Topic Introduction

Polymerase Chain Reaction

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The polymerase chain reaction (PCR) underlies almost all of modern molecular cloning. Using PCR, a defined target sequence that occurs once within a DNA of high complexity and large size—an entire mammalian genome, for example—can be rapidly and selectively amplified in a quasi-exponential chain reaction that generates millions of copies. The reaction is simple to set up, cheap, and undemanding, the only requirement being some knowledge of the nucleotide sequences of the target. In addition to its simplicity, PCR is robust, speedy, flexible, and sensitive.

THE BASIC POLYMERASE CHAIN REACTION

Since its initial development in the early 1980s (Saiki et al. 1985; Mullis and Faloona 1987; Mullis 1997), the basic polymerase chain reaction (PCR) has been adapted to a wide variety of tasks in molecular cloning, including DNA sequencing, in vitro mutagenesis, mutation detection, cloning of cDNA and genomic DNA, and allelotyping. With such a wide repertoire of applications, it is not surprising that entire journals and books have been devoted to the technique. This introduction discusses the parameters that affect PCR.

PCR uses temperature cycling to initiate and end bursts of enzyme-catalyzed DNA synthesis (see Protocol: **The Basic Polymerase Chain Reaction** [Green and Sambrook 2018a]). Each cycle consists of three stages:

- *Denaturation* of the template DNA by heat (usually $>90^{\circ}\text{C}$)
- *Annealing* of two synthetic oligonucleotide primers to the denatured template DNA. These primers, usually 20–25 nucleotides in length, are designed using preexisting knowledge of the DNA sequence of the template. The two primers are complementary to sequences on opposite strands of the target DNA. The binding sites for the primers could be separated by just a few nucleotides or as many as several thousands, as the investigator desires.
- *Extension*, in which DNA synthesis is initiated at the 3' ends of the bound primers. Extension of the primers occurs at temperatures between $\sim 55^{\circ}\text{C}$ and 70°C in an enzymatic reaction catalyzed by a thermostable DNA polymerase.

This process, which is repeated about 25–35 times, takes place in a thermal cycler, a programmable device that controls the time and temperature of each step in the cycle.

The products of the first round of synthesis are two daughter DNA strands that then act as templates for the next round of primer-driven DNA synthesis, generating products whose length is equal to the number of nucleotides between binding sites of the 5' ends of the two primers. From then on, the PCR proceeds for 25 or more cycles, with copies of the target sequence doubling during every

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cycle, until the concentration of the primers and/or the deoxynucleotide triphosphates (dNTPs) becomes limiting (Liu and Saint 2002a,b). In practice, the probability that a target molecule will be duplicated in a particular cycle is a little <1 . The failure of PCRs to follow ideal kinetics can result from many factors, including the presence of inhibitors in the reaction, the characteristics of the thermostable polymerase used to catalyze the PCR, the use of partially degraded template DNA, mispriming at ectopic sites in the template DNA, etc.

Essential Components of PCRs

The following components are required to set up PCRs:

- *A thermostable DNA polymerase to catalyze template-dependent synthesis of DNA.* A wide choice of enzymes is now available that vary in their fidelity, efficiency, and ability to synthesize large DNA products. For routine PCRs, *Taq* polymerase (0.5–2.5 units per standard 25–50- μ L reaction), originally isolated from *Thermus aquaticus*, remains the enzyme of choice. The specific activity of most commercial preparations of *Taq* is $\sim 80,000$ units/mg of protein. Standard PCRs therefore contain $2\text{--}10 \times 10^{12}$ molecules of enzyme. Because the efficiency of primer extension with *Taq* polymerase is generally ~ 0.7 (e.g., see Gelfand and White 1990; Lubin et al. 1991), the enzyme becomes limiting when $1.4\text{--}7.0 \times 10^{12}$ molecules of the amplified product have accumulated in the reaction.
- *A pair of synthetic oligonucleotides to prime DNA synthesis.* Of the many factors that influence the efficiency and specificity of the amplification reaction, none is more crucial than the design of oligonucleotide primers. Careful design of primers is required to obtain the desired products in high yield, to suppress amplification of unwanted sequences, and to facilitate subsequent manipulation of the amplified product. Although the efficiency of primers heavily influences the success or failure of PCRs, the guidelines for their design are based more on common sense than on well-understood thermodynamic or structural principles. Compliance with these empirical rules does not guarantee success. Disregarding them, however, can lead to failure. For more information, see Table 1. In certain situations, it might be desirable to amplify several segments of target DNA simultaneously. In these cases, an amplification reaction termed “multiplex PCR” is used that includes more than one pair of primers in the reaction mix. (For further details on this variation, see Box 1.) Standard reactions contain nonlimiting amounts of primers, typically 0.1–0.5 μ M of each primer ($0.6\text{--}3.0 \times 10^{13}$ molecules). This quantity is enough for at least thirty cycles of amplification of a 1-kb segment of DNA. Higher concentrations of primers favor mispriming, which can lead to nonspecific amplification. Oligonucleotide primers synthesized on an automated DNA synthesizer can generally be used in standard PCRs without further purification. However, amplification of single-copy sequences from mammalian genomic templates is often more efficient if the oligonucleotide primers are purified by chromatography on commercially available resins or by denaturing polyacrylamide gel electrophoresis.
- *Deoxynucleoside triphosphates (dNTPs).* Standard PCRs contain equimolar amounts of dATP, dTTP, dCTP, and dGTP. Concentrations of 200–250 μ M of each dNTP are recommended for *Taq* polymerase in reactions containing 1.5 mM MgCl_2 . In a 50- μ L reaction, these amounts should allow synthesis of $\sim 6\text{--}6.5$ μ g of DNA, which should be sufficient even for multiplex reactions in which eight or more primer pairs are used at the same time. PCRs with dNTP concentrations as low as 20 μ M are capable of generating 0.5–1.0 pmol of a single amplified fragment ~ 1 kb in length. However, high concentrations of dNTPs (>4 mM) are inhibitory, perhaps because of Mg^{2+} sequestration. Many manufacturers (e.g., Roche, QIAGEN, GE Healthcare Life Sciences) sell dNTPs that have been purified by high-resolution high-performance liquid chromatography and are made specifically for use as substrates in PCRs. These dNTPs are free of tetra- and pyrophosphates that can inhibit PCRs. Stock solutions of dNTPs are sensitive to freezing and thawing. After a few freeze–thaw cycles, the efficiency of PCRs begins to decrease. To avoid problems, stocks of dNTPs (100–200 mM)—whether homemade or purchased—should be stored at -20°C in small aliquots (2–5 μ L) in 10 mM Tris (pH 8.0) that should be discarded

TABLE 1. Primer design^a

Property	Optimal design
Base composition	G + C content should be between 40% and 60%, with an even distribution of all four bases along the length of the primer (e.g., no polypurine or polypyrimidine tracts and no dinucleotide repeats). If possible, avoid GC-rich stretches, which are prone to forming secondary structures.
Length	The region of the primer complementary to the template should be 18–30 nucleotides in length. Members of a primer pair should not differ in length by more than three bases. Primers shorter in length than 18 nucleotides will tend to bind nonspecifically to complex template DNAs (e.g., genomic DNAs). Primers >30 nucleotides in length have an increased probability of forming secondary structures such as hairpin loops.
Internally repeated and self-complementary structures	Ensure that the primers contain no inverted repeat sequences or self-complementary sequences >3 bp in length. Sequences of this type tend to form hairpin structures that can suppress binding of the primer to its target sequence.
Complementarity between members of a primer pair	The 3′-terminal sequences of one primer should not be able to bind to any site on the other primer. Because primers are present in high concentrations in PCR, even weak complementarity between them can cause hybrid formation and the consequent amplification of primer dimers. These molecules can be a real nuisance because they can compete for DNA polymerase and dNTPs and can suppress amplification of the true target DNA. Formation of primer dimers can be reduced by careful primer design and by using a computer program (e.g., OligoAnalyzer [https://www.idtdna.com/calc/analyzet]) to screen pairs of oligonucleotides for self- and cross-complementarity. Formation of primer dimers can also be suppressed by use of hot start or touchdown PCR and/or by the use of specially formulated DNA polymerases (e.g., AmpliTaq Gold; Applied Biosystems). If all else fails, try adding formamide or dimethyl sulfoxide to the PCR mix and reoptimize the concentration of Mg ²⁺ in the PCR by setting up a series of test PCRs containing different amounts of the divalent cation.
Melting temperature (<i>T_m</i>)	The optimum <i>T_m</i> of the duplex formed between a primer and its target is between 55°C and 60°C. The <i>T_m</i> s of the primers in a PCR should not differ by >2–3 centigrade degrees. Most software for primer design uses equation-based nearest-neighbor thermodynamic theory. A first-order approximation of the melting temperature of oligonucleotides with >25 bases can be calculated from the Wallace rule (Wallace et al. 1979): $T_m = 2^{\circ}\text{C}(A + T) + 4^{\circ}\text{C}(G + C),$ where A, G, C, and T are the number of occurrences of each nucleotide. <p>The presence of G or C bases within the last five bases from the 3′ end of primers helps promote tight binding of the 3′ end of the target sequence because of the stronger hydrogen bonding of G and C bases. Priming efficiency and specificity are increased if the 3′-terminal residue is G. However, greater than three Gs or Cs should be avoided in the last five bases at the 3′ end of the primer. Useful sequences not complementary to the target DNA can be added to the 5′ termini of oligonucleotide primers. However, terminal and subterminal restriction sites are cleaved poorly by restriction enzymes; thus, the length of the primer should be extended by at least three nucleotides beyond the restriction site. The NEB catalog contains information on the efficiency with which different restriction enzymes cleave sites near the termini of DNA molecules.</p> <p>Target sequences should be searched using, e.g., BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) for cross-homology with the oligonucleotide primers. False priming at cross-homologous sites increases the level of nonspecific amplification.</p> <p>Contaminating genomic DNA causes many problems in reverse transcriptase PCR (RT-PCR), including an increased number of false positives. This problem is best avoided by designing primers that either span exon–exon junctions in mRNA or bind to the mRNA sequences flanking these junctions.</p>
GC clamp	
Adding restriction sites and other useful sequences to the 5′ termini of primers	
False priming	
cDNA-specific primers	

^aWeb-based tools are available that can assist in PCR primer design. These tools can reduce the cost and time involved in experimentation by lowering the chances of failure: Primer3-Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>), GeneFisher2 (<http://bibiserv.techfak.uni-bielefeld.de/genefisher2/>), and Primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). See also Chen et al. (2002).

BOX 1. MULTIPLEX PCR

“Multiplex PCR” is the term used when N target sequences are simultaneously amplified in a PCR containing N pairs of primers, where $N > 1$. Developed as an efficient method to screen human genomic DNAs for different mutations at the Duchenne muscular dystrophy locus (Chamberlain et al. 1988), multiplex PCR now is used for a variety of purposes: to screen for and identify multiple bacterial and viral pathogens in pathological samples (Elnifro et al. 2000); to screen human genomic DNAs for a variety of clinically significant mutations, genomic rearrangements, and polymorphisms (Beggs et al. 1990; Schuber et al. 1993; Peter et al. 2001); and, on a more mundane level, to measure the accuracy with which different brands of PCR machines control temperature (Schoder et al. 2003; Yang et al. 2005).

Multiplex PCR: Step-by-step optimization

Step 1. Design primer pairs, using primer-design software. Follow the rules in Table 1 in the main text. Make sure that the Tms of all of the primer pairs are within 2°C of each other. Make sure that the amplified products are sufficiently different in size to be displayed and identified on a gel.

Step 2. Use BLAST to check the specificity of the primers. Use primer-design software to test for possible homologies between different primer pairs, ability to form unwanted secondary structure, primer dimers, etc.

Step 3. Set up a series of Touchdown PCRs with single primer pairs, template DNA, *Taq* DNA polymerase, and standard amplification buffer supplemented with a cocktail of enhancers (see Table 1). Analyze the products of each individual PCR and a mixture of all of the products by gel electrophoresis.

Step 4. Using the same conditions as in Step 3, set up a multiplex PCR containing all of the primer pairs at the same molar concentration (between 0.1 and 0.4 μM). Analyze the products by gel electrophoresis, using the products of Step 3 as controls.

Problems and possible solutions

Weak amplification of all products

1. Because Mg^{2+} binds to nucleic acids, the high concentration of oligonucleotides in the multiplex PCR may have lowered the concentration of free Mg^{2+} to suboptimal levels. Set up a series of multiplex Touchdown PCRs containing different concentrations of Mg^{2+} (0.1–0.5 mM, in steps of 0.05 mM).
2. Set up a series of multiplex Touchdown PCRs containing the optimum concentration of Mg^{2+} and with increasing amounts of *Taq* DNA polymerase.
3. Set up a series of multiplex Touchdown PCRs with progressively longer extension times.

Weak amplification or shorter products

1. Set up a series of multiplex Touchdown PCRs in which the annealing and extension temperatures are progressively lowered by 1°C.
2. Increase the concentration of primers for weakly amplified products.

Weak amplification of longer products

1. Increase the extension time of Touchdown PCR.
2. Increase the annealing and extension temperatures by increments of 1°C.
3. Increase the concentration of primers for weakly amplified products.

Unacceptable amount of nonspecific amplification

1. Increase the annealing temperature of Touchdown PCR by increments of 1°C.
2. Decrease the amount of template DNA and *Taq* DNA polymerase.
3. If primer dimers are the problem, increase the annealing temperature by increments of 1°C and progressively decrease the concentration of Mg^{2+} . Try to identify which primers are responsible for dimer formation by setting up a series of multiplex PCRs that lack specific primers. Redesign the primers that are causing the problem.

FIGURE 1. Multiplex optimization.

BOX 1. Continued

Once optimized for a particular set of primer–template DNAs, multiplex PCR can save time and money, and it can efficiently extract a large amount of information from a valuable template DNA. However, optimization can be a lengthy and frustrating business, especially when the number of desired target sequences is large or when the template DNA is complex.

Great care must be taken to ensure that all of the primer pairs in the amplification reaction:

- have approximately the same melting temperature
- are specific for their target loci
- do not display significant homology to themselves or to one another
- generate amplified products that are approximately the same size but can be distinguished from one another by gel electrophoresis

The greater the value of N , the lower is the yield of the amplified product. As a general rule, up to eight primer pairs can be used simultaneously before the yield of the amplified products is reduced to the point of invisibility on an agarose gel. When $N > 8$, the amount and number of spurious amplified products (e.g., primer dimers) often become significant. The formation of these products is promoted by the high concentration of primers present in the early cycles of the amplification reaction. This problem can be prevented by careful primer design (see Table 1) and alleviated to some extent by adjusting the template primer:template ratio in the PCR.

Preferential amplification of some target sequences over others is a common problem. Multiplex PCR is essentially a competition for amplification between differing target sequences. In such a competitive environment, disparity in amplification efficiency can be caused by stochastic effects in the early stages of the PCR, particularly when the concentration of template DNA is very low. Preferential amplification can also result from differences inherent in the target sequences themselves: for example, the location of GC-rich tracts within the target and the propensity of the target to form secondary structures or to form transient duplexes with other regions of the template DNA. These problems can be alleviated by a careful choice of target sequences and by using a touchdown protocol (see Protocol: **Touchdown Polymerase Chain Reaction (PCR)** [Green and Sambrook 2018b]).

So many variables affect the efficiency and specificity of multiplex PCR that it would be impossible to generate an off-the-shelf protocol that would work well in all circumstances. The key to success is systematic optimization of each component and each step in the reaction. This is a considerable amount of work that will not be cost-effective unless the multiplex PCR protocol will be used many times.

Henegariu et al. (1997) published a useful step-by-step chart for avoiding, diagnosing, and solving problems that commonly occur with multiplex PCR. Figure 1 is a modified and updated version of their flowchart. Markoulatos et al. (2002) is also a useful source of advice about setting up and optimizing multiplex PCR.

after the second cycle of freezing–thawing. Storage in unbuffered H_2O can promote acid hydrolysis of dNTPs. During long-term storage at $-20^\circ C$, small amounts of water evaporate and then freeze on the walls of the vial. To minimize changes in concentration, vials containing dNTP solutions should be centrifuged for a few seconds in a microcentrifuge after thawing.

- **Divalent cations.** All thermostable DNA polymerases require free divalent cations—usually Mg^{2+} —for activity. Some polymerases will also work, albeit less efficiently, with buffers containing Mn^{2+} . Calcium ions are quite ineffective (Chien et al. 1976). Magnesium ions have two functions in PCR: reacting with dNTPs to form complexes that are the substrates for *Taq* polymerase, and stabilizing the primer–template complexes. Typically, the dependence of the PCR yield on Mg^{2+} concentration is a bell curve with a broad maximum. When Mg^{2+} concentration is too low, primers anneal inefficiently to the template DNA. When Mg^{2+} concentration is too high, base-pairing is stabilized to such an extent that duplexes formed during amplification are inefficiently denatured by heating. Because dNTPs and oligonucleotides bind Mg^{2+} , the molar concentration of the cation must exceed the molar concentration of phosphate groups contributed by dNTPs plus primers. It is therefore impossible to recommend a concentration of Mg^{2+} that is optimal in all

circumstances. Although a concentration of 1.5 mM Mg^{2+} is routinely used, increasing the concentration of Mg^{2+} to 4.5 mM or 6 mM has been reported to decrease nonspecific priming in some cases (Krawetz et al. 1989; Riedel et al. 1992) and to increase it in others (Harris and Jones 1997). The optimal concentration of Mg^{2+} must therefore be determined empirically for each combination of primers and template. Some companies (e.g., Life Technologies, Sigma-Aldrich, Roche, and Alliance Bio) sell PCR buffer optimization kits containing various buffer formulations that enable investigators to determine optimal reaction conditions for particular primer–template combinations. Once these conditions have been identified, the best buffer can then be purchased in volume or assembled in the laboratory. Alternatively, optimization can be achieved by comparing the yield obtained from a series of 10 PCRs containing concentrations of Mg^{2+} ranging from 0.5–5.0 mM, in 0.5 mM increments. Sometimes a second round of optimization is necessary using a narrower range of Mg^{2+} , in 0.2 mM increments. If possible, preparations of template DNA should not contain significant amounts of chelating agents such as ethylenediaminetetraacetic acid (EDTA) or negatively charged ions, such as PO_4^{3-} , which can sequester Mg^{2+} .

- *Buffer to maintain pH.* Tris-Cl, adjusted to a pH between 8.3 and 8.8 at room temperature, is included in standard PCRs at a concentration as low as 10 mM or as high as 66 mM. When incubated at 72°C (the temperature commonly used for the extension phase of PCR), the pH of the reaction mixture drops by more than a full unit, producing a buffer whose pH is ~ 7.2 .
- *Monovalent cations.* Standard PCR buffer contains 50 mM KCl and works well for amplification of segments of DNA >500 bp in length. Raising the KCl concentration to ~ 70 –100 mM often improves the yield of shorter DNA segments.
- *Template DNA.* Template DNA containing target sequences can be added to PCR in a single- or double-stranded form. Closed-circular DNA templates are amplified slightly less efficiently than linear DNAs. Although the size of the template DNA is not critical, amplification of sequences embedded in high-molecular-weight DNA (>10 kb) can be improved by digesting the template with a restriction enzyme that does not cleave within the target sequence.

In principle, PCR can detect a single target molecule in a reaction mixture. Typically, however, several thousand copies of the target DNA are seeded into the reaction. In the case of mammalian genomic DNA, up to 1.0 μ g of DNA is used per reaction, an amount that contains $\sim 3 \times 10^5$ copies of a single-copy autosomal gene. The typical amounts of yeast, bacterial, and plasmid DNAs used per reaction are 10 ng, 1 ng, and 1 pg, respectively.

Thermostable DNA Polymerases

Thermostable DNA polymerases are isolated from two classes of organisms: the thermophilic and hyperthermophilic eubacteria Archaeobacteria, whose most abundant DNA polymerases are reminiscent of DNA polymerase I of mesophilic bacteria, and thermophilic Archaea, whose chief DNA polymerases belong to the polymerase α -family *T. aquaticus*, an organism from the thermophilic Archaea family (Brock 1995a,b, 1997).

The choice among enzymes should be determined by the purpose of the experiment. For example, if the goal is to make faithful copies of a gene, an enzyme with proofreading function is required, whereas if the goal is to clone an amplified product, an enzyme that generates blunt ends might be advantageous. Until recently, these choices often involved compromise on the part of the investigator. However, mixtures of two or more DNA polymerases can significantly increase yield and enhance amplification, particularly of longer target DNAs (Barnes 1994; Cheng et al. 1994a,b; Cohen 1994). This improvement is presumed to be due to the capacity of one enzyme to complement the inability of another to extend a primer through potential obstructions on the template strand. These obstructions include regions of high secondary structure (Eckert and Kunkel 1993), abasic gaps that cannot be bridged by polymerases lacking terminal transferase activity (Hu 1993), and mispaired bases that cause nonproofreading polymerases to stall and dissociate from the primer–template (Barnes 1994).

Several manufacturers now sell cocktails of thermostable polymerases that allow desirable features to be assembled in one reaction mixture. For example, cocktails of *T. brockianus* (*Tbr*) and *Taq* polymerases (sold under the trade name DyNAzyme) show high fidelity because of the proofreading function of *Tbr* and the high efficiency that is characteristic of *Taq*. Similarly, a mixture of *Taq* and *Pyrococcus furiosus* (*Pfu*) polymerases (e.g., Roche's Expand Long Template PCR System) generates high yields of long targets (up to 35 kb).

DNAs synthesized in amplification reactions catalyzed by *Taq* carry A (adenine) overhangs at their 3' ends. This can be useful in TA cloning, in which a cloning vector (such as a plasmid) is used that has a T (thymidine) 3' overhang that base-pairs with the A overhang of the PCR product, thus enabling ligation of the PCR product into the plasmid vector.

Taq DNA Polymerase

Taq, a thermostable DNA-dependent DNA polymerase, was first isolated from the thermophilic eukaryote *Thermus aquaticus* in 1976 (see Fig. 2) (Chien et al. 1976; Kaledin et al. 1980). Some years later, the enzyme became famous for its use in PCR (Saiki et al. 1988) and in 1989 was designated "Molecule of the Year" (Guyer and Koshland 1989); it remains the workhorse of PCR in most laboratories. An excellent review of the fascinating legal issues surrounding the patenting and commercialization of PCR catalyzed by *Taq* has been published by Fore et al. (2006). A more romantic account of its unusual origin has been composed by the inventor of the technique (Mullis 1990). Although the original patents for PCR expired in 2005–2006, patents remain on many PCR-related techniques, and in these cases, purchase of reagents from the patent holder provides license for use.

The gene for *Taq* (Lawyer et al. 1989) encodes an 832-amino-acid two-domain protein ($M_r = 93.9$ kDa) that displays three different enzymatic activities. The amino-terminal region (residues 1–290) is similar in sequence and structure to the 5' → 3' exonuclease domain of members of the polymerase I family of DNA polymerases (including *Escherichia coli* DNA polymerase I and related bacteriophage-encoded polymerases). Several commercial preparations of *Taq* are available that lack the 5' → 3' exonuclease activity. These include the Stoffel fragment and several site-directed mutants (Merkens et al. 1995). In general, these enzymes are less efficient and less processive than wild-type *Taq*. The polymerase subdomain (residues 424–831) of *Taq* is very similar to that of the Klenow fragment of

FIGURE 2. The Brock expedition. Thomas D. Brock, a microbial ecologist at the University of Wisconsin, Madison, is standing next to Mushroom Spring in Yellowstone National Park, June 23, 1967. *Thermophilus aquaticus* strain YT-1 was isolated from a sample taken in the previous year from the outflow channel (visible on the left side of the photo) by Tom Brock and his undergraduate student Hudson Freeze. Their work is elegantly and proudly described in autobiographical memoirs by Tom Brock (Brock 1995a,b, 1997). The subsequent impact of "extremophilic" microorganisms on the biotechnology industry is described by Madigan and Mairs (1997). (Reprinted, with permission, from Brock 1995b.)

E. coli DNA polymerase I. The amino acid residues critical for catalytic activity are conserved in both polymerases (for reviews, see Joyce and Steitz 1994, 1995; Pelletier 1994; Perler et al. 1996). *Taq* polymerase, like several other thermostable DNA polymerases, also possesses an independent but sluggish transferase activity, which adds a nontemplated residue to the 3' ends of amplified DNAs (Table 2) (Clark 1988; Mole et al. 1989; Hu 1993). Double-stranded, linear DNAs, with the exception of those with protruding 3' ends, can be converted by *Taq* to molecules having 3'-A overhangs. The presence of this unpaired (A) residue facilitates cloning of amplified DNA fragments into double-stranded vectors carrying an unpaired (T) residue. Finally, the carboxy-terminal domain of *Taq* (residues 294–422) contains a catalytically inactive 3' → 5' exonuclease. In consequence, the enzyme, like several other thermostable DNA polymerases, lacks a proofreading function, and its rate of misincorporation of dNTPs is high (Tindall and Kunkel 1988). More than 50% of the DNA molecules produced after 25 cycles of *Taq*-driven amplification of a 200-bp fragment can be expected to carry mutations of one sort or another. When a high fidelity of amplification is required, it is best to catalyze PCRs with a commercial mixture of thermostable polymerases. Platinum *Taq* (Thermo Fisher Scientific), for example, is a mixture of recombinant *Taq* DNA polymerase and *Pyrococcus* GB-D polymerase, which possesses a proofreading ability that increases fidelity approximately sixfold. Other mixtures of DNA polymerases include *Taq*Plus Precision PCR (Agilent), AccuPrime DNA polymerase (Thermo Fisher Scientific), and Expand High Fidelity PCR System (Roche).

The thermal stability of *Taq* DNA polymerase is thought to result from increased hydrophobicity of the core of the enzyme, improved stabilization of electrostatic forces, and enhanced interaction with solvent molecules, because of the presence of additional proline residues on the surface of the enzyme (Kim et al. 1995; Korolev et al. 1995). The thermostable DNA polymerase originally isolated by Chien et al. (1976) was smaller than the full-length *Taq* protein, had slightly different catalytic properties, and in all probability was a proteolytic fragment that lacked part of the amino-terminal domain. In *T. aquaticus*, *Taq* polymerase is expressed at such low levels (0.01%–0.02% of the cellular protein) that commercial production is not a viable proposition. These days, the enzyme is produced from versions of the *Taq* gene that have been engineered so as to obtain high levels of expression in *E. coli*. Most of these alterations involve modification of the DNA sequences that precede and immediately follow the initiating ATG codon (e.g., see Engelke et al. 1990; Lawyer et al. 1993; Ishino et al. 1994; Desai and Pfaffle 1995). Because the clones used by various commercial manufacturers might have been engineered in different ways and because the protocols used for purification of the enzyme might also differ, preparations obtained from different manufacturers do not necessarily deliver identical

TABLE 2. Conditions for standard PCRs catalyzed by *Taq* DNA polymerase

DNA template	10 ⁴ –10 ⁵ copies of target sequence
Number of cycles	25–30
Primers (18–25 nucleotides in length)	0.1–0.5 μ M
Mg ²⁺	Generally 1.5–2.0 mM, but optimization might be necessary to achieve efficient amplification.
dNTPs	Each of the four dNTPs at a concentration of 200 μ M. Higher concentrations of dNTP reduce polymerase accuracy and require higher concentrations of Mg ²⁺ in the reaction mix.
<i>Taq</i> DNA polymerase	1.0–1.25 units/reaction (50 μ L)
Denaturation conditions	For templates whose G + C content is >55%, 30 sec at 95°C is usually sufficient. GC-rich templates can require longer denaturation times (up to 4 min).
Annealing conditions	Normally between 45°C and 65°C, depending on the calculated <i>T_m</i> of the primer pair.
Extension conditions	Although the optimal temperature of <i>Taq</i> DNA polymerase is 75°C–80°C, extension reactions are generally performed at a slightly lower temperature (68°C–70°C). The time of the extension phase depends on the length of the target sequences. Because the rate of polymerization of <i>Taq</i> is 35–100 nucleotides/sec, an extension time of 1 min per kilobase of template DNA is usually adequate.
Additives	Additives can be used if the efficiency of amplification remains low after optimization of PCR. Commonly used additives include glycerol (5%–10%) and bovine serum albumin (up to 0.8 μ g/ μ L). Formamide (1%–5%), dimethyl sulfoxide (2%–10%), or betaine (0.5–2 μ M) can be added for amplification of template DNAs with high GC content (>55%). When using betaine, reduce the denaturation melting temperature to 92°C–93°C, and lower the annealing temperature by 1°C–2°C.

results. Indeed, variations have been reported in the yield, length, and fidelity of the amplified product generated by different commercial preparations of *Taq* in standardized PCRs (e.g., Linz et al. 1990). However, homemade *Taq* polymerase, which is simple to prepare (Engelke et al. 1990; Pluthero 1993; Desai and Pfaffle 1995), is consistently of high quality and shows little batch-to-batch variation. Nevertheless, it is always good practice to optimize PCRs every time for each new batch of *Taq*.

Preparations of *Taq* DNA polymerase typically display the following properties:

Optimal reaction temperature:	75°C–80°C
Optimal reaction conditions:	1.5 mM MgCl ₂ , 50–55 mM KCl (pH 7.8–9.0)
<i>K_m</i> dNTPs:	10–15 μM
<i>K_m</i> DNA:	1.5 nM
Extension rates (dNTPs/sec per enzyme molecule):	
75°C	150
70°C	<60
55°C	24
37°C	1.25
22°C	0.25
Processivity (dNTPs/sec per enzyme molecule):	42
Half-life of enzyme:	
97.5°C	5–6 min
95°C	40 min
92.5°C	130 min

Taq DNA polymerase also accepts modified deoxyribonucleoside triphosphates as substrates and can be used to label DNA fragments with radionucleotides, digoxigenin, fluorescein, or biotin (Innis et al. 1988; Lo et al. 1988).

To initiate DNA synthesis, *Taq* polymerase, like other DNA polymerases, requires a primer that is annealed to the template strand and carries a hydroxyl group at its 3' end. During the extension reaction in vitro, *Taq* polymerase removes oligonucleotides carrying a 5'-hydroxyl group that are annealed to the template strand ahead of the growing strand. However, 5'-phosphorylated oligonucleotides cannot be displaced from the template strand of DNA by *Taq* polymerase. The enzyme is not able to continue synthesis when it encounters a depurinated base in the template. Because depurination occurs at a significant rate when DNA is incubated at high temperatures, this can limit the length of DNA that can be amplified by *Taq* polymerase (Barnes 1994).

One unit of *Taq* DNA polymerase incorporates 10 nmol of dNTP into acid-precipitable material in 30 min at 74°C. Because the specific activity of most commercial preparations of a 94-kDa *Taq* polymerase is ~80,000 units/mg of protein, one unit of the enzyme contains ~8 × 10¹⁰ *Taq* molecules. A typical PCR contains one unit of *Taq*—a vast excess of polymerase molecules relative to the DNA template. By the end of the amplification reaction, however, the number of amplified DNA molecules can exceed the number of enzyme molecules by a factor of 100.

Although *Taq* polymerase remains the enzyme of choice for routine amplification of small segments of DNA, it lacks a 3' → 5' proofreading function (Lawyer et al. 1993) and has a high misincorporation rate (one nucleotide in ~9000 nucleotides) (Tindall and Kunkel 1988). Thermostable DNA polymerases isolated from other thermophilic bacteria and archaea, such as *Pfu* DNA polymerase, possess a proofreading activity (Cline et al. 1996) and can be used instead of (or in combination with) *Taq* when greater fidelity is required, when the length of the target amplicon exceeds a few thousand bases, or when cloning mRNA by reverse transcription PCR (RT-PCR).

Programming Polymerase Chain Reactions

PCR is an iterative process, consisting of three elements: denaturation of the template by heat, annealing of the oligonucleotide primers to the single-stranded target sequence(s), and extension of the annealed primers by a thermostable DNA polymerase (Fig. 3).

FIGURE 3. Sequence of amplification in the PCR. The diagram shows the steps involved in the first few rounds of a PCR. The original template (top) is double-stranded DNA, and the leftward and rightward oligonucleotide primers are shown as ← and →, respectively. The products of the first few rounds of the amplification reaction are heterogeneous in size; however, the tract of DNA lying between the two primers is preferentially amplified and quickly becomes the dominant product of the amplification reaction.

- **Denaturation.** Double-stranded DNA templates denature at a temperature that is determined in part by their G + C content. The higher the proportion of G + C, the higher is the temperature required to separate the strands of template DNA. The longer the DNA molecules are, the greater is the time required at the chosen denaturation temperature to separate the two strands completely. If the temperature for denaturation is too low or if the time is too short, only AT-rich regions of the template DNA will be denatured. When the temperature is reduced later in the PCR cycle, the template DNA will reanneal into a fully native condition.

In PCRs catalyzed by *Taq* DNA polymerase, denaturation is performed at 94°C–95°C, which is the highest temperature that the enzyme can endure for 30 or more cycles without sustaining excessive damage. In the first cycle of PCR, denaturation is sometimes performed for 5 min to increase the probability that long molecules of template DNA are fully denatured. However, in our experience, this extended period of denaturation is unnecessary for linear DNA molecules and can sometimes be deleterious (Gustafson et al. 1993). We recommend denaturation for 45 sec at 94°C–95°C for routine amplification of linear DNA templates whose content of G + C is 55% or less.

Higher temperatures might be required to denature template and/or target DNAs that are rich in G + C (>55%). DNA polymerases isolated from Archaea are more heat-tolerant than *Taq* and are therefore preferred for amplification of GC-rich DNAs.

- **Annealing of primers to template DNA.** The temperature used for the annealing step (T_a) is critical. If the annealing temperature is too high, the oligonucleotide primers anneal poorly, if at all, to the template, and the yield of amplified DNA is very low. If the annealing temperature is too low, nonspecific annealing of primers can occur, resulting in the amplification of unwanted segments of DNA. Annealing is usually performed 3°C–5°C lower than the calculated melting temperature at which the oligonucleotide primers dissociate from their templates. Many formulas exist to determine the theoretical melting temperature, but none of them are accurate for oligonucleotide primers of all lengths and sequences (see “Calculating Melting Temperatures of Hybrids between Oligonucleotide Primers and Their Target Sequences” below). It is best to optimize the annealing

conditions by performing a series of trial PCRs at temperatures ranging from 2°C to 10°C below the lower of the melting temperatures calculated for the two oligonucleotide primers. Alternatively, the thermal cycler can be programmed to use progressively lower annealing temperatures in consecutive pairs of cycles (Don et al. 1991; see also Protocol: **Touchdown Polymerase Chain Reaction (PCR)** [Green and Sambrook 2018b]). Instead of surveying a variety of annealing conditions in separate PCRs, optimization is achieved by exposing a single PCR to a sequential series of annealing temperatures in successive cycles of the reaction. For many investigators, touchdown PCR bypasses the need to determine the optimum annealing temperature for every pair of primers and is used to obtain acceptable yields of amplified products in routine PCR (Peterson and Tjian 1993; Hecker and Roux 1996; Roux and Hecker 1997).

- *Extension of oligonucleotide primers* is performed at or near the optimal temperature for DNA synthesis catalyzed by the thermostable polymerase. In the first two cycles, extension from one primer proceeds beyond the sequence complementary to the binding site of the other primer. In the next cycle, the first molecules are produced whose length is equal to the segment of DNA delimited by the binding sites of the primers. From the third cycle onward, this segment of DNA is amplified geometrically, whereas longer amplification products accumulate arithmetically (Mullis and Faloona 1987). As a rule of thumb, extension is performed for 1 min for every 1000 bp of product. For the last cycle of PCR, many investigators use an extension time that is three times longer than in the previous cycles, ostensibly to allow completion of all amplified products. However, in our experience, the result of the PCR is not significantly altered by tinkering with the extension time in this way.
- *Number of cycles.* The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction and the efficiency of primer extension and amplification. Once established in the geometric phase, the reaction proceeds until one of the components becomes limiting. At this point, the yield of specific amplification products should be maximal, whereas nonspecific amplification products should be barely detectable, if at all. This is generally the case after ~30 cycles in PCRs containing ~10⁵ copies of the target sequence and *Taq* DNA polymerase. At least 25 cycles are required to achieve acceptable levels of amplification of single-copy target sequences in mammalian DNA templates.

Optional Components of Polymerase Chain Reactions

Several cosolvents and additives have been reported to reduce unacceptably high levels of mispriming and to increase the efficiency of amplification of G + C-rich templates. Cosolvents include organic amides such as formamide (1.25%–10% [v/v]; Sarkar et al. 1990; Varadaraj and Skinner 1994; Chakrabarti and Schutt 2001), dimethyl sulfoxide (up to 15% [v/v]; Bookstein et al. 1990), and glycerol (1%–10% [v/v]; Lu and Nègre 1993). Additives include tetramethylammonium chloride (Hung et al. 1990; Chevet et al. 1995), betaine (Henke et al. 1997), potassium glutamate (10–200 mM), ammonium sulfate, nonionic and cationic detergents (e.g., Tween 20 and NP-40; Bachmann et al. 1990; Pontius and Berg 1991), and specificity enhancers such as Perfect Match PCR Enhancer (Agilent) and GC-Melt (Clontech). Many of these additives and cosolvents inhibit PCR when used at high concentrations, and the optimum concentration must be determined empirically for each combination of primers and template DNA. Rather than reaching for these enhancers at the first sign of trouble, it is far better in our view to optimize the regular components of the reaction, particularly the concentrations of Mg²⁺ and K⁺ ions (Krawetz et al. 1989; Riedel et al. 1992). The one exception to this general rule concerns the use of GC-Melt, which in our hands often overcomes problems of low-efficiency amplification with uncooperative G + C-rich templates.

Inhibitors

Almost anything will inhibit PCRs if present in excess. The common culprits include proteinase K (which, if given the opportunity, can degrade thermostable DNA polymerase), phenol, and EDTA.

Other substances that can cause problems are ionic detergents (Weyant et al. 1990), heparin (Beutler et al. 1990), polyanions such as spermidine (Ahokas and Erkkilä 1993), hemoglobin, and gel-loading dyes such as bromophenol blue and xylene cyanol (Hoppe et al. 1992). In many cases, the chief cause of low or erratic yields is contaminants in the template DNA, which is often the only component of the reaction supplied by the investigator (see “Contamination in PCR” below). Many problems with PCR can be cured simply by cleaning up the template by dialysis, ethanol precipitation, extraction with chloroform, and/or chromatography through a suitable resin.

DESIGN OF OLIGONUCLEOTIDE PRIMERS FOR BASIC PCR

The chief goal of primer design is specificity, which is achieved only when each member of a primer pair anneals in a stable fashion to its target sequence in the template DNA. As a rule of thumb, the longer an oligonucleotide, the higher is its specificity for a particular target. The following equation can be used to calculate the probability that a sequence exactly complementary to a string of nucleotides will occur by chance within a DNA sequence space that consists of a random sequence of nucleotides (Nei and Li 1979):

$$K = [g/2]^{G+C} \times [(1-g)/2]^{A+T},$$

where K is the expected frequency of occurrence within the sequence space, g is the relative $G + C$ content of the sequence space, and G , C , A , and T are the number of specific nucleotides in the oligonucleotide. For a double-stranded genome of size N (in nucleotides), the expected number (n) of sites complementary to the oligonucleotide is $n = 2NK$.

These equations predict that an oligonucleotide of 15 nucleotides would be represented only once in a mammalian genome where $N = \sim 3.0 \times 10^9$. In the case of a 16-mer, there is only one chance in 10 that a typical mammalian cDNA library (with a complexity of $\sim 10^7$ nucleotides) will fortuitously contain a sequence that exactly matches that of the oligonucleotide. However, these calculations are based on the assumption that the distribution of nucleotides in mammalian genomes is random. This is not the case because of bias in codon usage and because a significant fraction of the genome is composed of repetitive DNA sequences and gene families. To minimize problems of nonspecific annealing, it is advisable to use oligonucleotide primers longer than the statistically indicated minimum. Because of the presence of repetitive elements, no >85% of the mammalian genome can be targeted precisely, even by primers that are twenty or more nucleotides in length. Before synthesizing an oligonucleotide primer, it is prudent to scan DNA databases to check that the proposed sequence occurs only in the desired gene and not in vectors, undesired genes, or repetitive elements.

Table 1 presents information on the design of oligonucleotide primers for basic PCR. Failures will be rare if the advice provided in the table is followed carefully.

Selecting PCR Primers

Several steps are involved in the selection of oligonucleotide primers.

1. Analyze the target gene for potential priming sites that are free of homopolymeric tracts, have no obvious tendency to form secondary structures, are not self-complementary, and have no significant homology with other sequences on either strand of the target genome. (See also Protocol: **Optimizing Primer and Probe Concentrations for Use in Real-Time Polymerase Chain Reaction (PCR)** [Green and Sambrook 2018c].)
2. Create lists of possible forward and reverse primers based on the criteria provided in Table 1. Calculate the melting temperatures of the oligonucleotides from the formulas given in the section “Calculating Melting Temperatures of Hybrids between Oligonucleotide Primers and Their Target Sequences,” below.

3. Select well-matched pairs of forward and reverse primers that are similar in their content of G + C and will generate an amplified product of the appropriate size and base composition. The GC content of both primers and the amplified product should be similar and lie between 40% and 60%.
4. Refine the length and/or placement of the oligonucleotides so that the 3'-terminal nucleotide is a G or a C. Check that the two oligonucleotides do not display significant complementarity. As a rule of thumb, no more than three consecutive nucleotides on one primer should be complementary to the other primer.

Computer-Assisted Design of Oligonucleotide Primers

To save time and minimize problems, use computer programs to optimize the design, selection, and placement of oligonucleotide primers (for review, see Chen et al. 2002). Many stand-alone computer programs are available to search sequences for priming sites that fit a set of user-defined parameters and are free of potential hairpins, self-dimers, and other problematic structures. Such programs generate a hierarchy of potentially specific primers whose melting temperatures have been calculated, generally using the nearest-neighbor method, in which the thermodynamic stability of the primer–template duplex is derived from the sum of the stacking interactions of neighboring bases.

Most of the programs use graphic tools and user-friendly interfaces and rank potential primers and primer pairs according to the weight assigned to various parameters. Some of the programs contain, for example, facile searching of databases for unintentional matches to the primer, optimization of conditions for the amplification reaction, translation of amino acid sequences into populations of degenerate oligonucleotides, and elimination of primers capable of forming stable secondary structures. All of the popular DNA analysis packages contain sophisticated modules for primer design; the website “PCR Primer Design and PCR Setup” (http://www.humgen.nl/primer_design.html) provides links to a wide range of available software packages.

Calculating Melting Temperatures of Hybrids between Oligonucleotide Primers and Their Target Sequences

Several equations are available to calculate the melting temperature of hybrids formed between an oligonucleotide primer and its complementary target sequence. None of these programs is perfect, and thus the choice between them is largely a matter of personal preference. The melting temperature of each member of a primer pair should obviously be calculated using the same equation.

- An empirical and convenient equation, known as “The Wallace Rule” (Suggs et al. 1981; Thein and Wallace 1986), can be used to calculate the melting temperature for perfect duplexes 15–20 nucleotides in length in solvents of high ionic strength (e.g., 1 M NaCl):

$$T_m = 2(A + T) + 4(G + C),$$

where T_m is the melting temperature expressed in °C, $(A + T)$ is the sum of the A and T residues in the oligonucleotide, and $(G + C)$ is the sum of the G and C residues in the oligonucleotide.

- The equation of Baldino et al. (1989) predicts reasonably well the melting temperature of oligonucleotides, 14–70 nucleotides in length, in cation concentrations of 0.4 M or less:

$$T_m = 81.5^\circ\text{C} + 16.6(\log_{10}[K^+]) + 0.41(\%[G + C]) - (675/n),$$

where n is the number of bases in the oligonucleotide. This equation can also be used to calculate the melting temperature of an amplified product whose sequence and size are both known. When PCR amplification is performed under standard conditions, the calculated melting temperature of the amplified product should not exceed ~85°C, which will ensure complete separation of its strands during the denaturation step. Note that the “denaturation temperature” in PCR is

more accurately defined as “the temperature of irreversible strand separation of a homogeneous population of molecules”; the temperature of irreversible strand separation is several degrees higher than the melting temperature (typically, 92°C for DNA whose content of G + C is 50%) (Wetmur 1991).

Neither of the above equations takes into account the effect of base sequence (as opposed to base composition) on the melting temperature of oligonucleotides. A more accurate estimate of melting temperature can be obtained by incorporating nearest-neighbor thermodynamic data into the equations (see, e.g., Rychlik 1995). However, the Wallace Rule and the Baldino algorithm are far simpler to apply and are perfectly adequate for most purposes.

DETECTING, ANALYZING, AND QUANTIFYING mRNAs

Over the years, many methods have been developed to detect, analyze, and quantify mRNAs and other types of cellular transcripts (see Table 3). In chronological order of their development, these methods include the following:

- *Northern hybridization* (Alwine et al. 1977). A population of RNAs is (1) separated by electrophoresis under denaturing conditions through an agarose gel, (2) transferred to a solid support (nylon or nitrocellulose), and (3) hybridized to a labeled probe. The size of the RNA of interest is estimated from its migration relative to controls of known size, its abundance from the intensity of hybridization.
- *Ribonuclease (RNase) protection* (Zinn et al. 1983; Melton et al. 1984). An antisense-labeled probe specific for the target RNA(s) is hybridized to a population of RNAs. All the unhybridized sequences are hydrolyzed by digestion with RNase. The amount of labeled probe remaining after digestion is a measure of the amount of target RNA in the original RNA population.
- *Reverse transcription PCR (RT-PCR)* (Wang et al. 1989). A population of mRNAs is used as the substrate for reverse transcriptase. A pair of oligonucleotides specific for the target RNA is then used to amplify the target by conventional PCR. These two enzymatically catalyzed steps can be performed as a single-step coupled reaction or as a two-step uncoupled reaction. During the PCR phase of RT-PCR, the reaction is stopped at a cycle number where the amplification is assumed to be in the exponential phase. The amplified product is then analyzed by gel electrophoresis and by Southern blotting. The amount of DNA in the band is then estimated against a set of standards generated in parallel from PCRs spiked with different amounts of a known mRNA. (See Protocol: **Amplification of cDNA Generated by Reverse Transcription of mRNA: Two-Step Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** [Green and Sambrook 2019a]).
- *Real-time quantitative PCR (real-time qPCR)*. A population of mRNAs is used as the substrate for reverse transcriptase. A pair of oligonucleotides specific for the target RNA is then used to amplify the target by conventional PCR. These two enzymatically catalyzed reactions can be performed as a single-step coupled reaction or as a two-step uncoupled reaction. Detection and quantitation of the mRNA under study is performed using fluorescent markers that emit light in proportion to the amount of PCR product generated during the amplification reaction. Measurements of fluorescent intensity are made in real time. (See Protocol: **Quantification of RNA by Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** [Green and Sambrook 2018d]). These days, the standard method for quantifying cellular RNAs is real-time RT-qPCR (VanGuilder et al. 2008). RT-qPCR is performed in a thermal cycler equipped with a system to measure the fluorescence emitted by a detector molecule. Many different technologies are available, the most popular of which are TaqMan (Applied Biosystems), LightCycler (Roche), LUX (Thermo Fisher Scientific), Molecular Beacons, and SYBR Green.

TABLE 3. Detecting, analyzing, and quantifying mRNAs

Method	Type(s) of target RNA	Number of target RNAs quantified simultaneously	Uses	Disadvantages
Northern hybridization	mRNA	Usually one; at most, a few	Used chiefly to estimate the size of the target mRNA in different types of cells or tissues; the amount of target mRNA can be roughly quantified.	Requires a large amount of RNA. One or at most a few species of mRNAs can be detected simultaneously. Insensitive and time-consuming compared with PCR-based methods.
RNase protection	mRNA	Usually one; but by using a mixture of probes, up to 12 mRNAs can be detected simultaneously.	Used chiefly to detect and quantify the amount of target mRNA in different types of cells or tissues.	Requires a specific antisense hybridization probe (usually radioactive). RNase protection is far more sensitive than northern hybridization but still relatively insensitive compared with PCR-based methods.
Conventional reverse-transcription PCR (RT-PCR)	mRNA	Usually one, but with effort can be converted to a multiplex system	Highly sensitive method for detecting target RNAs, even those that are present at very low numbers of copies in cells. Used to measure the relative abundance of mRNAs in different cells or tissues.	Because the specificity of RT-PCR is determined by the primers used for reverse transcription and subsequent amplification, false positives are always a possibility. The reverse transcription step is highly variable. In addition, end-point measurement of the amount of product has many defects, including low resolution and poor sensitivity.
Real-time quantitative PCR (real-time qPCR)	mRNA; miRNAs	Usually one, but the method can be multiplexed; see, e.g., Stanley and Szewczuk (2005), who analyzed 72 mRNAs in a single multiplex reaction.	A highly sensitive and accurate method for detecting target RNAs, even those that are present at very low numbers of copies in cells. Real-time qPCR is more sensitive, faster, and more accurate than any other method and has become a dominant technique to measure the relative abundance of mRNAs in different cells or tissues. Real-time qPCR is not limited to mRNAs and can also be used to measure the abundance of microRNAs (see, e.g., Chen et al. 2005; Benes and Castoldi 2010).	The several commercial devices on the market use different detection methods. In addition, hundreds of different protocols describing variants of real-time qPCR have been published. The resulting lack of standardization at every step of real-time qPCR makes comparison of reliability and reproducibility difficult if not impossible. However, the publication of sensible advice about the planning and execution of real-time qPCR experiments (Nolan et al. 2006; Derveaux et al. 2010) and of guidelines suggesting a minimal set of information required for publication of results of real-time qPCR experiments should help to resolve a confusing situation (Bustin et al. 2009; Bustin 2010).

Other PCR-based techniques for determining the 5' or 3' ends of mRNAs are 5'-RACE (Protocol: **Rapid Amplification of Sequences from the 5' Ends of mRNAs: 5'-RACE** [Green and Sambrook 2019b]) and 3'-RACE (Protocol: **Rapid Amplification of Sequences from the 3' Ends of mRNAs: 3'-RACE** [Green and Sambrook 2019c]).

PCR IN THEORY

The course of PCRs can be represented graphically in two ways:

- A standard x - y linear plot of the amount of amplified product (or fluorescence) versus cycle number. Because the amount of amplified product increases geometrically—ideally doubling in each cycle of the reaction—a linear x - y plot should display an exponential component for several cycles after the signal emerges from the background.
- In contrast, the exponential phase of the reaction appears as a straight line when the $\log_{10}$ of the amplified product (or fluorescence) is plotted against cycle number. The slope of the line can be used to calculate the efficiency of the reaction. Displaying the data in a semilog fashion allows a value of the “threshold cycle” (C_t) to be determined (for a fuller discussion, see Introduction: **Analysis and Normalization of Real-Time PCR Experimental Data** [Green and Sambrook 2018e]).

During quantitative PCR, the C_t value is defined as the point at which a fluorescent signal is first detected above the baseline and is inversely correlated with the $\log_{10}$ of the initial copy number of the target sequence. In most commercial machines, the C_t value is set automatically within the exponential phase of the PCR at a point where the signal is 10-fold higher than the average baseline signal. The higher the initial copy number, the earlier the C_t value is reached during the amplification reaction amplification.

The efficiency of a PCR eventually begins to drop as a result either of product inhibition or because the concentration of one or more of the reagents becomes limiting. The reaction then enters a linear phase, which appears as a straight line in the linear x - y plot. The plateau is the end point of the PCR. In the logarithmic plot, reactions that contain different amounts of template seem to reach the same plateau. But this is solely the result of the log scaling of the plot. In linear plots, reactions containing different amounts of template show a clear difference in the levels of their plateau phases.

The earliest methods of quantitative PCR used end-point measurement, which is time-consuming, has low sensitivity and accuracy, has a restricted dynamic range, and requires extensive post-PCR processing. In addition, the level of the plateau phase is affected by variables that have nothing to do with the initial concentration of template. For example, the efficiency of the exponential portion of the amplification reaction depends on the quality of the thermostable DNA polymerase. Because of the geometric nature of PCR, the penalty for using an inefficient enzyme is severe. For example, Linz et al. (1990) found that after twenty cycles of exponential amplification, the amount of product varied over a 200-fold range depending on the polymerase used. This large difference in yield was attributed to a twofold difference in the efficiency of the enzymes during the exponential phase of the reaction. For further discussion of the reaction efficiency of PCRs, please see Peccoud and Jacob (1996), Liu and Saint (2002a,b), Jagers and Klebaner (2003), and Lalam (2006).

These days, quantitative PCR relies on kinetic analysis of the early phases of the amplification reaction. This method of measurement directly reflects the initial abundance of the template and avoids the variables that can contaminate calculations based on the final level of the plateau phase.

CONTAMINATION IN PCR

A problem commonly encountered in PCR is contamination with exogenous DNA sequences that can be amplified by the oligonucleotide primers. In every case, this contamination is the fault of sloppy

work by investigators or their colleagues, who inadvertently introduce potential target sequences into equipment, solutions, and enzymes used in PCR. The first sign of trouble is generally the appearance of an amplification product in the negative controls that lack template DNA. From that moment on, all amplified products obtained in the reactions containing test DNAs must be regarded as suspect. In our experience, little is gained in searching for the source(s) of the contamination. Instead, it is simpler, less expensive, and less disruptive for all concerned to discard all solutions and reagents and all disposables, to decontaminate instruments, and to take steps such as those described below to reduce the risk of contamination in the future.

Laboratory Space

In an ideal world, PCRs would be assembled in a separate laboratory that has its own set of equipment and freezers for storing buffers and enzymes. A more practical alternative for most investigators, however, is to designate a particular section of the laboratory for setting up PCRs. The assembly of PCRs is best performed in a laminar flow hood equipped with ultraviolet (UV) lights. These lights should be turned on whenever the hood is not in use. Keep in the hood a microcentrifuge, disposable gloves, supplies, and sets of pipetting devices used to handle *only* reagents for PCR. Because the barrels of automatic pipetting devices are common sources of contamination, positive-displacement pipettes equipped with disposable tips and plungers should be used to prepare and handle reagents.

Alternatively, use preplugged, sterile, disposable pipette tips (e.g., ART Aerosol Resistant Tips; Research Products International) on automatic air-displacement pipetting devices. Disposable items such as pipette tips and tubes should be used directly from the manufacturer's packaging and should not be autoclaved before use. Thermal cyclers should be located in a separate area of the laboratory, well separated from the hood used for assembly of PCR and for preparation of reagents.

Rules for Assembling and Performing PCRs

Below is a list of rules for assembling and performing PCRs. Investigators setting up PCRs must have an understanding of these rules and agree to follow them:

- Keep to a minimum all traffic in and out of the laboratory area designated for assembly of PCRs.
- Wear gloves when working in the area, and change them frequently. Use face masks and head caps to reduce contamination from facial skin and hair cells.
- Prepare your own set of reagents and disposable items (including PCR tubes, mineral oil, and wax beads). Use new glassware, plasticware, and pipettes that have not been exposed to DNA in the laboratory to prepare and store solutions. Store buffers and enzymes in small aliquots, in a designated section of a freezer located near the flow hood. Discard aliquots of reagents after use. Never use PCR reagents for other purposes.
- Before opening microcentrifuge tubes containing reagents used in PCRs, centrifuge them briefly (10 sec) in the microcentrifuge located in the laminar flow hood. This centrifugation deposits the fluid in the base of the tube and reduces the possibility of contamination of gloves or pipetting devices.
- Prepare dilutions of DNA used as templates in PCR at your own laboratory bench, and take only as much of the dilution as needed into the PCR area.
- At the end of the PCR, do not take the tubes containing amplified DNA into the PCR area. Instead, open the tubes and perform postamplification processing at your laboratory bench.
- PCR-grade water should be used in all PCR experiments. PCR-grade water is free of ions, salts, and nucleases and has a balanced pH. It is commercially available from a number of suppliers.

Decontamination of Solutions and Equipment

Contaminating DNA can be inactivated by irradiating certain reagents (buffers without dNTPs and H₂O) with UV light at 254-nm wavelength. UV light forms dimers between adjacent pyrimidine residues in contaminating DNAs and renders them inactive as templates in the PCR. Irradiation (200–300 mJ/cm² for 5–20 min) is most readily accomplished in translucent white microcentrifuge tubes using a commercially available UV cross-linker (e.g., Stratalinker; Agilent). dNTPs are resistant to UV, but *Taq* DNA polymerase is not. The sensitivity of primers to UV is variable and unpredictable (Ou et al. 1991).

UV irradiation can also be used to decontaminate the outer surfaces of small pieces of equipment (e.g., racks, pipettes). Work areas, nonmetallic surfaces of microcentrifuges, and thermal cyclers can be decontaminated with weak solutions of bleach (e.g., 10% Clorox) (Prince and Andrus 1992) or with a commercial product such as DNAzap.

Preventing Contamination of One PCR by the Products of Another

Uracil *N*-glycosylase (Ung) can be used to destroy amplified DNAs that are unintentionally carried from one PCR to another (Longo et al. 1990; Thornton et al. 1992; for review, see Hartley and Rashtchian 1993). This enzyme will cleave uracil–glycosidic bonds in DNA that contains dU residues incorporated in place of dT residues but will not cleave RNA or double-stranded DNA that contains rU or dT residues, respectively. The contamination protocol is initiated by routinely substituting dUTP for dTTP in PCR. This substitution has little effect on the specificity of the PCR or the analysis of PCR products. However, the yield of amplified products might be reduced slightly (Persing 1991). Nevertheless, when subsequent sets of PCRs are briefly treated with Ung, contaminating DNA containing uracil residues is destroyed. The use of dUTP and uracil *N*-glycosylase to reduce contamination is most helpful when a small number of DNA fragments are to be amplified from many hundreds of samples, e.g., as part of a large genetic screen. It is important to note that decontamination by Ung is helpful but not completely effective (Niederhauser et al. 1994); it should therefore be viewed only as a single component of a more comprehensive program to manage and prevent contamination.

RELATED INFORMATION

A number of variations of the basic PCR protocol have been developed to address specific issues. Protocol: **Hot Start Polymerase Chain Reaction (PCR)** (Green and Sambrook 2018f) suppresses nonspecific amplification by withholding an essential component of the reaction (e.g., the DNA polymerase) until the reaction mixture has reached a temperature inhibitory to nonspecific hybridization or the formation of primer dimers. Protocol: **Polymerase Chain Reaction (PCR) Amplification of GC-Rich Templates** (Green and Sambrook 2018g) uses a cocktail of cosolvents and additives to enhance the amplification of templates with high G + C content. Protocol: **Long and Accurate Polymerase Chain Reaction (LA PCR)** (Green and Sambrook 2018h) uses two different DNA polymerases with different efficiencies and activities to achieve high yields and greater accuracy when amplifying larger templates. Protocol: **Inverse Polymerase Chain Reaction (PCR)** (Green and Sambrook 2019d) uses circularized restriction fragments to amplify for characterization of unknown sequences of DNA that are adjacent to known sequences. Protocol: **Nested Polymerase Chain Reaction (PCR)** (Green and Sambrook 2019e) enhances the sensitivity of the reaction by using two sequential rounds of PCR, each with its own set of primers, and uses the product of the first reaction as the template for the second. Finally, Protocol: **Screening Colonies by Polymerase Chain Reaction (PCR)** (Green and Sambrook 2019f) provides a simple means to screen recombinant bacterial colonies for plasmids of interest.

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