Real-Time PCR

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INTRODUCTION

The polymerase chain reaction (PCR) is a well-established method in clinical laboratories. In a series published in 2014, authors highlighted 12 transformative molecular diagnostic assays, including several infectious disease tests such as PCR for the detection of the human immunodeficiency virus (HIV) and the herpes simplex virus (HSV) (1). That series underscored the significant impact that PCR has had on medicine. While the impact of PCR on the diagnosis of diseases is undeniable, early PCR tests were cumbersome and not readily transferrable from basic research benches to high-complexity clinical laboratories. Early works that preceded and laid the foundation for the development of PCR focused on enzymatic replication of synthetic DNA, a process coined "replication repair" (2). Further efforts followed, using only two cycles of amplifications and taking several hours to amplify a 30-nucleotide target (3). That initial approach was labor-intensive, low-yield and did not achieve the potential of PCR to generate thousands of copies of DNA from a single molecule.

In 1985, Kary Mullis, a chemist working at Perkin-Elmer Cetus Corporation in California, first described his development of PCR, which was built upon several existing concepts including dideoxy DNA sequencing as well as DNA repair and replication using DNA polymerases (4). In his approach, a PCR reaction included two oligonucleotide primers, dideoxynucleotide phosphates (ddNTPs), a DNA target, and DNA polymerase. The repeating nature of the experiment would exponentially amplify the target DNA by using prior amplicons as templates for the following amplification cycles. PCR is a cyclic process with the following steps: i) denaturation of the DNA template from double strands to single strands at 95°C; ii) annealing of oligonucleotide primers at a lower temperature (50–65°C); iii) primer extension by *Taq* polymerase. The cycle is repeated 30–45 times, resulting in detectable concentrations of DNA. Key improvements in several processes, including the use of the thermostable *Taq* DNA polymerase, improved oligonucleotides (oligos) chemistry, and laboratory automation (e.g., thermocyclers), resulted in the development of the modern PCR test.

The development of thermal cyclers that enabled rapid switching between different temperatures significantly reduced the time to complete a PCR reaction, which initially required manual transfer of PCR reactions between water baths kept at different temperatures. Other technical advances included the use of capillary tubes, which allowed more rapid heat exchange in the PCR reaction mix and the addition of ethidium bromide (EtBr) in the PCR reaction mix, which allowed simultaneous amplification and detection of the amplified DNA template (5–7). Several variations on this technique, including reverse transcriptase (RT)-PCR to amplify RNA templates, were subsequently developed. Today, real-time PCR assays are central to the diagnosis of infectious diseases and have evolved from tests performed only in high-complexity laboratories by highly skilled medical laboratory scientists to Clinical Laboratory Improvement Amendments of 1988 (CLIA)-waived tests that can be performed at the point-of-care (POC) by nonlaboratory personnel.

This chapter will review the basics of real-time PCR, including instrumentation and detection chemistries, primer design and assay development, and quality control and quality assurance issues. Finally, clinical applications of real-time PCR will be presented.

REAL-TIME PCR

Basic Principles

Real-time PCR builds upon the basic principle of endpoint PCR. In endpoint PCR, the detection of the amplification products (amplicons) occurs once the PCR process is complete. Amplicons are mixed with an intercalating dye such as EtBr, which binds to double-stranded DNA (dsDNA) and fluoresces when visualized on an agarose gel under ultraviolet light (Fig. 1A). In real-time PCR, however, amplification and detection of amplicons occur simultaneously, obviating the need for postamplification manipulation of PCR products (Fig. 1B) (8).

Real-time PCR is performed on thermocycler instruments that are designed to rapidly achieve and maintain targeted

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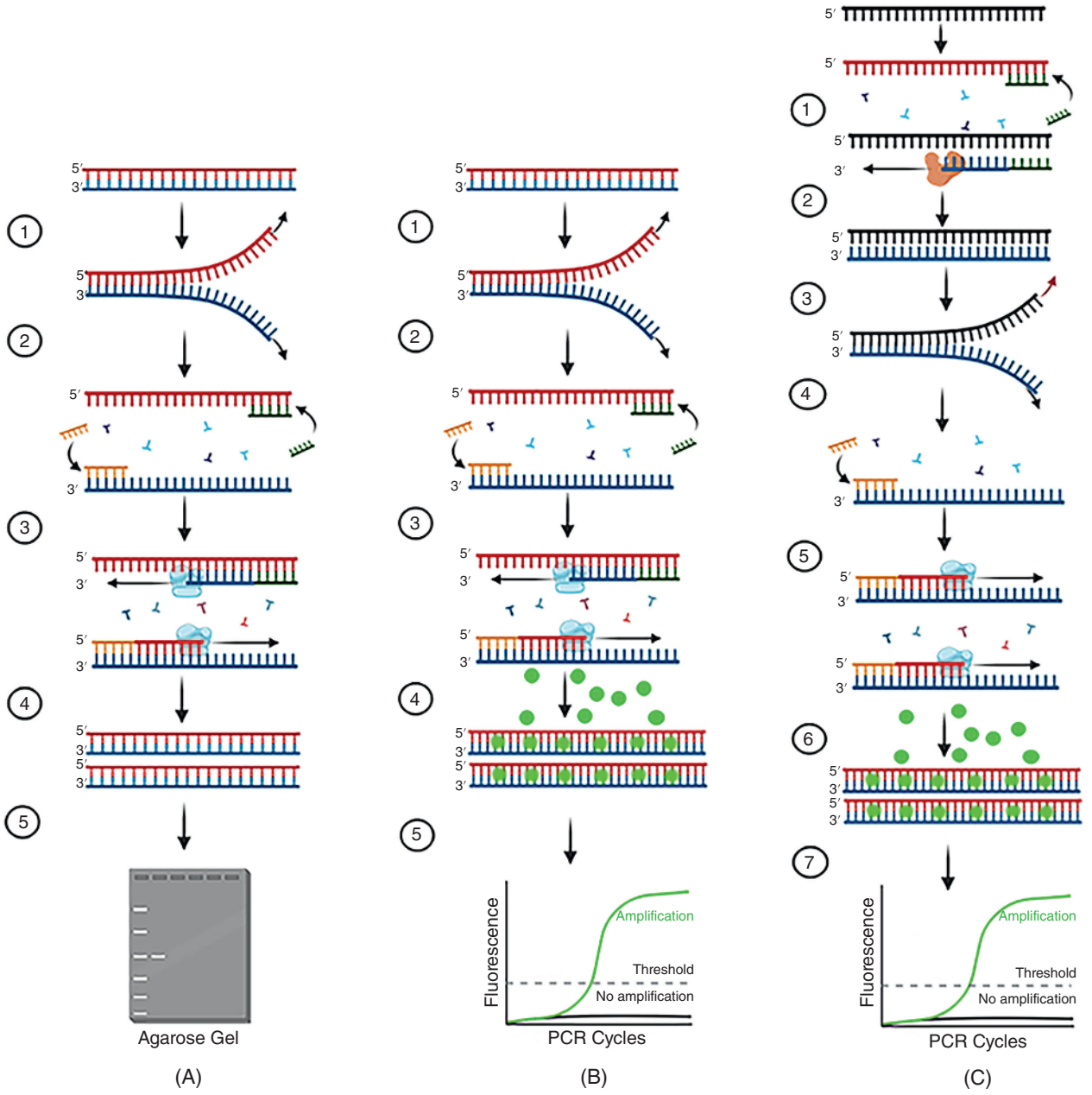

FIGURE 1 Thermal nucleic acids amplification methods. (A) Conventional PCR. Step 1, denaturation; step 2, primers annealing step; step 3, primer extension by *Taq* DNA polymerase; step 4: repeat of steps 1–3 (30–40 cycles) to yield dsDNA; step 5, visualization of PCR amplicons by agarose gel electrophoresis. (B) Real-time PCR. Same steps 1–3 as in (A); step 4: binding of DNA intercalating dye (dark green) to dsDNA; step 5, real-time increase and detection of fluorescence (bright green). (C) Reverse transcriptase real-time PCR. Step 1: primer annealing to RNA; step 2, reverse transcription of RNA to cDNA; step 3: degradation of the RNA strand, steps 4–7 correspond to steps 3–5 of (B). Created with BioRender.com.

temperatures using different approaches, including heating blocks (Peltier blocks) where heat is transferred through semiconductors, or surrounding air where heat is generated either using a light bulb or heating coil (9, 10) . Thermocyclers for real-time PCR additionally contain a fluorescence excitation source (e.g., a laser, a light-emitting diode, or a halogen lamp) and photodetectors (e.g., charge-coupled devices (CCDs) cameras, photodiodes, fluorimeters) to detect the fluorophore light-emission (11). Real-time PCR instruments come in a variety of formats to accommodate various laboratory needs. Instrumentation formats range from "sample-to-answer" platforms to high-throughput instruments that are able to perform thousands of PCR assays per day.

Detection Chemistry

In addition to the reaction components required for endpoint PCR, including primers, DNA polymerase, deoxynucleotides (dNTPs) and buffer, real-time PCR requires the addition of fluorescent dyes. Fluorescent signals are visualized using

specialized software that plot the fluorescence intensity with each cycle of amplification that produces dsDNA (Fig. 2A). The generation of fluorescence during amplification is achieved through binding of sequence-independent or sequence-dependent dyes or probes to the PCR products.

Fluorescent Dyes

Intercalating fluorescent dyes are sequence-independent dyes that bind nonspecifically to dsDNA, particularly to the DNA minor groove, and thus do not discriminate between amplicons and primer-dimers artifacts (12). When bound to dsDNA, the dye molecules fluoresce, and that fluorescence can be measured at a specific wavelength. Although EtBr was initially used to demonstrate real-time PCR (13), currently, the most widely used intercalating dye is thiazole green (most commonly sold as SYBR green I) and related compounds. When unbound, SYBR green emits little to no fluorescence, however, when bound to dsDNA, its fluorescence intensity increases by 1000-fold (Fig. 2A and 3A) (14). Further analysis of amplified nucleic acids when using intercalating dyes includes melt curve analysis (Fig. 2B and 2C) to confirm specificity and detect potential contaminants or artifacts. Use of intercalating dyes such as SYBR green presents several advantages. Because the dyes bind to dsDNA in a sequence-independent, nonspecific manner, they can be used generically in any real-time PCR since the specificity is dependent on primer selection for the desired target. This property allows real-time PCR assays to be cheap and easy to develop and design (15). Another advantage of intercalating dyes is their broad compatibility with typical thermocyclers on the market since the excitation and emission spectra of the dyes can be detected by the optical settings in most standard instruments (16). Utilizing intercalating dyes may require some optimization initially to determine optimal concentrations in order to prevent PCR inhibition or changes in DNA structure that could affect melting temperatures (17). Although intercalating dyes are nonspecific, some dyes preferentially bind with certain sequences; for example, SYBR green I preferentially binds to G-C rich sequences (15). To address some of these limitations, third- or next-generation intercalating dyes such as EvaGreen have been developed (18). EvaGreen has been optimized to be nonsaturating, produce low background fluorescence, and eliminate almost all inhibitory effects on the PCR reaction (16, 18). Common dyes used for real-time PCR are listed in Table 1.

Fluorescent Probes

Sequence-specific fluorescent probes are necessary to increase the specificity of real-time PCR assays and to perform more elaborate testing and analysis, including detection of single nucleotide polymorphisms (SNPs), multiplexing to detect multiple targets, and quantitation to determine pathogen burden. These probes are oligos with a sequence

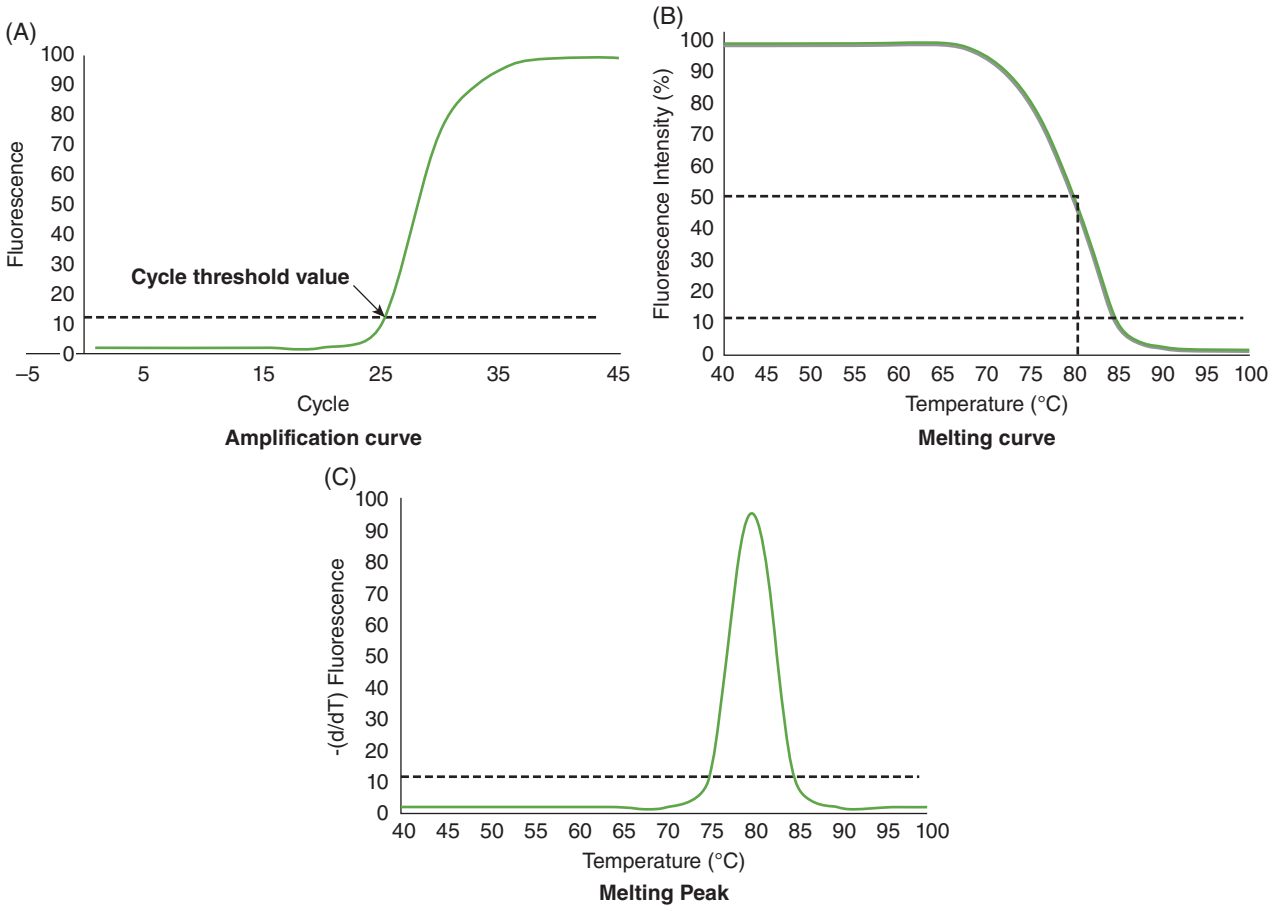

FIGURE 2 Real-time PCR. (A) Amplification curve. Increase in fluorescence and the resulting sigmoidal curve and cycle threshold value. (B) Melt curve analysis. After amplification is completed, the temperature is incrementally increased and the change in fluorescence of the PCR product plotted. (C) Melt curve. The Tm is the maximum peak of the first derivative of the melt curve.

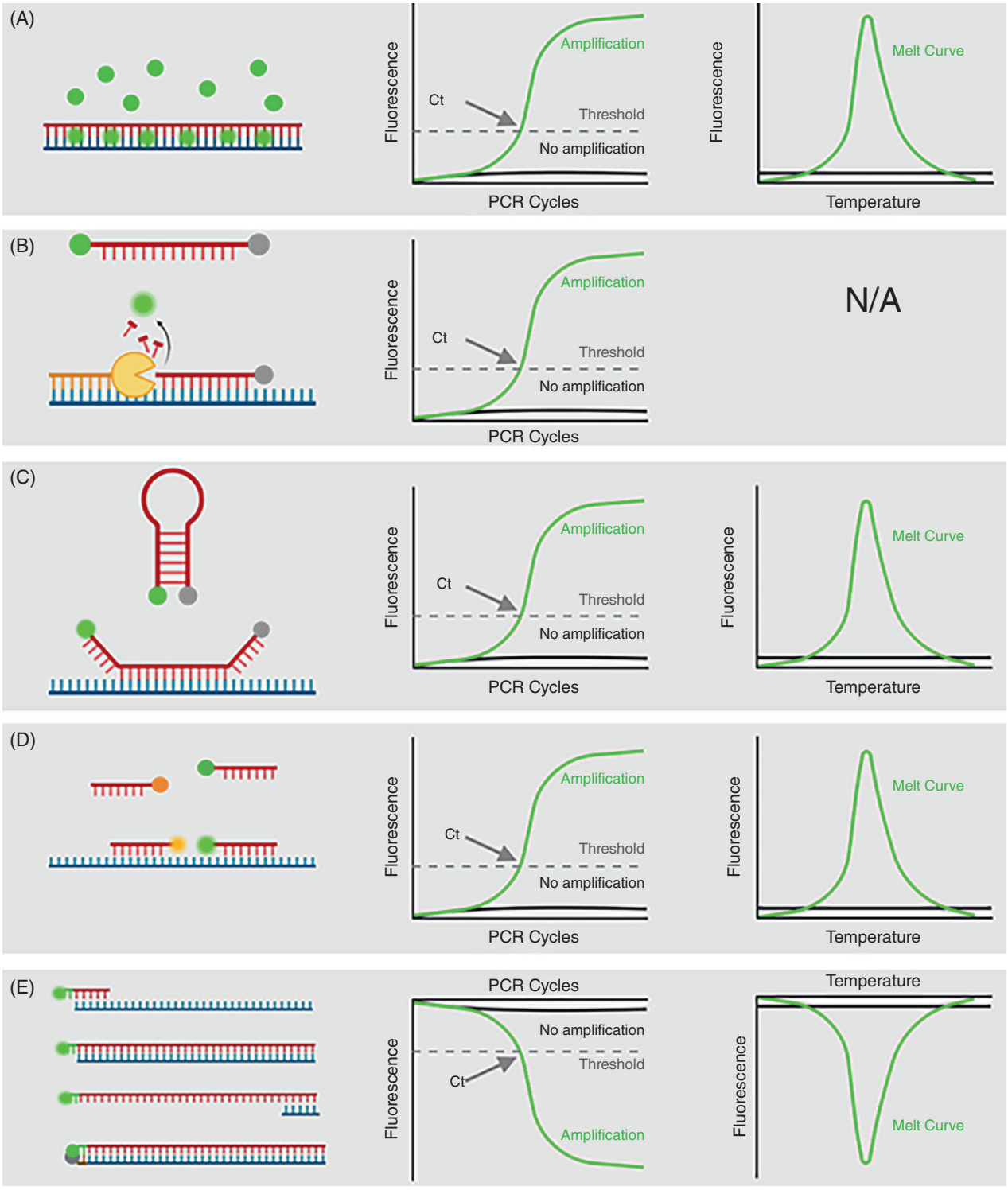

FIGURE 3 Real-time PCR detection chemistries. (A) Intercalating dye. Binding of dye (dark green) to dsDNA results in fluorescence (bright green) that is detected in real-time (amplification curve) and further analyzed using a melt curve analysis. (B) Hydrolysis probes. Binding of probes to the complementary DNA results in fluorescence (bright green) during extension step when the fluorophore is cleaved off away from the quencher (dark grey). Fluorescence is detected in real time (amplification curve) but no melt curve is performed since the probes are hydrolyzed. (C) Molecular beacon. Binding of probes to the complementary DNA results in fluorescence (bright green) during extension step when the fluorophore is separated from the quencher (dark grey). Fluorescence is detected in real time (amplification curve) and further analyzed using melt curve analysis. (D) Hybridization probes. Binding of the donor probe (dark orange) and the acceptor probe (dark green), results in transfer of energy from the excited donor (bright orange) to the acceptor that then emits fluorescence (bright green), which can be detected in real time (amplification curve) and further analyzed using melt curve analysis. (E) MultiCode primers. Labeled primers (bright green) bind to the complementary DNA and, as amplification and extension occur, incorporation of the quencher results in a decrease in fluorescence, which can be detected in real time (amplification curve) and further analyzed using a melt curve analysis. Created with BioRender.com.

TABLE 1 Examples of commercially available DNA intercalating dyes used for real-time PCR

Dye	Excitation peak (nm)	Emission peak (nm)	Manufacturer
SYBR Green	~498[a]	~520	ThermoFisher Scientific
ResoLight	487	503	Roche Life Science
EvaGreen	500	530	Biotium
LCGreen Plus	440–470	470–520	BioFire

[a] Main peak, additional peaks at ~290 nm and ~380 nm.

complementary to a region of the target internal to the forward and reverse primers. Fluorescent probes depend on the physical process known as fluorescence (or Förster) resonance energy transfer (FRET), which relies on the transfer of energy from an excited donor fluorophore to an acceptor fluorophore. Alternatively, a fluorophore and a quencher, instead of two fluorophores, may form the pair in the process. FRET is dependent on the distance between the donor and the acceptor fluorophore as energy is transferred through intermolecular long-range dipole-dipole coupling and an overlap in emission and absorption spectrums of the donor and the acceptor is necessary (19).

There are several types of fluorescent probes. Among the most widely used are the hydrolysis probes, also known as TaqMan probes. These probes are labeled with a fluorescent reporter dye at the 5′ end and a quencher at the 3′ end (Fig. 3B). When the probe is intact, close proximity of the quencher prevents the reporter dye from fluorescing and producing a detectable signal. During the annealing step, the probes hybridize to the complementary target sequence. As the *Taq* polymerase extends and synthesizes the new complementary strand, the 5′–3′ exonuclease activity of the *Taq* DNA polymerase cleaves the probe, releasing the quencher and allowing the reporter to fluoresce (8, 20). The detectable fluorescence is proportional to the quantity of amplified product. With each cycle, fluorescence is expected to increase if the target sequence is present. Hydrolysis probes are widely utilized for diagnostic assays because they increase the PCR specificity, are easy to multiplex, and do not require melt curve analysis. The downside of hydrolysis probe-based assays is the cost to synthesize custom probes for each assay in comparison to utilizing nonspecific intercalating dyes. This cost also increases with multiplexing.

In contrast to hydrolysis probes, molecular beacons do not depend on a polymerase with exonuclease activity. Molecular beacons are sequence-specific oligos that are folded in a hairpin structure with the loop section complementary to the target sequence (Fig. 3C). The two stem regions are complementary sequences with a reporter dye and a quencher attached at the 5′ and 3′ ends, respectively. The proximity of the reporter and the quencher in the hairpin prevents fluorescence of the reporter dye. During the annealing phase of amplification, the increased temperature melts the stem of the hairpin, allowing the target sequence to hybridize with the loop. Hybridization of the molecular beacon to the complementary target causes the reporter to separate from the quencher, allowing the reporter to fluoresce (21). Molecular beacons are advantageous for real-time PCR assays with short target sequences, for assays that require high specificity such as SNP detection, and for high-throughput testing and multiplexing. However, molecular beacons are challenging to design and optimize because the sequence and the melting temperature of the hairpin must be precise to allow for optimal denaturation and hybridization to the target sequence.

Dual hybridization probes utilize two sequence-specific probes: one probe contains a fluorophore at the 3′ end and acts as a donor probe, while the second probe contains a fluorophore at its 5′ end and acts as an acceptor probe (Fig. 3D). If the desired PCR target is present, the two probes hybridize to adjacent sequences on the target, about 1-5 bases apart. Following excitation, the donor fluorophore transfers emitted energy to the acceptor fluorophore, which absorbs it, becomes excited, and in turn emits fluorescence at a longer, third wavelength. The increase in fluorescence of the acceptor is proportional to the amount of target sequences present.

Modified Nucleotides

MultiCode-RTx (Luminex/Diasorin Molecular, Stillwater, MN) is an example of an alternative approach to real-time detection of PCR amplicons. This chemistry utilizes two modified bases, 5′-methyl-isocytosine (Iso-C) and isoguanine (Iso-G), labeled with a reporter fluorophore and a quencher, respectively (Fig. 2E) (22, 23). These two bases can only pair with each other. The iso-C nucleotide is linked to the 5′ end of the primer and during amplification the iso-G, bound to the quencher molecule, is incorporated into the complementary DNA strand, resulting in a decrease of fluorescence. Therefore, increase in PCR products results in a decrease in fluorescence detected, a reverse to other chemistries described earlier. Similar to fluorescent dyes and hybridization probes, a melt curve analysis can be performed with MultiCode reagents. Since this approach does not require the use of oligonucleotide probes, MultiCode chemistry is great for multiplexing assays as it only requires the modified bases to be labeled with a different reporter dye and linked to the 5′ end of a primer. Thus, the specificity is primarily dependent on the primers' sequences and several targets can be detected in one reaction. However, postamplification analysis requires a specific software to interpret the reverse spectra.

Postamplification Analysis

Postamplification analyses may vary depending on the chemistry used to detect the PCR amplicons as well as the design of the assay (e.g., laboratory-developed tests or commercial, *in vitro* diagnostics (IVD) tests). The detection of amplified products in real-time PCR occurs as soon as enough DNA is produced to bind to the fluorescent probes/dyes and produce a signal that can be measured by the photodetectors on the instrument.

In the early cycles of PCR, reaction components are abundant and the concentration of PCR products doubles with each cycle. As PCR progresses, the intensity of the fluorescent signal increases and produces a sigmoidal curve (Fig. 2A). The cycle at which the fluorescent signal crosses an established background threshold is referred to as the cycle threshold value or Ct value. The Ct value is usually set in the exponential phase of the amplification curve. The Ct value is inversely proportional to the concentration of initial DNA in the sample; the lower the Ct value the higher the DNA concentration (i.e., high concentration of DNA requires fewer PCR cycles to become detectable). In a well-optimized assay, every 3.3 cycles of a PCR run correlates to a 1-log increase in the target concentration. Although amplification continues past the Ct value point, the reaction reagents (e.g., primers, probes, dNTPs) begin to deplete and become limited, resulting in plateauing of the fluorescent

signal (Fig. 3A). This typically occurs around 40–45 cycles of amplification. Ct values are used in both quantitative and qualitative real-time PCR assays to establish analytical performance characteristics of tests including, for example, the lower limit of detection (LOD) and linear range of the tests. For quantitative real-time PCR, the Ct value can be used to determine the absolute amount of the amplified PCR target by extrapolating from a standard curve made using reference material of known quantities. For qualitative real-time PCR however, where the test is not calibrated to a standard curve, the Ct value is only used as a relative measure of the concentration of amplified PCR target. The interpretation of real-time PCR assays, whether quantitative or qualitative, is dependent on the performance specifications determined during the assay development.

For real-time PCR assays utilizing intercalating dyes or probes that are not hydrolyzed during the amplification, a melt curve analysis is performed immediately after amplification. The melting temperature (Tm) of a target DNA is defined as the temperature at which 50% of the dsDNA strands are denatured into single strands (Fig. 2B). As the dsDNA denatures, fluorescence of the intercalating dye decreases back to baseline. To determine the Tm of DNA amplicons, after amplification is completed, the temperature is incrementally increased and the change in fluorescence of the PCR product plotted. The Tm is calculated as the maximum peak of the first derivative of the melt curve and is unique to an oligonucleotide sequence (Fig. 2C). This process is automated on most real-time PCR thermocyclers.

Sequences with high G-C contents tend to have higher Tm. Theoretically, if a single PCR product is present, a single peak at the expected melting point is indicative of amplification of the desired target. The presence of multiple peaks reflects nonspecific amplification of untargeted DNA sequences or detection of PCR artifacts such as primer dimers (24).

BRIEF ASSAY DESIGN CONSIDERATIONS

There are several key components that must be considered when designing a real-time PCR test, from the selection of the gene to target to the design of the primers and/or probes. See also chapters 12–14 for more details.

Target(s) Selection

A key variable in designing a real-time PCR assay is the selection of the genomic (DNA or RNA) region that will be used as the amplification target. Whether the intent of the assay is to identify, quantitate, or genotype, target characteristics are important to understand, including: copy number, the existence of closely related paralogues, the existence of transcript or splice variants for mRNA targets, the presence of a potential secondary structure, the target mutational rate, and the effects of sequences directly upstream or downstream of the target (25). These characteristics can have a considerable influence on the sensitivity, specificity, and efficiency of the PCR assay. Other considerations when selecting a PCR target, particularly for clinical assays, include the specimens' source(s), the course of disease or infection and the overall clinical utility of detecting the selected target (26–29). An ideal genetic target should be unique and conserved in the organism of interest. Database resources such as the National Center for Biotechnology Information (NCBI) GenBank nucleic acid sequence database or the European Molecular Biology Laboratory Nucleotide Sequence Database (EMBL-Bank) can be used to view and analyze sequences, to identify conserved or highly variable regions, and to perform multiple sequence alignments, all of which can be informative in the target selection process.

Primers and Probes Design

Primers and probes are critical for both the sensitivity and the specificity of a real-time PCR test. Once the target has been selected, the design of the primers and probes will depend in part on the type of PCR detection chemistry that will be used as well as the assay intent. Primers and probes are short oligos approximately 18–24 bases long. The basic rules of primer design include choosing oligos that have a G-C content ranging from 40–60%, the inclusion of G-C pairs on the 5′ and 3′ ends, and a target melting temperature between 50–60°C. The annealing temperature of the oligos is also a critical parameter to prevent nonspecific amplification and secondary structures such as hairpin loops (25). Probes are designed to complement the target sequence between the primer pairs and usually have a Tm approximately 5°C higher than the Tm of the primers. There are numerous open-source primer and probe design programs including the NCBI's basic local alignment search tool (BLAST) (30) and commercial options from both vendors of real-time PCR thermocyclers and oligos manufacturers. These programs help ensure that all necessary parameters are included for optimal primer and probe design. The design of primers for multiplexed real-time PCR may be more complex depending on the number of expected targets. Interference between the various primer pairs or probes must be minimized. For example, the efficiency of a multiplexed PCR can be diminished due to primer dimers, which occur when complementary primers hybridize to one another instead of the target sequence (25).

Real-Time PCR Optimization

Once the set(s) of primers and probes have been designed, further assay optimization is necessary to ensure highest PCR efficiency. Optimization includes determining the best concentrations of each of the master mix components and the best thermal cycling temperatures. Traditionally, this process involved the manual and stepwise optimization of the concentration of each component of the master mix, including primers and probes (or DNA-binding fluorescent dyes), Mg^{2+}, *Taq* DNA polymerases, and dNTPs. Several ready-made master mixes are now commercially available that can simplify the process by providing solutions that include optimized concentrations of common real-time PCR components, such as dNTPs, polymerases, Mg^{2+}, buffers, and fluorescent dyes, depending on the type of desired assay. With these ready-made master mixes, the optimization process can focus primarily on finding the best concentration of primers and probes and on cycling parameters. The initial starting concentration is often provided by the oligos manufacturers (or the primers/probes design software) and typically ranges between 0.1 and 0.5 nM. Cycling parameters are optimized to ensure highest PCR efficiency. The initial cycling parameters often used are the default programs available on most real-time PCR instruments. Elements that are adjusted include the number of PCR cycles, the duration of each PCR step, and the temperatures, particularly for the annealing and extension steps, which will depend on the primers/probes melting temperatures and on amplicon size, respectively (11, 31).

Finally, the amount of template nucleic acids can also be varied. The amount of template nucleic acids will impact the sensitivity of the real-time PCR. Ideally, the maximum amount of the target of interest will be added to the PCR reaction to ensure maximum amplification and detection. However, high concentration of the template may result in PCR inhibition and false negative results. In addition to the quantity of input nucleic acids, the quality of the nucleic acids used in the PCR reaction will have a significant impact on the performance of the test, a topic discussed in subsequent chapters (see chapters 11 and 12). The hallmarks of a fully optimized assay include high amplification efficiency and high reproducibility over a wide dynamic range (25).

Quality Control

Quality control (QC) measures are essential to ensure the accuracy and reliability of real-time PCR assays (see also chapter 15). These measures include both QC reagents and QC protocols. Several guidance and regulatory documents (e.g., CLIA'88; College of American Pathologists [CAP] checklist, Clinical and Laboratory Standards Institute [CLSI] guidelines) provide information on the number and types of controls that should be used for real-time PCR tests, which will vary depending on the type of tests (e.g., qualitative versus quantitative PCR) (32–34). For qualitative real-time PCR assays, at least two levels of QC controls should be used, including a positive control and a negative control. These controls, whether commercially available or laboratory-developed, should closely mimic clinical samples, be in similar matrices, and be processed similar to clinical samples in order to assess all the steps of the assay from nucleic acid extraction to amplification and detection. For quantitative real-time PCR, at least three levels of controls are necessary, including negative controls as well as low and high positive controls to assess the quantitative range of the assay.

Two additional types of controls that can be used include the inhibition control and the no-template control (11, 28). The inhibition control is typically tested for in the same reaction as the clinical sample and can be either a human housekeeping gene (e.g., beta-globin) or a known genetic sequence seeded into the clinical sample prior to processing. Detection of the inhibition control at the expected cycle number confirms that all steps of the process, from extraction to amplification, occurred as expected. Lack of detection (or significantly reduced detection) of the inhibition control may reflect inhibition of the PCR reaction and is particularly important to distinguish between two potential reasons a sample would be negative for the target of interest: either the sample does not contain the target (i.e., the inhibition control is detected) or the PCR reaction was inhibited (i.e., inhibition control was not detected). The no-template control is used to monitor for potential contamination. As the name implies, this control includes all the components of the PCR reaction, except for a template, which is usually replaced by PCR-grade water or buffer and is used to identify contamination of the reagents with the target of interest. Unlike the negative control, the no-template control is used only for the PCR reaction and does not go through the nucleic acid extraction process. A no-template control should be negative for the PCR target.

While real-time PCR assays are performed in relatively closed systems compared to endpoint PCR, contamination with either the PCR target or the PCR amplicon may still occur (35). Several practices and procedures can be utilized in the laboratory to prevent and minimize contamination. The design of the molecular laboratory space is key in preventing contamination (see also chapter 10 for more details). Ideally, testing should follow a unidirectional workflow in which reagent preparation occurs in a "clean room" where specimens or amplified products are never brought in, followed by sample preparation and extraction in a separate space, and finally, amplification in a third space, often referred to as the "dirty" room due to the presence of PCR amplicons (Fig. 4) (36). The dirty room should have negative pressure compared to the space outside to prevent potential amplicon contamination. Each room should

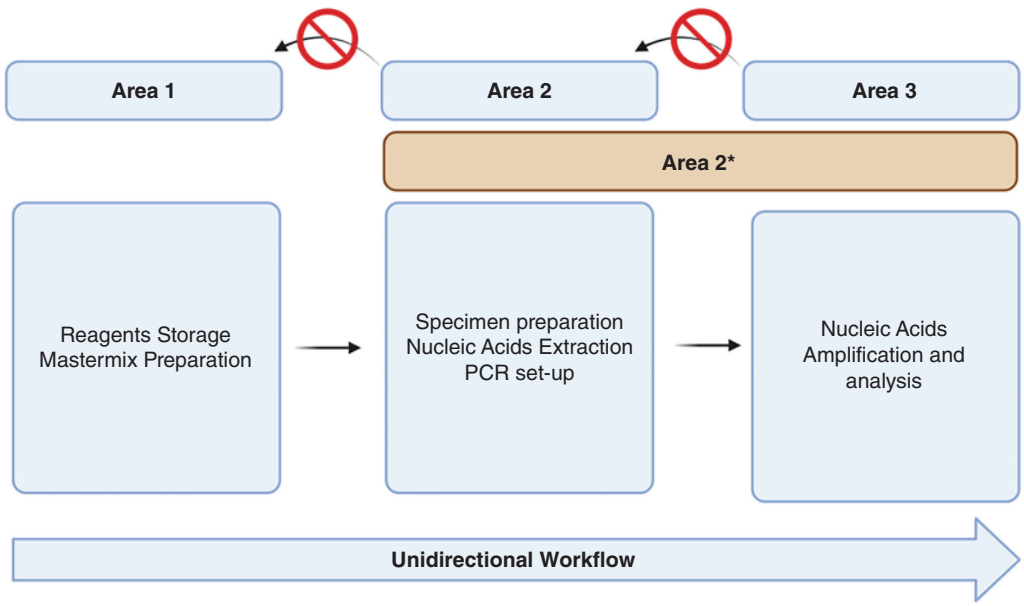

FIGURE 4 Recommended unidirectional workflow. Area 1, pre-PCR processes, clean room; Area 2, pre-PCR processes, specimen manipulation; Area 3: "dirty" room, PCR and post-PCR analysis. With "sample-to-answer" closed systems, Areas 2 and3 are combined as these processes occur within one instrument (Area 2*). Created with BioRender.com.

ideally have dedicated equipment, supplies, and personal protective equipment to mitigate carryover between rooms. If separate rooms are not available, dedicated spaces or areas of the laboratory and use of dead air boxes or biosafety cabinets (BSC) can be implemented to create unidirectional flow (37). Of note, with many of the current "sample-to-answer" PCR platforms where specimen processing, nucleic acid extraction, amplification, and detection are all integrated, the need for multiple testing areas has been reduced or eliminated, but precautions should still be maintained (Fig. 4).

In addition to the unidirectional workflow, practices such as changing gloves between samples and aliquoting reagents also help prevent and mitigate the opportunity for contamination. The use of consumables such as aerosol barrier pipette tips and low-binding, DNase- and RNase-free plastic consumables adds an extra layer of contamination prevention. Addition of uracil-DNA-glycosylase (UNG) and deoxyuridine triphosphate (dUTP) in the master mix can help mitigate carryover of amplified product (i.e., contamination by amplified DNA) (36). dUTP is incorporated into the PCR product during amplification and subsequently the uracil base is cleaved from the phosphodiester backbone of uracil-containing DNA by UNG, blocking DNA polymerase activity and preventing amplification of contaminating amplicons (37, 38).

Performing periodic wipe tests or environmental swabbing of laboratory surfaces and equipment can help identify areas of amplicon contamination. Decontamination of laboratory surfaces should be routinely performed using 10% bleach followed by ethanol, special decontamination products such as DNA AWAY and RNase AWAY and/or using UV light fixtures in BSC and dead-air boxes (28, 37).

VARIATIONS ON REAL-TIME PCR

While real-time PCR is a powerful tool in infectious disease diagnostics, variations on this technology have further enhanced its capabilities and utility in the clinical laboratory. Some of these variations include multiplex real-time PCR, reverse transcriptase real-time PCR, nested real-time PCR, and quantitative real-time PCR. Many real-time PCR assays currently in use in clinical microbiology laboratories utilize one or several of these variations on real-time PCR.

Multiplex Real-Time PCR

Multiplexing allows for the amplification and detection of more than one target in a single PCR reaction. Each genetic target is amplified using separate primers and probes. One of the first applications of multiplex PCR was in prenatal screening to detect multiple gene deletions in Duchenne muscular dystrophy (39). Today, multiplex real-time PCR is a mainstay of infectious disease diagnostics and includes a wide range of testing, from duplex assays (e.g., *Chlamydia trachomatis/Neisseria gonorrhoeae* PCR) to some large syndromic panels with more than thirty targets (e.g., pneumonia panels). Prior to the advent of real-time PCR, amplicons were separated via gel electrophoresis. However, current multiplex PCR reactions take one of two approaches for detection. For assays with fewer targets, primers and probes for different targets utilize different fluorophores. As these fluorophores emit energy at different wavelengths, reactions for the different targets can be separately monitored and detected. The number of targets that can be tested using this approach is limited by the number of fluorophores that can be utilized without overlapping emission spectrums and the number of detection channels available on real-time thermocyclers (Table 2).

TABLE 2 Select common dyes used for real-time PCR

Dye	Maximum excitation wavelength (nm)	Maximum detection wavelength (nm)
FAM	494	520
HEX	535	560
JOE	520	548
TET	521	538
VIC	538	552
TAMRA	560	582
Texas Red	598	617
Cy3	550	564
Cy5	648	668
Quasar 705	672–684	705–730
ROX	587	607

Utilizing multiplex PCR has added significant functionality to real-time PCR. However, there are a few unique challenges that must be considered when designing multiplexed PCR reactions. First, the primers must be designed to have similar annealing temperatures, as one annealing temperature will be used for the overall reaction (40). The primers must also lack internal homology to avoid primer dimer formation. With a greater number of targets, the specificity of multiplex PCR may be difficult to achieve. Competition for common reagents to amplify multiple targets may impact the sensitivity of a multiplex PCR compared to the corresponding singleplex PCR and should be considered in the assay design.

Microarrays

Microarrays can be used to multiplex for a higher number of targets (41, 42). In a microarray, multiple copies of a target-specific DNA capture probe are either fixed in a specific order to a slide or chip (solid-phase microarrays) (43) or on microsphere beads (suspension microarrays) which have been internally labeled with two or three fluorophores (44). PCR products of the target of interest are fluorescently labeled and hybridize with these capture probes based on sequence complementarity. The identity of the amplified target is determined by the location of the spots for solid-phase microarrays or the beads to which the amplicon hybridized. Amplification is measured either optically or by flow cytometry. Each target may be represented by multiple microarray spots to increase specificity. The large number of spots or beads allows for the high multiplexing capacity of the microarrays (42).

Most microarrays use endpoint PCR rather than real-time PCR for target detection, even if done using fluorescence. The large number of possible probes or fluorescent dyes mixtures allows for significant increase in multiplexing with limited or no amplicon manipulation, similar to real-time PCR tests. Guidance for developing and validating microarrays has been published, however, this can be a challenging effort (45). Numerous commercial assays based on microarray technology are available for several clinical syndromes including respiratory infections (e.g., NxTAG Respiratory Pathogen Panel), gastrointestinal infections (e.g., BioCode Gastrointestinal Pathogen Panel) and bloodstream infections (e.g., ePlex Blood Culture Identification Panels).

Reverse Transcriptase Real-Time PCR

When the target sequence is RNA (e.g., ribosomal RNA) instead of DNA, the target has to first be converted to DNA in order for amplification to occur. This process is accomplished using enzymes with reverse transcriptase (RT) activity to convert the RNA sequence into a complementary DNA (cDNA) sequence that is used as the template for real-time PCR (Fig. 1C) (46, 47). RT-PCR is useful for monitoring genetic expression via mRNA or when detecting or quantifying RNA viruses. Initially, RT-PCR required two separate steps: a reverse transcription step using a reverse transcriptase enzyme followed by a separate PCR powered by a DNA-dependent DNA polymerase. RT-PCR can now be accomplished in one tube using the *Thermus thermophilus* DNA polymerase, which has both reverse transcriptase and DNA polymerase activity (48). Prior to reverse transcription, RNA must first be denatured via heating to unwind secondary structures and to ensure the enzyme can access all parts of the RNA strand (49). For the reverse transcription reaction, gene-specific primers can be used to selectively reverse transcribe targets of interest, or poly-dT oligos can be used (49). These bind to the poly-A tails of mRNA and facilitate reverse transcription of all poly-A tailed mRNA. Several commercial tests, both singleplex and multiplex RT-PCR are available and FDA-cleared for routine diagnosis of many respiratory viruses, including influenza and SARS-CoV-2.

Nested Real-Time PCR

In nested PCR, two sequential PCR reactions are performed, wherein the PCR product from the first reaction is used as the template for the second PCR (50). Two sets of primers are used, and the set of primers used in the second reaction must be internal to those used in the first reaction (48). This approach increases both the sensitivity of the PCR, as there are more overall cycles in the two successive PCR reactions, and the specificity of the PCR, as primer sets from both reactions must bind (rather than just one set of primers in non-nested PCR). When one of the primers used in the first reaction is also used in the second reaction, the PCR is termed semi-nested PCR (51). When the second reaction set up is performed manually, nested PCR is prone to contamination issues, as the amplicon resulting from the first reaction must be manipulated during set up of the second reaction. The use of fluorescent probes and dsDNA binding dyes in some commercially available technologies have circumvented this concern by incorporating nested PCR in sample-to-answer testing systems (52).

Quantitative Real-Time PCR

In quantitative real-time PCR, quantitation of a target is performed by comparing the Ct value of the target in one sample to either another target in the same sample or to the Ct value of the same target in a different sample (53). In the first case, the Ct value of the target of interest may be normalized to the Ct value of a housekeeping gene with stable expression in the same specimen. This quantitation strategy is commonly used in gene expression studies. Alternatively, a standard curve can be created by testing specimens with known concentrations of an analyte or prepared standards (54). A linear relationship can then be determined between analyte concentration and Ct value, and this can be used to extrapolate the concentration of the analyte in the clinical specimen based on the Ct value measured. This approach is commonly used for viral loads measurements. As quantitation is relative, extraction efficiency, reverse transcription efficiency (if applicable), and amplification efficiency must ideally be similar between standards and samples being tested. Small sample-to-sample or sample-to-standard variations in efficiency can dramatically alter results (55). Using added exogenous materials as an internal control detected via multiplex reaction can help detect and correct any variation that may affect the quality of the results.

ADVANTAGES AND LIMITATIONS OF REAL-TIME PCR

Sensitivity and Specificity

Due to the exponential amplification that occurs during PCR, these assays are typically very sensitive. In a hypothetical assay consisting of forty cycles with 100% amplification efficiency, one input copy would result in over 1 trillion copies by the end of the reaction. This sensitivity is especially valuable when the target organism is difficult to culture or requires special media (e.g., *Bordetella pertussis*, *Mycoplasma* species) (56). As amplification relies on sequence homology with primers/probes, analytical specificity is generally high. It should be noted, however, that while PCR is a sensitive method, the clinical sensitivity might be low if the disease process does not result in the presence of pathogen nucleic acid in sufficient concentration in the specimen being evaluated at the time of collection. For example, serology is the preferred testing methodology for *Borrelia burgdorferi* and *Treponema pallidum* in blood samples, as PCR testing lacks clinical sensitivity (56).

While enhanced sensitivity can be a major advantage when designing an assay for an organism that is considered pathogenic when present in any amount, interpretation of PCR results may be challenging if the organism of interest can also cause asymptomatic colonization. For example, *Clostridioides difficile* and *Pneumocystis jirovecii* PCR can detect asymptomatic colonization, which may confound diagnoses; in these cases, test stewardship, patient selection, and clinical correlation are essential (57, 58). The reader is referred to chapter 31 for additional discussion of this topic.

Turnaround Time

Real-time PCR is universally faster when compared to culture-based techniques. For fast-growing pathogens, this difference may seem minor (1–2 hours for PCR versus 18–24 hours for culture). However, even these minor differences can result in faster time to patient isolation, fewer nosocomial infections, and fewer doses of broad-spectrum antibiotics (59, 60). Turnaround time (TAT) differences are even more dramatic for pathogens that may be difficult to culture or slow growing. For example, viral culture of respiratory specimens required at least 2 days using shell vials and up to 2 weeks with traditional viral tube culture (61). In contrast, multiplex respiratory panels can detect more than twenty pathogens in 1 to 2 hours. Similarly, detection of *Mycobacterium tuberculosis* complex via smear typically requires multiple hours of hands-on time by trained technologists and results are often not available until the following day (62) and recovery in culture and antimicrobial susceptibility testing may require an additional 6–8 weeks. In contrast, commercially available real-time PCR assays can yield results in approximately 2 hours by technologists without specific mycobacteriology expertise and provide

information on rifampin resistance as well. The rapid TAT of PCR is a major consideration when considering the cost-effectiveness of real-time PCR.

Costs

While many large multiplex molecular panels can cost more than $100 for reagents, even simpler PCR tests are significantly more expensive than comparable culture-based methods. Early *Bordetella pertussis* PCR tests were 3–6 times the cost of culture, though results were available 2.7 days faster (63). Similarly, group B *Streptococcus* screening via PCR was 45–300% more expensive than culture-based testing (64). Despite increased costs, many published studies show that molecular methods, including real-time PCR, are cost-effective alternatives to culture-based methods due to the patient care implications resulting from increased performance and decreased TAT (65–67). As novel PCR assays are developed, a focus on costs and not just test performance will be necessary to ensure test equity for all patients.

Differentiation between Viable and Nonviable Organisms

As real-time PCR detects nucleic acids only, the method cannot distinguish between viable and nonviable organisms. In contrast, culture-based methods detect only organisms that are both viable and replication competent. In research settings, propidium monoazide can be used in real-time PCR assays to detect only viable organisms, though this is not routinely performed in clinical practice (68, 69). This limitation of PCR must be considered when interpreting results. Real-time PCR can be used to monitor response to therapy over time, but this is typically only recommended where quantitation is possible, asymptomatic disease is still clinically relevant, and guidelines are established for result interpretations. This is clearly illustrated with viral loads monitoring of HIV, hepatitis B virus, and hepatitis C virus (70, 71). In contrast, assays primarily used for diagnosis where quantitation is not possible should not be used as a test-of-cure, as nucleic acids may be detected from nonviable organisms. This can result in extended test positivity with questionable clinical relevance (58, 72, 73).

Required Expertise

As PCR has become a mainstay diagnostic technique in the clinical microbiology laboratory, knowledge, expertise, and range of skills needed has expanded to cover the wide range of available PCR methods with different complexity levels. Many sample-to-answer PCR systems are now available that require little hands-on time and few manual manipulation steps, and thus molecular expertise may not be necessary. Additionally, due to simple assay setup and rapid TAT, molecular testing is being performed in settings where traditional (culture-based) microbiology would be performed (small hospital labs, physician offices, etc.). While open-system lab developed tests are more prone to contamination, false positives have also been noted in some sample-to-answer based systems (74, 75). As these systems are more likely to be utilized in labs where microbiology-specific oversight is limited or even as POC devices (76, 77), ensuring all users have adequate understanding of potential pitfalls will be essential for quality results. For moderate and high-complexity real-time PCR assays, the availability of high-throughput real-time PCR platforms has simplified the workflow for many pathogens and decreased the level of skill and expertise needed to perform PCR. However, for many other, more complex commercial tests, as well for laboratory-developed real-time PCR tests, a high degree of skill, knowledge, and expertise is still required.

CLINICAL APPLICATIONS OF REAL-TIME PCR

Sample-to-Answer Platforms

Developments in microfluidics, microengineering, and improved probe chemistry have enabled miniaturization of real-time PCR instrumentation into platforms that combine nucleic acid extraction, amplification, and detection. In these simplified PCR methods, the sample is added to a cartridge or a pouch, loaded on the platform and, with no further user interaction, results are generated and interpreted. These "sample-to-answer" PCR platforms have expanded PCR testing access to a wider range of laboratory settings, including POC locations. One of the first of these platforms to receive FDA clearance was the GeneXpert with approval of the Xpert Group B *Streptococcus* assay in 2006 (78). The test menu for the GeneXpert has significantly expanded in the last 20 years and other manufacturers have introduced similar commercial platforms.

Initially, many "sample-to-answer" platforms offered primarily singleplex assays or small panels that targeted two or three pathogens. In 2008, the first broadly multiplexed PCR test, the xTAG Respiratory Viral Panel (RVP) (Luminex Molecular Diagnostics, Toronto, Canada) was FDA-cleared. The xTAG RVP targeted twelve viruses, a significant leap in the diagnosis of respiratory infections and the beginning of syndromic panel testing. However, the test was labor-intensive, included several steps, and carried a high-risk of contamination, which limited testing to high-complexity laboratories (79). Approval of the xTAG RVP was followed by the FDA clearance of the Idaho Technologies FilmArray respiratory syndromic panel (bioMérieux, Salt Lake City, UT) that included the same viruses as the xTAG RVP plus Coronaviruses NL63 and HKU1 and Parainfluenza virus 4 but in a "sample-to-answer" platform. The assay was further expanded to include additional viruses as well as bacterial targets associated with atypical pneumonia including *B. pertussis*, *C. pneumoniae*, and *M. pneumoniae*. The FilmArray combines automated sample preparation, total nucleic acid extraction with nested multiplex PCR, reverse transcriptase PCR, and automated detection of amplified targets through DNA melting curve analysis (52). All reagents and controls are contained within the FilmArray pouch and results are available in about one hour (52, 79, 80).

Today, these "sample-in, answer-out" singleplex and syndromic multiplexed tests are used in several clinical applications that benefit from a rapid TAT including health care-associated infections (e.g., *C. difficile*, MRSA), meningitis/encephalitis (e.g., Enteroviruses), and respiratory infections (e.g., influenza viruses). The panels vary in the numbers of pathogens targeted (2 to 30), the type of pathogens included (e.g., bacteria, viruses, or yeasts), the level of complexity (low versus high) and the TAT to results (from 1 hour to 12 hours). In general, performance characteristics show comparable sensitivity and specificity greater than 90% when compared to culture or bidirectional sequencing as the reference methods (81). While their impact is clear for some indications (e.g., upper respiratory tract infections) and have become the standard of

care, for many other syndromes, their clinical utility continues to be explored (82).

Testing at the Point of Care (Including CLIA-Waived Testing)

Prior to the coronavirus disease 2019 (COVID-19) pandemic, a handful of real-time PCR based tests had been developed for testing in POC settings including core laboratories or emergency room departments (Table 3). Many of these POC PCR tests were developed as an alternative to the less sensitive lateral-flow immunoassays (LFA) that had been widely available for several years for the detection of influenza (Flu) and respiratory syncytial virus (RSV) antigens. While the specificity of antigen tests has generally been high (i.e., >97%), the pooled sensitivity as reported in various meta-analyses ranged from 75–80% for RSV, 54.4–80% for FluA and 53.2–76.8% for FluB, with higher sensitivity observed when using a digital reader (83). Given this limitation of antigens tests, the FDA reclassified Flu antigen tests in 2017 from class I to class II devices, with a minimum requirement for sensitivity of ≥ 80% when compared to molecular tests (84).

The first CLIA-waived molecular test was approved in 2015. The Alere i Influenza A&B (Alere Scarborough, Scarborough, ME) used an isothermal amplification method (nicking endonuclease amplification reaction [NEAR]) to detect and differentiate FluA and FluB in nasopharyngeal swab (NPS) specimens (85). This test was followed soon after by multiple PCR tests including the Roche Liat (Roche), the Xpert Xpress Flu (Cepheid) and the BioFire EZ (BioFire) (83). The availability of these waived PCR tests marked a significant shift in the evolution of PCR tests for infectious diseases diagnosis and management, by

TABLE 3 Clinical indications with CLIA-waived PCR tests[a]

Manufacturer	Test name	Indications	Target	Methodology
binx health limited	binx health *io* CT/NG Assay	STI	*Chlamydia trachomatis* and *Neisseria gonorrhoeae*	Ultra-rapid PCR, electrochemical detection
BioFire Diagnostics limited	FilmArray Respiratory2.1 EZ	URTI	Adenovirus, coronavirus, human metapneumovirus, influenza A (H1, H1 2009, H3), influenza B, parainfluenza virus, RSV, *Bordetella pertussis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*	Nested multiplex PCR, high resolution melting curve analysis
	BioFire Spotfire Respiratory (R) Panel Mini	URTI	SARS-CoV-2, human rhinovirus, influenza A, influenza B, RSV	Nested multiplex PCR, high resolution melting curve analysis
	BioFire Spotfire Respiratory (R) Panel	URTI	Adenovirus, coronavirus (seasonal), SARS-CoV-2, human metapneumovirus, human rhinovirus/enterovirus, influenza A (H1 2009, H3), influenza B, parainfluenza virus, RSV, *Bordetella pertussis*, *Bordetella parapertussis*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*	Nested multiplex PCR, high resolution melting curve analysis
Cepheid	Xpert Xpress MVP	Bacterial vaginosis, vulvovaginal candidiasis, trichomoniasis	Bacterial vaginosis, *Candida group* (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. dubliniensis*), *C. glabrata*/*C. krusei*, *T. vaginalis*	Multiplexed real-time PCR
	Xpert Xpress Strep A	Pharyngitis	Group A *Streptococcus*	Real-time PCR
	Xpert Xpress CoV-2 plus	URTI	SARS-CoV-2	Real-time RT-PCR
	Xpert Xpress CoV-2/Flu/RSV plus	URTI	SARS-CoV-2, influenza A, influenza B, RSV	Multiplexed real-time RT-PCR
	Xpert Xpress Flu	URTI	Influenza A, influenza B	Multiplexed real-time RT-PCR
Roche Molecular	cobas Liat Influenza A/B & RSV	URTI	Influenza A, influenza B, RSV	Multiplexed real-time RT-PCR
	cobas Liat SARS-CoV-2 & Influenza A/B	URTI	SARS-CoV-2, influenza A, influenza B, RSV	Multiplexed real-time RT-PCR
	cobas Liat Strep A	Pharyngitis	Group A *Streptococcus*	Real-time PCR
Visby Medical	Visby Medical Respiratory Health Test	URTI	SARS-CoV-2, influenza A, influenza B	Multiplexed real-time RT-PCR
	Visby Medical Sexual Health Test	STI	*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*	Multiplexed real-time PCR

[a] This list was compiled based on data available as of March 1st, 2024. A complete list of FDA-cleared nucleic acid-based tests can be found on the FDA's website: https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests with additional information for waived tests at https://www.cms.gov/files/document/r12415cp.pdf.

bringing testing closer to the patient, similar to antigen testing, while still maintaining accuracy comparable to standard laboratory real-time PCR assays. In studies comparing the performance of CLIA-waived and POC PCR tests, the sensitivity and specificity for the detection of FluA, FluB, and RSV was greater than 95% for all three targets (86).

The COVID-19 pandemic resulted in a substantial increase in the number of molecular tests for use at the POC, including the first at-home molecular tests. While many of these POC tests used isothermal amplification methods for detection of SARS-CoV-2 RNA, several were based on RT-PCR. These waived PCR tests maintained the rapid turnaround time of LFAs, however, implementation presented some challenges. For example, unlike antigen tests, PCR tests are significantly more expensive and while these assays are provided in closed systems, the potential for environmental contamination and false positives is not zero and thus, appropriate quality control and quality assurance processes still need to be considered (83, 87).

The menu of POC PCR tests has now expanded to combine detection of SARS-CoV-2 with FluA, FluB, and/or RSV. Beyond testing for respiratory viruses, current clinical indications for PCR testing at the POC include bacterial pharyngitis (i.e., group A *Streptococcus*), women's health (e.g., bacterial vaginosis), vaginitis (e.g., candidiasis) and sexually transmitted infections (e.g., *Neisseria gonorrhoeae*, *Chlamydia trachomatis*) (Table3).

High-Throughput Testing Platforms

Real-time PCR forms the basis of several FDA-cleared high-throughput platforms used for the monitoring of viral loads (e.g., cytomegalovirus, Epstein-Barr virus) in transplant patients, the detection and management of bloodborne viral pathogens (e.g., HIV, HBV, HCV) and testing for sexually transmitted infections (e.g., human papillomavirus, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*) (28, 88) (Table 4). For viral load testing, one of the biggest challenges has been the lack of standardization across laboratory-developed tests due to several factors including differences in gene target selection, extraction, and PCR platforms, as well as calibration methods (88, 89). These variables limit comparison of viral loads obtained by different tests and make it challenging to establish viral thresholds for management of these infections (88, 90–95). The availability of the WHO international standards (96, 97), as well as increased availability of commercial, FDA-cleared assays, offer increasing opportunity to improve standardization of viral load testing across centers. In one study, the use of the EBV WHO standards to convert viral loads from copies/ml to IU/ml resulted in lower EBV values and reduced, but did not eliminate, variability in viral load measurements (98). As there continue to be increasing numbers of commercially available FDA-approved tests performed on systems requiring minimal sample manipulation with results calibrated to WHO standards, it is expected that this will improve the comparability and standardization of viral tests results (99).

Emerging Applications

In 2019, The WHO issued REASSURED (**R**eal-time connectivity, **E**ase of sample collection, **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free and **D**eliverable) criteria to guide the development of diagnostic tests that could be available in resources-limited settings (100). The same criteria are driving innovation for more point-of-need testing, even in resource-rich settings, where issues such as losing patients to follow-up while waiting for laboratory results can have a significant impact on both the individual and public health (e.g., hepatitis testing [101]).

Improvement in reagent chemistry, instrumentation, and microfluidics are resulting in faster TAT for real-time PCR. A recent report described the development of an ultra-rapid real-time PCR that could complete 40 PCR cycles in less than 10 minutes (26, 102). The authors accomplished this TAT by building "an ultra-fast mechatronic real-time PCR with injection molded polycarbonate microfluidic chips" which allowed them to rapidly ramp up heating, improve heat transfer, and simultaneously perform thermal cycling and fluorescence measurement. A proof-of-principle test on a NPS positive for SARS-CoV-2 demonstrated that their method reduced the viral RNA detection time from 56 minutes to less than 15 minutes (102).

In addition to faster PCR, novel approaches are allowing for increased capability for multiplexing. One example is high-definition PCR (HDPCR), a method that has now been commercialized by ChromaCode, Inc. HDPCR uses standard reagents and TaqMan probes to support multiplexing of up to 20 targets in a single well (103). Instead of target identification based on detection of a specific fluorophore, each target is assigned an endpoint amplitude through modulation of the TaqMan probe concentrations in the various PCR channels. A research-use-only HDPCR tick-borne panel that targets nine tick-borne pathogens has been evaluated on whole blood samples (104, 105). Overall agreement with standard real-time PCR was generally high for most targets (i.e., >95%), although in one study performance for detecting *Borrelia burgdorferi* was low (44.4%) (105), suggesting that further improvements are needed.

TABLE 4 Select FDA-cleared high-throughput real-time PCR platforms

Manufacturer	Platforms	Maximum throughput (number of samples/8–10 hours)	Examples of sample types	Examples of targets
Abbott Molecular	Alinity m	300 in ~8 hours	Plasma, serum, urine, vaginal swabs, cervical swabs, throat swabs, nasopharyngeal swabs	Cytomegalovirus, Epstein-Barr virus, hepatitis B virus, hepatitis C virus, HIV, *Chlamydia trachomatis/Neisseria gonorrhoeae/Trichomonas vaginalis/Mycoplasma genitalium*, SARS-CoV-2
Becton, Dickinson and Company	BD Viper	120 in ~9.5 hours	Cervical swabs	Human papilloma virus
Roche Molecular Systems, Inc.	Cobas 5800 Cobas 6800 Cobas 8800	144 in ~8 hours 384 in ~8 hours 1056 in ~8 hours	Plasma, serum, urine, vaginal swabs, cervical swabs, throat swabs, nasopharyngeal swabs	BK polyomavirus, cytomegalovirus, Epstein-Barr virus, hepatitis B virus, hepatitis C virus, HIV, human papilloma virus, *Chlamydia trachomatis/Neisseria gonorrhoeae*, SARS-CoV-2

SUMMARY

The implementation of real-time PCR in clinical laboratories has had a major impact on infectious diseases diagnostics and for many syndromes, these tests are now the gold standard (106). Improvements in reagent chemistry and instrumentation have provided faster TAT. However, the need for faster results, more affordable and readily accessible tests for a greater number of conditions, will continue to grow and will drive innovations in real-time PCR methods.

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