CHAPTER

2

Laboratory approaches in molecular pathology: the polymerase chain reaction

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Abstract

The polymerase chain reaction (PCR) represents a rapid, sensitive, and specific method for in vitro amplification of nucleic acid sequences. Using specific oligodeoxynucleotide primers and a DNA polymerase, the PCR can identify a target sequence and amplify millions of copies (amplicons) of the target. PCR was first described in the mid-1980s. Since then methodological modifications, developments, and new instrumentation have combined to enhance the technology, which has evolved into a reliable, affordable, user-friendly method that is performed in laboratories worldwide. The major technological breakthrough in the development of PCR was the introduction of a thermostable DNA polymerase. The second major technological breakthrough in the development of the PCR was the introduction of the programmable heat block that automatically changes the reaction temperature during each amplification cycle known as the thermocycler. PCR is now used as the basis for numerous clinical molecular diagnostic tests in laboratories worldwide. Real-time PCR, reverse-transcriptase quantitative PCR (RT-qPCR), and digital PCR (dPCR) all allow for the quantification of the target with varying applications that depend on the method's sensitivity. The most recent methodological modification, dPCR provides the absolute quantification and is sensitive enough to detect rare alleles and perform copy number analysis.

Introduction

The polymerase chain reaction (PCR) represents a rapid, sensitive, and specific method for in vitro amplification of nucleic acid sequences. Using specific oligodeoxynucleotide primers, PCR can identify a target sequence and then a DNA polymerase is used to amplify millions of copies (amplicons) of the target. PCR was first described in the mid-1980s and made its initial impact on the research laboratory [1–3]. Once clinical laboratories realized the power of this technique, it quickly became the basis for numerous applications in clinical testing. Since then, developments related to methodological modifications and new forms of instrumentation have combined to enhance the technology, which has evolved into a reliable, affordable, user-friendly method performed in laboratories worldwide. There is little question that PCR has had an extraordinary impact as a modern technology in molecular diagnostics. PCR methodology has become routine and the instrumentation required is standard/available to most/all laboratories. Thus, it is easy to underestimate and take for granted the significant impact of PCR on the day-to-day operation of both clinical molecular diagnostics laboratories and basic science research laboratories.

The PCR offers a sensitive and specific method to perform quantitative and qualitative analyses of target sequences. Developing various chemistries for primer and probe labeling has produced a unique technology with reliable performance characteristics. Early PCR methods used the Klenow fragment of *Escherichia coli* DNA polymerase I for DNA synthesis during each amplification cycle [1]. However, the Klenow fragment is not thermostable. Therefore this method required the addition of fresh enzymes after each denaturation step as samples were quickly cooled to avoid heat denaturation of the enzyme. In addition, the primer annealing and DNA synthesis steps were carried out at 30°C to preserve the activity of the polymerase enzyme. Low annealing temperatures caused primers to hybridize to nontarget sequences and contributed to nonspecific amplification [4]. The first major technological breakthrough in the development of PCR was the introduction of a thermostable DNA polymerase [3]. *Thermus aquaticus* is a bacterium found in hot springs

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which has adapted to variations in temperature that are suitable for its survival and accompanying environment. Taq polymerase is the DNA polymerase enzyme expressed in *T. aquaticus*. Taq polymerase exhibits robust polymerase activity that is relatively unaffected by rapid fluctuations in temperature over a wide range [5]. Introduction of Taq polymerase to PCR improved the practicality of this methodology because the polymerase enzyme can survive extended incubation at the elevated temperatures required for DNA denaturation (93°C–95°C) and eliminating the need for the addition of fresh enzyme after each cycle [5]. The second major technological breakthrough in the development of PCR was the introduction of the thermocycler. The thermocycler is a programmable heat block that ramps up or down to the reaction temperature during each amplification cycle. The thermocycler instrument enabled the automation of the PCR [6].

As a basic molecular technique, PCR is used to amplify nucleic acid fragments in research and clinical laboratories worldwide. PCR technology has significantly improved over time for three generations. The first-generation technology depended heavily on agarose gel electrophoresis to study the end-product of PCR making it a qualitative assay. However, the limitations of this approach include (1) laborious processes, (2) low detection limits, and (3) single applications [7]. The second-generation technologies are real-time quantitative PCR (real-time qPCR) and reverse-transcriptase quantitative PCR (RT-qPCR). In both technologies, the end-product is directly analyzed within the reaction tube in real time and is used to quantify the end-product of the target sequence using the standard curve [8]. Real-time qPCR is the gold standard method adopted in most clinical laboratories for diagnosing infectious, genetic, and other disease types [9]. Due to certain pre-analytical and technical limitations, it is critical to assess alternative approaches to overcome the limitation of traditional PCR, real-time qPCR, and RT-qPCR technologies. The third-generation technology is digital PCR (dPCR) which allows for absolute quantification by partitioning the PCR reaction. These alterations to the original PCR methodology have allowed for PCR to be a critical technology regularly used in research and clinical laboratories for a variety of applications.

The polymerase chain reaction

In a typical PCR, successive cycles are performed in which a DNA polymerase copies target DNA sequences from a template molecule in vitro. The amplification products produced during each cycle provide new templates for the successive rounds of amplification (Fig. 2.1). Hence, the concentration of the target DNA increases exponentially over the course of the PCR. The typical PCR reaction mixture contains the following: (1) a thermostable DNA polymerase (Taq polymerase), (2) target-specific forward and reverse oligodeoxynucleotide primers, (3) the four deoxynucleotide triphosphates (dNTPs: dATP, dTTP, dGTP, and dCTP), (4) reaction buffer, and (5) a template DNA source (genomic DNA, cDNA, or cell lysate).

The target sequence is defined by the specificity of the oligodeoxynucleotide primers that anneal to complementary sequences on opposite template strands flanking the region of interest. During the PCR, the primers are extended in the 5′→3′ direction by the DNA polymerase enzyme to yield overlapping copies of the original template. The PCR proceeds through four phases: (1) initial denaturation, (2) denaturation, (3) primer annealing, and (4) primer extension (Fig. 2.2). The time and temperature of the initial denaturation and denaturation step is optimized based on the template DNA, DNA polymerase, and buffer components being used. The initial denaturation step is commonly performed at 94°C–98°C for 1–3 minutes to linearize the template and make the template single-stranded. The subsequent denaturation steps are typically accomplished by incubating samples for 0.5–2 minutes at 94°C–98°C to render the template single-stranded. For example, long and/or GC-rich DNA templates may benefit from prolonged incubation and/or a higher temperature. The primer annealing step is accomplished at a temperature specific to the PCR primers such that the oligodeoxynucleotide primers recognize and hybridize (hydrogen bond) to the target sequence contained in the single-stranded template. The annealing temperature is determined by calculating the melting temperature (T_m) of the selected primers for PCR amplification. The primer extension step is accomplished at 72°C. During this step, the polymerase enzyme catalyzes the polymerization of dNTPs in a 5′→3′ DNA-directed DNA synthesis reaction. The actual times used for each cycle (and each step in the cycle) will vary from 15 seconds to 1–2 minutes depending upon the type of thermocycler used and its temperature ramping speed. A target sequence is amplified through repetition of these incubations for 25–30 (or more) cycles. The exact number of cycles necessary to produce sufficient amplicons for detection will depend upon the starting concentration of the target sequence. By the end of the third cycle of amplification (in a typical 25–30 cycle PCR), a new double-stranded template molecule is formed in which the 5′ and 3′ ends coincide exactly with the oligodeoxynucleotide primers used. Because the copy number theoretically doubles after each successive amplification cycle, an exponential increase of 2^n (where n is the total number of cycles of PCR performed) is accomplished during the complete reaction. Accumulation of amplicons

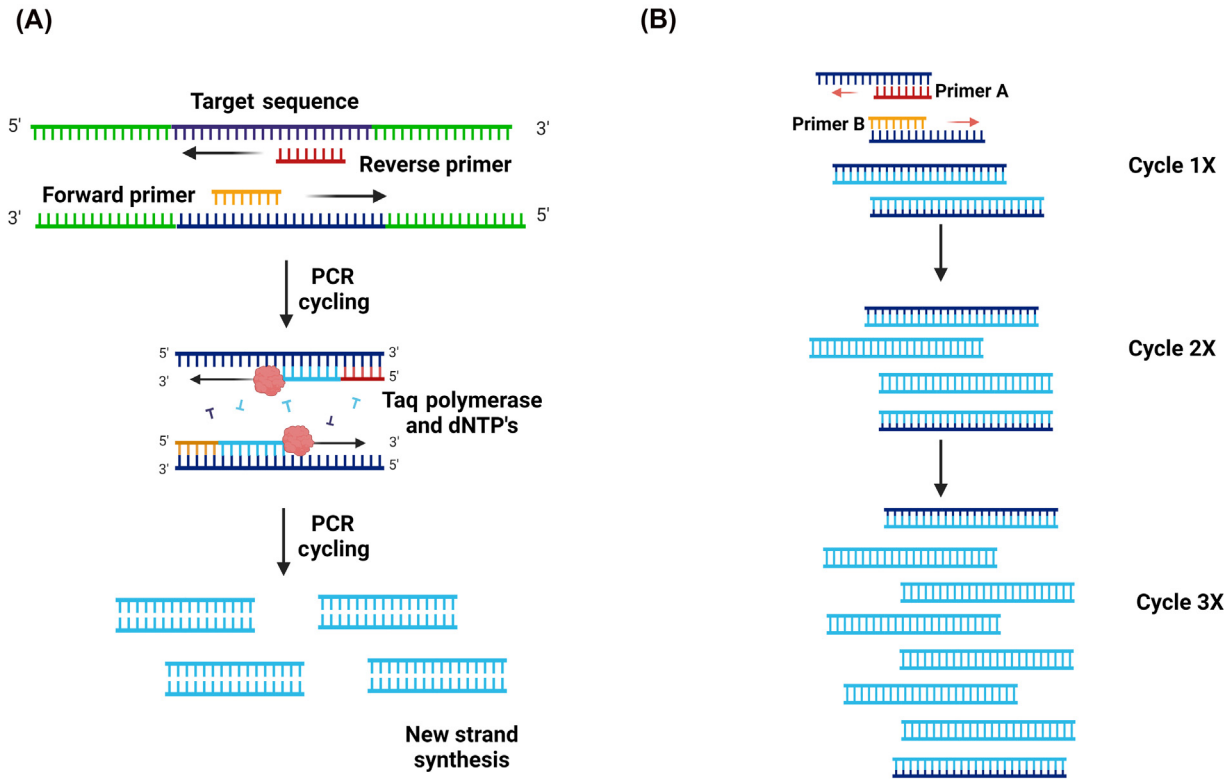

FIGURE 2.1 **Schematic representation of the polymerase chain reaction (PCR).** (A) This simplified schematic depicts two cycles of PCR amplification from a single target sequence (*light blue* and *dark blue bars*) found in a DNA template (*green bar*). Primers are depicted in yellow (forward primer) and red (reverse primer) with black arrows showing the direction of DNA synthesis by DNA polymerase. (B) This schematic depicts the PCR products in the initial rounds of PCR amplification from a single target sequence. Forward primers are shown as yellow lines and reverse primers are depicted as red lines. As shown the number of PCR products in each round corresponds to 2^ number of PCR cycles. Source*: Images created with http://www.BioRender.com*.

corresponding to the target sequence eventually reaches a plateau and depends on the initial number of target sequences contained within the template sample, the efficiency of primer extension, and the number of PCR cycles performed.

Components of the polymerase chain reaction

The PCR depends on the successful synthesis of target sequences through a series of amplification cycles, beginning with the first cycle and continuing through the final elongation step. Similarly, each PCR is performed in the presence of reagents that are critical to the reaction performance.

DNA template

The PCR amplifies specific sequences from DNA templates (genomic DNA or cDNA derived from RNA) that can be prepared from various sample sources. Clinical specimens may be derived from various bodily fluids (i.e., blood, urine, or amniotic fluid) or surgical samples (i.e., frozen cancer specimens) [10,11]. Forensic specimens may be derived from blood, semen, hair, or tissue (i.e., skin cells). In addition to fresh specimens, DNA derived from fixed tissues (formalin-fixed paraffin-embedded specimens) can be used routinely in PCR applications [12]. Most PCR reactions amplify small targets from the template sample (100−500 bp in size). Hence, high-molecular-weight DNA is unnecessary, and highly fragmented DNA (like that obtained from formalin-fixed paraffin-embedded tissues) can be used. However, certain tissue fixatives (such as Bouin solution, which contains picric acid) and tissue treatments (such as tissue decalcification) damage DNA and can render tissue samples useless as a source of DNA for molecular analysis. In addition, RNA can be prepared from fresh tissues or from formalin-fixed paraffin-embedded samples and converted to cDNA for use in PCR.

DNA polymerase enzyme

The active component of the PCR is a DNA polymerase enzyme that is required for DNA synthesis

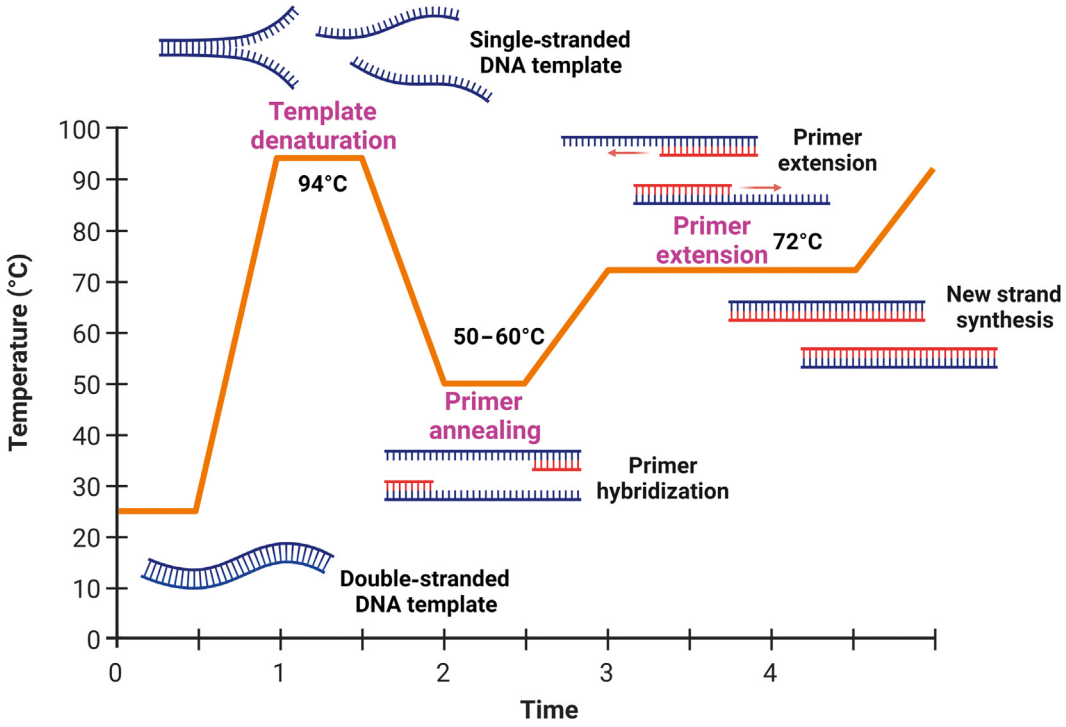

FIGURE 2.2 **Steps and temperatures for a single polymerase chain reaction (PCR) cycle.** One cycle of PCR amplification is depicted. Each cycle consists of three segments: (1) template denaturation, (2) primer annealing, and (3) primer extension. Typical temperatures are indicated for each segment. Each segment will typically occur over 15–30 s, but some instruments and applications call for longer segment times (up to 1–2 mins each). Source: *Images created with* ***http://www.BioRender.com***.

during the primer extension step of the PCR. Contemporary PCR uses Taq polymerase (isolated from *T. aquaticus*) or similar [5,12]. Taq polymerase exhibits 5′→3′ polymerase activity, 5′→3′ exonuclease activity, thermostability, and optimum performance at 70°C–80°C. The activity of Taq polymerase can be influenced by temperature, pH, and ion concentrations (Mg^{2+}). The half-life of Taq polymerase activity at 95°C is approximately 40–60 minutes. Extremely high denaturation temperatures (>97°C) will significantly reduce the activity of Taq polymerase. Because time and temperature are the critical variables for the maintenance of Taq polymerase activity, lowering the denaturation temperature or reducing the denaturation time can prolong the enzyme activity during a PCR reaction. The optimum pH for a given PCR reaction is between 8.0 and 10.0 but must be determined empirically. The typical PCR reaction is carried out in a buffer (usually Tris-Cl) that is pH 8.3. Taq polymerase activity requires divalent cations in the form of Mg^{2+}. Lower divalent cation (Mg^{2+}) concentrations decrease the enzyme dissociation rate from the template by stabilizing the enzyme-nucleic acid interaction. Most PCR mixtures contain at least 1.5 mM $MgCl_2$. However, $MgCl_2$ titration is recommended for any new template–primer combination. Although Taq polymerase is ideal for routine PCR applications, several other thermostable DNA polymerases with unique qualities are available [13]. The properties of these alternative thermostable polymerase enzymes make them useful for specialized applications such as the amplification of long stretches of DNA sequence or high-fidelity amplification.

Oligodeoxynucleotide primers

The oligodeoxynucleotide primers used in a PCR determine the target specificity of the amplification reaction. Effective oligodeoxynucleotide primers for PCR are highly sequence-specific, free of secondary structure, and form stable duplexes with target sequences. Four variables need to be considered when designing oligodeoxynucleotide primers: (1) size of the target sequence to be amplified, (2) the location of the target sequence within the overall genomic DNA (or cDNA) sequence, (3) secondary structure within the target sequence and flanking regions, and (4) specificity of amplification.

The target sequence size should be selected such that the PCR products produced range from 100 to 500 bp in length. Primer length can influence target specificity and efficiency of the hybridization. A long oligodeoxynucleotide primer may be more specific for the target sequence but is less efficient at hybridization. A short

oligodeoxynucleotide primer is efficient at hybridization but is less specific for the target sequence. As a general guideline, oligodeoxynucleotide primers should be 17–30 nucleotides in length. Whenever possible, both primers should be the same length because primer length influences the calculated optimal annealing temperature for a specific primer. The base composition of the oligodeoxynucleotide primers is also important because the annealing temperature is governed in part by the G + C content of the primers. Ideally, G + C content should be 50%–60%, and the percent G + C should be the same or very similar for both oligodeoxynucleotide primers in any given primer pair. The 3′ terminus of an oligodeoxynucleotide primer should always contain a G, C, GC, or CG. In addition, avoid repetitive or palindromic sequences, and primer pairs should not contain sequences that are complementary to one another. Likewise, oligodeoxynucleotide primers should not anneal elsewhere in the gene of interest or in the genome. Determining the optimal annealing temperature for a given oligodeoxynucleotide primer set is critical for designing an effective and specific PCR. The T_m of an oligodeoxynucleotide primer can be calculated using a simplified formula that is generally valid for primers that are 18–24 nucleotides in length [14]: $T_m = 69.3 + 0.41(\%G + C) - (650/L)$. In this formula, L is the primer length in bases and the result is the theoretical annealing temperature in degrees Celsius. Online tools are now available to assist with the prediction of properties of oligodeoxynucleotides, including annealing temperatures (see http://biotools.nubic.northwestern.edu/OligoCalc.html).

Polymerase chain reaction buffer

Components of a PCR reaction can often be combined into a single reaction mixture, most of which are commercially available. A typical PCR mixture will include a reaction buffer, oligodeoxynucleotide primers, Taq polymerase, and an appropriate DNA template. The PCR buffer consists of 50 mM KCl, 1.5 mM $MgCl_2$, 10–50 mM Tris-Cl (pH 8.3), and 50–200 μM dNTPs. Concentrations of KCl >50 mM can inhibit the enzymatic activity of Taq polymerase and should be avoided. However, the presence of KCl is necessary to encourage oligodeoxynucleotide primer annealing to target sequences in the DNA template. Likewise, excessive NaCl concentrations in the PCR mixture can adversely affect the enzymatic activity of Taq polymerase. The amount of $MgCl_2$ that is optimal for a given PCR reaction must be empirically determined. However, most standard PCR amplification reactions can be accomplished using 1.5–2 mM $MgCl_2$. The final concentration of dNTPs is 200 μM for a typical PCR, but some applications can be accomplished using much lower concentrations. Higher concentrations of dNTPs (or $MgCl_2$) can encourage errors related to dNTP misincorporation by Taq polymerase and should be avoided. The concentration of oligodeoxynucleotide primers should not exceed 1 μM unless the primers used contain a high degree of degeneration. Commercial suppliers provide Taq polymerase enzyme at 5 U/μL. One unit (U) of enzymatic activity is defined as the amount of enzyme required to catalyze the incorporation of 10 nmol of dNTP into acid-insoluble material in 30 minutes under standard reaction conditions. A 50-μL reaction will typically require 2.5 U of enzyme activity, while a 10-μL reaction will only require 0.5 U of enzyme activity. The amount of DNA template included in a PCR reaction will vary with the nature of the template source and the target sequence. Amplification from genomic DNA may require as much as 100 ng of DNA for a 50-μL reaction, whereas amplification from a plasmid template (for example) may only need 5 ng of DNA. Including gelatin or bovine serum albumin (BSA) in a PCR reaction can enhance the efficiency of the PCR amplification because these agents stabilize the polymerase enzyme. Gelatin or BSA can be included in the PCR mixture at ≤100 μg/mL. The addition of helix destabilizing chemicals may be necessary if the PCR target sequence is located in a DNA region with high G + C content. For example, the PCR reaction may include dimethylsulfoxide (DMSO), dimethyl formamide (DMF), formamide, or urea for destabilization. In most cases, these additives are included in the reaction mixture at 10% (w/v or v/v). These additives are thought to lower the T_m of the target sequence. However, care must be exercised when additives of this type are included in PCR reaction mixtures because high concentrations of these chemicals can adversely affect polymerase activity.

Optimization of polymerase chain reaction amplification reactions

Several factors can significantly affect PCR sensitivity and specificity, including (1) oligodeoxynucleotide primer design, (2) PCR cycling variables (number of cycles, cycle times, and temperatures), and (3) composition of the PCR reaction mixture (divalent cation concentration).

For most PCR applications, the most critical variable is the annealing temperature for the oligodeoxynucleotide primers. The primer with the lowest T_m determines the maximum annealing temperature. Exceeding this T_m by more than a few degrees will diminish the ability of the oligodeoxynucleotide primers to anneal to the target sequence and may prevent the amplification of the product of interest. If an annealing temperature equal to the T_m of the oligodeoxynucleotide primers fails to make the

desired amplification product, then it may be necessary to lower the annealing temperature. If the desired amplicon is produced at a lower T_m, but the amount of background products is high, then the annealing temperature should be increased. Salt concentrations also affect several aspects of the PCR reaction. Mg^{2+} concentration can affect oligodeoxynucleotide primer annealing to the target sequence, the T_m of the oligodeoxynucleotide-template complexes, enzyme activity, and enzyme fidelity. Taq polymerase requires free Mg^{2+} for activity, and sufficient $MgCl_2$ must be included in the PCR reaction to provide adequate Mg^{2+} for the enzyme after some of the cations are lost to chelation by the oligodeoxynucleotide primers and the template DNA. The concentration of other salts can also affect the PCR reaction (including KCl). However, optimization of most PCR applications can be achieved through modification of Mg^{2+} concentration. Complete optimization of the reaction conditions may require several adjustments to the annealing temperature, PCR cycle variables, and salt concentrations. Several commercial kits are available that provide a range of PCR reaction mixtures for simple and rapid optimization of specific PCR conditions for a particular target sequence and its primers. Likewise, several manufacturers offer gradient thermocyclers that feature heating blocks where temperatures can be varied across the samples, enabling the optimization of temperatures in a single PCR run.

Increasing polymerase chain reaction specificity and sensitivity

Taq polymerase has substantial enzymatic activity at 37°C, although its optimal activity is expressed at a much higher temperature (approximately 72°C). This low-temperature polymerase activity is the basis for nonspecific amplification associated with mispriming events that occur during the initial phase of the PCR reaction. Extension resulting in incorrect PCR products can occur from oligodeoxynucleotide primers that anneal nonspecifically to template DNA before the first denaturation step at 93°C–95°C. Because of this, several modified polymerase enzymes have been created to avoid this nonspecific primer extension activity. One example of this is Platinum Taq Polymerase (from Invitrogen Life Technologies, http://www.thermofisher.com). By including a thermolabile inhibitor of Taq polymerase in the form of a monoclonal antibody, the enzyme does not become active until the inhibitor is heat-inactivated. Hence, the Taq polymerase becomes active after the elevated temperature destroys the monoclonal antibody during the initial denaturation phase of the PCR reaction resulting in the release of the functional enzyme. The antibody-mediated inhibition of Taq polymerase allows for room-temperature assembly of the PCR reaction mixture. Nonspecific amplification associated with primer extension from mispriming events is eliminated or reduced by holding the Taq polymerase functionally inactive until the critical temperature is reached.

Polymerase chain reaction contaminants

When performing PCR amplification, it is critical to be aware of potential sources of DNA contamination and to employ procedures to ensure contamination-free working conditions. The power of PCR to amplify minimal quantities of DNA to produce detectable amplification products, demands that special care be taken to prevent cross-contamination between different samples. This cross-contamination can be especially problematic when PCR targets are expected to be present in low amounts because these reactions typically require more amplification to be detected. Sources of contamination include (1) genomic DNA contamination in RNA samples, (2) cross-contamination among different nucleic acid samples processed simultaneously, (3) laboratory contamination of cloned target sequences (genomic DNA or cDNA), and (4) carryover of PCR products.

In general, working in a clean laboratory and using good laboratory practices (such as wearing sterile gloves at all times) substantially reduces the likelihood of contamination. Carryover products from other PCR reactions can be effectively controlled by the use of aerosol-free pipette tips, dedicated pipettes and solutions, and by maintaining separate areas to handle pre-PCR and post-PCR solutions and samples. In all PCR applications, it is essential to include proper positive-control and negative-control reactions to guard against systemic contamination of PCR reagents and to ensure that the desired amplicon is produced in positive reactions.

Inhibitors of polymerase chain reaction

Organic and inorganic compounds that inhibit PCR amplification of nucleic acids are common contaminants in DNA samples from various origins. These contaminating substances can interfere with the PCR reaction at several levels, leading to different degrees of attenuation and/or complete inhibition. Many PCR inhibitors have been reported, and they appear to be particularly abundant in complex samples such as bodily fluids and samples containing high numbers of bacteria. Most of these contaminants (i.e., polysaccharides, urea, humic acids, hemoglobin) exhibit a similar solubility in aqueous solutions as DNA. Consequently, these contaminants are not entirely removed by typical extraction

procedures used to prepare the template DNA (i.e., detergent, protease, and phenol-chloroform treatments). Several methods have been developed to avoid these contaminating substances. Some of these simple methods used to remove forms of contamination can lead to the loss of significant amounts of the original sample and/or may require expensive reagents.

Analysis of polymerase chain reaction products

There are numerous methods for the analysis of PCR products and the method of choice will depend on the desired information (Fig. 2.3). Typical analysis of PCR products involves electrophoretic separation of amplicons on an agarose gel and visualization with ethidium bromide staining (or similar DNA dye). Agarose gel electrophoresis effectively separates DNA products over a wide range of sizes (100 bp to >25 kbp). PCR products from 200 to 2000 bp can be separated quickly on a 1.6% agarose gel. When greater resolution or separation power is required, such as in the analysis of small PCR products (<100 bp), polyacrylamide gel electrophoresis is the method of choice. DNA products are easily visualized by ultraviolet illumination after ethidium bromide staining. Another method often used to quantify products is the incorporation of radioactive, fluorescent, or biotinylated markers. PCR products may be labeled by incorporating labeled nucleotides or through the use of labeled oligodeoxynucleotide primers. Labeled PCR products are separated by electrophoresis on agarose or polyacrylamide gels and visualized using appropriate techniques (i.e., autoradiography for radiolabeled products). If multiplex PCR analysis is performed or for the most accurate sizing of amplicons within banding patterns, laboratories now employ capillary electrophoresis systems. In some cases, a simple analytical gel separation (i.e., in assays where the presence or absence of a PCR product answers the question) can obtain the desired information from a PCR reaction using commercially available pre-casted agarose gels. In other cases, additional information is required (i.e., DNA sequencing to detect a gene mutation). Hence, PCR products can be cloned and used for sequence analysis, construction of molecular probes, mutation analysis, in vitro mutagenesis, and in gene expression studies (Fig. 2.3).

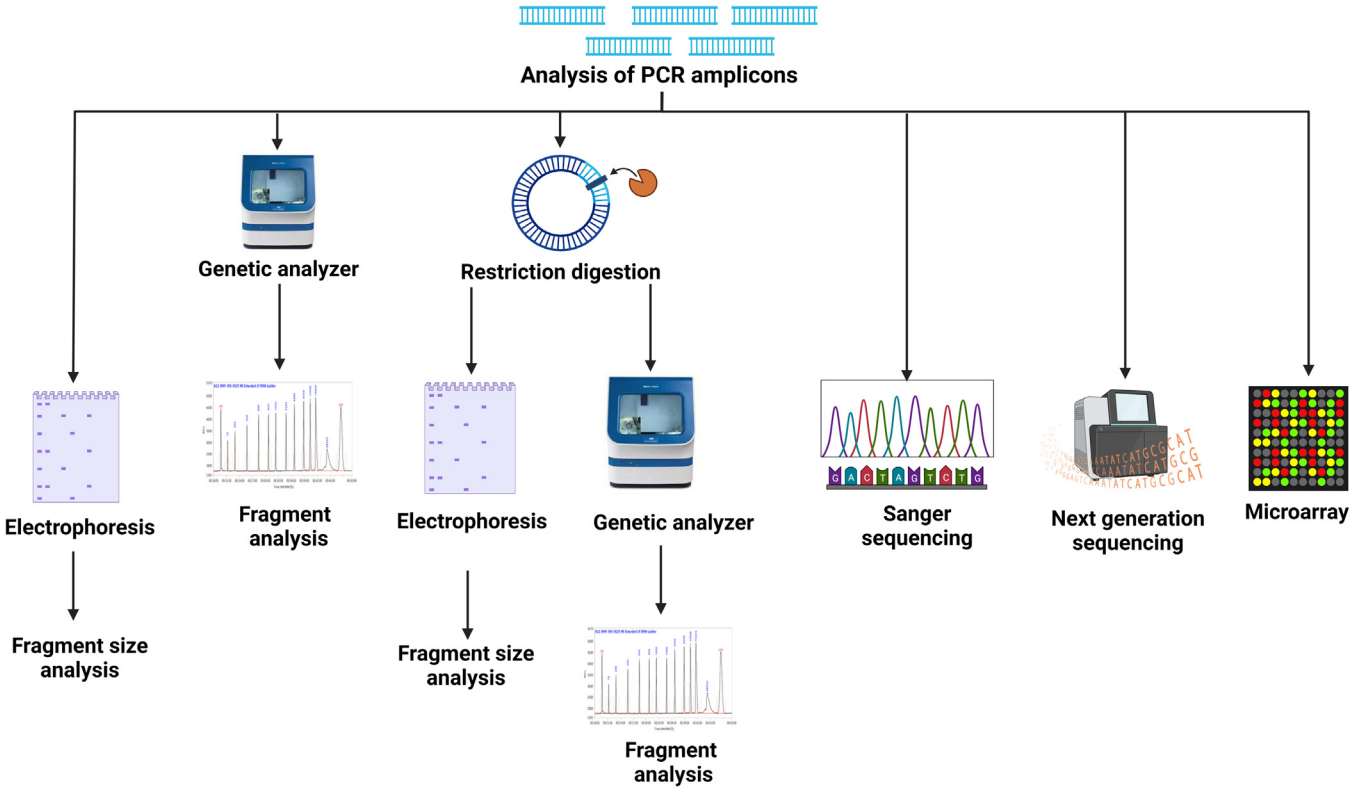

FIGURE 2.3 **Methodologies for analysis of polymerase chain reaction (PCR) products.** PCR products (amplicons) generated through traditional PCR reactions can be analyzed in various ways using several methodologies. For some questions, the presence or absence of a PCR product answers the question, and a simple electrophoretic analysis with visualization of the product will suffice. For other questions, the amplicon may require fragment analysis using a genetic analyzer, restriction analysis, or direct sequencing to obtain the desired information. PCR products can also be analyzed using various next-generation sequencing and array-based platforms. Source: *Images created with http://www.BioRender.com.*

Variations of the typical polymerase chain reaction amplification reaction

Over the years, many modifications have been made to the standard PCR reaction. Some of the more significant improvements include (1) hot-start PCR, (2) nested PCR, (3) RT-PCR, (4) real-time PCR and (5) dPCR.

Hot-start and nested polymerase chain reaction

Hot-start PCR was developed to reduce background from nonspecific amplification. Initial hot-start PCR was performed by limiting the Mg^{2+}, dNTP, or enzyme concentration. Alternatively, a hot-start can be achieved by separating the reaction components with a wax bead barrier that melts as the mixture is heated during the initial denaturation step of the PCR. Hot-start is used to prevent the polymerization of new DNA during the initial phase of the reaction when nonspecific binding may occur between primers and nonspecific DNA targets [15,16]. More recently, chemical or antibody-engineered polymerases have become commercially available that are activated once a specific temperature is reached. Nested PCR can increase both the sensitivity and specificity of amplification [17]. The amplification product(s) generated in the first PCR reaction are used as the template for a second PCR reaction, in which primers are used that are internal, or nested, within the first primer pair.

Polymerase chain reaction analysis of RNA

RT-PCR is an excellent method for analyzing RNA transcripts, especially for measuring low-abundance species or working with limited amounts of starting material. RT-PCR couples the tremendous DNA amplification powers of the PCR with the ability of RT to reverse transcribe small quantities of total RNA. RT-PCR is a four-step process: (1) RNA isolation, (2) reverse transcription, (3) PCR amplification, and (4) PCR product analysis. RNA is isolated from cells or tissue using various chemical-based extraction techniques or affinity-based (column) methods to eliminate contaminating DNA. This RNA is then used as a template in a reverse transcription reaction that produces cDNA, which serves as a template for the PCR reaction. RT (retroviral RNA-directed DNA polymerase) is the enzyme used to catalyze cDNA synthesis. The RT reaction consists of (1) cDNA synthesis primer, (2) an appropriate RT reaction buffer, (3) dNTPs, (4) RNA template (total RNA or mRNA), and (5) the RT enzyme.

Several commercially available RT enzyme preparations can be used in standard RT-PCR reactions. These include Moloney murine leukemia virus (MMLV) RT and avian myeloblastosis virus (AMV) RT. More recently, recombinant derivatives of these RT enzymes have become available that offer advantages over native enzymes. One example of these recombinant enzymes is SuperScript IV RT from Invitrogen (http://www.thermofisher.com). These recombinant enzymes are engineered for higher thermostability and processivity in the presence of inhibitors to provide robust cDNA synthesis and greater cDNA yields.

RT-PCR is an excellent method for the analysis of RNA transcripts, especially for measuring low-abundance species or working with the limited starting material (such as those obtained from formalin-fixed paraffin-embedded samples). Traditional blotting and hybridization assays require much more RNA for analysis and lack the speed and ease of technique afforded by PCR-based applications. Some conventional methods (such as northern blotting) require high-quality, intact RNA species, whereas RT-PCR approaches can tolerate some RNA degradation. RT-PCR combines the tremendous DNA amplification power of PCR with the ability of RT to reverse transcribe minimal quantities of total RNA (<1 ng) into cDNA. The use of total RNA preparations rather than poly(A)-purified RNA reduces the possibility of losing specific rare or low-abundance mRNAs during the purification process and allows for the use of minimal quantities of starting material (cells or tissues). Additional advantages of RT-PCR include versatility, sensitivity, rapid turnaround time, and the ability to compare multiple samples simultaneously.

Real-time polymerase chain reaction

Real-time PCR combines the amplification steps of traditional PCR with simultaneous detection steps that do not require post-PCR manipulation or interrogation of amplified products (Fig. 2.4). The PCR product is directly examined within the reaction tube. During real-time PCR, the exponential phase of PCR is monitored as it occurs using fluorescently labeled probes [18]. During the exponential phase, the amount of PCR product in the reaction tube is directly proportional to the amount of emitted fluorescence and the amount of the initial target sequence [19,20]. Thus, these reactions can also be quantitative. There are two types of detection chemistries for real-time PCR: (1) those which use intercalating DNA-binding dyes such as ethidium bromide or SYBR green, and (2) those that use various types of fluorescently labeled probes.

Intercalating DNA-binding dyes allow for the simple determination of the presence or absence of an amplicon. SYBR green, like ethidium bromide, is a dye that emits fluorescence when it is bound to

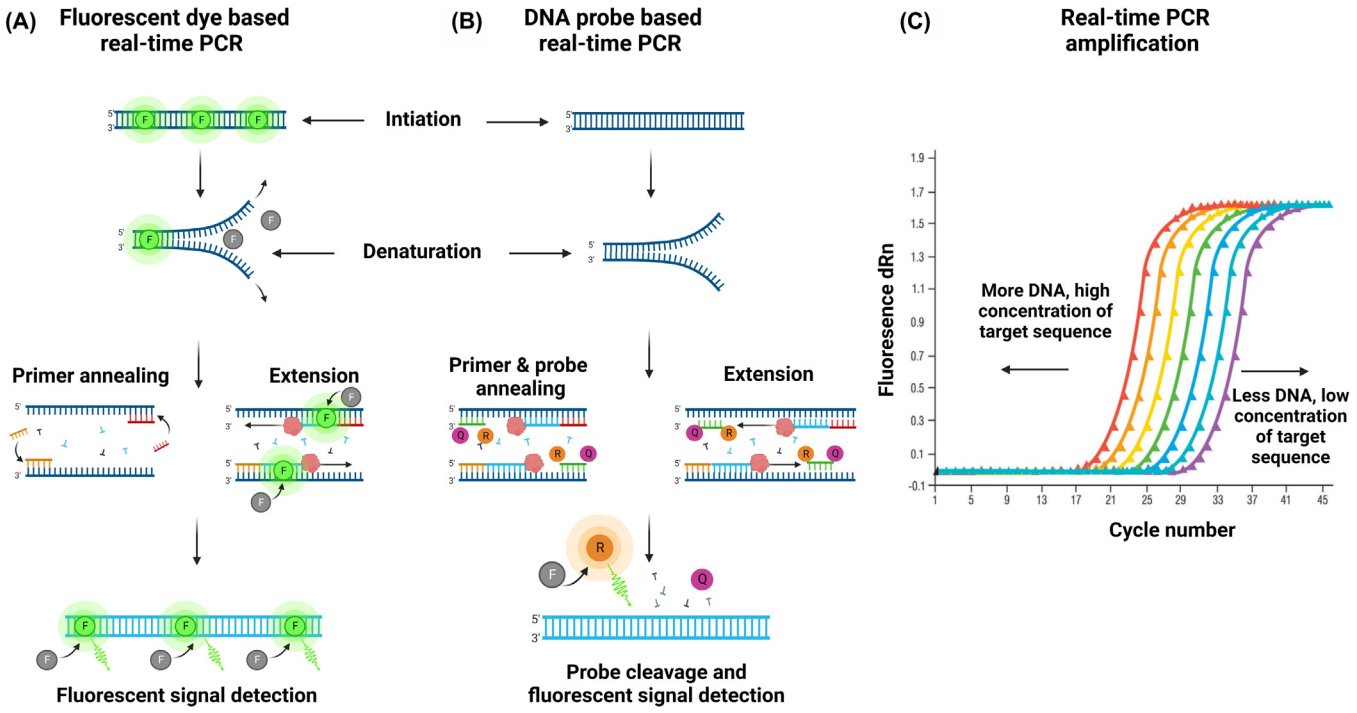

FIGURE 2.4 **Real-time polymerase chain reaction (PCR) amplification of target sequences.** (A) Depiction of a fluorescent dye-based real-time PCR with the target sequence shown in blue and fluorescent intercalating dye shown in green. The real-time primers (red and orange) are illustrated with a pink ball (DNA polymerase) and a black arrow showing the direction of synthesis. During extension, the intercalating dye binds double-stranded DNA and emits a fluorescent signal that is detected providing a measure of abundance of the target sequence. (B) Depiction of a DNA probe-based real-time PCR. Similar to Panel A except the real-time probe is depicted as a green line with a fluorescent reporter tag (R; orange ball) and quencher (Q; pink ball). With primer extension and exonuclease activity, the fluorescent tag is released from the quencher. Accumulation of the fluorescent signal provides a measure of abundance of the target sequence. (C) Results from a real-time amplification run indicate the abundance of the target sequence. Samples with curves that look more like the red line have a higher concentration of the target sequence because a fluorescent label for the target sequence is detected with fewer amplification cycles. Samples with curves that look more like purple have a low concentration of target sequence because more amplification cycles are required to detect the fluorescent label for the target sequence. Source: *Images created with http://www.BioRender.com.*

double-stranded DNA. During the PCR reaction, there is an increase in the copy number of the amplicon and a simultaneous increase in the amount of intercalated SYBR green dye (Fig. 2.4A). This will increase the amount of emitted fluorescent signal proportionally to the copy number [21]. One disadvantage of DNA-binding dyes of this type is that they are nonspecific and will bind to any double-stranded DNA.

Detection of real-time PCR products can also be accomplished using various types of fluorescently labeled probes. There are three primary detection chemistries for these probes: (1) cleavage-based (5′ exonuclease) probes, (2) molecular beacons, and (3) fluorescence resonance energy transfer (FRET) probes. Cleavage-based probes depend upon the 5′→3′ exonuclease activity of the Taq polymerase. These assays are commercially available as Taqman assays (Fig. 2.4B). Molecular beacons are self-complimentary single-stranded oligonucleotides that form a hairpin loop structure and consist of a probe homologous to the target sequence flanked by sequences that are homologous to each other. Molecular beacons have the reporter dye attached to one end (FAM or TAMRA) and a quencher (DABCYL) on the other. When the beacon binds to the target sequence, the quencher and reporter are separated, and fluorescence is emitted. FRET probes are composed of two separate fluorescently labeled oligonucleotides, one with a 5′ donor molecule and the other with a 3′ acceptor molecule attached. When these probes hybridize with close proximity, energy can be transferred from the donor to the acceptor and results in fluorescence emission.

Real-time PCR is rapidly becoming the method of choice for most molecular diagnostics laboratories because of its increased sensitivity/specificity and turnaround times. This technology can be used for quantitative and qualitative assessment of target sequences and for distinguishing mutant from wild-type sequences. For single nucleotide polymorphism (SNP) genotyping and small mutation testing, two different labeled probes are designed—one for the wild-type allele and one for the mutant allele. The mismatch

between the wild-type allele and the mutant probe facilitates competitive hybridization and fluorescence will only be detected when the allele-specific probe binds to its corresponding target sequence (Fig. 2.5A). If binding dye chemistries are used, another powerful feature of most real-time PCR instruments is the ability to perform melting curve analyses (Fig. 2.5B) [21]. An additional thermal step on the PCR product in the same reaction tube can identify the T_m of a specific amplicon. Real-time PCR can also determine the copy number of specific target sequences in infectious disease and oncology [22]. A relative quantification reaction can be used to accurately assess the target copy number by multiplexing the primers and probes for the target sequence with primers and probes for a control sequence.

In contrast, using external standards of known concentration to create a standard curve enables the determination of absolute quantities of target copy number. The main advantages of real-time PCR are the speed with which samples can be analyzed (because no post-PCR processing steps are required) and the closed single-tube nature of the technology. The analysis of results via amplification curve and melting curve analysis is very straightforward and contributes to overall increases in the speed of PCR analysis (Fig. 2.4C). With respect to potential contamination issues, another major advantage of having no post-PCR steps is that real-time PCR is a closed-tube method of analysis which greatly reduces (1) the chance that a sample will be contaminated, (2) from mistakes in tube transfers, or (3) the possibility of amplicons aerosolizing into the laboratory environment.

Some investigators argue that a limitation of real-time PCR is the initial capital investment for instrumentation. However, since the first real-time PCR instruments were introduced on the market, several new real-time platforms have become available that are very cost-effective. In fact, the pricing of some instruments may be less than a conventional thermocycler and different detection systems. One technical limitation that should be noted is when using DNA-binding dyes to detect real-time PCR products, nonspecific amplicons may also be detected. Such nonspecific amplicons can be monitored through traditional post-PCR product analysis using electrophoresis. Hence, optimization of reaction conditions and melting curve analysis should be performed to distinguish between the desired (correct) amplicon and those corresponding to nonspecific amplification products.

Digital polymerase chain reaction

The development of dPCR is a highly sensitive and accurate third-generation technology that is beneficial for detecting low nucleic acid levels, rare mutations, and genetic and copy number variations [23]. Sykes et al. first introduced the concept of dPCR in 1992 [24]. dPCR is performed by diluting the nucleic acid template to a single molecule level and quantifying DNA molecules using the Poisson distribution [24]. Today this technology is used to quantify different nucleic acid types and can be classified into two methods which are based on how the reaction mixture is separated: (1) chip digital PCR (cdPCR) (Fig. 2.6A–C), and

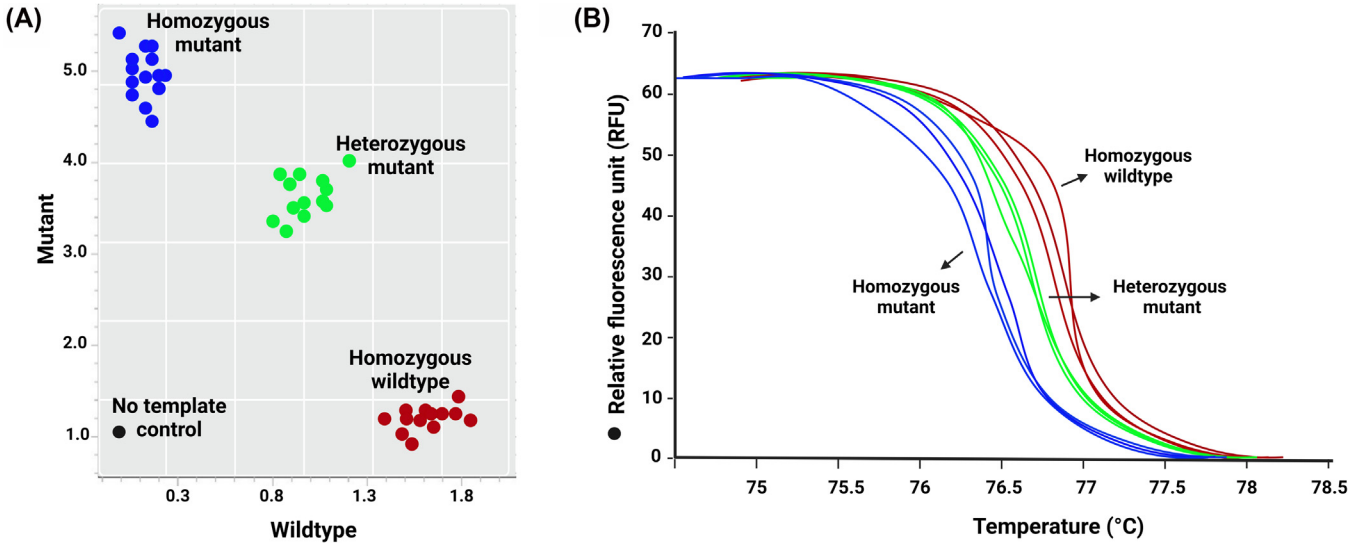

FIGURE 2.5 **Representation of allelic discrimination and high-resolution melt curve analysis using quantitative real-time polymerase chain reaction method.** (A) Representative image of allelic discrimination plot. (B) Example of high-resolution melt curve analysis for gene expression studies. Source: *Images created with http://www.BioRender.com.*

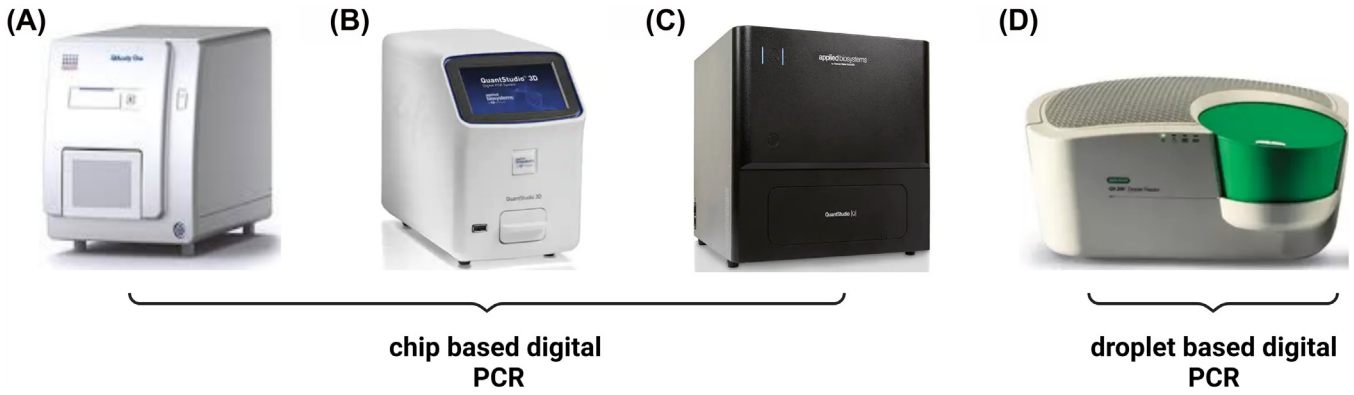

FIGURE 2.6 **Types of digital polymerase chain reaction (dPCR) systems available commercially.** (A–C) represent chip-based dPCR systems. D represents a droplet-based digital PCR system. Source: *A—Qiagen, B, C—Thermo Fisher Scientific, and D—Bio-Rad.*

(2) droplet digital PCR (ddPCR) (Fig. 2.6D). The cdPCR depends entirely on the integrated fluidic circuits, arrayed lipid bilayer chamber system, and nanoliter self-priming compartmentalization chip. In cdPCR, the reaction is divided into nanoliter reaction chambers. Then the fluorescence signals are captured with an imaging system integrated with the inverted scope. This differs from droplet digital PCR (ddPCR) where each PCR reaction is separated into thousands of individual droplets using water-oil emulsion droplet technology that was developed in 2011. Each droplet contains a small amount of template DNA or RNA. The amplification and the subsequent quantification of the absorbance of each droplet are measured independently [25]. This technology allows for the absolute quantification of the target sequence such that a droplet containing a reaction with no target molecules is considered 0, and a droplet containing a reaction with one target molecule is counted as 1. This technology allows for much more precise and accurate quantification of the target nucleic acid [7,23].

The process of ddPCR involves multiple steps. First, the sample is prepared by extracting and purifying the nucleic acid of interest. Prior to droplet generation, ddPCR reactions are set up in the same way as the real-time qPCR reactions that use a TaqMan hydrolysis probe labeled with FAM and VIC reporter fluorophores or any intercalating dye such as SYBR green or EvaGreen in a 96-well plate (Fig. 2.7A). The plate containing the reactions is then placed into an automated droplet generator which divides each reaction into thousands of small droplets (approximately 20,000 nanoliter-sized droplets) (Fig. 2.7B). This step generates uniform droplets containing a small amount of template DNA or RNA enabling precise target quantification. The droplets are transferred to a ddPCR-specific 96-well plate and placed in a thermal cycler for amplification (Fig. 2.7C). Post-amplification, the plate is placed in a droplet reader to analyze individual droplets using either the two-color or the six-color detection system (Fig. 2.7D). Positive droplets containing at least one copy of the target DNA molecule exhibit increased fluorescence compared to negative droplets. The data can be viewed using proprietary software in a 1D or 2D plot (Fig. 2.7D).

In the era of precision medicine, precise nucleic acid measurement is becoming critical. The ddPCR splits templates into individual reactions allowing for improved sensitivity to detect rare alleles. In addition, target sequences are quantified by directly counting PCR-positive droplets among all reactions. In contrast, qPCR determines DNA amounts by quantifying PCR-amplified fluorescent signals at a specific time point known as the cycle threshold (Ct) and comparing Ct values to a standard curve produced by well-defined samples [26]. The benefit of ddPCR is that it provides absolute quantification, is highly precise, and does not need a standard curve [27,28]. Moreover, ddPCR can concurrently amplify different targets in the given sample, allowing the more accurate and efficient study of complex samples in clinical settings. Overall, ddPCR has higher sensitivity, better accuracy, and more stable replications for extremely low input samples.

Increased accuracy and sensitivity associated with dPCR make it an attractive method for use in diagnostic, prognostic, and predictive tests in clinical laboratories. dPCR has applications in infectious disease testing, prenatal/newborn screening, genetic disease testing, and oncology. In clinical laboratories, dPCR and real-time qPCR have been used to quantify DNA, RNA viruses, bacteria, and parasites. Specifically, for infectious disease testing, ddPCR has been used to detect COVID-19, Hepatitis B, HIV, bacterial infections (i.e., Staphylococcus, Salmonella, and Listeria), Tuberculosis, Human herpes, and Malaria [26]. dPCR is especially useful in quantifying pathogen load when reference material is unavailable, low pathogen loads are expected, samples could be contaminated with inhibitors, or targets have high sequence variability [29]. dPCR has been a helpful method for

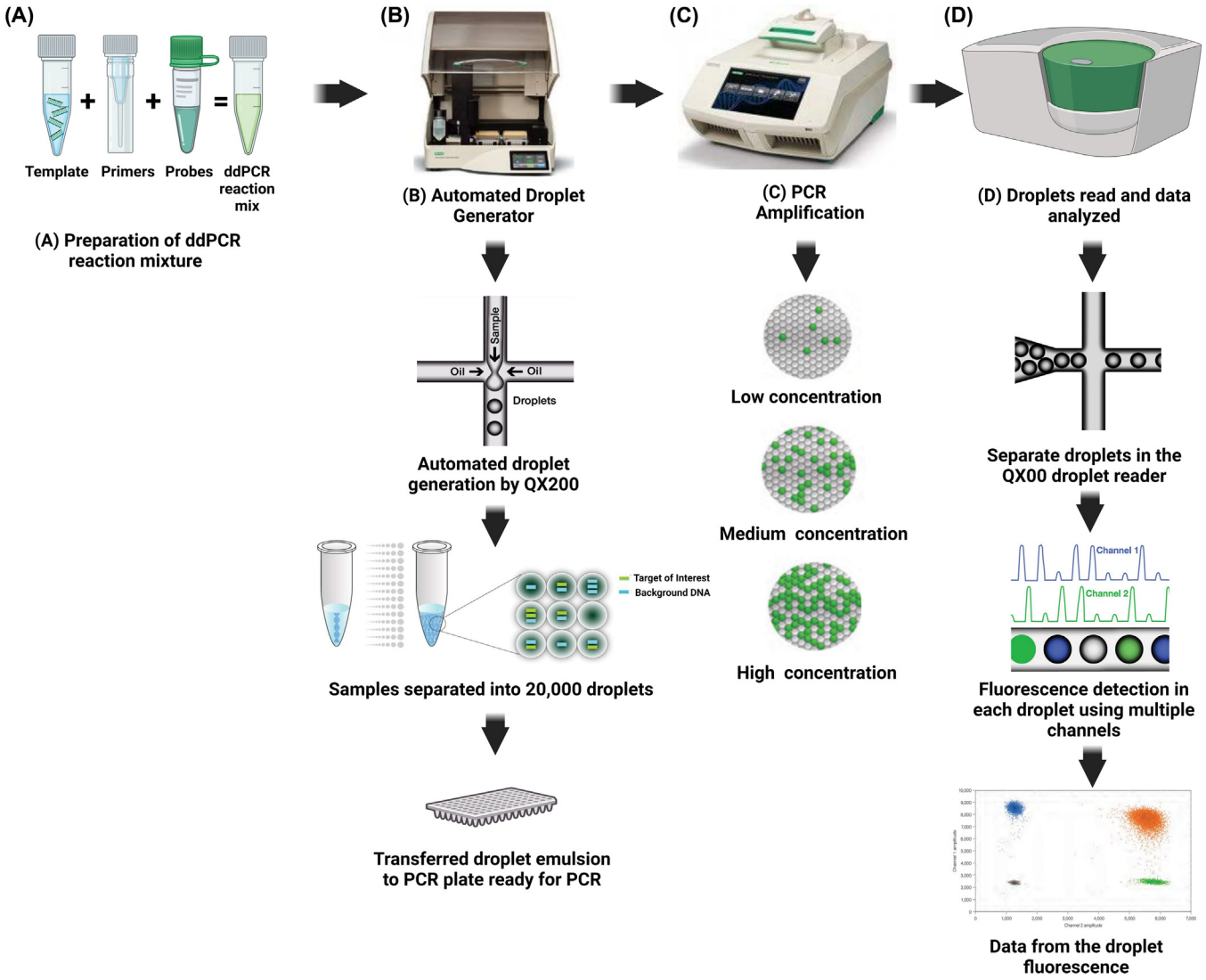

FIGURE 2.7 **Overview of droplet digital polymerase chain reaction (PCR) workflow.** (A) Standard ddPCR reaction mixtures are prepared in tubes. (B) The reaction mixture is separated into 20,000 droplets using an automated droplet generator, and the droplet emulsion is transferred into a 96-well PCR plate for PCR amplification. (C) PCR amplification is done, and droplets with green were designated as positive reaction and gray color as negative. (D) Using a two- or six-channel digital droplet reader, the fluorescence detection in each droplet is measured and the data is visualized using proprietary software in 1D or 2D plots. Source: *Bio-Rad and images created with http://www.BioRender.com.*

noninvasive prenatal testing using cell-free fetal DNA because it is sensitive despite the limited input DNA. For noninvasive prenatal testing, dPCR is used to screen for chromosome aneuploids, single gene inheritance diseases (i.e., Sickle cell disease), and autosomal recessive disorders (i.e., Spinal muscular atrophy) [7]. In oncology, ddPCR can detect specific variants, gene amplifications, and epigenetic modifications (such as methylation) from circulating tumor DNA (ctDNA) found in peripheral blood and other liquid matrices. ctDNA studies have used ddPCR to detect known mutations that inform treatment or prognosis in melanoma, non-small cell lung carcinoma, oral cancer, breast cancer, chronic myelogenous leukemia, ovarian cancer, and other cancers [7,9]. Given the many potential clinical applications of ddPCR, it is a foreseeable method of choice for clinical laboratories.

With the increased sensitivity and reproducibility of dPCR methods compared to qPCR methods, the slow uptake of this technology by the clinical laboratory is surprising. The lack of automation, the cost of acquiring new instrumentation, the need to retrain personal, and the lack of FDA-approved assays have likely prevented clinical laboratories from switching from qPCR to ddPCR [30]. In addition, the only commercially available ddPCR instrument was 2-plex until recently with the commercially available 6-plex instrument. Furthermore, in some applications (i.e., liquid biopsy) dPCR competes with increased sensitivity, decreased

costs, and shorter turnaround times compared to next-generation sequencing methods (NGS). NGS does not require amplification of known sequences, positioning dPCR as a confirmatory and complementary technique for NGS testing when target variants are unknown. ddPCR can best be used in clinical laboratories to detect copy number variations and known rare alleles that the wild-type allele would otherwise obscure. In addition, ddPCR could be used to detect low levels of viral and/or bacterial infections in patients before illness may be detected by culture allowing early intervention by clinicians. dPCR methods are helpful in clinical testing when a small number of known targets are being assessed, especially when input material is low and to detect rare alleles.

The PCR is a rapid, sensitive, and specific method for in vitro amplification of nucleic acid sequences and produces millions of copies of the target sequence. The introduction of a thermostable DNA polymerase and the thermocycler has made PCR a method that is now used as the basis for numerous clinical molecular diagnostic tests in laboratories worldwide. PCR technology has significantly improved over three generations, including real-time qPCR, RT-qPCR, and dPCR. These advanced technologies, with alterations to the original PCR methods, have allowed traditional and advanced PCR methods to be a critical technology regularly used in clinical laboratories for numerous testing and applications.

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