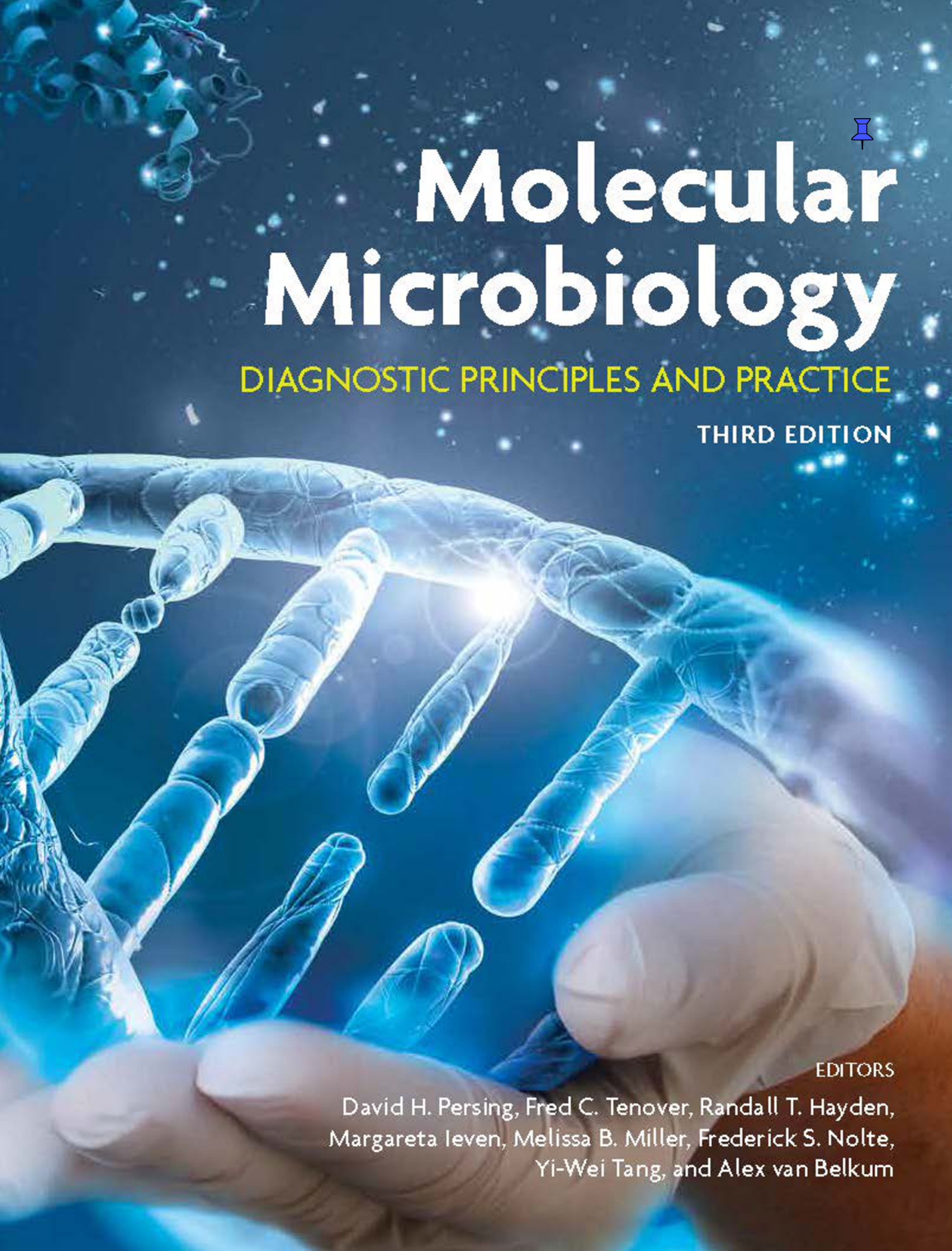
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DIAGNOSTIC PRINCIPLES AND PRACTICE
THIRD EDITION
EDITORS
David H. Persing, Fred C. Tenover, Randall T. Hayden,
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Preface

In the 5 years since the 2011 edition of this book, the molecular diagnostics landscape has changed dramatically. In the 1990s, molecular diagnostics was the domain of only a few reference laboratories; it took almost 20 years for these techniques to make their way into about half of the CLIA high-complexity laboratories in the United States. The full potential of this technology was slow to be realized largely because the methods used by these laboratories were not capable of delivering on-demand results or being conducted at the point of care. Over the past year, with the advent of CLIA-waived molecular testing spurred on by the inexorable force of innovation, molecular diagnostics have become increasingly democratized to the extent that physician office laboratories and sexual health clinics are now performing molecular testing on the premises, often delivering results in minutes or a few hours.

Laboratory professionals may at times find themselves a bit bewildered in this rapidly evolving landscape. Adding to this, enter next-generation sequencing (NGS) technology, as described in several chapters in this book (chapters 2, 3, 5, 6, 10–14, and 53). NGS-based analysis of microbial genomes and populations is in some ways similar to where PCR was in 1987: full of opportunities and challenges. For the first time, identification of the full range of pathogens—viruses, bacteria, fungi, and protozoa—can be addressed by using the same core technology. Microbial population analysis can be carried out at unprecedented depth, opening up the field of metagenomics (chapters 10–14). Whole-genome analysis goes beyond organism identification to predict drug resistance and detect pathogenic determinants. As diagnosticians, it seems likely that as this field evolves, so will our job descriptions. Still, much progress remains to be made before NGS can move beyond its current status as a research tool. NGS systems need to become more automated and less expensive to operate. The analysis of complex data sets provided by these systems needs to be simplified; the interpretation of results cannot require a PhD in bioinformatics for delivery of routine results. However, as complex as it is now, NGS too will eventually become democratized by the integration of workflow automation, improvements in sequencing technology, and information technology (IT).

Speaking of which, IT itself is about to play an increasing role in how and to whom our results are delivered (section X). A rapid molecular result is only as good as the downstream action taken in the treatment and management of patients. As we speak, patients in London, along with providers, are getting "push notifications" of results from their sexual health tests, resulting in a dramatically shortened time to therapy. Cloud-based aggregation of molecular test data is providing snapshots of emerging pathogens and drug resistance in real time by collecting de-identified test data directly from testing platforms. From the respiratory cloud to the digital cloud, we are watching the emergence of a new generation of global surveillance capabilities which will be of enormous public health benefit. Rapid detection technologies are also likely to evolve in the direction of on-demand multiplexing for simultaneous detection of treatment-informing targets. The convergence of rapid molecular assays with improvements in IT to deliver actionable information to health care providers is becoming a reality.

In 2015, the White House announced a $20 million prize for innovative diagnostic tests that will lead to more precise antimicrobial therapeutic decisions. In addition, the United Kingdom has announced the Longitude Prize, a challenge with a £10 million award for developing a point-of-care diagnostic test that also will identify when antibiotics are needed and which one to use. Thus, it seems that the importance of molecular diagnostic testing is finally being appreciated at the highest levels, especially to address the global problem of antimicrobial resistance. Let's not disappoint them.

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section I

Novel and Emerging Technologies

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Nucleic Acid Amplification Methods Overview

FREDERICK S. NOLTE AND CARL T. WITTWER

1

The development of the polymerase chain reaction, or PCR, by Saiki et al. (1) was a milestone in biotechnology and heralded the beginning of the modern era of molecular diagnostics. Although PCR is the most widely used nucleic acid amplification strategy, other strategies have been developed, and several have clinical utility. These strategies are based on either signal or target amplification. Examples of each category will be discussed in the sections that follow. These techniques have sensitivity unparalleled in laboratory medicine, have created new opportunities for the clinical laboratory to impact patient care, and have become the new "gold standards" for laboratory diagnosis of many infectious diseases.

SIGNAL AMPLIFICATION TECHNIQUES

In signal amplification methods, the concentration of the probe or target does not increase. The increased analytical sensitivity comes from increasing the concentration of labeled molecules attached to the target nucleic acid. Multiple enzymes, multiple probes, multiple layers of probes, and reduction of background noise have all been used to enhance target detection (2). Target amplification systems generally have greater analytical sensitivity than signal amplification methods, but technological developments, particularly in branched DNA (bDNA) assays, lowered the limits of detection to levels that rivaled those of some earlier target amplification assays (3).

Signal amplification assays have several advantages over target amplification assays. In signal amplification systems, the number of target molecules is not altered, and as a result, the signal is directly proportional to the amount of the target sequence present in the clinical specimen. This reduces concerns about false-positive results due to cross-contamination and simplifies the development of quantitative assays. Since signal amplification systems are not dependent on enzymatic processes to amplify the target sequence, they are not affected by the presence of enzyme inhibitors in clinical specimens. Consequently, less cumbersome nucleic acid extraction methods may be used. Typically, signal amplification systems use either larger probes or more probes than target amplification systems and, consequently, are less susceptible to errors resulting from target sequence heterogeneity. Finally, RNA levels can be measured directly without the synthesis of a cDNA intermediate.

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bDNA

The bDNA signal amplification system is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes (4). The key to this technology is the amplifier molecule, a bDNA molecule with 15 identical branches, each of which can bind to three labeled probes.

The bDNA signal amplification system is illustrated in Fig. 1. Multiple target-specific probes are used to capture the target nucleic acid onto the surface of a microtiter well. A second set of target-specific probes also binds to the target and to preamplifier molecules, which in turn bind to up to eight bDNA amplifiers. Three alkaline phosphatase-labeled probes hybridize to each branch of the amplifier. Detection of bound labeled probes is achieved by incubating the complex with dioxetane, an enzyme-triggerable substrate, and measuring the light emission in a luminometer. The resulting signal is directly proportional to the quantity of the target in the sample. The quantity of the target in the sample is determined from an external standard curve.

Nonspecific hybridization of any of the amplification probes and nontarget nucleic acids leads to amplification of the background signal. To reduce potential hybridization to nontarget nucleic acids, isocytidine (isoC) and isoguanosine (isoG) were incorporated into the preamplifier, and labeled probes were used in the third-generation bDNA assays (5). IsoC and isoG form base pairs with each other but not with any of the four naturally occurring bases (6).

The use of isoC- and isoG-containing probes in bDNA assays increases target-specific signal amplification without a concomitant increase in the background signal, thereby greatly enhancing the detection limits without loss of specificity. The detection limit of the third-generation bDNA assay for human immunodeficiency virus type 1 (HIV-1) RNA is 75 copies/ml. bDNA assays for the quantification of hepatitis B virus DNA, hepatitis C virus (HCV) RNA, and HIV-1 RNA are commercially available (Siemens Healthcare Diagnostics, Deerfield, IL). The SiemensVersant 440 analyzer for bDNA assays automates the incubation, washing, reading, and data-processing steps.

Hybrid Capture

The hybrid capture system is a solution hybridization-antibody capture method that uses chemiluminescence

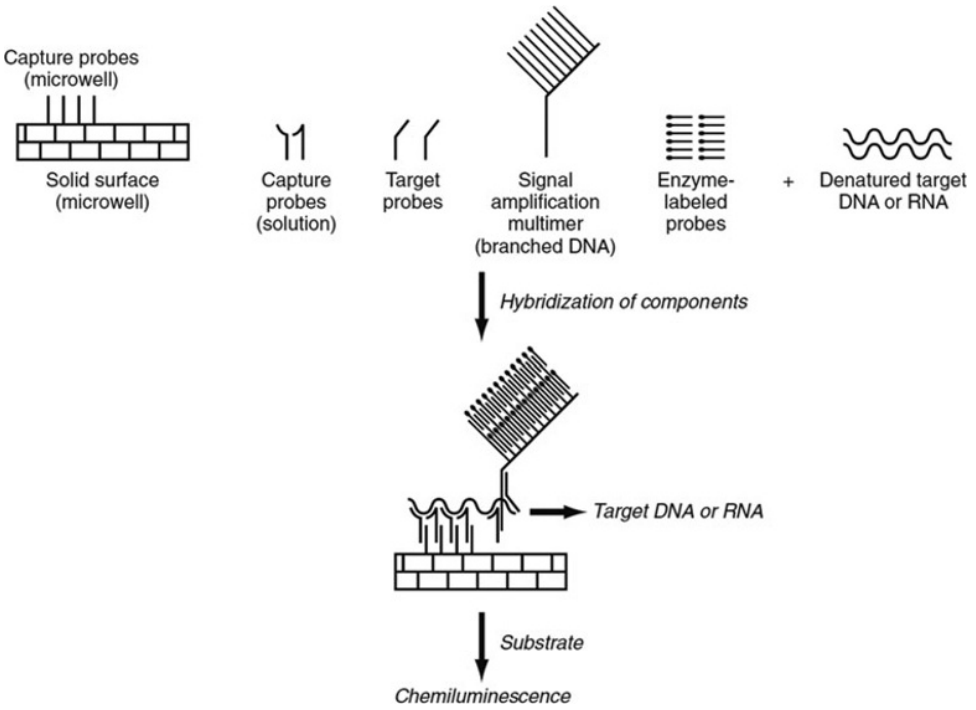

FIGURE 1 Branched DNA signal amplification. Reprinted with permission from reference 70.

detection of hybrid DNA-RNA duplexes (Fig. 2). The target DNA in the specimen is denatured and then hybridized with a specific RNA probe. The DNA-RNA hybrids are captured by antihybrid antibodies that are used to coat the surface of a tube. Alkaline phosphatase-conjugated antihybrid antibodies bind to the immobilized hybrids. The bound antibody conjugate is detected with a chemiluminescent substrate, and the light emitted is measured in a luminometer. Multiple alkaline phosphatase conjugates bind to each hybrid molecule, amplifying the signal. The intensity of the emitted light is proportional to the amount of target DNA in the specimen. Hybrid capture assays for detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and human papillomavirus in clinical specimens are available from Qiagen, Germantown, MD (7). There are manual and automated (rapid capture system) versions of these assays.

Cleavase-Invader Technology

Invader assays (Hologic/Gen-Probe, San Diego, CA) are based on a signal amplification method that relies upon the specific recognition and cleavage of particular DNA structures by cleavase, a member of the FEN-1 family of DNA polymerases. These polymerases will cleave the 5′ single-stranded flap of a branched base-paired duplex. This enzymatic activity likely plays an essential role in the elimination of the complex nucleic acid structures that arise during DNA replication and repair. Since these structures may occur anywhere in a replicating genome, the enzyme recognizes the molecular structure of the substrate without regard to the sequence of the nucleic acids making up the DNA complex (8, 9).

In the invader assays, two probes are designed which hybridize to the target sequence in an overlapping fashion (Fig. 3). Under the proper annealing conditions, the probe oligonucleotide binds to the target sequence. The invader oligonucleotide probe is designed such that it hybridizes upstream of the probe with a region of overlap between the 3′ end of the invader and the 5′ end of the probe. Cleavase cleaves the 5′ end of the probe and releases it. It is in this way that the target sequence acts as a scaffold upon which the proper DNA structure can form. Since the DNA structure necessary to serve as a cleavase substrate will occur only in the presence of the target sequence, the generation of cleavage products indicates the presence of the target. Use of a thermostable cleavase enzyme allows reactions to be run at temperatures high enough for a primer exchange equilibrium to exist. This allows multiple cleavase products to form off of a single target molecule. FRET probes and a second invasive cleavage reaction are used to detect the target-specific products. FDA-cleared assays for detection of pools of high-risk genotypes and types 16 and 18 of human papillomavirus in cervical samples are available from Hologic/Gen-Probe (10, 11).

TARGET AMPLIFICATION TECHNIQUES

All of the target amplification systems share certain fundamental characteristics. They use enzyme-mediated processes, in which a single enzyme or multiple enzymes synthesize copies of a target nucleic acid. In all of these techniques, amplification is initiated by two oligonucleotide primers that bind to complementary sequences on opposite strands of double-stranded targets. These techniques result in the production of millions to billions of copies of the targeted sequence in a matter of minutes to hours, and in each case, the amplification products can serve as templates for subsequent rounds of amplification. Because of this, these techniques are sensitive to contamination with product molecules that can lead to false-positive reactions. The potential for contamination should be adequately addressed before these techniques are used in the clinical laboratory. However, the occurrence of false-positive reactions

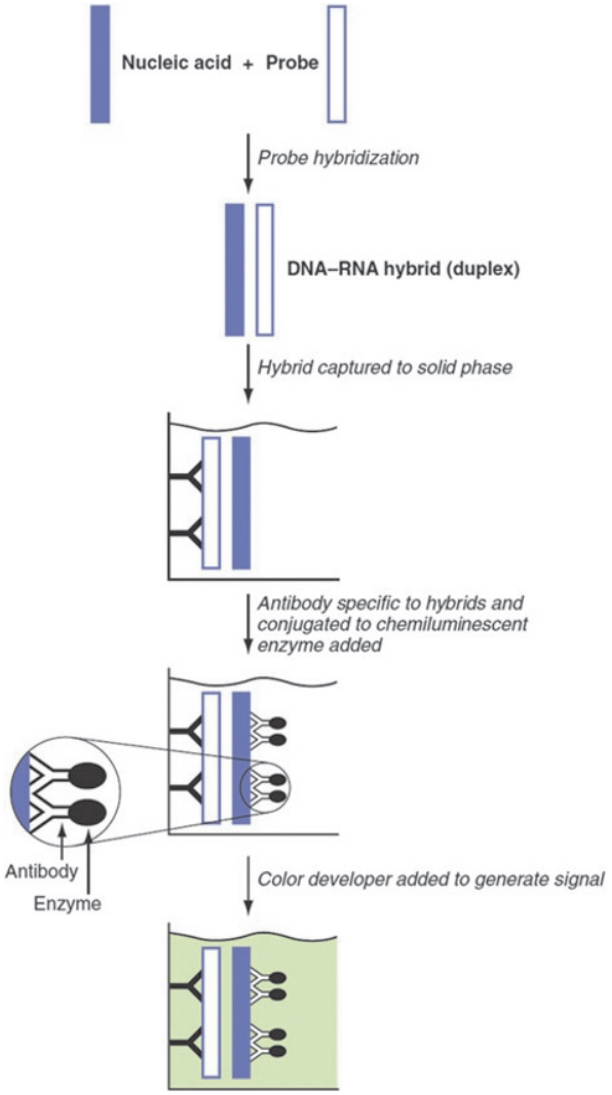

FIGURE 2 Hybrid capture signal amplification. Reprinted with permission from reference 70.

can be reduced through special laboratory design, practices, and workflow (12). In addition, amplification products can be modified by UV light or enzymes into forms that cannot be replicated. For example, if T is replaced with U during the PCR, it can be treated later by an enzyme that degrades U containing carryover products to prevent false-positive reactions (13). The growing use of closed systems where products are not exposed to the environment also helps to greatly reduce the threat of carryover contamination.

PCR

PCR was the first target amplification technique and remains the most popular today, for both research and clinical applications. It deserves such recognition and use because of its simplicity. Kary Mullis received the Nobel Prize in 1993 for its invention. The evolution and development of PCR is covered nicely by many books dedicated to the subject (14–16).

PCR requires a thermostable polymerase, two oligonucleotide primers to select the region to be amplified, a mixture of deoxynucleotide monomers (dNTPs), and template DNA. The polymerase is typically from *Thermus aquaticus*, originally obtained from Yellowstone National Park and later cloned into expression vectors for production. The two primers anneal to opposite DNA strands, typically placed 50 to 1,000 bases apart to select the region to be amplified. Typical reactant concentrations for PCR are shown in Table 1.

PCR is driven by temperature changes. The initial template is denatured or separated by heat (typically 90 to 95°C), lowering the temperature is required for primer annealing (55 to 65°C), and enzyme extension is typically performed at 65 to 75°C. Three-step cycling is performed if all three temperatures are different, although two-step cycling with a combined annealing/extension step is also common in diagnostics. Repeated temperature cycling through denaturation, annealing, and extension accumulates many identical products of defined length (Fig. 4). The products are most commonly detected by agarose gel electrophoresis, hybridization to complementary nucleic acids on solid supports, or probe interaction in solution. For example, if products are sampled during one cycle of PCR and separated on a gel, the process within each cycle can be observed visually (Fig. 5).

The advantages of PCR include simplicity, speed (17), and cost. Basic PCR is off-patent, and most forms of real-time PCR will be off-patent by the time this chapter goes to print. PCR as a process is very similar to bacterial growth. Both processes begin with exponential growth that eventually plateaus (Fig. 6). Growth curves follow a familiar S-curve shape tracking the logistic model of population growth. Although the endpoints of bacterial growth in media and amplification of DNA *in vitro* by PCR are different, they follow the same curve shape. Accurate quantification of the initial template is enabled by controlling denaturation, annealing, and extension by temperature cycling so that each amplification cycle can be measured and overall efficiency calculated.

PCR is clinically used in most laboratory-developed tests and *in vitro* diagnostic tests for infectious diseases. A complete list of all FDA-cleared or -approved nucleic acid amplification tests for detection, quantification, and genotyping of microorganisms can be found at http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm.

Reverse Transcriptase-PCR

When the initial template is RNA instead of DNA, an initial conversion of RNA into DNA is necessary for PCR. This conversion is performed by an RNA-dependent DNA polymerase, and the combined process is called reverse transcriptase PCR or RT-PCR. It can be performed in one or two steps. Two-step RT-PCR is typical of most research studies with two different enzymes and conditions optimized for each. One-step RT-PCR is more common for clinical assays where both the reverse transcription and the PCR are performed in a single tube. RT-PCR enables PCR to amplify common RNA targets, including HIV-1, HCV, enterovirus, and many respiratory viruses. The added complexity does require greater care, especially for viral load and other quantification assays. The MIQE guidelines (Minimum Information for Quantitative PCR Experiments) ensure the integrity of the scientific literature, promote consistency between laboratories, and increase

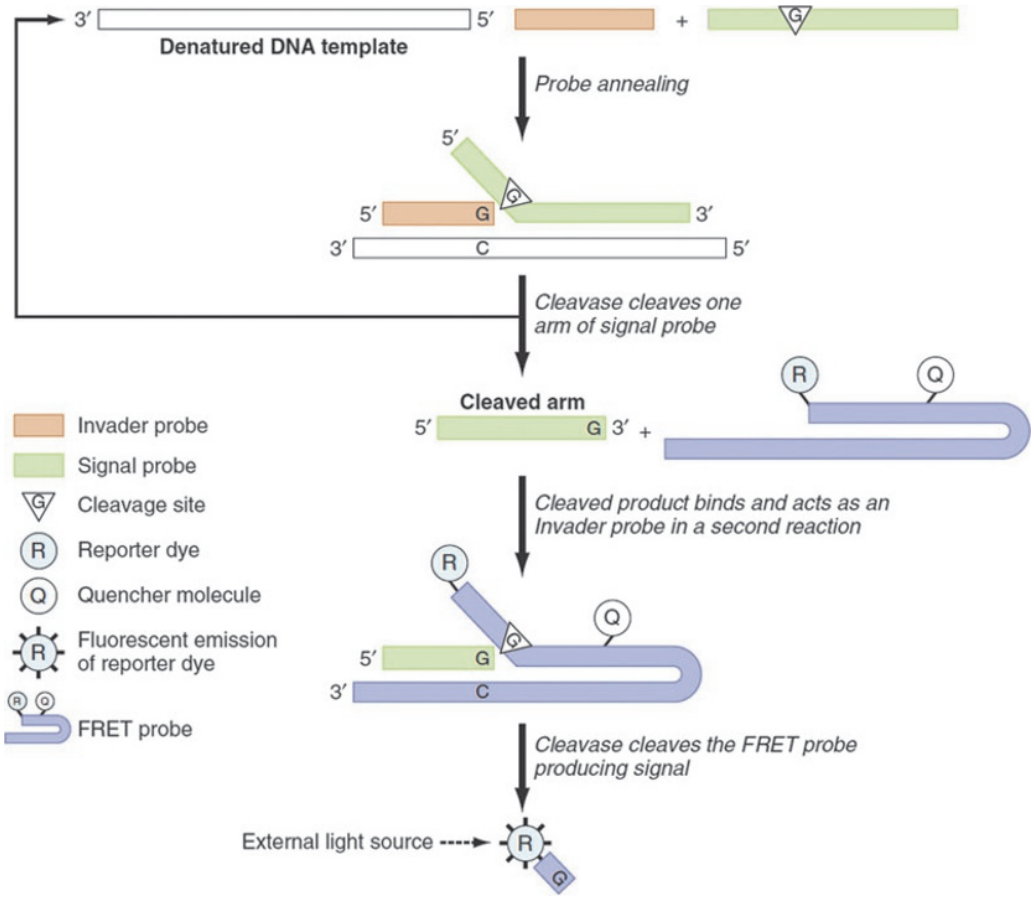

FIGURE 3 Cleavase invader signal amplification. Reprinted with permission from reference 70.

experimental transparency (18). Although written for the research community, these guidelines remain relevant for clinical assays.

Nested PCR

If PCR is followed by a second round of PCR on the products of the first, it is called nested PCR. Typically, both primers in the second PCR are internal to the first, so successful amplification depends on four primers rather than two. However, if one of the primers in the second PCR is the same as the first, it is called "hemi-nested" PCR. The advantage of nested or hemi-nested PCR is a further increase in sensitivity and specificity. The main disadvantage is an increased risk of carryover contamination, and the only nested tests that are FDA-approved are closed-tube real-time systems. The Cepheid MTB/RIF test is hemi-nested and detects *Mycobacterium tuberculosis* and rifampin resistance in <2 h (19). Nested, multiplex panels for respiratory agents (20), positive blood culture bottles (21), and gastrointestinal microbes are also FDA-approved with sample-to-answer results in about an hour and were developed by BioFire Diagnostics, Salt Lake City, UT/bioMérieux, Durham, NC.

Multiplex PCR

When more than one target is amplified by PCR, the process is called "multiplex." Multiplexing can save reagents and sample and is often used when a more complete answer can be obtained by including additional targets. Multiplexing is analyzed by separating products by size on a gel, by spatial separation on a surface or beads, or by probe color in real-time PCR. Real-time PCR is typically limited

TABLE 1 Typical reactant amounts in PCR (10-µl reaction mixture)

Reactant	Type	No. of copies/10 µl
Template DNA	50 ng of human DNA	1.6×10^4
	50 pg of bacterial DNA (3 Mb)	
	0.17 pg of viral DNA (10 kb)	
Polymerase	0.4 U of *Taq*	8.8×10^9
Primers	0.5 µM (each)	3.0×10^{12} (each)
Deoxynucleoside triphosphates	0.2 mM (each)	4.8×10^{15} (total)

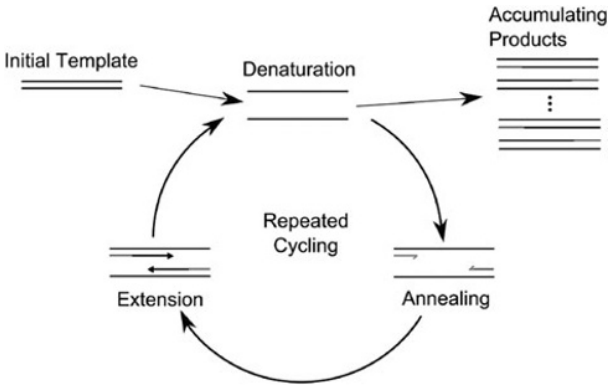

FIGURE 4 The PCR cycle. The initial template DNA is first denatured by heat. The reaction is then cooled to anneal two oligonucleotide primers to opposite strands with their 3′ ends pointed inward. A polymerase then extends each primed template to double the amount of targeted DNA. The cycle is repeated 20 to 40 times through successive steps of denaturation, annealing, and extension, accumulating double-stranded PCR products. Reprinted with permission from reference 16.

to two to six colors, but greater multiplicity is possible by combining color with the melting temperatures of the probes.

One example of multiplexed PCR with clinical utility is for upper respiratory infection. Many viruses and bacteria can cause flu-like illness, and a panel may provide a definitive answer in one multiplexed test. The first multiplexed respiratory panel was FDA-approved in 2008 with 10 viruses (Luminex, Austin, TX). Additional PCR-based respiratory panels are now offered by many companies including Cepheid, Sunnyvale, CA; GenMark Dx, Carlsbad, CA; Nanosphere, Northbrook, IL; Gen-Probe/Hologic, San Diego, CA; and BioFire/biomérieux. BioFire/biomérieux's nested multiplex respiratory panel is most inclusive, with 17 viruses and 3 bacteria (20).

Real-Time PCR

"Real time" implies that data collection and analysis occur as a reaction proceeds. Required reagents for analysis, such as DNA dyes or fluorescent probes, are added to the PCR mixture before amplification. Data are collected during amplification in the same tube and in the same instrument. There are no sample transfers, reagent additions, or gel separations. Real-time PCR is powerful, simple, and rapid and is replacing many conventional techniques in the microbiology laboratory.

Fluorescence is the indicator of choice for real-time PCR. Dyes can be used to monitor double-stranded PCR products, acquiring fluorescence once each cycle (22). If target DNA is present, the fluorescence increases. How soon this rise occurs depends on the initial amount of target DNA. The full power of real-time PCR goes beyond monitoring only once each cycle (23). When fluorescence is monitored as the temperature is changing, melting curves can verify the product amplified and detect sequence variants down to a single base. An example of the data generated from real-time PCR with melting analysis is shown in Fig. 7.

dsDNA Binding Fluorescent Dyes

In research, most real-time PCR is performed with dyes that fluoresce in the presence of double-stranded DNA because of their low cost and convenience (23). However, FDA-approved assays typically use probes instead of dyes. With dyes, any double-stranded product that is formed is detected, including primer dimers and other unintended products. Unless melting analysis of the product is performed, false positives are common (24). Multiplexing is possible by melting temperature discrimination rather than color (25). The mechanism of dye fluorescence during real-time PCR is compared to several probes in Fig. 8.

Hydrolysis (TaqMan) Probes

The most common probes used in FDA-approved real-time PCR assays are hydrolysis probes. If a probe labeled with a

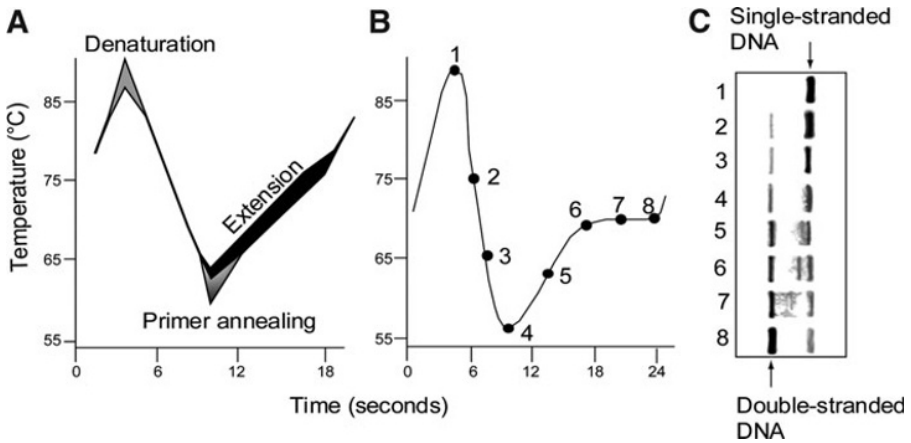

FIGURE 5 Visualization of PCR kinetics. The three phases of PCR (denaturation, annealing, and extension) occur as the temperature is continuously changing (A). Toward the end of PCR the reaction contains single- and double-stranded PCR products. When different points of the cycle are sampled (by snap-cooling the mixture in ice water) (B) and analyzed, the transition from denatured single-stranded DNA to double-stranded DNA is revealed as a continuum (C). Progression of the extension reaction can be followed by additional bands appearing between the single- and double-stranded DNA (time points 5 to 7). Modified with permission from reference 71.

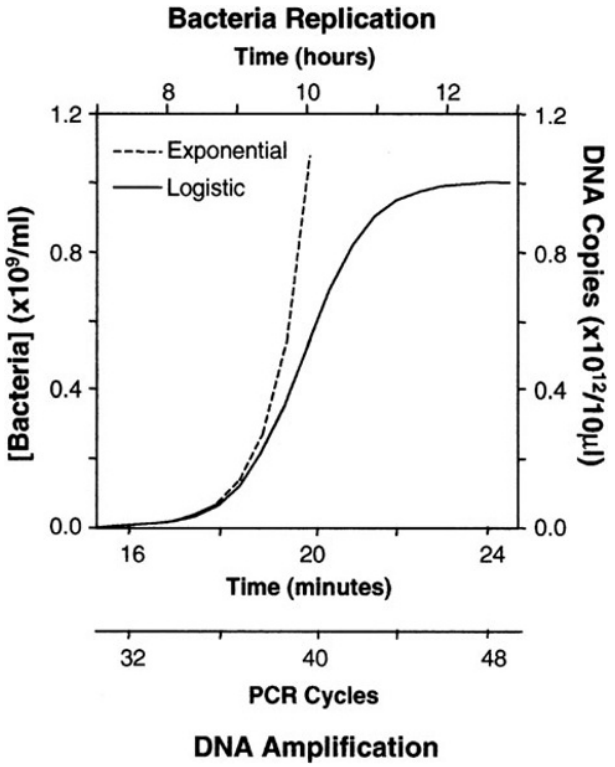

FIGURE 6 Model exponential and logistic curves for bacterial growth and PCR. Doubling times of 20 min and 30 s are assumed for bacteria and PCR, respectively. That is, given the equation $Nt = N_0e^{rt}$, r is 0.0347 min^{-1} for bacteria and 1.386 min^{-1} for PCR. The carrying capacity for bacteria was set at 10^9/ml. Assuming that PCR is primer limited at one-third the primer concentration (Table 1), a carrying capacity of 10^{12} copies of PCR product/10 µl was used. The shapes of the curves for bacteria and DNA are identical, with only the axis scales specific to each method. Starting with a single bacterium, growth plateaus after 11 to 12 h, while PCR takes only 23 min (46 cycles) to amplify a single copy to saturation.

fluorophore and a quencher is hydrolyzed during PCR and the labels are separated, fluorescence will increase. The most frequent implementation uses the 5′-exonuclease activity of a DNA polymerase to hydrolyze the probe and dissociate the labels (26). Another interesting way to hydrolyze fluorescent probes is to produce a DNAzyme during PCR (27). The fluorescence generated by hydrolysis probes is irreversible, and melting analysis is typically not useful. Hydrolysis probes are diagrammed in Fig. 8B.

Dual Hybridization Probes

Hybridization probes change fluorescence on hybridization, usually by fluorescence resonance energy transfer. Two interacting fluorophores are typically placed on adjacent probes (23) so that when they both hybridize, the fluorophores are brought together and energy transfer occurs, changing the color of the emitted fluorescence. Dual hybridization probes were used in the first FDA-approved genetic tests and, along with hydrolysis probes and molecular beacons, are found in many laboratory-developed microbiology tests (28). They are also used in the Roche (Indianapolis, IN) FDA-approved methicillin-resistant *Staphylococcus aureus* (MRSA) test. In contrast to hydrolysis probes, the fluorescence change of hybridization probes is reversible, and melting analysis can be very informative for strain typing and/or antibiotic resistance. Dual hybridization probes are shown in Fig. 8C.

Molecular Beacons

Molecular beacons (hairpin probes) fluoresce when they hybridize to a target (29). A fluorophore and a quencher are present on opposite strands of the stem, typically at the 3′ and 5′ ends of the probe. When the loop hybridizes to the target of interest, the fluorophore and quencher are separated, enhancing fluorescence. Molecular beacons of different colors can be combined with melting temperature for highly multiplexed assays (30). Molecular beacons are used in FDA-approved assays for *M. tuberculosis* and MRSA (Cepheid) and are shown in Fig. 8D.

Scorpion Probes

The fluorescence generated during PCR from self-probing amplicons (31) also depends on separating a fluorophore and a quencher on opposite ends of a hairpin stem. With scorpions, the primer is modified at its 5′ end to include a labeled hairpin similar to a molecular beacon. A blocker prevents copying of the hairpin region during PCR. The hairpin loop is complementary to the primer's extension product, so intramolecular hybridization occurs, replacing one hairpin with another that has a longer stem and is more stable. This separates the fluorophore from the quencher, and fluorescence is increased (Fig. 8E). Scorpion probes are used in FDA-approved assays for group B *Streptococcus* (BD Diagnostics, Franklin Lakes, NJ), *Clostridium difficile* (Focus Diagnostics, Cypress, CA), and some molecular oncology assays.

Dark Quencher Probes

Dark quencher (Pleiades) probes have a minor-groove binder and fluorophore at their 5′ end with a 3′ nonfluorescent quencher. Background fluorescence is very low because hydrophobic attraction between the quencher and minor groove binder ensures efficient quenching, further augmented by the minor groove binder (Fig. 8F). When bound to a target, the fluorophore and quencher are separated, similar to molecular beacons or scorpion primers. The minor groove binder also increases probe stability, making shorter probes possible. Short probes can be an advantage when sequence variation is high. Dark quencher probes are not degraded during PCR and can generate melting curves. Dark quencher probes (ELITech Group, Princeton, NJ) are available as analyte-specific reagents for cytomegalovirus, Epstein-Barr virus, and BK polyomavirus.

Partially Double-Stranded Probes

Partially double-stranded linear probes consist of two complementary oligonucleotides of different length (32). The longer target-specific strand has a 5′ fluorescent label that is effectively quenched by a 3′ quencher on the shorter negative strand (Fig. 8G). When a target is present the longer strand preferentially binds to the target, the shorter strand is displaced, and fluorescence is enhanced. These probes are tolerant to mismatches and are used in FDA-approved assays for HIV-1 and HCV (Abbott Molecular, Des Plaines, IL).

Melting Curve Analysis

Continuous monitoring of PCR (Fig. 9) suggests that hybridization can be followed during temperature cycling

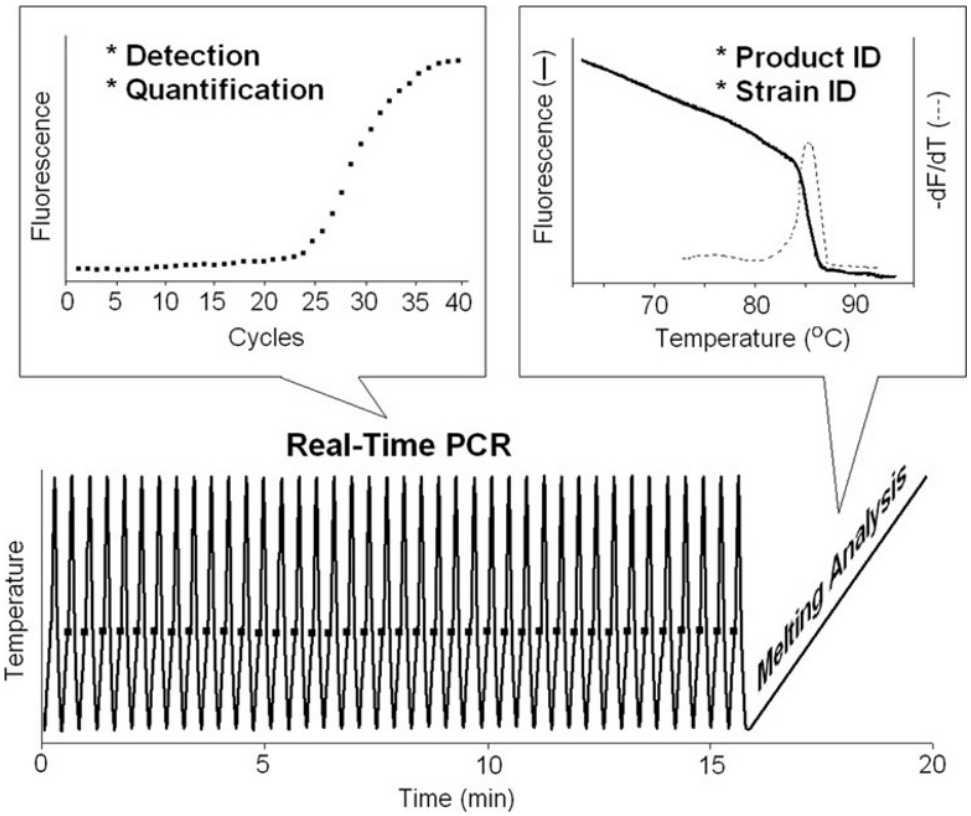

FIGURE 7 Real-time PCR with melting analysis. Detection and quantification are enabled by monitoring fluorescence once each cycle at the end of extension (solid squares). Amplification is immediately followed by melting-curve acquisition. Melting-curve analysis identifies PCR products, microbial strains and sequence alterations by melting temperature. The original melting-curve data (solid line) can also be plotted as a derivative melting curve (dotted line). Reprinted from reference 72 with permission from the American Society of Investigative Pathology and the Association for Molecular Pathology.

with dyes and most probes. Hydrolysis probes are the exception because they are destroyed during signal generation. Instead of monitoring hybridization throughout PCR, a single melting analysis after PCR is typically performed (Fig. 7). The midpoint of melting, called the melting temperature, or T_M, is determined mainly by the GC content and size of the duplex region. DNA melting curve analysis takes advantage of the fluorimeters and temperature control of real-time PCR instruments (17, 23, 24).

Product melting with dyes is useful to confirm PCR specificity by T_M and curve shape. Both T_M and curve shape can be predicted (33). PCR products of >200 bp often have multiple melting domains, and heterozygous products create heteroduplexes, both affecting curve shape. High-resolution melting analysis uses subtle differences in T_M and curve shape for genotyping and mutation scanning (34). Although usually a research technique, high-resolution melting is used in FDA-approved nested, multiplex assays for upper respiratory, blood culture, and gastrointestinal microbes (BioFire/bioMérieux).

Probe melting distinguishes variants only under the probe as opposed to the entire PCR product. For example, single nucleotide variants can be genotyped with hybridization probes because different sequences are revealed by different T_Ms. Irrelevant sequence variants under the probe can be masked by a deletion, mismatch, or universal base (35). Labeled hybridization probes include the dual hybridization probes of Fig. 8C and several single hybridization probes including molecular beacons (Fig. 8D), scorpion primers (Fig. 8E), dark quenchers (Fig. 8F), and partially double-stranded probes (Fig. 8G). Genotyping with labeled hybridization probes is shown in Fig. 10A and B. In parallel to labeled probes, melting and genotyping can also be performed with simple dyes rather than covalent labels. Examples include unlabeled probes (Fig. 10C) and snapback primers (Fig. 10D).

Unlabeled probes have no fluorescent labels but are 3′ blocked with a phosphate or other blocker (36). Unlabeled probes have been used for herpes simplex virus detection and typing (37) and in model studies have distinguished up to 10 variants (34). Similar to scorpion primers, "snapback primers" (Fig. 10D) generate a self-probing amplicon that forms a hairpin (38). Snapback primers achieve probe specificity with only two primers, one of which has a simple 5′ extension without any covalently attached fluorophores. Only amplicon melting is conceptually simpler (Fig. 10E), but the smaller differences between variants usually require high-resolution melting. Melting curves of unlabeled probe and snapback primers show both product and probe melting transitions, providing synergistic information for PCR variant identification (39).

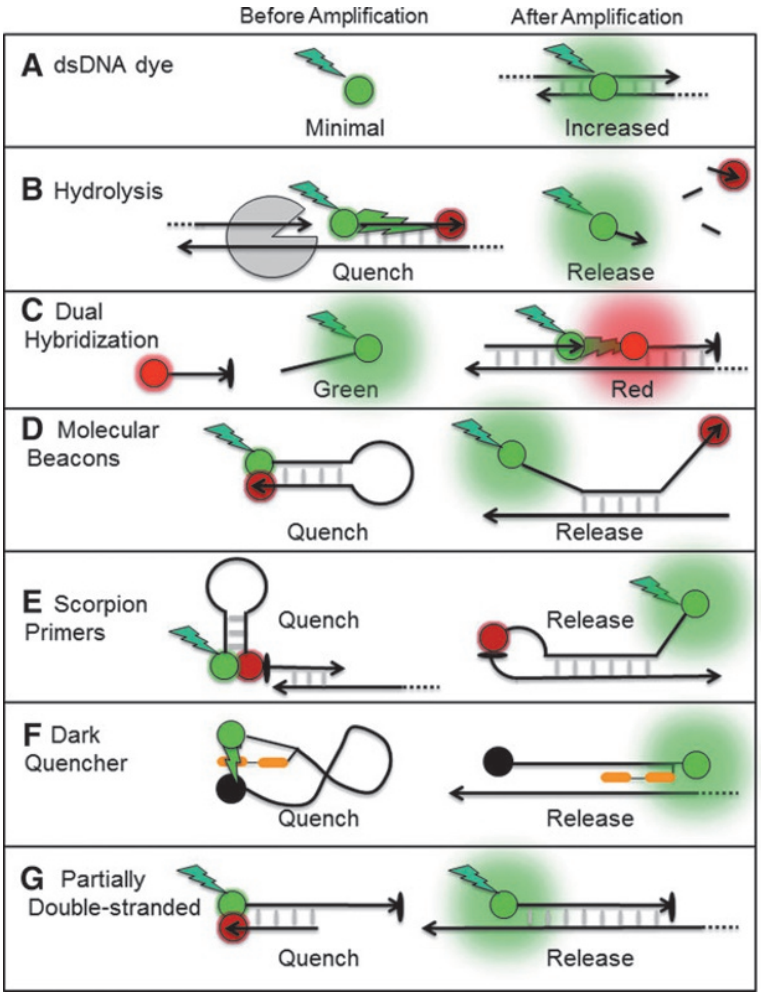

FIGURE 8 Common probes and dyes for real-time PCR. The green lightning bolt is the excitation light. The green circles are fluorophores, the dark red circles are quenchers, and the black circles are dark quenchers. The large hungry gray circle is a polymerase with 5′ to 3′ exonuclease activity. The thin black ovals are blockers, and the orange sausages are minor groove binders. (A) Double-stranded DNA dyes show a significant increase in fluorescence when bound to DNA. (B) Hydrolysis probes are cleaved between a fluorescent reporter and a quencher, resulting in increased fluorescence. (C) Dual hybridization probes change color by resonance energy transfer when hybridized. (D) The molecular beacon hairpin quenches fluorescence until target binding that separates the quencher from the flourophore. (E) Scorpion primers are quenched in the native conformation but increase in fluorescence when the original hairpin loop is hybridized to its extension product. (F) Dark quencher probes are initially quenched by a minor groove binder and the dark quencher. Hybridization to the target releases the fluorescence. (G) The short strand of partially double-stranded probes is displaced in the presence of target, releasing fluorescence from quenching.

Digital PCR

The sensitivity of real-time PCR, defined as a 95% detection rate, cannot be better than three copies per reaction because of variable partitioning of templates into any particular reaction (18). Digital PCR, however, uses partitioning to its advantage by running many PCRs with an average copy number typically between 0 and 1 (40). Each reaction is either positive or negative. Digital PCR can precisely determine the number of copies of a template (or variant) present at less than one copy per reaction if enough reactions are performed. Instruments that divide microliter PCR volumes into hundreds or millions of nanoliter to picoliter partitions on microfluidic chips or droplets are now available, promising highly sensitive and precise quantification. Digital MIQE guidelines defining the minimal information for publication of quantitative digital PCR experiments emphasize the unique requirements of digital PCR (41). The main uses of digital PCR in microbiology are (i) absolute quantification of reference materials, (ii) quantification of rare variants, for example, the emergence of a drug-resistant variant, and (iii) viral load testing.

Because digital PCR does not depend on a standard curve for absolute quantification, it is an ideal method to establish quantitative reference materials. For example, the U.S. National Institute of Standards and Technology produced a standard reference material for cytomegalovirus quantification by digital PCR (42), and many more are

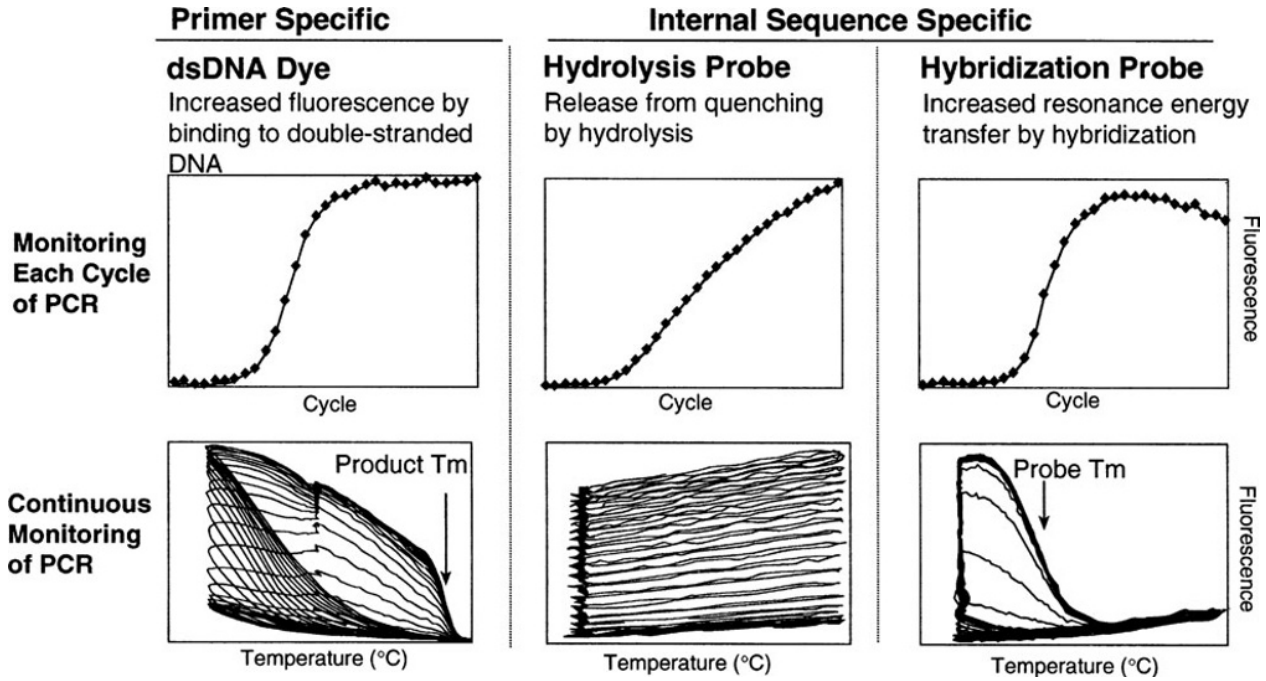

FIGURE 9 Typical real-time PCR amplifications monitored with SYBR Green I, hydrolysis probes, and hybridization probes. Both once-per-cycle and continuously monitored displays are shown. Note the hybridization information inherent in reactions monitored with SYBR Green I and hybridization probes.

likely to follow. Please see the chapter on digital PCR in this book for more details on the methods and clinical applications.

Detecting a small percentage of drug-resistant microbes in a population, or heteroresistance, is challenging by conventional methods. Digital PCR was successfully applied to heteroresistance in *M. tuberculosis*, targeting variants in four genes associated with isoniazid, rifampin, fluoroquinolone, and aminoglycoside resistance (43). Variants were detected at 0.01%, much more sensitive than real-time PCR or sequencing. Similar studies in HIV-1, HCV, and other viruses and bacteria are sure to follow.

Digital PCR for viral load testing has been compared to real-time PCR in several studies. In addition to the more common chip and droplet systems, novel rotational systems provide greater dynamic range, as demonstrated for HIV-1 and HCV (44). The proportion of chromosomally integrated human herpesvirus type 6 (HHV-6) to genomic DNA was precisely determined by digital PCR to prevent misdiagnosis and unnecessary treatment of active HHV-6 (45). Two studies comparing digital to real-time PCR for viral load testing of cytomegalovirus concluded that although there are theoretical advantages to digital PCR, practically clinical results are similar (46, 47).

Transcription-Based Amplification Methods

Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are both isothermal RNA amplification methods modeled after retroviral replication (48–50). These methods are similar in that the RNA target is reverse transcribed into cDNA and then RNA copies are synthesized with an RNA polymerase. NASBA uses avian myeloblastosis virus RT, RNase H, and T7 bacteriophage RNA polymerase, whereas TMA uses an RT enzyme with endogenous RNase H activity and T7 RNA polymerase.

Amplification involves the synthesis of cDNA from the RNA target with a primer containing the T7 RNA polymerase promoter sequence (Fig. 11). The RNase H then degrades the initial strand of target RNA in the RNA-cDNA hybrid. The second primer then binds to the cDNA and is extended by the DNA polymerase activity of the RT, resulting in the formation of double-stranded DNA containing the T7 RNA polymerase promoter. The RNA polymerase then generates multiple copies of single-stranded, antisense RNA. These RNA product molecules reenter the cycle, with subsequent formation of more double-stranded cDNA molecules that can serve as templates for more RNA synthesis. A 10^9-fold amplification of the target RNA can be achieved in less than 2 h by this method.

The single-stranded RNA products of TMA in the Hologic/Gen-Probe tests are detected by the hybridization protection assay. Oligonucleotide probes are labeled with modified acridinium esters with either fast or slow chemiluminescence kinetics so that signals from two hybridization reactions can be analyzed simultaneously in the same tube. The probes are added after amplification and hybridize to the amplicons. A selection reagent is then added which differentiates between hydridized and unhybridized probes by inactivating the label on the unhybridized probes. The NASBA products in the bioMérieux tests are detected by hybridization with probes that are added after amplification, labeled with tris (2,2′-bispyridine)ruthenium and detected by electrochemiluminescence. NASBA has also been used with molecular beacons to create a homogeneous, kinetic amplification system similar to real-time PCR (51).

Transcription-based amplification systems have several strengths, including no requirement for a thermal cycler,

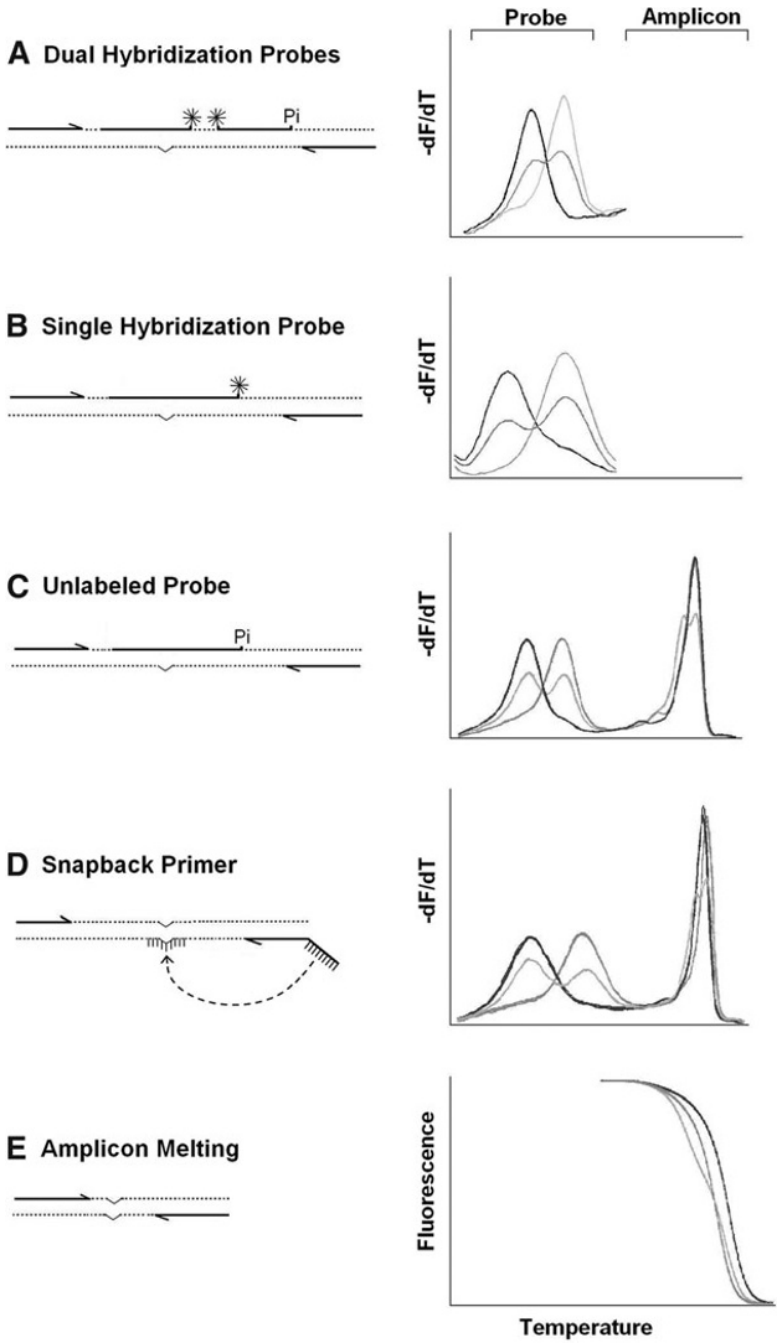

FIGURE 10 Variant typing by melting analysis. Primer and probe designs are shown on the left with typical data on the right. Dual (A) and single (B) hybridization probes use covalent fluorescent labels (asterisks), and typing is solely derived from the probe signal. Single hybridization probes discussed here include molecular beacons, scorpion primers, dark quencher probes, and partially double-stranded probes. Unlabeled probes (C) and snapback primers (D) require no covalent labels because fluorescence is provided by a dye that binds to dsDNA. With unlabeled probes and snapback primers, both probe and PCR product melting transitions are observed and can contribute to typing. Any free 3′ ends on the probes are terminated with a phosphate (Pi) or other blocker to prevent probe extension by the polymerase. The snapback primer (D) incorporates an unlabeled probe into the 5′ end of one primer, generating a self-probing amplicon that forms a hairpin. In panel E, no probe is present, but typing of the PCR product is still possible by high-resolution melting. High-resolution melting identifies heterozygotes by a change in curve shape and distinguishes homozygotes by T_m.

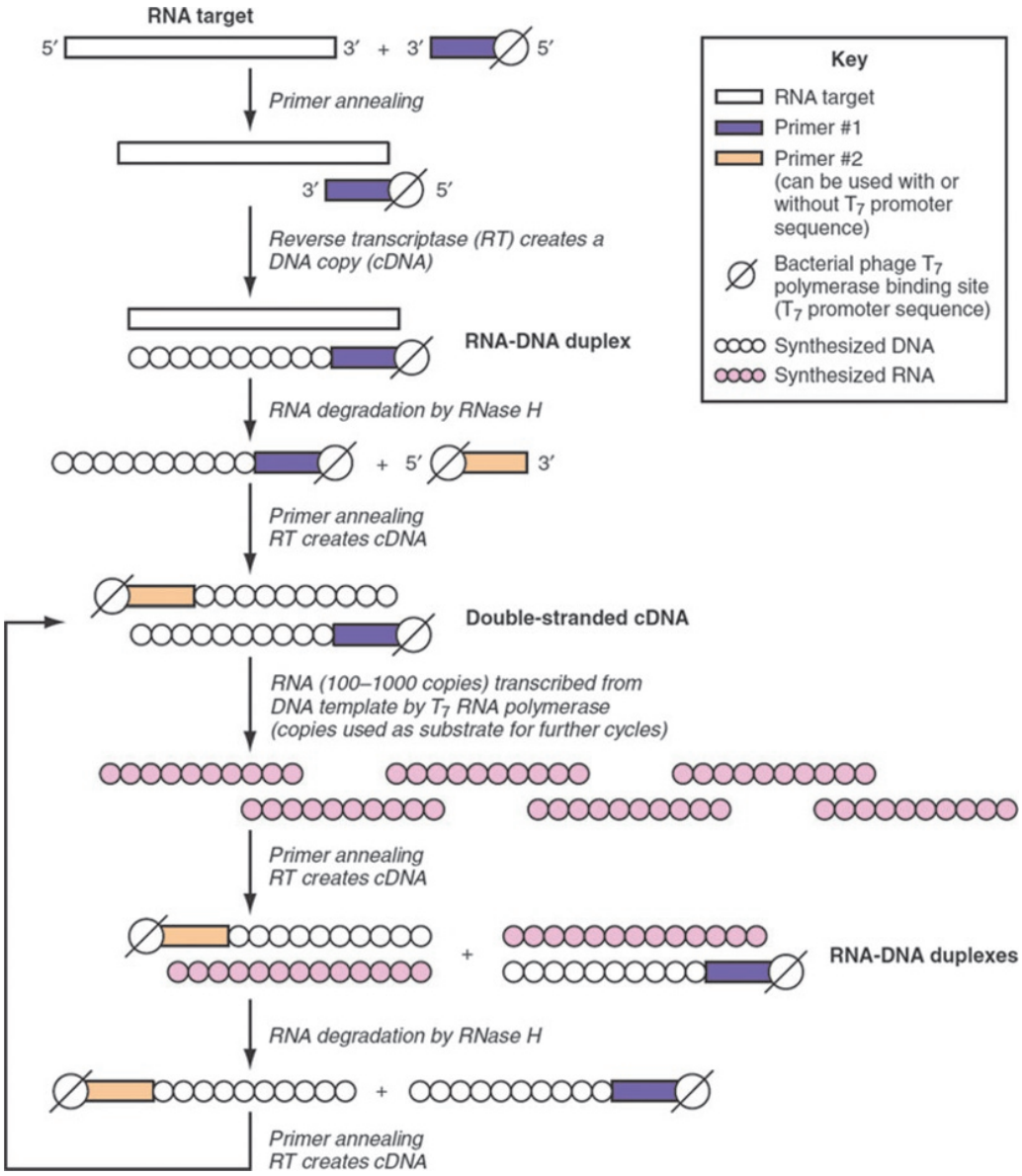

FIGURE 11 Transcription-based target amplification. NASBA and TMA are examples of transcription-based amplification systems. Reprinted with permission from reference 70.

rapid kinetics, and a single-stranded RNA product that does not require denaturation prior to detection. Also, single-tube clinical assays and a labile RNA product may help minimize contamination risks. Limitations include the poor performance with DNA targets and concerns about the stability of complex multienzyme systems. Hologic/Gen-Probe has developed FDA-cleared, TMA-based assays for detection of *M. tuberculosis*, *C. trachomatis*, *N. gonorrhoeae*, human papillomavirus, and *Trichomonas vaginalis*. NASBA-based kits (bioMérieux) for the detection and quantification of HIV-1 RNA and detection of enterovirus and MRSA were developed but are no longer commercially available. A basic NASBA kit is also available for the development of other applications defined by the user. In its latest iteration, NucliSens EasyQ, NASBA is coupled with molecular beacons for real-time amplification and detection of target nucleic acids (52).

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal template amplification technique that can be used to detect trace amounts of DNA or RNA of a particular sequence. SDA, as it was first described, was a conceptually straightforward amplification process with some technical limitations (53). Since its initial description, however, it has evolved into a highly versatile tool that is technically simple to perform but conceptually complex. SDA is the intellectual property of BD Diagnostics.

In its current iteration, SDA occurs in two discrete phases: target generation and exponential target amplification (54). Both are illustrated in Fig. 12. In the target generation phase, a double-stranded DNA target is denatured and hybridized to two different primer pairs, designated as bumper and amplification primers. The amplification primers include the single-stranded restriction endonuclease

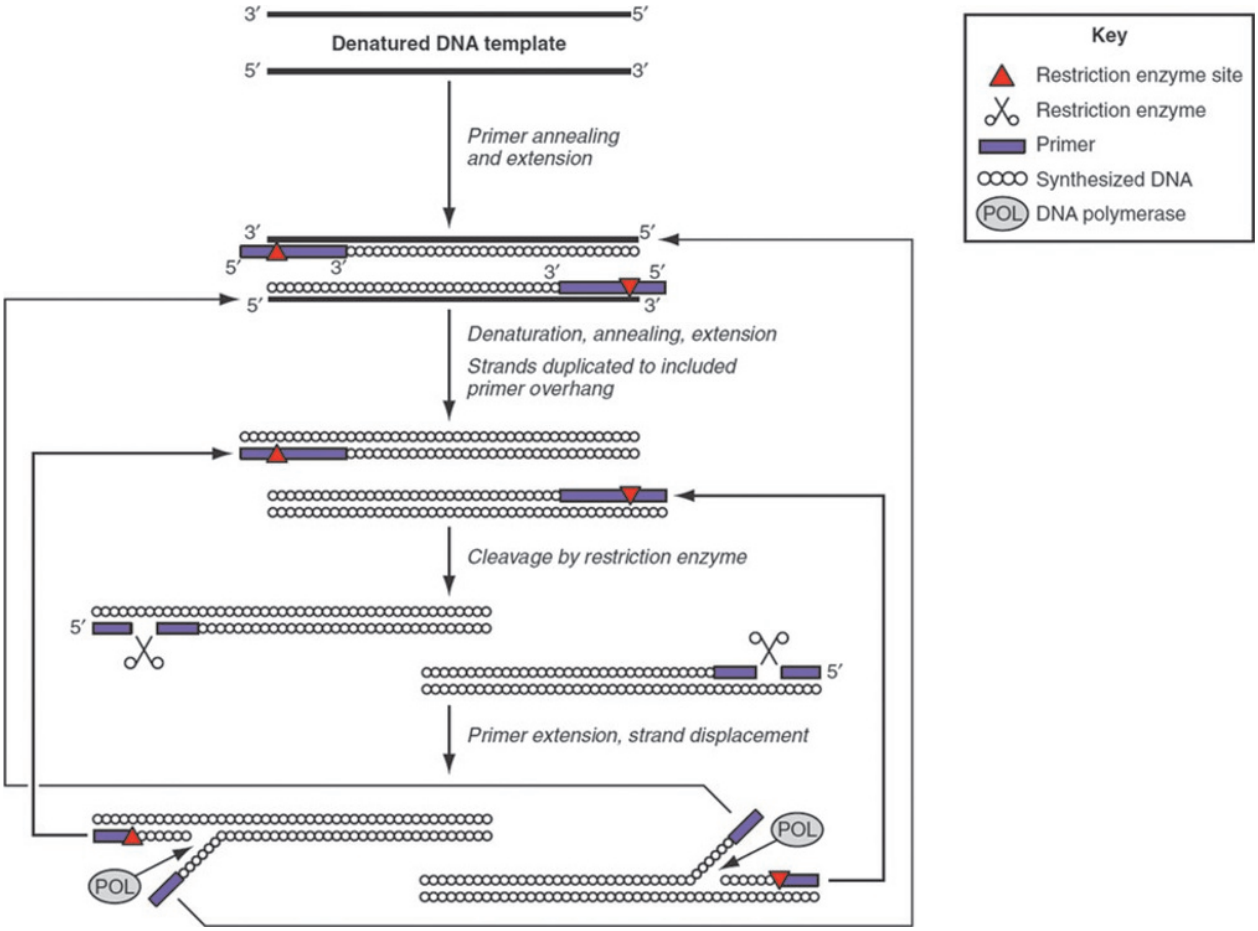

FIGURE 12 Strand displacement target amplification. The process is shown for only one strand of a double-stranded DNA target, but amplification occurs on both strands simultaneously. Reprinted with permission from reference 70.

enzyme sequence for BsoB1 located at the 5′ end of the target binding sequence. The bumper primers are shorter and anneal to the target DNA just upstream of the region to be amplified. In the presence of BsoB1, an exonuclease-free DNA polymerase, and a dNTP mixture consisting of dUTP, dATP, dGTP, and thiolated dCTP (C_s), simultaneous extension products of both the bumper and amplification primers are generated. This process displaces the amplification primer products, which are available for hybridization with the opposite-strand products with the opposite-strand bumper and amplification primers.

The simultaneous extension of opposite-strand primers produces strands complementary to the product formed by extension of the first amplification primer with C_s incorporated into the BsoB1 cleavage site. This product enters the exponential target amplification phase of the reaction. The BsoB1 enzyme recognizes the double-stranded site, but because one strand contains C_s, it is nicked rather than cleaved by the enzyme. The DNA polymerase then binds to the nicked site and begins synthesis of a new strand while simultaneously displacing the downstream strand. This step re-creates the double-stranded species with the hemimodified restriction endonuclease recognition sequence, and the iterative nicking and displacement process repeats. The displaced strands are capable of binding to opposite-strand primers, which produces exponential amplification of the target sequences.

These single-stranded products also bind to detector probes for real-time detection. The detector probes are single-stranded DNA molecules with fluorescein and rhodamine labels. The region between the labels includes a stem-loop structure. The loop contains the recognition site for the BsoB1 enzyme. The target-specific sequences are located 3′ of the rhodamine label. In the absence of a specific target, the stem-loop structure is maintained with the fluorescein and rhodamine labels in close proximity. The net effect is that very little emission for the fluorescein is detected after excitation. After SDA, the probe is converted to a double-stranded species, which is cleaved by BsoB1. The cleavage causes physical separation of the fluorescein and rhodamine labels, which results in an increase in emission from the fluorescein label.

SDA has a reported sensitivity high enough to detect as few as 10 to 50 copies of a target molecule (53). By using a primer set designed to amplify a repetitive sequence with 10 copies in the M. *tuberculosis* genome, the assay is sensitive enough to detect 1 to 5 genome copies from the bacterium. SDA has also been adapted to quantify RNA by adding an RT step (RT-SDA). In this case, a primer hybridizes to the target RNA and an RT synthesizes a cDNA molecule. This cDNA can then serve as a template for