when DNA polymerase is used to extend a second probe. In the original method (98), the target DNA underwent initial cleavage into 47-bp fragments and heat denaturation. A probe containing a HincII recognition site on the 5' end was hybridized to the target fragments. When it is bound to the target site, the probe possessing the HincII recognition site (6 nucleotides) and several more bases that do not hybridize to the target nucleic acid is extended past the end of the target fragments (Fig. 11). DNA polymerase then generates a nucleic acid strand complemen-
terminating to the target and free end of the probe, incorporating a modified dATP (dATPαS). The HincII enzyme then nicks the probe strand at the HincII recognition site (the hemi-phosphorothioate HincII recognition site prevents complete cleavage of the dsDNA). DNA polymerase then generates a nucleic acid strand that is complementary to the target nucleic acid strand and that displaces the original probe segment from the target fragment. Because of the orientation of the HincII recognition site, the complementary probe sequence generated by DNA polymerase retains a functional HincII recognition site. This allows continual nicking of the probe by HincII and subsequent displacement through the action of DNA polymerase.

To partially overcome background amplification of non-target DNA, the method was modified to eliminate initial target cleavage (97). Using four probes, the reaction proceeds as shown in Fig. 11. The simultaneous extension of all four primers displaces two probes that can act as targets for the opposite probes. The amplification then proceeds with the nicking of the probe HincII recognition site and displacement by the original method. The resulting action of DNA polymerase on the probes amplifies nucleic acid exponentially.

Strand displacement amplification has been used to amplify Mycobacterium tuberculosis genomic DNA to $10^7$ copies in 2 h (97). Like PCR, certain experimental parameters impose some limitations (97) on the procedure.

**FIG. 10.** Molecular switch. RNase III is an enzyme that destroys dsRNA. Incorporation of the probe sequence into the end of a complementary loop structure will cause the loop structure to become linear on hybridization. Loss of the RNase-binding site (the complementary double-stranded region) on hybridization will make the probe resistant to destruction. Probe sequences that are not bound become susceptible to destruction and prevent nonspecific signal generation.

**FIG. 11.** Strand displacement amplification. Strand displacement amplification is based on the displacement of an enzymatically extended DNA probe by the extension of another enzymatically extended DNA probe located upstream (5' to the first probe). With the incorporation of a restriction endonuclease site that can be nicked, creating a second probe system, the reaction becomes cyclic without the need for temperature changes after the initial denaturation step. Two probes hybridize each strand of the target sequence in the initial step. Both probes are enzymatically extended at the same time, with the upstream probe displacing the downstream probe. The displaced probe becomes the target for the opposite strand’s probe. The restriction site is oriented in such a way that the dsDNA is only nicked instead of cleaved. This nick allows the nick site to act as an extension site for polymerization of cDNA that displaces the downstream DNA. The displaced DNA becomes another target sequence. The creation of displaced strands generates the amplification effects of the method.

**SIGNAL AMPLIFICATION AND DETECTION METHODS**

Amplification of the detectable signal also improves the sensitivity of nucleic acid probes. A properly constructed probe system that hybridizes even one target can be detected if the signal is amplified sufficiently. Current probe assays cannot detect a single hybridization reaction, but the following methods increase the sensitivity of signal detection and, in some instances, provide very substantial signal amplification.

Several nonisotopic test detection systems that are being
developed are based on chemiluminescence. One system uses acridinium ester-labeled probes. The reaction is initiated by the addition of hydrogen peroxide (77, 87). Light is produced by decay of the ester as a result of the effect of hydrogen peroxide, and the amount of light is proportional to the amount of target nucleic acid present. This reaction is completed within seconds; however, a chemiluminometer is required to detect the small quantities of light emitted (77). Another chemiluminescent compound is phenylphosphate-substituted dioxetane (84). This compound undergoes conversion to a luminescent form in the presence of alkaline phosphatase; it is reportedly 10 times more sensitive than colorimetric detection with o-phenylenediamine or horseradish peroxidase (19). Chlorine- and bromine-substituted dioxetanes have also been described (12); these improve chemiluminescent detection by eliminating nonenzymatically activated chemiluminescence, and they have short enzyme activation times. Another approach being developed is the use of a photochemical sensitizer as a label (64). Oxygen transfer of light excitation molecules to olefin results in generation of a dioxetan that, when heated, emits light. By using filters, background noise can be reduced; this increases the sensitivity of the assay.

The generation of a chemiluminescent reaction at the surface of an electrode by means of an electrical and chemical excitation is termed electrochemiluminescence. This process provides another rapid (<25 min) and sensitive (200 fmol/liter) detection method for nucleic acids (9, 43). The commercially available label uses a Tris-ruthenium bipyridyl complex (9, 43). The Tris-ruthenium bipyridyl complex and a reductant such as tripropylamine undergo oxidation-reduction at the electrode surface, and a photon (detected at 620 nm) is emitted (9). The Tris-ruthenium bipyridyl complex can be regenerated to form the basis of a cyclic type of reaction that can amplify the signal (9). Instrumentation for detecting this reaction is reported to be simple, and the process can be automated (9).

Another nonisotopic method involves the use of enzyme-labeled antibodies. Labeled antibodies are targeted against a compound that is covalently linked to or incorporated into the probe. For example, probes with modified cytidine residues are labeled with fluorescein. High-affinity antifluo-

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**FIG. 12.** Branched DNA signal amplification. Signal amplification, based on the use of branched DNA oligonucleotides, involves target cell lysis and denaturation of the target nucleic acid, addition of the capture and label probes, solid-phase extraction and washing, addition of the branched DNA multimer, another washing step, addition and hybridization of an enzyme-labeled probe, and detection of the enzymatic signal.
resin antibodies are labeled with horseradish peroxidase and used to detect the probe (77). Instead of fluorescein, sulfonation of cytosine, introduction of 2-acetylaminofluorene groups into guanine and its derivatives, and incorporation of digoxigen-labeled dUTP into DNA have been used with enzyme- or fluorophore-conjugated antibodies (24, 44, 77, 89, 99).

A nucleic acid-based method of amplifying the detection signal involves the use of branched oligonucleotides (91, 93). Branched oligonucleotides that have a primary sequence conceived to directly or indirectly hybridize to a target nucleic acid have been synthesized (only the indirect method is shown in Fig. 12). The secondary sequences of the synthesized oligonucleotide serve as hybridization sites for an enzyme-labeled oligonucleotide. The primary and secondary sequences are covalently linked through branch points (39). Signal amplification results from the attachment of 60 to 300 enzyme molecules (92) to each nucleic acid target (Fig. 12). The method is dependent on solid-phase separation and washing to reduce background interference before the addition of the branched oligonucleotide. When this method was combined with the sensitivity of chemiluminescence, as few as 1,000 viral hepatitis genomes were detected directly in human serum or plasma (91). The method has also been applied to the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and penicillin and tetracycline resistance plasmids (92).

Although not a true signal amplification, probe-based signal generation is a unique method of generating a signal on the basis of nucleic acid hybridization. In probe-based signal generation (Fig. 13), a single-stranded probe is cut in half and each piece of the probe is labeled with one component of a two-component interacting fluorophore or enzyme system (15, 95). When the two separate probes hybridize beside each other, excited energy of the fluorophore is transferred to the now-adjacent acceptor fluorophore or enzyme system. The acceptor is activated, and a signal is created. A separation step is not required because without hybridization, the two components are not close enough to each other to generate a signal.

**AUTOMATION**

Several authors (3, 9, 38, 60, 64, 73) include the subject of automation in their discussions of the use of probes. Hybridization reactions definitely have the potential to be automated. At this time, except for one automated slide stainer that can be set up for in situ DNA hybridization, no completely automated systems are commercially available. Until automated systems become available, the use of routine nucleic acid amplification-detection methods in clinical laboratories outside of the academic or major medical center will be limited.

One method that holds promise for automation of DNA-based detection methods is based on a class of sensors called biosensors. A biosensor is an electronic-based device that generates a signal, either electrical or optical, when biological molecules bind to a sensor. These biological molecules can be carbohydrates, nucleic acids, or proteins such as enzymes, hormones, antigens, and antibodies. One application, for DNA hybridization detection, is based on surface plasmon resonance (86). This method is based on reduction in the intensity of reflected light and changes in refractive index that occur during hybridization of DNA. The system is sensitive enough to detect DNA hybridization on the surface of a sensor that is being illuminated with laser light and scanned with photodiode detectors. The system can detect 320 fg (3.2 x 10^-13 g) of a 97-bp molecule or 24 fg of a 7,200-bp DNA molecule (compared with 100 fg of DNA on a Southern blot).

Analogies between immunoassay development and progress in nucleic acid detection methods are appropriate. The first immunoassays were research laboratory methods that were subsequently transferred to clinical laboratories. Still, immunoassay automation probably has not peaked. The ultimate conception of an automated DNA probe analyzer would be like a “black box” that could be loaded with a clinical specimen and started and that would, in less than 1 h, indicate that each of multiple target organisms was or was not present. Whether or not this black box evolves as Lizardi and Kramer’s conception of a multiplex diagnostic assay (60) with a membrane strip that includes an arrangement of defined DNAs that bind amplified products, the future of automation of DNA probe methods holds much exciting promise.

**SUMMARY AND CONCLUSIONS**

Tenover (89) summarized the use of DNA probes as follows: “The goal of DNA probe technology is to eliminate routine cultures, whether they be bacterial, viral, or fungal.” This is a very ambitious statement but one that might someday be fulfilled if the technology continues to progress as it has. Currently, these methods are more suitable for research laboratories than for routine clinical laboratories. As patent disputes are resolved and the processes become more refined, automated, and standardized, nucleic acid
detection methods will displace or supplement current methods of rapid clinical laboratory diagnosis.

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