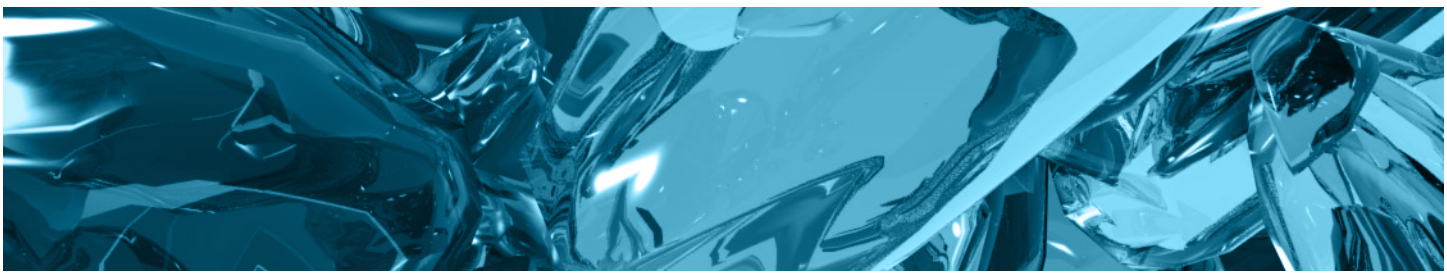
CONTENTS



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I. Introduction

The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro. Traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell often require days or weeks of work, but amplification of DNA sequences by PCR requires only hours. While most biochemical analyses, including nucleic acid detection with radioisotopes, require the input of significant amounts of biological material, the PCR process requires very little. Thus, PCR can achieve more sensitive detection and higher levels of amplification of specific sequences in less time than previously used methods. These features make the technique extremely useful, not only in basic research, but also in commercial uses, including genetic identity testing, forensics, industrial quality control and in vitro diagnostics. Basic PCR is commonplace in many molecular biology labs where it is used to amplify DNA fragments and detect DNA or RNA sequences within a cell or environment. However, PCR has evolved far beyond simple amplification and detection, and many extensions of the original PCR method have been described. This chapter provides an overview of different types of PCR methods, applications and optimization. A detailed treatment of these methods is beyond the scope of this publication. However, an extensive bibliography is provided in the References section for researchers who require more comprehensive information.

A. Basic PCR

The PCR process was originally developed to amplify short segments of a longer DNA molecule (Saiki *et al*. 1985). A typical amplification reaction includes target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Once assembled, the reaction is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures for set amounts of time. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. Ten cycles theoretically multiply the amplicon by a factor of about one thousand; 20 cycles, by a factor of more than a million in a matter of hours.

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension (Figure 1.1). The initial step denatures the target DNA by heating it to 94°C or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase. In the next step of a cycle, the temperature is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts approximately 15–60 seconds. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 70–74°C. The extension step lasts approximately 1–2 minutes. The next cycle begins with a return to 94°C for denaturation.

Each step of the cycle should be optimized for each template and primer pair combination. If the temperature during the annealing and extension steps are similar, these two steps can be combined into a single step in which both primer annealing and extension take place. After 20–40 cycles, the amplified product may be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.

An animated presentation illustrating the PCR process is available.

Additional Resources for Basic PCR

Technical Bulletins and Manuals

TB254	*GoTaq® PCR Core Systems Technical Bulletin*
9PIM750	*PCR Master Mix Promega Product Information*
9PIM300	*GoTaq® DNA Polymerase Promega Product Information*
9PIM829	*GoTaq® Flexi DNA Polymerase Promega Product Information*

Promega Publications

Ensuring successful PCR using online resources

GoTaq® Green Master Mix: From amplification to analysis

Introducing GoTaq® DNA Polymerase: Improved amplification with a choice of buffers

Performance advantages designed into Promega's PCR Master Mix

Online Tools

Amplification Product Selector

Citations

Bermejo-Alvarez, P. *et al.* (2008) Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially? *Biol. Reprod.* **79**, 594–7.

To determine whether oocytes are able to select X-bearing or Y-bearing spermatozoa, the authors performed in vitro fertilization of bovine oocytes with X-sorted semen, Y-sorted semen, a mixture of X- and Y-sorted semen, and unsorted semen. The gender of the resulting embryos was determined by amplifying two DNA targets: a Y chromosome-specific target for gender assignment and a bovine-specific satellite sequence as a control. PCRs were performed using GoTaq® Flexi DNA Polymerase (1 unit per 25µl reaction), and amplified products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining.

PubMed Number: 18579751

Staniszewska, I. *et al.* (2008) Integrin alpha9 beta1 is a receptor for nerve growth factor and other neurotrophins. *J. Cell Sci* **121**, 504–13.

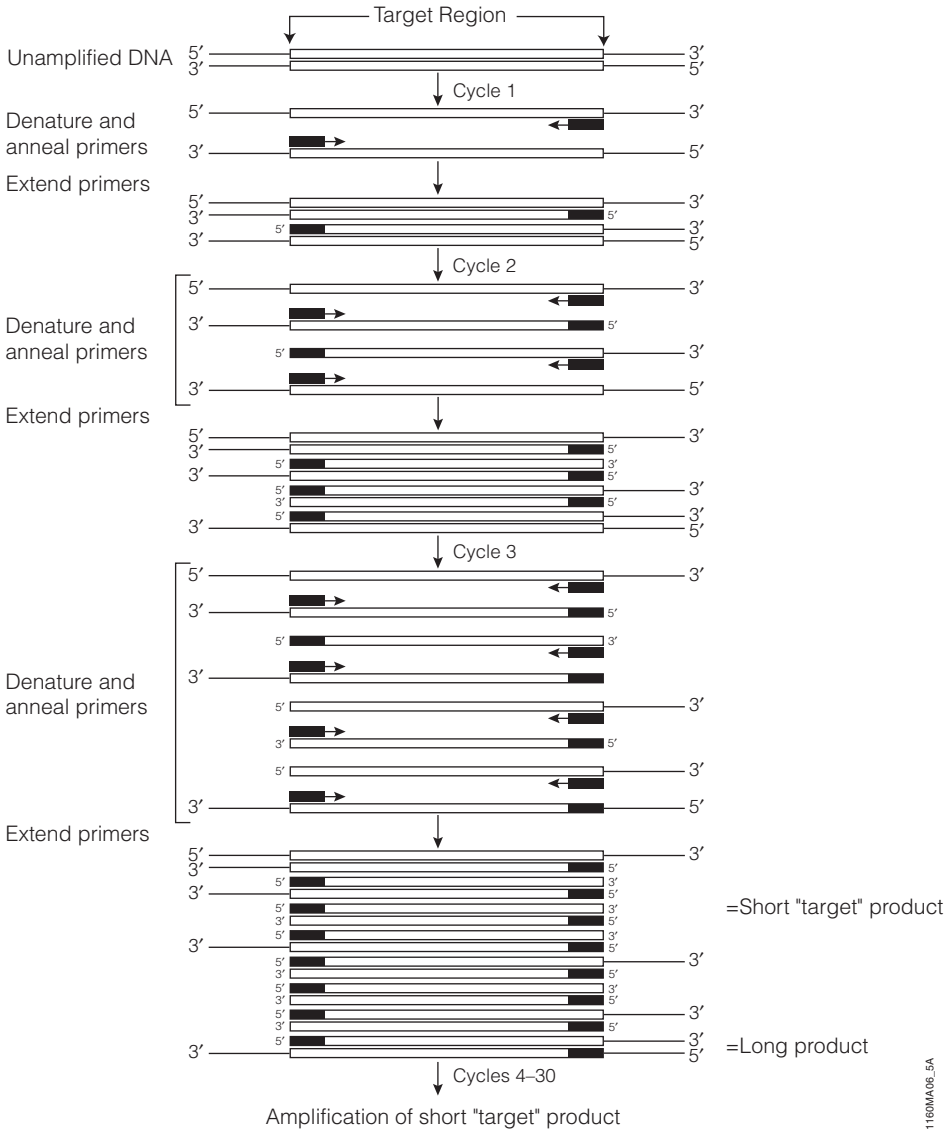

Figure 1.1. Schematic diagram of the PCR process.

The authors investigated the ability of $\alpha 9\beta 1$ integrin to act as a neurotrophin receptor and affect cell signaling pathways. As part of the study, RT-PCR was used to detect the presence of other neurotrophin receptors in their model cell line SW480. Reverse transcription was performed using the Reverse Transcription System and 1µg of total RNA isolated using the SV Total RNA Isolation System. The resulting cDNA (5µg) was amplified for 35 cycles (β-actin as a control) or 40 cycles (TrkA and p75NTR) using GoTaq® Green Master Mix. RT-PCR results were confirmed by Western blot analysis.

PubMed Number: 18230652

B. RT-PCR

Thermostable DNA polymerases used for basic PCR require a DNA template, and as such, the technique is limited to the analysis of DNA samples. Yet numerous instances exist in which amplification of RNA would be preferred. To apply PCR to the study of RNA, the RNA sample must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase (Figure 1.2). This process is called reverse transcription (RT), hence the name RT-PCR.

Avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (M-MLV or MuLV) reverse transcriptases are generally used to produce a DNA copy of the RNA template using either random primers, an oligo(dT) primer or sequence-specific primers. Promega offers GoScript™ Reverse Transcriptase (Cat.# A5003) and ImProm-II™ Reverse Transcriptase (Cat.# A3801). GoScript™ Reverse Transcriptase is qualified for use in qPCR and is compatible with GoTaq® qPCR and Plexor® qPCR Systems for performing RT-qPCR. Alternatively, some thermostable DNA polymerases (e.g., *Tth* DNA polymerase) possess a

reverse transcriptase activity, which can be activated by using manganese instead of magnesium as a cofactor (Myers and Gelfand, 1991). After this initial reverse transcription step to produce the cDNA template, basic PCR is carried out to amplify the target sequence.

The quality and purity of the RNA template is crucial to the success of RT-PCR. Total RNA or poly(A)+ RNA can be used as the starting template, but both must be intact and free of contaminating genomic DNA. Specific capture of poly(A)+ RNA will enrich a targeted message so that less of the reverse transcription reaction is needed for subsequent amplification. The efficiency of the first-strand synthesis reaction, which can be related to the RNA quality, also will significantly affect amplification results.

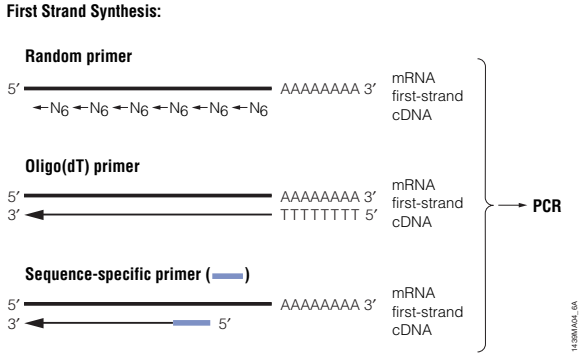

Figure 1.2. Schematic diagram of RT-PCR.

Additional Resources for RT-PCR

Technical Bulletins and Manuals

TB220	*Access RT-PCR System Technical Bulletin*
TM316	*GoScript™ Reverse Transcriptase Technical Manual*
TM236	*ImProm-II™ Reverse Transcription System Technical Manual*
TB099	*Reverse Transcription System Technical Bulletin*
9PIA170	*AccessQuick™ RT-PCR System Promega Product Information*

Promega Publications

AccessQuick™ RT-PCR System: Simple, stable and sensitive

Using ImProm-II™ Reverse Transcription System for coupled RT-PCR

Technically speaking: Promega RT-PCR systems explained

Using the Access RT-PCR System: Reaction parameters that affect efficient amplification

Citations

Nanashima, N. *et al.* (2008) The hairless phenotype of the Hirosaki hairless rat is due to the deletion of an 80-kb genomic DNA containing five basic keratin genes. *J. Biol. Chem.* **283**, 16868–75.

The mutation responsible for the hairless phenotype was linked to a 80kb deletion of chromosome 7q36. Because many basic keratin genes are located at 7q36, the authors examined keratin gene expression in the Hirosaki rat using RT-PCR. Expression of kb21, kb23 and kb26 was not detected, whereas other basic keratin genes were expressed. RT-PCR was performed using the AccessQuick™ RT-PCR System and 0.5µg of total RNA isolated from rat skin for 21–30 cycles.

PubMed Number: 18420582

Capozzo, A.V. *et al.* (2003) Development of DNA vaccines against hemolytic-uremic syndrome in a murine model. *Infect. Immun.* **71**, 3971–8.

Researchers used the pGEM®-T Vector System to clone the entire 1.4kb Shiga toxin type 2 gene (Stx2) from *E. coli* O157-H7 C600 (933W). The resultant construct, named pGEMTStx2, was used as a template in PCR to amplify each region of the gene corresponding to Shiga toxin type 2 subunits A and B. Each PCR product was digested with BamHI and EcoRI, then ligated into pCDNA 3.1+ to create pStx2ΔA and pStx2B. Mice then were immunized with either one or both of these constructs and another construct expressing murine granulocyte-macrophage colony-stimulating factor. Expression of each subunit in mouse tissue was verified by RT-PCR using specific primers and the AccessQuick™ RT-PCR System.

PubMed Number: 12819084

C. Hot-Start PCR

Hot-start PCR is a common technique to reduce nonspecific amplification due to assembly of amplification reactions at room temperature. At these lower temperatures, PCR primers can anneal to template sequences that are not perfectly complementary. Since thermostable DNA polymerases have activity at these low temperatures (although in most cases the activity is less than 25%) the polymerase can extend misannealed primers. This newly synthesized region then acts as a template for primer extension and synthesis of undesired amplification products. However, if the reaction is heated to temperatures >60°C before polymerization begins, the stringency of primer annealing is increased, and synthesis of undesired PCR products is avoided or reduced.

Hot-start PCR also can reduce the amount of primer-dimer synthesized by increasing the stringency of primer annealing. At lower temperatures, PCR primers can anneal to each other via regions of complementarity, and the DNA polymerase can extend the annealed primers to produce primer dimer, which often appears as a diffuse band of approximately 50–100bp on an ethidium bromide-stained gel. The formation of nonspecific products and primer-dimer can compete for reagent availability with amplification of the desired product. Thus, hot-start PCR can improve the yield of specific PCR products.

To perform manual hot-start PCR, reactions are assembled on ice or at room temperature, but one critical component is omitted until the reaction is heated to 60–65°C, at which point the missing reagent is added. This omission prevents the polymerase from extending primers until the critical

component is added at the higher temperature where primer annealing is more stringent. However, this method is tedious and increases the risk of contamination. A second, less labor-intensive approach involves the reversible inactivation or physical separation of one or more critical components in the reaction. For example, the magnesium or DNA polymerase can be sequestered in a wax bead, which melts as the reaction is heated to 94°C during the denaturation step, releasing the component only at higher temperatures (Carothers *et al*. 1989; Krishnan *et al*. 1991; Clark, 1988). The DNA polymerase also can be kept in an inactive state by binding to an oligonucleotide, also known as an aptamer (Lin and Jayasena, 1997; Dang and Jayasena, 1996) or an antibody (Scalice *et al*. 1994; Sharkey *et al*. 1994). This bond is disrupted at the higher temperatures, releasing the functional DNA polymerase. Finally, the DNA polymerase can be maintained in an inactive state through chemical modification (Moretti, T. *et al* 1998).

Additional Resources for Hot-Start PCR

Technical Bulletins and Manuals

9PIM500 *GoTaq® Hot Start Polymerase Promega Product Information*

9PIM512 *GoTaq® Hot Start Green Master Mix Promega Product Information*

9PIM513 *GoTaq® Hot Start Colorless Master Mix Promega Product Information*

Promega Publications

Get the convenience of hot-start PCR with the new GoTaq® Hot Start Polymerase

D. Long PCR

Amplification of long DNA fragments is desirable for numerous applications such as physical mapping applications (Rose, 1991) and direct cloning from genomes. While basic PCR works well when smaller fragments are amplified, amplification efficiency (and therefore the yield of amplified fragments) decreases significantly as the amplicon size increases over 5kb. This decrease in yield can be attributed to the accumulation of truncated products, which are not suitable substrates for additional cycles of amplification. These products appear as smeared, as opposed to discrete, bands on a gel.

In 1994, Wayne Barnes (Barnes, 1994) and other researchers (Cheng *et al*. 1994) examined factors affecting polymerization across larger regions of DNA by thermostable DNA polymerases and identified key variables affecting the yield of longer PCR fragments. They devised an approach using a mixture of two thermostable polymerases to synthesize longer PCR products. The first polymerase lacks a 3′→5′ exonuclease (proofreading) activity; the second enzyme, present at a reduced concentration, contains a potent proofreading activity. Presumably, when the nonproofreading DNA polymerase (e.g., *Taq* DNA polymerase) misincorporates a dNTP, subsequent extension of the newly synthesized DNA either proceeds very slowly or stops completely. The proofreading polymerase (e.g., *Pfu* DNA polymerase or *Tli* DNA polymerase) serves to remove the misincorporated nucleotide, allowing the DNA polymerases to continue extension of the new strand.

Although the use of two thermostable DNA polymerases can significantly increase yield, other conditions can have a significant impact on the yield of longer PCR products (Cheng *et al*. 1995). Logically, longer extension times can increase the yield of longer PCR products because fewer partial products are synthesized. Extension times depend on the length of the target; times of 10–20 minutes are common. In addition, template quality is crucial. Depurination of the template, which is promoted at elevated temperatures and lower pH, will result in more partial products and decreased overall yield. In long PCR, denaturation time is reduced to 2–10 seconds to decrease depurination of the template. Additives, such as glycerol and dimethyl sulfoxide (DMSO), also help lower the strand-separation and primer-annealing temperatures, alleviating some of the depurination effects of high temperatures. Cheng *et al*. also found that reducing potassium concentrations by 10–40% increased the amplification efficiency of longer products (Cheng *et al*. 1995).

E. Quantitative Endpoint PCR

PCR and RT-PCR are generally used in a qualitative format to evaluate biological samples. However, a wide variety of applications, such as determining viral load, measuring responses to therapeutic agents and characterizing gene expression, would be improved by quantitative determination of target abundance. Theoretically, this should be easy to achieve, given the exponential nature of PCR, because a linear relationship exists between the number of amplification cycles and the logarithm of the number of molecules. In practice, however, amplification efficiency is decreased because of contaminants (inhibitors), competitive reactions, substrate exhaustion, polymerase inactivation and target reannealing. As the number of cycles increases, the amplification efficiency decreases, eventually resulting in a plateau effect.

Normally, quantitative PCR requires that measurements be taken before the plateau phase so that the relationship between the number of cycles and molecules is relatively linear. This point must be determined empirically for different reactions because of the numerous factors that can affect amplification efficiency. Because the measurement is taken prior to the reaction plateau, quantitative PCR uses fewer amplification cycles than basic PCR. This can cause problems in detecting the final product because there is less product to detect.

To monitor amplification efficiency, many applications are designed to include an internal standard in the PCR. One such approach includes a second primer pair that is specific for a "housekeeping" gene (i.e., a gene that has constant expression levels among the samples compared) in the reaction (Gaudette and Crain, 1991; Murphy *et al*. 1990). Amplification of housekeeping genes verifies that the target

nucleic acid and reaction components were of acceptable quality but does not account for differences in amplification efficiencies due to differences in product size or primer annealing efficiency between the internal standard and target being quantified.

The concept of competitive PCR—a variation of quantitative PCR—is a response to this limitation. In competitive PCR, a known amount of a control template is added to the reaction. This template is amplified using the same primer pair as the experimental target molecule but yields a distinguishable product (e.g., different size, restriction digest pattern, etc.). The amounts of control and test product are compared after amplification. While these approaches control for the quality of the target nucleic acid, buffer components and primer annealing efficiencies, they have their own limitations (Siebert and Larrick, 1993; McCulloch *et al*. 1995), including the fact that many depend on final analysis by electrophoresis.

Numerous fluorescent and solid-phase assays exist to measure the amount of amplification product generated in each reaction, but they often fail to discriminate amplified DNA of interest from nonspecific amplification products. Some of these analyses rely on blotting techniques, which introduce another variable due to nucleic acid transfer efficiencies, while other assays were developed to eliminate the need for gel electrophoresis yet provide the requisite specificity. Real-time PCR, which provides the ability to view the results of each amplification cycle, is a popular way of overcoming the need for analysis by electrophoresis.

F. Quantitative Real-Time PCR

The use of fluorescently labeled oligonucleotide probes or primers or fluorescent DNA-binding dyes to detect and quantitate a PCR product allows quantitative PCR to be performed in real time. Specially designed instruments perform both thermal cycling to amplify the target and fluorescence detection to monitor PCR product accumulation. DNA-binding dyes are easy to use but do not differentiate between specific and nonspecific PCR products and are not conducive to multiplex reactions. Fluorescently labeled nucleic acid probes have the advantage that they react with only specific PCR products, but they can be expensive and difficult to design. Some qPCR technologies employ fluorescently labeled PCR primers instead of probes. One example, which will be discussed in more detail below, is the Plexor® technology, which requires only a single fluorescently labeled primer, is compatible with multiplex PCR and allows specific and nonspecific amplification products to be differentiated (Sherrill *et al*. 2004; Frackman *et al*. 2006).

The use of fluorescent DNA-binding dyes is one of the easiest qPCR approaches. The dye is simply added to the reaction, and fluorescence is measured at each PCR cycle. Because fluorescence of these dyes increases dramatically in the presence of double-stranded DNA, DNA synthesis can be monitored as an increase in fluorescent signal. However, preliminary work often must be done to ensure that the PCR conditions yield only specific product. In subsequent reactions, specific amplification can verified by a melt curve analysis. Thermal melt curves are generated by allowing all product to form double-stranded DNA at a lower temperature (approximately 60°C) and slowly ramping the temperature to denaturing levels (approximately 95°C). The product length and sequence affect melting temperature (T_m), so the melt curve is used to characterize amplicon homogeneity. Nonspecific amplification can be identified by broad peaks in the melt curve or peaks with unexpected T_m values. By distinguishing specific and nonspecific amplification products, the melt curve adds a quality control aspect during routine use. The generation of melt curves is not possible with assays that rely on the 5′→3′ exonuclease activity of *Taq* DNA polymerase, such as the probe-based TaqMan® technology.

The GoTaq® qPCR Master Mix (Cat.# A6001) is a qPCR reagent system that contains a proprietary fluorescent DNA-binding dye that often exhibits greater fluorescence enhancement upon binding to double-stranded DNA and less PCR inhibition than the commonly used SYBR® Green I dye. The dye in the GoTaq® qPCR Master Mix enables efficient amplification, resulting in earlier quantification cycle (C_q, formerly known as cycle threshold [C_t]) values and an expanded linear range using the same filters and settings as SYBR® Green I. The GoTaq® qPCR Master Mix is provided as a simple-to-use, stabilized 2X formulation that includes all components for qPCR except sample DNA, primers and water. For more information, view the GoTaq® qPCR Master Mix video.

Real-time PCR using labeled oligonucleotide primers or probes employs two different fluorescent reporters and relies on energy transfer from one reporter (the energy donor) to a second reporter (the energy acceptor) when the reporters are in close proximity. The second reporter can be a quencher or a fluor. If the second reporter is a quencher, the energy from the first reporter is absorbed but re-emitted as heat rather than light, leading to a decrease in fluorescent signal. Alternatively, if the second reporter is a fluor, the energy can be absorbed and re-emitted at another wavelength through fluorescent resonance energy transfer (FRET, reviewed in Didenko, 2001), and the progress of the reaction can be monitored by the decrease in fluorescence of the energy donor or the increase in fluorescence of the energy acceptor. During the exponential phase of PCR, the change in fluorescence is proportional to accumulation of PCR product.

Examples of a primer-based approach are the Plexor® qPCR and qRT-PCR Systems, which require two PCR primers, only one of which is fluorescently labeled. These systems take advantage of the specific interaction between two modified nucleotides (Sherrill *et al*. 2004; Johnson *et al*. 2004; Moser and Prudent, 2003). The two novel bases, isoguanine (iso-dG) and 5′-methylisocytosine (iso-dC), form a unique base pair in double-stranded DNA (Johnson *et al*. 2004). To perform fluorescent quantitative PCR using this new

technology, one primer is synthesized with an iso-dC residue as the 5′-terminal nucleotide and a fluorescent label at the 5′-end; the second primer is unlabeled. During PCR, this labeled primer is annealed and extended, becoming part of the template used during subsequent rounds of amplification. The complementary iso-dGTP, which is available in the nucleotide mix as dabcyl-iso-dGTP, pairs specifically with iso-dC. When the dabcyl-iso-dGTP is incorporated, the close proximity of the dabcyl quencher and the fluorescent label on the opposite strand effectively quenches the fluorescent signal. This process is illustrated in Figure 1.3. The initial fluorescence level of the labeled primers is high in Plexor® System reactions. As amplification product accumulates, signal decreases.

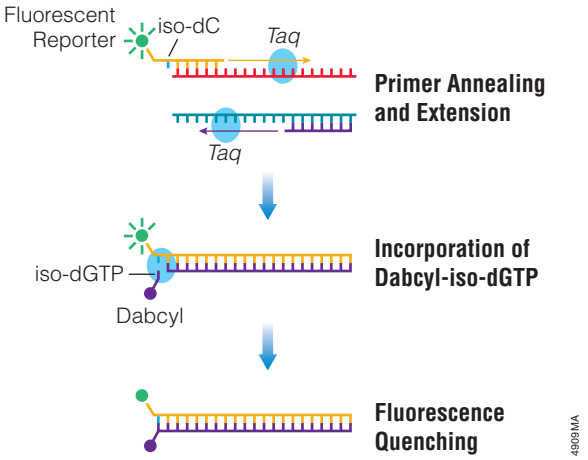

Figure 1.3. Quenching of the fluorescent signal by dabcyl during product accumulation.

Quenching of the fluorescent label by dabcyl is a reversible process. Fluorescence is quenched when the product is double-stranded. Denaturing the product separates the label and quencher, resulting in an increased fluorescent signal. Consequently, thermal melt curves can be used to characterize amplicon homogeneity.

A benefit of the Plexor® technology over detection using simple DNA-binding dyes is the capacity for multiplexing. The labeled primer can be tagged with one of many common fluorescent labels, allowing two- to four-color multiplexing, depending on the instrument used. The simplicity of primer design for the Plexor® technology is a distinct advantage over probe-based quantitative PCR approaches. Also, the Plexor® technology does not rely on enzymatic cleavage to generate signal and does not have the complex hybridization kinetics that can be typical of other approaches to real-time PCR. The Plexor® technology also can be used for quantitative RT-PCR by incorporating a reverse transcription step.

Some qPCR strategies employ complementary nucleic acid probes to quantify the DNA target. These probes also can be used to detect single nucleotide polymorphisms (Lee *et al*. 1993; Bernard *et al*. 1998). There are several general categories of real-time PCR probes, including hydrolysis, hairpin and simple hybridization probes. These probes contain a complementary sequence that allows the probe to anneal to the accumulating PCR product, but probes can differ in the number and location of the fluorescent reporters.

Hydrolysis probes are labeled with a fluor at the 5′-end and a quencher at the 3′-end, and because the two reporters are in close proximity, the fluorescent signal is quenched. During the annealing step, the probe hybridizes to the PCR product generated in previous amplification cycles. The resulting probe:target hybrid is a substrate for the 5′→3′ exonuclease activity of the DNA polymerase, which degrades the annealed probe and liberates the fluor (Holland *et al*. 1991). The fluor is freed from the effects of the energy-absorbing quencher, and the progress of the reaction and accumulation of PCR product is monitored by the resulting increase in fluorescence. With this approach, preliminary experiments must be performed prior to the quantitation experiments to show that the signal generated is proportional to the amount of the desired PCR product and that nonspecific amplification does not occur.

Hairpin probes, also known as molecular beacons, contain inverted repeats separated by a sequence complementary to the target DNA. The repeats anneal to form a hairpin structure, where the fluor at the 5′-end and a quencher at the 3′-end are in close proximity, resulting in little fluorescent signal. The hairpin probe is designed so that the probe binds preferentially to the target DNA rather than retains the hairpin structure. As the reaction progresses, increasing amounts of the probe anneal to the accumulating PCR product, and as a result, the fluor and quencher become physically separated. The fluor is no longer quenched, and the level of fluorescence increases. One advantage of this technique is that hairpin probes are less likely to mismatch than hydrolysis probes (Tyagi *et al*. 1998). However, preliminary experiments must be performed to show that the signal is specific for the desired PCR product and that nonspecific amplification does not occur.

The use of simple hybridization probes involves two labeled probes or, alternatively, one labeled probe and a labeled PCR primer. In the first approach, the energy emitted by the fluor on one probe is absorbed by a fluor on the second probe, which hybridizes nearby. In the second approach, the emitted energy is absorbed by a second fluor that is incorporated into the PCR product as part of the primer. Both of these approaches result in increased fluorescence of the energy acceptor and decreased fluorescence of the energy donor. The use of hybridization probes can be simplified even further so that only one labeled probe is required. In this approach, quenching of the fluor by deoxyguanosine is used to bring about a change in fluorescence (Crockett and Wittwer, 2001; Kurata *et al*. 2001). The labeled probe anneals so that the fluor is in close proximity to G residues within the target sequence, and as probe annealing increases, fluorescence decreases due to deoxyguanosine quenching. With this approach, the

location of probe is limited because the probe must hybridize so that the fluorescent dye is very near a G residue. The advantage of simple hybridization probes is their ability to be multiplexed more easily than hydrolysis and hairpin probes through the use of differently colored fluors and probes with different melting temperatures (reviewed in Wittwer *et al*. 2001).

Additional Resources for Real-Time PCR

Technical Bulletins and Manuals

TM318	*GoTaq® qPCR Master Mix Technical Manual*
TM262	*Plexor® qPCR System Technical Manual*
TM263	*Plexor® One-Step qRT-PCR System Technical Manual*
TM264	*Plexor® Two-Step qRT-PCR System Technical Manual*

Promega Publications

Introducing GoTaq® qPCR Master Mix: The bright choice for dye-based qPCR

The Plexor™ Systems provide accurate quantitation in multiplex qPCR and qRT-PCR

Plexor™ technology: A new chemistry for real-time PCR

G. Rapid Amplified Polymorphic DNA Analysis

Genetic analysis of organisms at the molecular level is an important and widely practiced scientific tool. Several techniques developed over more than a decade offer the opportunity to identify each individual or type of individual in a species unambiguously.

One important PCR-based genetic analysis is random amplified polymorphic DNA analysis (RAPD; reviewed in McClelland and Welsh, 1994; Power, 1996; Black, 1993). RAPD uses small, nonspecific primers to amplify seemingly random regions of genomic DNA. Successful primer pairs produce different banding profiles of PCR products between individuals, strains, cultivars or species when analyzed by gel electrophoresis.

Slight modifications to the basic PCR method are made for RAPD. First, the primers are approximately 10 bases in length compared to the 17- to 23-base primer length of normal PCR. Because primers are shorter, the annealing temperature is reduced to less than 40°C.

As with most PCR techniques, RAPD requires very little material for analysis and is relatively insensitive to template integrity. No blotting techniques are required, thus eliminating the use of ^{32}P, bypassing probe generation and decreasing the amount of time required to obtain results.

H. Rapid Amplification of cDNA Ends (RACE)

Rapid amplification of cDNA ends (RACE) is a variation of RT-PCR that amplifies unknown cDNA sequences corresponding to the 3′- or 5′-end of the RNA. Numerous variations of the original protocols have been published (Troutt *et al*. 1992; Edwards *et al*. 1991; Edwards *et al*. 1993; Liu and Gorovsky, 1993; Fromont-Racine *et al*. 1993; reviewed in Schaefer, 1995) but will not be discussed in detail here.

Two general RACE strategies exist: one amplifies 5′ cDNA ends (5′ RACE) and the other captures 3′ cDNA end sequences (3′ RACE). In either strategy, the first step involves the conversion of RNA to single-stranded cDNA using a reverse transcriptase. For subsequent amplification, two PCR primers are designed to flank the unknown sequence. One PCR primer is complementary to known sequences within the gene, and a second primer is complementary to an "anchor" site (anchor primer). The anchor site may be present naturally, such as the poly(A) tail of most mRNAs, or can be added in vitro after completion of the reverse transcription step. The anchor primer also can carry adaptor sequences, such as restriction enzyme recognition sites, to facilitate cloning of the amplified product. Amplification using these two PCR primers results in a product that spans the unknown 5′ or 3′ cDNA sequence, and sequencing this product will reveal the unknown sequence. The information obtained from partial cDNA sequences then can be used to assemble the full-length cDNA sequence (Frohman *et al*. 1988; Loh *et al*. 1989; Ohara *et al*. 1989).

In 5′ RACE (Figure 1.4), the first-strand cDNA synthesis reaction is primed using an oligonucleotide complementary to a known sequence in the gene. After removing the RNA template, an anchor site at the 3′-end of the single-stranded cDNA is created using terminal deoxynucleotidyl transferase, which adds a nucleotide tail. A typical amplification reaction follows using an anchor primer complementary to the newly added tail and another primer complementary to a known sequence within the gene.

The 3′-RACE procedure uses a modified oligo(dT) primer/adaptor as the reverse transcription primer. This oligo(dT) primer/adaptor is comprised of an oligo(dT) sequence, which anneals to the poly(A)+ tail of the mRNA, and an adaptor sequence at the 5′ end. A single G, C or A residue at the 3′ end ensures that cDNA synthesis is initiated only when the primer/adaptor anneals immediately adjacent to the junction between the poly(A)+ tail and 3′ end of the mRNA. This oligo(dT) primer/adaptor is used as the anchor primer in the subsequent amplifications along with a primer complementary to known sequences within the gene. See Figure 1.5.

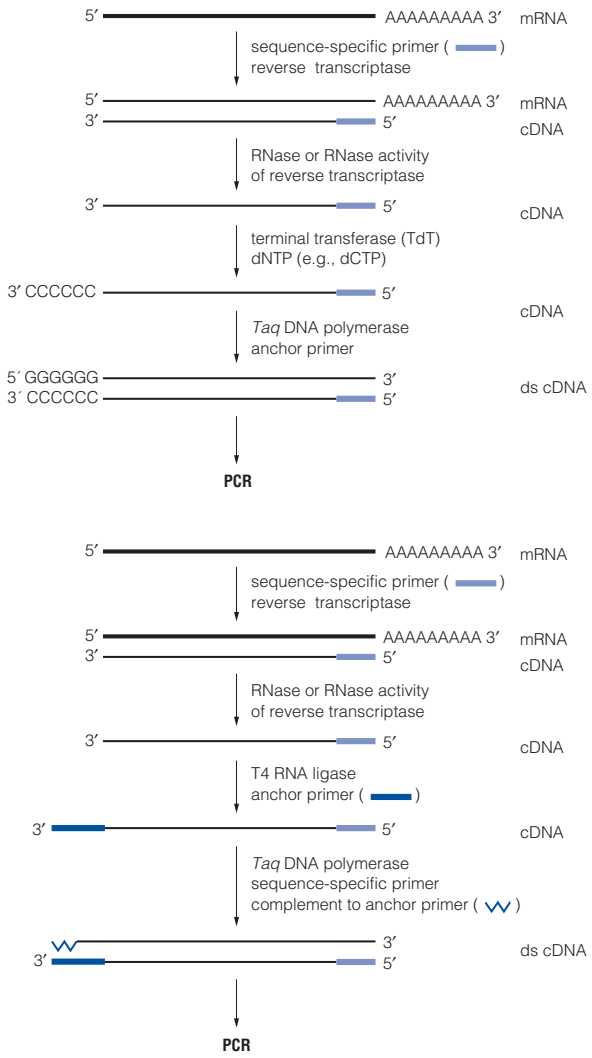

Figure 1.4. Schematic diagram of two 5′ RACE methods.

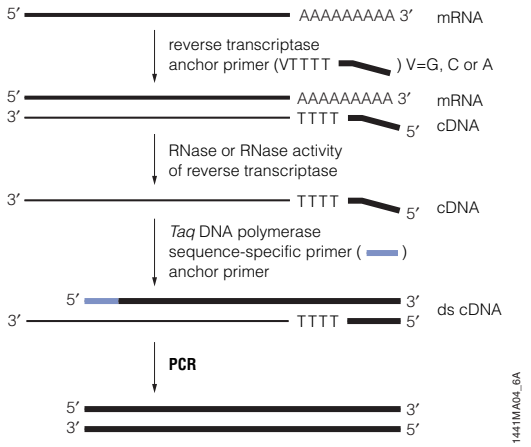

Figure 1.5. Schematic diagram of a typical 3′-RACE protocol.

I. Differential Display PCR

Differential display PCR is another variation of RT-PCR and is used to identify differences in mRNA expression patterns between two cell lines or populations. In one example of this procedure, cDNA synthesis is primed using a set of modified oligo(dT) primers, which anneal to the poly(A)+ tail of mRNA (Liang and Pardee, 1992). Each of the oligo(dT) primers carries an additional two nucleotides at the 3′-end. This ensures that extension only occurs if the primer anneals immediately adjacent to the junction between the poly(A)+ tail and 3′ end of the mRNA. Because the two additional nucleotides will only anneal to a subset of the mRNA molecules, this also reduces the complexity of the RNA population that is reverse transcribed. The RNA is first reverse transcribed with one of the modified oligo(dT) primers to synthesize first-strand cDNA, which is then amplified by PCR using two random 10mer primers. After amplification, the reaction products are visualized by gel electrophoresis, and banding patterns for the two cell populations are compared to identify differentially expressed cDNAs.

Another form of analyzing differences between complex genomes is representational difference analysis (RDA). This method combines "subtractive" library techniques (Lisitsyn *et al*. 1993) with PCR amplification to find differences in complex genomes. A variation of this is cDNA RDA, where total RNA from the cell populations is first converted into cDNA, subtractive techniques are performed and the products are amplified by PCR (Hubank and Schatz, 1994). By using cDNA, the complexity is significantly reduced, providing another method to analyze differences in expression between cell types or in response to various treatments.

J. In situ PCR

In situ PCR, first described in 1990, combines the sensitivity of PCR or RT-PCR amplification with the cellular or histological localization associated with in situ hybridization techniques (Haase *et al*. 1990). These features make in situ PCR a powerful tool to detect proviral DNA, oncogenesis and localization of rare messages.

The technique is amenable to analysis of fixed cells or tissue cross-sections. Detection of amplified products can be accomplished indirectly by subsequent hybridization using either radiolabeled, fluorescently labeled or biotin-labeled nucleic acid probes. PCR products also can be detected directly by incorporating a labeled nucleotide, although this method is subject to higher background levels.

The use of in situ PCR requires altering some of the reaction parameters typical of basic PCR (Nuovo *et al*. 1993; Thaker, 1999). For example, increased Mg^{2+} concentrations (approximately 4.5mM versus the normal 1.5–2.5mM) are used for in situ PCR. An increased amount of DNA polymerase is also required unless BSA is added to the reaction, presumably because the polymerase binds to the glass plate and coverslip.

Tissue preparation also plays a significant role in the success of in situ PCR. A strong relationship exists between the time of fixation and protease digestion and the intensity of PCR signal. Tissue preparation also affects the level of side reactions, resulting in primer-independent signals, which are not normally present in basic PCR. These primer-independent signals often arise from *Taq* DNA polymerase-mediated repair of single-stranded gaps in the genomic DNA.

As the use of the technique has spread, the process has been further optimized. Numerous publications (reviewed in Nuovo, 1995; Staskus *et al*. 1995) describe process improvements that increase sensitivity and decrease nonspecific amplification products.

K. High-Fidelity PCR

For some applications, such as gene expression, mutagenesis or cloning, the number of mutations introduced during PCR needs to be minimized. For these applications, we recommend using a proofreading polymerase. Proofreading DNA polymerases, such as *Pfu* and *Tli* DNA polymerases, have a 3′→5′ exonuclease activity, which can remove any misincorporated nucleotides so that the error rate is relatively low. The accuracy of *Pfu* DNA polymerase is approximately twofold higher than that of *Tli* DNA polymerase and sixfold higher than that of *Taq* DNA polymerase (Cline *et al*., 1996).

The most commonly used DNA polymerase for PCR is *Taq* DNA polymerase, which has an error rate of approximately 1×10^{-5} errors per base. This error rate is relatively high due to the enzyme's lack of 3′→5′ exonuclease (proofreading) activity. The error rate of *Tfl* DNA polymerase, another nonproofreading polymerase, is similar to that of *Taq* DNA polymerase.

Reaction conditions can affect DNA polymerase fidelity, and DNA polymerases may be affected in different ways or to different degrees. In general, excess magnesium or the presence of manganese will reduce the fidelity of DNA polymerases (Eckert and Kunkel, 1990). Unequal nucleotide concentrations also can affect fidelity; nucleotides that are present at higher concentrations will be misincorporated at a higher frequency (Eckert and Kunkel, 1990). Reaction pH also can have a big effect on fidelity (Eckert and Kunkel, 1990; Eckert and Kunkel, 1991). For example, the fidelity of *Taq* DNA polymerase increases as pH decreases, with the lowest error rate occurring in the range of pH 5–6 (Eckert and Kunkel, 1990), but the opposite is true for *Pfu* DNA polymerase. *Pfu* DNA polymerase has higher fidelity at higher pH (Cline *et al*, 1996). Finally, exposing the DNA template to high temperatures (i.e., 94°C) for extended periods of time can lead to DNA damage, specifically the release of bases from the phosphodiester backbone. The resulting abasic sites can cause some DNA polymerases to stall but also can result in a higher rate of mutations, most frequently transversions, as the DNA polymerase adds a random nucleotide at an abasic site (Eckert and Kunkel, 1991).

Additional Resources for High-Fidelity PCR

Promega Publications

Pfu DNA Polymerase: A high fidelity enzyme for nucleic acid amplification

L. PCR and DNA Sequencing: Cycle Sequencing

The PCR process also has been applied to DNA sequencing in a technique called cycle sequencing (Murray, 1989; Saluz and Jost, 1989; Carothers *et al*. 1989; Krishnan *et al*. 1991). Cycle sequencing reactions differ from typical PCR amplification reactions in that they use only a single primer, resulting in a linear (as opposed to theoretically exponential) amplification of the target molecule. Other reaction components are comparable, and either radioactive or fluorescent labels are incorporated for detection.

M. Cloning PCR Products

Amplification with a DNA polymerase lacking 3′→5′ (proofreading) exonuclease activity (e.g., *Taq* DNA polymerase) yields products that contain a single 3′-terminal nucleotide overhang, typically an A residue (Clark, 1988; Hu, 1993). These PCR products can be conveniently cloned into T-vectors, which contain a single T overhang (reviewed in Mezei and Storts, 1994; Guo and Bi, 2002).

DNA polymerases that possess proofreading activity (e.g., *Tli* DNA polymerase or *Pfu* DNA polymerase) generate blunt-ended PCR products. These products are compatible with standard blunt-end cloning strategies. Conversely, blunt-end PCR products can be tailed with *Taq* DNA polymerase and dATP prior to cloning into a T-vector (Zhou *et al*. 1995).

Additional Resources for Cloning PCR Products

Technical Bulletins and Manuals

TM042	*pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual*
TM044	*pTARGET™ Mammalian Expression Vector System Technical Manual*

Promega Publications

Technically speaking: T-vector cloning

Rapid ligation for the pGEM®-T and pGEM®-T Easy Vector Systems

Cloning blunt-end *Pfu* DNA polymerase-generated PCR fragments into pGEM®-T Vector Systems

Technically speaking: Optimized cloning with T vectors

Digestion of PCR and RT-PCR products with restriction endonucleases without prior purification or precipitation

Vector Maps

pGEM®-T Vector

pGEM®-T Easy Vector

pTARGET™ Mammalian Expression Vector

Citations

Kurth, E.G. *et al.* (2008) Involvement of BmoR and BmoG in n-alkane metabolism in *Pseudomonas butanovora*. *Microbiology* **154**, 139–47.

The authors characterized five open-reading frames flanking the alcohol-inducible alkane monooxygenase (BMO) structural gene of *Pseudomonas butanovora*. Strains with mutated bmoR, encoding a putative transcriptional regulator, or bmoG, encoding a putative chaperonin, were created by gene inactivation. The bmoR gene was amplified and cloned into the pGEM®-T Vector for disruption with a kanamycin cassette. The two termini of the bmoG gene were amplified separately, ligated to the kanamycin cassette and cloned into the pGEM®-T Easy Vector. Plasmids encoding the disrupted genes were transformed into *Pseudomonas butanovora* by electroporation.

PubMed Number: 18174133

Bröker, D. *et al.* (2008) The genomes of the non-clearing-zone-forming and natural-rubber-degrading species *Gordonia polyisoprenivorans* and *Gordonia westfalica* harbor genes expressing Lcp activity in *Streptomyces* strains. *Appl. Environ. Microbiol.* **74**, 2288–97.

Natural rubber-degrading bacteria fall into two categories: those forming clearing zones on latex overlay plates and those that do not. To investigate this degradation process, the authors amplified latex-clearing protein (*lcp*) homologs from non-clearing-zone-forming bacteria using degenerate PCR primers based on *lcp* sequences from clearing-zone forming species. The 3′ region of the *lcp* gene in *G. westfalica* was amplified by nested PCR using biotinylated primers, and the amplified products were cloned in the pGEM®-T Easy Vector and sequenced using universal M13 forward and reverse primers.

PubMed Number: 18296529

II. General Considerations for PCR Optimization

This discussion focuses on the use of *Taq* DNA polymerase in PCR, since this is the enzyme most commonly used in PCR. Many of these suggestions also apply when using other DNA polymerases.

A. Magnesium Concentration

Magnesium is a required cofactor for thermostable DNA polymerases, and magnesium concentration is a crucial factor that can affect amplification success. Template DNA concentration, chelating agents present in the sample (e.g., EDTA or citrate), dNTP concentration and the presence of proteins all can affect the amount of free magnesium in the reaction. In the absence of adequate free magnesium, *Taq* DNA polymerase is inactive (Figure 1.6). Excess free magnesium reduces enzyme fidelity (Eckert and Kunkel, 1990) and may increase the level of nonspecific amplification (Williams, 1989; Ellsworth *et al*. 1993). For these reasons, researchers should empirically determine the optimal magnesium concentration for each target. To do so, set up a series of reactions containing 1.0–4.0mM Mg^{2+} in 0.5–1mM increments and visualize the results to determine which magnesium concentration produced the highest yield of product and the minimal amount of nonspecific product. The effect of magnesium concentration and the optimal concentration range can vary with the particular DNA polymerase. For example, the performance of *Pfu* DNA polymerase seems depend less on magnesium concentration, but when optimization is required, the optimal concentration is usually in the range of 2–6mM.

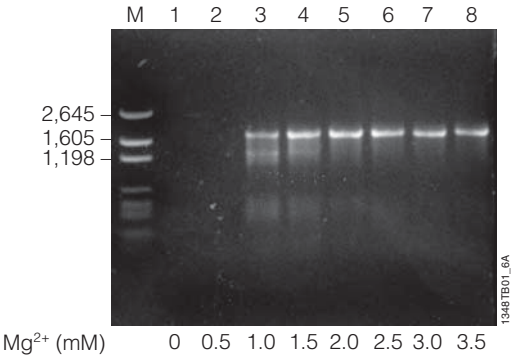

Figure 1.6. Effects of magnesium concentration on amplification. Amplifications were performed using various Mg^{2+} concentrations to demonstrate the effect on the amplification of a 1.8kb target luciferase gene. The reaction products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. Lane M, Promega pGEM® DNA Markers (Cat.# G1741); lane 1, 0mM Mg^{2+}; lane 2, 0.5mM Mg^{2+}; lane 3, 1mM Mg^{2+}; lane 4, 1.5mM Mg^{2+}; lane 5, 2mM Mg^{2+}; lane 6, 2.5mM Mg^{2+}; lane 7, 3mM Mg^{2+} and lane 8, 3.5mM Mg^{2+}.

Many DNA polymerases are supplied with a magnesium-free reaction buffer and a tube of 25mM $MgCl_2$ so that you can adjust the Mg^{2+} concentration to the optimal level for each reaction. Before assembling the reactions, be sure to thaw the magnesium solution completely prior to use and vortex the magnesium solution for several seconds before pipetting. Magnesium chloride solutions can form concentration gradients as a result of multiple freeze-thaw cycles, and vortex mixing is required to obtain a uniform solution. These two steps, though seemingly simple, eliminate the cause of many failed experiments.

Some scientists prefer to use reaction buffers that already contain $MgCl_2$ at a final concentration of 1.5mM. It should be noted, however, that Hu *et al*. reported performance variability of reaction buffer solutions containing magnesium (Hu *et al*. 1992). The free magnesium changes of 0.6mM observed in their experiments dramatically affected amplification yields in an allele-specific manner. The authors found that heating the buffer at 90°C for 10 minutes restored the homogeneity of the solution. They postulated that magnesium chloride precipitates as a result of multiple freeze-thaw cycles.

B. Buffer Considerations

Most reaction buffers consist of a buffering agent, most often a Tris-based buffer, and salt, commonly KCl. The buffer regulates the pH of the reaction, which affects DNA polymerase activity and fidelity. Modest concentrations of KCl will increase DNA polymerase activity by 50–60% over activities in the absence of KCl; 50mM KCl is considered optimal (Gelfand, 1989).

GoTaq® DNA Polymerase contains native *Taq* DNA polymerase in a proprietary formulation. It is supplied with 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer. The 5X Green GoTaq® Reaction Buffer contains two dyes (blue and yellow) that separate during electrophoresis to monitor migration progress. The buffer also contains a compound that increases the density of the sample so that it will sink into the well of the agarose gel, allowing reactions to be directly loaded onto an agarose gel without the need for loading dye. The blue dye comigrates at the same rate as a 3–5kb DNA fragment in a 1% agarose gel. The yellow dye migrates at a rate faster than primers (<50bp) in a 1% agarose gel. The 5X Colorless GoTaq® Reaction Buffer and 5X Green GoTaq® Reaction Buffer have the same formulation, except for the dyes. The 5X Colorless GoTaq® Reaction Buffer is recommended for any applications where absorbance or fluorescence measurements of the PCR amplimer will be taken without prior cleanup. Both buffers are supplied at pH 8.5 and contain $MgCl_2$ at a concentration of 7.5mM for a final concentration of 1.5mM.

GoTaq® Flexi DNA Polymerase is supplied with 5X Green GoTaq® Flexi Reaction Buffer and 5X Colorless GoTaq® Flexi Reaction Buffer. The compositions are identical to the 5X Green GoTaq® Reaction Buffer and 5X Colorless GoTaq® Reaction Buffer, except that the GoTaq® Flexi reaction buffers do not contain $MgCl_2$. Instead, the GoTaq® Flexi DNA Polymerase is supplied with a tube of 25mM $MgCl_2$ so that reactions can be supplemented with varying concentrations of magnesium.

C. Enzyme Concentration

We recommend using 1–1.25 units of *Taq* DNA polymerase in a 50µl amplification reaction. In most cases, this is an excess of enzyme, and adding more enzyme will not significantly increase product yield. In fact, increased amounts of enzyme increase the likelihood of generating artifacts associated with the intrinsic 5′→3′ exonuclease activity of *Taq* DNA polymerase, resulting in smeared bands in an agarose gel (Longley *et al*. 1990; Bell and DeMarini, 1991).

Pipetting errors are a frequent cause of excessive enzyme levels. Accurate dispensing of small volumes of enzyme solutions in 50% glycerol is difficult, so we strongly recommend preparing a reaction master mix, which requires a larger volume of each reagent, to reduce pipetting errors.

D. PCR Primer Design

PCR primers define the target region to be amplified and generally range in length from 15–30 bases. Ideally primers will have a GC-content of 40–60%. Avoid three G or C residues in a row near the 3′-end of the primer to minimize nonspecific primer annealing. Also, avoid primers with intra- or intermolecular complementary sequences to minimize the production of primer-dimer. Intramolecular regions of secondary structure can interfere with primer annealing to the template and should be avoided.

Ideally, the melting temperature (T_m), the temperature at which 50% of the primer molecules are annealed to the complementary sequence, of the two primers will be within 5°C so that the primers anneal efficiently at the same temperature. Primers can be designed to include sequences that are useful for downstream applications. For example, restriction enzyme sites can be placed at the 5′-ends of PCR primers to facilitate subsequent cloning of the PCR product, or a T7 RNA polymerase promoter can be added to allow in vitro transcription without the need to subclone the PCR product into a vector.

E. Template Quality

Successful amplification depends on DNA template quantity and quality. Reagents commonly used to purify nucleic acids (salts, guanidine, proteases, organic solvents and SDS) are potent inactivators of DNA polymerases. For example, 0.01% SDS will inhibit *Taq* DNA polymerase by 90%, while 0.1% SDS will inhibit *Taq* DNA polymerase by 99.9% (Konat *et al*. 1994). A few other examples of PCR inhibitors are phenol (Katcher and Schwartz, 1994), heparin (Beutler *et al*. 1990; Holodniy *et al*. 1991), xylene cyanol, bromophenol blue (Hoppe *et al*. 1992), plant polysaccharides (Demeke and Adams, 1992), and the polyamines spermine and spermidine (Ahokas and Erkkila, 1993). In some cases, the inhibitor is not introduced into the reaction with the nucleic acid template. A good example of this is an inhibitory substance that can be released from polystyrene or polypropylene upon exposure to ultraviolet light (Pao *et al*. 1993; Linquist *et al*. 1998).

If an amplification reaction fails and you suspect the DNA template is contaminated with an inhibitor, add the suspect DNA preparation to a control reaction with a DNA template and primer pair that has amplified well in the past . Failure to amplify the control DNA usually indicates the presence

of an inhibitor. Additional steps to clean up the DNA preparation, such as phenol:chloroform extraction or ethanol precipitation, may be necessary.

F. Template Quantity

The amount of template required for successful amplification depends upon the complexity of the DNA sample. For example, of a 4kb plasmid containing a 1kb target sequence, 25% of the input DNA is the target of interest. Conversely, a 1kb target sequence in the human genome (3.3×10^9bp) represents approximately 0.00003% of the input DNA. Thus, approximately 1,000,000-fold more human genomic DNA is required to maintain the same number of target copies per reaction. Common mistakes include using too much plasmid DNA, too much PCR product or too little genomic DNA as the template. Reactions with too little DNA template will have low yields, while reactions with too much DNA template can be plagued by nonspecific amplification. If possible, start with $>10^4$ copies of the target sequence to obtain a signal in 25–30 cycles, but try to keep the final DNA concentration of the reaction ≤10ng/µl. When reamplifying a PCR product, the concentration of the specific PCR product is often not known. We recommend diluting the previous amplification reaction 1:10 to 1:10,000 before reamplification.

1µg of 1kb RNA = 1.77×10^{12} molecules

1µg of 1kb dsDNA = 9.12×10^{11} molecules

1µg of pGEM® Vector DNA = 2.85×10^{11} molecules

1µg of lambda DNA = 1.9×10^{10} molecules

1µg of *E. coli* genomic DNA = 2×10^8 molecules

1µg of human genomic DNA = 3.04×10^5 molecules

G. Cycling Parameters

The two most commonly altered cycling parameters are annealing temperature and extension time. The lengths and temperatures for the other steps of a PCR cycle do not usually vary significantly. However in some cases, the denaturation cycle can be shortened or a lower denaturation temperature used to reduce the number of depurination events, which can lead to mutations in the PCR products.

Primer sequence is a major factor that determines the optimal annealing temperature, which is often within 5°C of the melting temperature of the primers. Using an annealing temperature slightly higher than the primer T_m will increase annealing stringency and can minimize nonspecific primer annealing and decrease the amount of undesired products synthesized. Using an annealing temperature lower than the primer T_m can result in higher yields, as the primers anneal more efficiently at the lower temperature. We recommend testing several annealing temperatures, starting approximately 5°C below the T_m, to determine the best annealing conditions. In many cases, nonspecific amplification and primer-dimer formation can be reduced through optimization of annealing temperature, but if undesirable PCR products remain a problem, consider incorporating one of the many hot-start PCR methods.

Oligonucleotide synthesis facilities will often provide an estimate of a primer's T_m. The T_m also can be calculated using the Biomath Calculators. Numerous formulas exist to determine the theoretical T_m of nucleic acids (Baldino, Jr. *et al*. 1989; Rychlik *et al*. 1990). The formula below can be used to estimate the melting temperature for oligonucleotides:

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

where $[Na^+]$ is the molar salt concentration and n = number of bases in the oligonucleotide

Example:

To calculate the melting temperature of a 22mer oligonucleotide with 60% G+C in 50mM KCl:

$$T_m = 81.5 + 16.6 \times (\log_{10}[0.05]) + 0.41 \times (60) - 675/22$$

$$= 81.5 + 16.6 \times (-1.30) + 24.60 - 30.68 = 54°C$$

The length of the extension cycle, which may need to be optimized, depends on PCR product size and the DNA polymerase being used. In general, allow approximately 1 minute for every 1kb of amplicon (minimum extension time = 1 minute) for nonproofreading DNA polymerases and 2 minutes for every 1kb of amplicon for proofreading DNA polymerases. Avoid excessively long extension times, as they can increase the likelihood of generating artifacts associated with the intrinsic 5′→3′ exonuclease activity of *Taq* DNA polymerase (Longley *et al*. 1990; Bell and DeMarini, 1991).

PCR typically involves 25–35 cycles of amplification. The risk of undesirable PCR products appearing in the reaction increases as the cycle number increases, so we recommend performing only enough cycles to synthesize the desired amount of product. If nonspecific amplification products accumulate before sufficient amounts of PCR product can be synthesized, consider diluting the products of the first reaction and performing a second amplification with the same primers or primers that anneal to sequences within the desired PCR product (nested primers).

H. PCR Enhancers and Additives

Addition of PCR-enhancing agents can increase yield of the desired PCR product or decrease production of undesired products. There are many PCR enhancers, which can act through a number of different mechanisms. These reagents will not enhance all PCRs; the beneficial effects are often template- and primer-specific and will need to be determined empirically. Some of the more common enhancing agents are discussed below.

Addition of betaine, DMSO and formamide can be helpful when amplifying GC-rich templates and templates that form strong secondary structures, which can cause DNA polymerases to stall. GC-rich templates can be problematic due to inefficient separation of the two DNA strands or the

tendency for the complementary, GC-rich primers to form intermolecular secondary structures, which will compete with primer annealing to the template. Betaine reduces the amount of energy required to separate DNA strands (Rees *et al*. 1993). DMSO and formamide are thought to aid amplification in a similar manner by interfering with hydrogen bond formation between two DNA strands (Geiduschek and Herskovits, 1961).

Some reactions that amplify poorly in the absence of enhancers will give a higher yield of PCR product when betaine (1M), DMSO (1–10%) or formamide (1–10%) are added. Concentrations of DMSO greater than 10% and formamide greater than 5% can inhibit *Taq* DNA polymerase and presumably other DNA polymerases as well (Varadaraj and Skinner, 1994).

In some cases, general stabilizing agents such as BSA (0.1mg/ml), gelatin (0.1–1.0%) and nonionic detergents (0–0.5%) can overcome amplification failure. These additives can increase DNA polymerase stability and reduce the loss of reagents through adsorption to tube walls. BSA also has been shown to overcome the inhibitory effects of melanin on RT-PCR (Giambernardi *et al*. 1998). Nonionic detergents, such as Tween®-20, NP-40 and Triton® X-100, have the added benefit of overcoming inhibitory effects of trace amounts of strong ionic detergents, such as 0.01% SDS (Gelfand and White, 1990). Ammonium ions can make an amplification reaction more tolerant of nonoptimal conditions. For this reason, some PCR reagents include 10–20mM $(NH_4)_2SO_4$. Other PCR enhancers include glycerol (5–20%), polyethylene glycol (5–15%) and tetramethyl ammonium chloride (60mM).

I. Nucleic Acid Cross-Contamination

It is important to minimize cross-contamination between samples and prevent carryover of RNA and DNA from one experiment to the next. Use separate work areas and pipettors for pre- and postamplification steps. Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Wear gloves, and change them often.

There are a number of techniques that can be used to prevent amplification of contaminating DNA. PCR reagents can be treated with isopsoralen, a photo-activated, cross-linking reagent that intercalates into double-stranded DNA molecules and forms covalent, interstrand crosslinks, to prevent DNA denaturation and replication. These interstrand crosslinks effectively render contaminating DNA unamplifiable.

Treatment of PCR reagents with uracil-N-glycosylase (UNG), a DNA repair enzyme that hydrolyzes the base-ribose bond at uracil residues, eliminates one of the most common sources of DNA contamination: previously amplified PCR products. UNG treatment prevents replication of uracil-containing DNA by causing the DNA polymerase to stall at the resulting abasic sites. For UNG to be an effective safeguard against contamination, the products of previous amplifications must be synthesized in the presence of dUTP. This is easily accomplished by substituting dUTP for some or all of the dTTP in the reaction. Nonproofreading polymerases will readily incorporate dUTP into a PCR product, but proofreading polymerases incorporate dUTP much less efficiently (Slupphaug *et al*. 1993; Greagg *et al*. 1999; Lasken *et al*. 1996). Since dUTP incorporation has no noticeable effect on the intensity of ethidium bromide staining or electrophoretic mobility of the PCR product, reactions can be analyzed by standard agarose gel electrophoresis. While both methods are effective (Rys and Persing, 1993), UNG treatment has the advantage that both single-stranded and double-stranded DNA templates will be rendered unamplifiable (Longo *et al*. 1990).

III. General Considerations for RT-PCR

Please also read General Considerations for PCR Optimization. Many of the important parameters discussed there also apply to RT-PCR. For a discussion of reverse transcriptases commonly used for RT-PCR, see the Reverse Transcription section.

A. Overview of the Access and AccessQuick™ RT-PCR Systems

The Access RT-PCR System and AccessQuick™ RT-PCR System are designed for the reverse transcription and amplification of a specific target RNA from either total RNA or mRNA (Miller and Storts, 1995; Knoche and Denhart, 2001). These one-tube, two-enzyme systems provide sensitive, quick and reproducible analysis of even rare RNAs (Miller and Storts, 1996). The systems use AMV Reverse Transcriptase for first-strand cDNA synthesis and the thermostable *Tfl* DNA Polymerase from *Thermus flavus* (Kaledin *et al*. 1981) for second-strand cDNA synthesis and DNA amplification. The systems include an optimized single-buffer system that permits sensitive detection of RNA transcripts without the need for buffer additions between reverse transcription and PCR amplification steps. This simplifies the procedure and reduces the potential for contamination. The elevated reaction temperature (45°C) possible with AMV reverse transcriptase minimizes problems encountered with RNA secondary structures (Brooks *et al*. 1995).

B. Template Considerations

For RT-PCR, successful reverse transcription depends on RNA integrity and purity. Procedures for creating and maintaining a ribonuclease-free (RNase-free) environment to minimize RNA degradation are described in Blumberg, 1987. The use of an RNase inhibitor (e.g., Recombinant RNasin® Ribonuclease Inhibitor) is strongly recommended. For optimal results, the RNA template, whether a total RNA preparation, an mRNA population or a synthesized RNA transcript, should be DNA-free to avoid amplification of contaminating DNA. The most commonly used DNA polymerases for PCR have no reverse transcriptase activity under standard reaction conditions, and thus, amplification products will be generated only if the template contains trace amounts of DNA with similar sequences.

Successful RT-PCR also depends on RNA quantity, which may need to be varied to determine the optimal amount. Excellent amplification results can be obtained with the Access and AccessQuick™ RT-PCR Systems using total RNA template levels in the range of 1pg–1µg per reaction (Figure 1.7) or poly(A)+ RNA template levels in the range of 1pg–100ng.

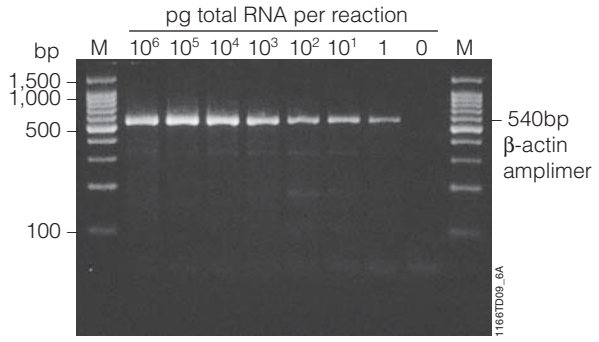

Figure 1.7. Amplification of a specific message in total RNA. RT-PCR amplifications containing the indicated amounts of mouse liver total RNA were performed using the Access RT-PCR System as described in the Access RT-PCR protocol using oligonucleotide primers specific to the mouse β-actin transcript. The specific 540bp amplicon is indicated. Equivalent aliquots of each amplification reaction were separated on a 3% NuSieve®/ 1% agarose gel in 1X TAE buffer containing 0.5µg/ml ethidium bromide. Lanes M, 100bp DNA Ladder (Cat.# G2101).

C. Reverse Transcription Primer Design

Selection of an appropriate primer for reverse transcription depends on target mRNA size and the presence of secondary structure. For example, a primer that anneals specifically to the 3′-end of the transcript (a sequence-specific primer or oligo(dT) primer) may be problematic when reverse transcribing the 5′-ends of long mRNAs or molecules that have significant secondary structure, which can cause the reverse transcriptase to stall during cDNA synthesis. Random hexamers prime reverse transcription at multiple points along the transcript. For this reason, they are useful for either long mRNAs or transcripts with significant secondary structure.

Whenever possible, we recommend using a primer that anneals only to defined sequences in particular RNAs (sequence-specific primers) rather than to entire RNA populations in the sample (e.g., random hexamers or oligo(dT) primer). To differentiate between amplification of cDNA and amplification of contaminating genomic DNA, design primers to anneal to sequences in exons on opposite sides of an intron so that any amplification product derived from genomic DNA will be much larger than the product amplified from the target cDNA. This size difference not only makes it possible to differentiate the two products by gel electrophoresis but also favors the synthesis of the smaller cDNA-derived product (amplification of smaller fragments is often more efficient that that of long fragments).

Regardless of primer choice, the final primer concentration in the reaction is usually within the range of 0.1–1.0µM, but this may need to be optimized. We recommend using a final concentration of 1µM primer (50pmol in a 50µl reaction) as a starting point for optimization. More information on PCR primer design is provided in the PCR Primer Design section.

D. Cycle Parameters

Efficient first-strand cDNA synthesis can be accomplished in a 20- to 60-minute incubation at 37–45°C using AMV reverse transcriptase or at 37–42° for M-MLV reverse transcriptase. When using AMV RT we recommend using a sequence-specific primer and performing reverse transcription at 45°C for 45 minutes as a starting point. The higher reaction temperature will minimize the effects of RNA secondary structure and encourage full-length cDNA synthesis. First-strand cDNA synthesis with random hexamers and oligo(dT) primer should be conducted at room temperature (20–25°C) and 37°C, respectively.

The Access and AccessQuick™ RT-PCR Systems do not require RNA denaturation prior to initiation of the reverse transcription reaction. If desired, however, a denaturation step may be incorporated by incubating a separate tube containing the primers and RNA template at 94°C for 2 minutes. Do not incubate AMV reverse transcriptase at 94°C; it will be inactivated. The template/primer mixture then can be cooled to 45°C and added to the RT-PCR mix for the standard reverse transcription incubation at 45°C. Following the reverse transcription, we recommend a 2-minute incubation at 94°C to denature the RNA/cDNA hybrid, inactivate AMV reverse transcriptase and dissociate AMV RT from the cDNA. It has been reported that AMV reverse transcriptase must be inactivated to obtain high yields of amplification product (Sellner *et al*. 1992; Chumakov, 1994).

Most RNA samples can be detected using 30–40 cycles of amplification. If the target RNA is rare or if only a small amount of starting material is available, it may be necessary to increase the number of cycles to 45 or 50 or dilute the products of the first reaction and reamplify.

IV. Thermostable DNA Polymerases

Prior to the use of thermostable DNA polymerases in PCR, researchers had to laboriously replenish the reaction with fresh enzyme (such as Klenow or T4 DNA polymerase) after each denaturation cycle. Thermostable DNA polymerases revolutionized and popularized PCR because of their ability to withstand the high denaturation temperatures. The use of thermostable DNA polymerases also allowed higher annealing temperatures, which improved the stringency of primer annealing.

Thermostable DNA polymerases can be used for either one-enzyme or two-enzyme RT-PCR (Myers and Gelfand, 1991; Chiocchia and Smith, 1997). For example, *Tth* DNA polymerase can act as a reverse transcriptase in the presence of Mn^{2+} for one-enzyme RT-PCR (Myers and Gelfand, 1991).

All of the DNA polymerases mentioned below can be used to amplifye first-strand cDNA produced by a reverse transcriptase, such as AMV RT, in two-enzyme RT-PCR.

Thermostable DNA polymerases can be divided into two groups: those with a 3′→5′ exonuclease (proofreading) activity, such as *Pfu* DNA polymerase, and those without the proofreading function, such as *Taq* DNA polymerase. These two groups have some important differences. Proofreading DNA polymerases are more accurate than nonproofreading polymerases due to the 3′→5′ exonuclease activity, which can remove a misincorporated nucleotide from a growing DNA chain. When the amplified product is to be cloned, expressed or used in mutation analysis, *Pfu* DNA polymerase is a better choice due to its high fidelity. However, for routine PCR, where simple detection of an amplification product is the goal, *Taq* DNA polymerase is the most commonly used enzyme because yields tend to be higher with a nonproofreading DNA polymerase.

Amplification with nonproofreading DNA polymerases results in the template-independent addition of a single nucleotide to the 3′-end of the PCR product, whereas the use of proofreading DNA polymerases results in blunt-ended PCR products (Clark, 1988; Hu, 1993). The single-nucleotide overhang can simplify the cloning of PCR products.

Proofreading DNA polymerases also are used in blends with nonproofreading DNA polymerases, or amino-terminally truncated versions of *Taq* DNA polymerase, to amplify longer stretches of DNA with greater accuracy than the nonproofreading DNA polymerase alone (Barnes, 1994; Cline *et al*. 1996). See Long PCR.

A. Taq DNA Polymerase

Taq DNA polymerase is isolated from *Thermus aquaticus* and catalyzes the primer-dependent incorporation of nucleotides into duplex DNA in the 5′→3′ direction in the presence of Mg^{2+}. The enzyme does not possess 3′→5′ exonuclease activity but has 5′→3′ exonuclease activity.

Taq DNA polymerase is suitable for most PCR applications that do not require a high-fidelity enzyme, such as detecting specific DNA or RNA sequences. The error rate of *Taq* DNA polymerase is approximately 1×10^{-5} errors/base, although the fidelity does depend somewhat on the reaction conditions. The fidelity is slightly higher at lower pH, lower magnesium concentration and relatively low dNTP concentration (Eckert and Kunkel, 1990; Eckert and Kunkel, 1991). See High-Fidelity PCR.

Taq DNA polymerase is commonly used to amplify PCR products of 5kb or less. PCR products in the range of 5–10kb can be amplified with *Taq* DNA polymerase but often require more optimization than smaller PCR products. For products larger than approximately 10kb, we recommend an enzyme or enzyme mix and reaction conditions that are designed for long PCR.

Taq DNA polymerase is a processive enzyme with an extension rate of >60 nucleotides/second at 70°C (Innis *et al*. 1988), so an extension step of 1 minute per 1kb to be amplified should be sufficient to generate full-length PCR products. The enzyme has a half-life of 40 minutes at 95°C (Lawyer *et al*. 1993). Because *Taq* DNA polymerase is a nonproofreading polymerase, PCR products generated with *Taq* DNA polymerase will contain a single-nucleotide 3′ overhang, usually a 3′ A overhang.

Additional Resources for *Taq* DNA Polymerase

Technical Bulletins and Manuals

9PIM300 *GoTaq® DNA Polymerase Promega Product Information*

9PIM829 *GoTaq® Flexi DNA Polymerase Promega Product Information*

Promega Publications

GoTaq® Green Master Mix for quick and easy two-step RT-PCR

B. Tfl DNA Polymerase

Tfl DNA polymerase catalyzes the primer-dependent polymerization of nucleotides into duplex DNA in the presence of Mg^{2+}. In the presence of Mn^{2+}, *Tfl* DNA polymerase can use RNA as a template. *Tfl* DNA polymerase exhibits a 5′→3′ exonuclease activity but lacks a 3′→5′ exonuclease activity. This enzyme is commonly used in PCR (Gaensslen *et al*. 1992), where its activity is similar to that of *Taq* DNA polymerase. *Tfl* DNA polymerase is used in the Access and AccessQuick™ RT-PCR Systems.

C. Tth DNA Polymerase

Tth DNA polymerase catalyzes polymerization of nucleotides into duplex DNA in the 5′→3′ direction in the presence of $MgCl_2$. The enzyme can use an RNA template in the presence of $MnCl_2$ (Myers and Gelfand, 1991; Ruttimann *et al*. 1985). *Tth* DNA polymerase exhibits a 5′→3′ exonuclease activity but lacks detectable 3′→5′ exonuclease activity. The error rate of *Tth* DNA polymerase has been measured at 7.7×10^{-5} errors/base (Arakawa *et al*. 1996). *Tth* DNA polymerase can amplify target DNA in the presence of phenol-saturated buffer (Katcher and Schwartz, 1994) and has been reported to be more resistant to inhibition by blood components than other thermostable polymerases (Ehrlich *et al*. 1991; Bej and Mahbubani, 1992).

Tth DNA polymerase is commonly used for PCR (Myers and Gelfand, 1991; Carballeira *et al*. 1990) and RT-PCR (Myers and Gelfand, 1991; Chiocchia and Smith, 1997). For primer extension, RT-PCR and cDNA synthesis using RNA templates with complex secondary structure, the high reaction temperature of *Tth* DNA polymerase may be an advantage over more commonly used reverse transcriptases, such as AMV and M-MLV reverse transcriptases. Recombinant *Tth* DNA polymerase has been shown to exhibit RNase H-like activity (Auer *et al*. 1995).

Additional Resources for *Tth* DNA Polymerase

Technical Bulletins and Manuals

9PIM210 *Tth DNA Polymerase Promega Product Information*

D. Tli DNA Polymerase

Tli DNA polymerase replicates DNA through polymerization of nucleotides into duplex DNA in the 5′→3′ direction in the presence of Mg^{2+}. This enzyme also contains a 3′→5′ exonuclease activity, which results in increased fidelity of nucleotide incorporation. There is no detectable reverse transcriptase activity or 5′→3′ exonuclease activity. *Tli* DNA polymerase will promote strand displacement at 72°C but will not displace DNA at 55°C (Kong *et al.* 1993). Greater than 95% of the amplified products will be blunt-ended.

Tli DNA polymerase is commonly used for PCR and RT-PCR, where its proofreading activity makes it useful for high-fidelity and long PCR (Keohavong *et al*. 1993). Due to the 3′→5′ exonuclease activity of *Tli* DNA polymerase, the enzyme can degrade the oligonucleotide primers used to initiate DNA synthesis. This exonucleolytic attack can be effectively prevented by using hot-start PCR or introducing a single phosphorothioate bond at the 3′ termini of the primers (Byrappa *et al*. 1995). *Tli* DNA polymerase also can be used for primer extension, where the high optimal temperature of the enzyme may be an advantage for templates with complex secondary structure.

E. Pfu DNA Polymerase

Pfu DNA polymerase has one of the lowest error rates of all known thermophilic DNA polymerases used for amplification due to the high 3′→5′ exonuclease activity (Cline *et al*. 1996; Andre *et al*. 1997). For cloning and expressing DNA after PCR, *Pfu* DNA polymerase is often the enzyme of choice. *Pfu* DNA polymerase can be used alone to amplify DNA fragments up to 5kb by increasing the extension time to 2 minutes per kilobase. It is also used in blends with DNA polymerases lacking the proofreading function, such as *Taq* DNA polymerase, to achieve longer amplification products than with *Pfu* DNA polymerase alone (Barnes, 1994). However, the proofreading activity can shorten PCR primers, leading to decreased yield and increased nonspecific amplification. This exonucleolytic attack can be effectively prevented by using hot-start PCR or introducing a single phosphorothioate bond at the 3′-termini of the primers (Byrappa *et al*. 1995).

Additional Resources for *Pfu* DNA Polymerase

Technical Bulletins and Manuals

9PIM774 *Pfu DNA Polymerase Promega Product Information*

Promega Publications

Pfu DNA Polymerase: A high fidelity enzyme for nucleic acid amplification

V. Reverse Transcriptases

The discovery of reverse transcriptases, or RNA-dependent DNA polymerases, and their role in retrovirus infection (Baltimore, 1970; Temin and Mizutani, 1970) altered molecular biology's central dogma of DNA→RNA→protein. Reverse transcriptases use an RNA template to synthesize DNA and require a primer for synthesis, like other DNA polymerases. For in vitro applications, the primer can be either oligo(dT), which hybridizes to the poly(A)+ tails of eukaryotic mRNAs, random hexamers, which prime synthesis throughout the length of the RNA template, or a sequence-specific primer, which hybridizes to a known sequence within the RNA template. Polymerization from a primer then proceeds as for DNA-dependent DNA polymerases. The commonly used reverse transcriptases, AMV reverse transcriptase, M-MLV reverse transcriptase and M-MLV reverse transcriptase, RNase H minus, perform the same reaction but at different optimum temperatures (AMV, 42°C; M-MLV, 37°C; and M-MLV RT, RNase H–, 42°C).

Some reverse transcriptases also possess intrinsic 3′- or 5′-exoribonuclease (RNase) activity, which is generally used to degrade the RNA template after first strand cDNA synthesis. Absence of the 5′-exoribonuclease (RNase H) activity may aid production of longer cDNAs (Berger *et al*. 1983).

Some DNA-dependent DNA polymerases also possess a reverse transcriptase activity, which can be favored under certain conditions. For example, the thermostable, DNA-dependent *Tth* DNA polymerase exhibits reverse transcriptase activity when Mn^{2+} is substituted for Mg^{2+} in a reaction.

A. AMV Reverse Transcriptase

AMV RT catalyzes DNA polymerization using template DNA, RNA or RNA:DNA hybrids (Houts *et al*. 1979). AMV reverse transcriptase is the preferred reverse transcriptase for templates with high secondary structure due to its higher reaction temperature (up to 58°C). AMV RT is used in a wide variety of applications including cDNA synthesis (Houts *et al*. 1979; Gubler and Hoffman, 1983), RT-PCR and rapid amplification of cDNA ends (RACE; Skinner *et al*. 1994). Although the high optimal temperature (42°C) makes it the enzyme of choice for cDNA synthesis using templates with complex secondary structure, its relatively high RNase H activity limits its usefulness for generation of long cDNAs (>5kb). For these templates, M-MLV RT or M-MLV RT, RNase H minus, may be a better choice.

Additional Resources for AMV Reverse Transcriptase

Technical Bulletins and Manuals

9PIM510 *AMV Reverse Transcriptase Promega Product Information*

B. M-MLV Reverse Transcriptase

M-MLV RT is a single-polypeptide, RNA-dependent DNA polymerase. The enzyme also has DNA-dependent DNA polymerase activity at high enzyme levels (Roth *et al*. 1985). M-MLV RT is used in a variety of applications, including cDNA synthesis, RT-PCR and RACE (Gerard, 1983). Its relatively low RNase H activity compared to AMV RT makes M-MLV RT the enzyme of choice for generating long cDNAs (>5kb) (Sambrook and Russell, 2001). However, for short templates with complex secondary structure, AMV RT or M-MLV RT, RNase H minus, may be a better choice due to their higher optimal temperatures. M-MLV RT is less processive than AMV RT, so more units of M-MLV RT may be required to generate the same amount of cDNA (Schaefer, 1995).

Additional Resources for M-MLV Reverse Transcriptase

Technical Bulletins and Manuals

9PIM170 *M-MLV Reverse Transcriptase Promega Product Information*

C. M-MLV Reverse Transcriptase, RNase H Minus

M-MLV reverse transcriptase, RNase H minus, is an RNA-dependent, 5′→3′ DNA polymerase that has been genetically altered to remove the associated ribonuclease H activity, which causes degradation of the RNA strand of an RNA:DNA hybrid (Tanese and Goff, 1988). The absence of RNase H activity makes M-MLV, RNase H minus, the enzyme of choice for generating long cDNAs (>5kb). However, for shorter templates with complex secondary structure, AMV reverse transcriptase may be a better choice because it can be used at higher reaction temperatures.

There are two forms of M-MLV, RNase H minus: the deletion mutant and the point mutant. As the names suggest, the deletion mutant had a specific sequence in the RNase H domain deleted, and the point mutant has a point mutation introduced in the RNase H domain. While the native M-MLV RT has a recommended reaction temperature of 37°C, the deletion and point mutants are more stable at higher temperatures and can be used at reaction temperatures of up to 50°C and 55°C, respectively, depending upon the reverse transcription primers used. The point mutant is often preferred over the deletion mutant because the point mutant has DNA polymerase activity comparable to that of the wildtype M-MLV enzyme, whereas the deletion mutant has a slightly reduced DNA polymerase activity compared to that of the wildtype enzyme (Figure 1.8).

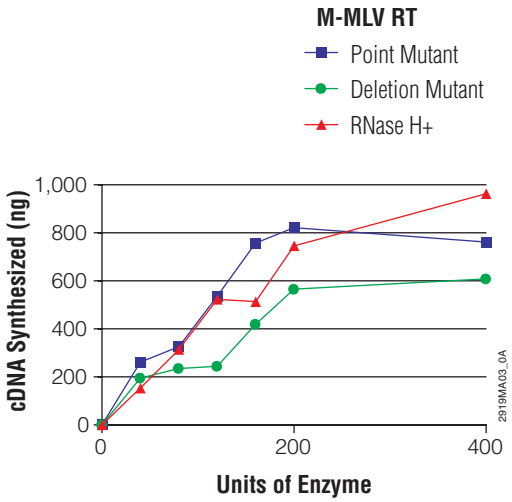

Figure 1.8. Comparison of the mass amount of total cDNA synthesized from 2µg of a 7.5kb RNA template by increasing amounts of three Promega M-MLV reverse transcriptases. Each first-strand cDNA reaction was performed using 2µg of a 7.5kb RNA template (1µl), 0.5µg of oligo(dT)$_{15}$ primer (1µl) and 14µl water. The RNA and oligo(dT)$_{15}$ primer were heated at 70°C for 5 minutes and cooled on ice for 5 minutes. Five microliters of M-MLV RT 5X Buffer, 1.25µl of 10µM dNTPs, 0.5µl of α-^{32}P dCTP (10µCi/µl, 400Ci/mmol) and either 25, 50, 100, 150, 200 or 400 units of M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant; M-MLV Reverse Transcriptase, RNase H Minus, Deletion Mutant; or native M-MLV Reverse Transcriptase (RNase H+) was used in a final volume of 25µl. Reactions were incubated at 42°C for 60 minutes. TCA precipitations were performed, and first-strand cDNA yields were calculated.

Additional Resources for M-MLV Reverse Transcriptase, RNase H Minus

Technical Bulletins and Manuals

9PIM530 *M-MLV Reverse Transcriptase, RNase H Minus, Promega Product Information*

9PIM368 *M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant, Promega Product Information*

VI. Example of a PCR Protocol

Materials Required:

(see Composition of Solutions section)

- template DNA
- downstream primer
- upstream primer
- GoTaq® DNA Polymerase (Cat.# M8291)
- $MgCl_2$, 25mM
- Nuclease-Free Water (Cat.# P1193)
- nuclease-free light mineral oil (e.g., Sigma Cat.# M5904) if using a thermal cycler without a heated lid; do not autoclave
- dNTP mix, 10mM of each dNTP

Note: To facilitate optimization, troubleshooting and validation, we strongly recommend including both positive and negative control reactions.

1. Combine the first five reaction components in the order listed below in a thin-walled 0.5ml reaction tube. Gently vortex the tube for 10 seconds, and briefly centrifuge in a microcentrifuge. Initiate the reaction by adding the template and primers.

Component	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
5X Green or Colorless GoTaq® Flexi Buffer	10µl	1X
dNTP mix, 10mM each dNTP	1µl	0.2mM each
GoTaq® DNA Polymerase (5u/µl)	0.25µl	0.025u/µl
25mM $MgCl_2$	3µl	1.5mM
downstream primer	50pmol[1]	1µM
upstream primer	50pmol	1µM
template[2]	Yµl	

[1]A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng × b; where b is the number of bases in the primer.

[2]If possible, start with >10^4 copies of the target sequence to obtain a signal in 25–30 cycles, but keep the final DNA concentration of the reaction at ≤10ng/µl. Less than 10 copies of a target can be amplified (Saiki, 1988), but more cycles may be required to detect a signal by gel electrophoresis. Additional cycles may increase nonspecific amplification, evidenced by smeared bands upon gel electrophoresis.

2. Overlay the reaction with 1–2 drops (20–40µl) of nuclease-free mineral oil to prevent condensation and evaporation. Mineral oil addition is not necessary if you are using a thermal cycler with a heated lid.

3. Place tube in a thermal cycler, and proceed with the thermal cycling profile chosen for your reactions.

4. Analyze 5µl of the PCR products by agarose gel electrophoresis. The products should be readily visible by UV transillumination of the ethidium bromide-stained gel.

5. Store reaction products at –20°C until needed.

VII. Example of an RT-PCR Protocol

A. Access RT-PCR Protocol

These conditions work well to detect the 323bp PCR product generated from the Positive Control RNA using the Upstream and Downstream Control Primers provided with the Access RT-PCR System. We recommend optimizing the parameters for each target RNA.

Materials Required:
(see Composition of Solutions section)

- template RNA
- downstream oligonucleotide primer
- upstream oligonucleotide primer
- Access RT-PCR System (Cat.# A1250)
- Nuclease-Free Water (Cat.# P1193)
- nuclease-free light mineral oil (e.g., Sigma Cat.# M5904) if using a thermal cycler without a heated lid

1. Prepare the reaction mix by combining the indicated volumes of Nuclease-Free Water, AMV/*Tfl* 5X Reaction Buffer, dNTP Mix, 25mM $MgSO_4$ and the specific upstream and downstream primers in a thin-walled 0.5ml reaction tube on ice. Mix the components by pipetting. Add AMV Reverse Transcriptase and *Tfl* DNA Polymerase to the reaction. Gently vortex the tube for 10 seconds to mix.

Component	Volume	Final Concentration
Nuclease-Free Water (to a final volume of 50µl)	Xµl	
AMV/*Tfl* 5X Reaction Buffer	10µl	1X
dNTP Mix, 10mM each dNTP	1µl	0.2mM each
downstream primer	50pmol[3]	1µM
upstream primer	50pmol	1µM
25mM $MgSO_4$	2µl	1mM
AMV Reverse Transcriptase (5u/µl)	1µl	0.1u/µl
Tfl DNA Polymerase (5u/µl)	1µl	0.1u/µl
RNA sample[4]	Yµl	

[3]A general formula for calculating the number of nanograms of primer equivalent to 50pmol is: 50pmol = 16.3ng × b; where b is the number of bases in the primer. For the positive control reaction, use 3.3µl of both the Downstream and Upstream Control Primers (50pmol).

[4]Use 10^3–10^6 copies of a specific target template or 1pg–1µg total RNA. Use 2µl of the Positive Control RNA with Carrier (2.5 attomoles or 1 × 10^6 copies).

2. Overlay the reaction with one or two drops (20–40µl) of nuclease-free mineral oil to prevent condensation and evaporation. Mineral oil addition is not necessary if you are using a thermal cycler with a heated lid.

3. Place tube in a thermal cycler equilibrated at 45°C, and incubate for 45 minutes.

4. Proceed directly to thermal cycling for second-strand cDNA synthesis and amplification (refer to Tables 1.1 and 1.2).

Table 1.1. First-Strand cDNA Synthesis.

1 cycle	45°C for >45 minutes	reverse transcription
1 cycle	94°C for >2 minutes	AMV RT inactivation and RNA/cDNA/primer denaturation

Table 1.2. Second-Strand cDNA Synthesis and PCR.

40 cycles	94°C for 30 seconds	denaturation
	60°C for 1 minute	annealing
	68°C for 2 minutes	extension
1 cycle	68°C for 7 minutes	final extension
1 cycle	4°C	soak

B. ImProm-II™ Reverse Transcription System Protocol

1. Place sterile, thin-walled dilution tubes and reaction tubes on ice. Thaw the experimental RNA or 1.2kb Kanamycin Positive Control RNA on ice, and return any unused portion to the freezer as soon as aliquots are taken.

2. On ice, combine RNA (up to 1µg) and primer in Nuclease-Free Water for a final volume of 5µl per reaction.

Experimental Reactions

Component	Volume
Experimental RNA (up to 1µg/reaction)[5]	Yµl
Oligo(dT)$_{15}$ Primer or Random Primers (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction)[6]	Xµl
Nuclease-Free Water to a final volume of	5µl

[5]Use 10^2–10^{10} copies of a specific target RNA template or 1pg–1µg total RNA or poly(A)+ mRNA.

[6]10–20pmol of primer in a 20µl reaction is equal to 0.5–1µM. A general formula for calculating nanograms of primer equivalent to 10pmol is 3.3 × b, where b is the number of bases in the primer.

Positive Control Reaction

Component	Volume
1.2kb Kanamycin Positive Control RNA, 0.5µg/µl	2µl
Oligo(dT)$_{15}$ Primer, 0.5µg/µl	1µl
Nuclease-Free Water	2µl
Final Volume	**5µl**

Negative (No Template) Control Reaction

Component	Volume
Oligo(dT)$_{15}$ Primer or Random Primers (0.5µg/reaction) or gene-specific primer (10–20pmol/reaction)	Xµl
Nuclease-Free Water to a final volume of	5µl

3. Close each tube of RNA tightly. Place tubes into a preheated 70°C heat block for 5 minutes. Immediately chill in ice-water for at least 5 minutes. Centrifuge each tube for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. Keep the tubes closed and on ice until the reverse transcription reaction mix is added.

4. Prepare the reverse transcription reaction mix by combining the following components of the ImProm-II™ Reverse Transcription System in the order listed in a sterile 1.5ml microcentrifuge tube on ice. Determine the volume of each component needed for the desired number of reaction, and combine components in the order listed. Vortex gently to mix, and keep on ice prior to dispensing into reaction tubes.

Experimental Reactions

Component	Volume
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$, 25mM (1.5–8.0mM final conc.) [7]	1.2–6.4µl
dNTP Mix, 10mM each dNTP (0.5mM final conc.)[8]	1.0µl
RNasin® Ribonuclease Inhibitor (optional)	20u
ImProm-II™ Reverse Transcriptase	1.0µl
Final Volume	**15.0µl**

[7]The final Mg^{2+} concentration should be optimized in the range of 1.5–8.0mM.

[8]If isotopic or nonisotopic incorporation is desired to monitor first-strand cDNA synthesis, $\alpha[^{32}P]$-dCTP or other modified nucleotides may be added to the dNTP mixture.

Positive Control Reaction

Component	Volume
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Buffer	4.0µl
$MgCl_2$, 25mM (6mM final conc.)	4.8µl
dNTP Mix, 10mM each dNTP (0.5mM final conc.)	1.0µl
RNasin® Ribonuclease Inhibitor (optional)	20u
ImProm-II™ Reverse Transcriptase	1.0µl
Final Volume	**15.0µl**

Negative (No Reverse Transcriptase) Control Reaction

Component	Amount
Nuclease-Free Water (to a final volume of 15µl)	Xµl
ImProm-II™ 5X Reaction Buffer	4.0µl
$MgCl_2$, 25mM (1.5–8.0mM final conc.)	1.2–6.4µl
dNTP Mix, 10mM each dNTP (0.5mM final conc.)	1.0µl
RNasin® Ribonuclease Inhibitor (optional)	20u
Final Volume	**15.0µl**

5. Dispense 15µl of reverse transcription reaction mix to each reaction tube on ice. Be careful to prevent cross-contamination. Add 5µl of RNA and primer mix to each reaction for a final reaction volume of 20µl per tube. If there is a concern about evaporation in subsequent steps, overlay the reaction with a drop of nuclease-free mineral oil.

6. Anneal: Place tubes in a controlled-temperature heat block equilibrated at 25°C, and incubate for 5 minutes.

7. Extend: Incubate tubes in a controlled-temperature heat block at 42°C for up to one hour. The extension temperature may be optimized between 37–55°C.

8. Inactivate reverse transcriptase: If the experimental goal is to proceed with PCR, the reverse transcriptase must be thermally inactivated prior to amplification. Incubate tubes in a controlled-temperature heat block at 70°C for 15 minutes.

9. Prepare the PCR mix by dispensing the appropriate volume of each component into a sterile, 1.5ml microcentrifuge tube on ice. Combine the components in the order listed, vortex gently to mix and keep on ice prior to dispensing to the reaction tubes. An aliquot of the first-strand cDNA (1µl or 20µl) from the reverse transcription reaction is added last to the PCR mix. See Notes 1–3.

Component	Volume per 100µl reaction (1µl RT reaction)	Volume per 100µl reaction (20µl RT reaction)
Nuclease-Free Water	55.2µl	45.6µl
5X Green or Colorless GoTaq® Flexi Buffer	19.8µl	16.0µl
$MgCl_2$, 25mM (2mM final conc.)[9]	7.8µl	3.2µl
PCR Nucleotide Mix, 10mM (0.2mM final conc.)	2.0µl	1.0µl
Upstream Control Primer (1µM final conc.)	6.6µl	6.6µl
Downstream Control Primer (1µM final conc.)	6.6µl	6.6µl
GoTaq® DNA Polymerase (5.0 units)	1.0µl	1.0µl
PCR mix per reaction	99µl	80µl
RT reaction per reaction	1.0µl	20.0µl
Total PCR Volume	100.0µl	100.0µl

[9]For experimental reactions, the final Mg^{2+} concentration should be optimized in the range of 1.5–2.5mM.

10. Overlay the reaction with two drops of nuclease-free mineral oil to prevent evaporation and condensation.

    Place the reactions in a thermal cycler that has been prewarmed to 94°C. An optimized program for amplification using the Upstream and Downstream Control Primers provided with the Access RT-PCR system is given in Table 1.3.

Table 1.3. Amplification Conditions for the Positive Control Reaction.

1 cycle	Denaturation: 94°C for 2 minutes
25 cycles	Denaturation: 94°C for 1 minute
	Annealing: 60°C for 1 minute
	Extension: 72°C for 2 minutes
1 cycle	Final extension: 72°C for 5 minutes
1 cycle	Hold 4°C

11. After the cycle is complete, analyze products or store amplifications at –20°C.

12. Analyze PCR products by agarose gel electrophoresis of 10% of the total reaction. The products will be readily visible by UV transillumination of an ethidium bromide-stained gel. The amplification product obtained using the Positive Control RNA with the Upstream and Downstream Control Primers is 323bp long.

13. Store reaction products at –20°C until needed.

Notes

1. In this example, the final volume of PCR mix should be sufficient for 100µl reactions once the cDNA volume is added. The volume of each component may be scaled for reactions of less than 100µl. Scale up volumes to accommodate the total number of PCR amplifications being performed.
2. The amount of reverse transcription reaction used in the PCR may be modified after experimental optimization.
3. The amounts of magnesium and dNTPs and volume of reaction buffer added to the PCR vary, depending on how much RT reaction is used as template. For example, for a 100µl PCR that contains 20µl of RT product, 8µl of 10X thermophilic polymerase reaction buffer is added to support the 80µl PCR mix addition. If 5µl of RT reaction were added to 95µl of PCR mix, 9.5µl of 10X thermophilic polymerase reaction buffer would be needed. Similar considerations must be given to the magnesium and dNTP additions. This example details the amplification conditions recommended to amplify either 1µl or 20µl of the cDNA synthesized in the positive control reverse transcriptase reactions containing the 1.2kb Kanamycin Positive Control RNA template in a 100µl PCR.

VIII. Troubleshooting PCR and RT-PCR

Symptoms	Solutions
Low yield or no amplification product (PCR or RT-PCR)	Template was degraded. Verify template integrity by electrophoresis. Repurify the DNA or RNA template if the nucleic acid appears degraded.
	Too much or too little template was used. Verify template concentration by comparing the staining intensity of the DNA template after agarose gel electrophoresis and ethidium bromide staining with that of DNA standards with known concentrations.
	Inhibitor was present in sample. Reduce template volume in the reaction. Perform an ethanol precipitation to remove inhibitors. Some common inhibitors are listed in the Template Quality section.
	Poor primer design. Make sure primers are not self-complementary or complementary to each other.
	Verify that primers are complementary to the appropriate strands.
	Insufficient number of cycles. Return reactions to thermal cycler for 5 more cycles.
	Primer concentration was too low. Verify primer concentration in the reaction. Increase primer concentration if necessary.
	Suboptimal reaction conditions. Optimize Mg^{2+} concentration, annealing temperature and extension time. Verify that primers are present at equal concentrations. Refer to General Considerations for PCR Optimization for more information about optimizing reaction conditions.
	Nucleotides were degraded. Keep nucleotides frozen in aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles.
	Target sequence was not present in target DNA or RNA. Redesign experiment, or try other sources of target DNA or RNA
	Reaction component was missing. Always perform a positive control reaction with a template/primer combination that has amplified well in the past to determine when a component was omitted. Check the reaction components, and repeat the reaction.
	Poor-quality mineral oil. The reaction must be overlaid with high-quality, nuclease-free light mineral oil when using a thermal cycler without a heated lid. Do not use autoclaved mineral oil.
	Thermal cycler was programmed incorrectly. Verify that times and temperatures are correct. Use step cycles, not hold segments.
	Thermal cycler did not reach the proper temperature. Calibrate the thermal cycler to be sure reactions are heated to the programmed temperatures. Depending on the primers and template, small changes in cycling conditions can affect yield.
	Temperature was too low in some positions of thermal cycler. Perform a set of control reactions to determine if certain positions in the thermal cycler give low yields.

Symptoms	Solutions
Nonspecific amplification products (PCR or RT-PCR)	Reaction conditions were suboptimal. Optimize Mg^{2+} concentration, annealing temperature, primer size, extension time and cycle number to minimize nonspecific priming. Refer to General Considerations for PCR Optimization for more information about optimizing reaction conditions. Perform hot-start PCR to minimize nonspecific amplification. If you are not using a DNA polymerase designed for hot-start PCR, such as GoTaq® Hot Start Polymerase, assemble reactions on ice and preheat the thermal cycler to 95°C before adding reaction tubes. Poor primer design. Make sure primers are not self-complementary or complementary to each other, especially near the 3′-ends. Avoid using three G or C nucleotides in a row at the 3′-end of a primer. Try a longer primer. Primer concentration was too high. Verify primer concentration in the reaction. Try a lower concentration. Reaction was contaminated by another RNA or DNA. Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Use a separate work area and pipettor for pre- and postamplification. Wear gloves, and change them often. Use UNG or another technique to prevent carryover of DNA produced in a previous amplification into subsequent reactions. See the Nucleic Acid Cross-Contamination section. Multiple target sequences exist. Design new primers with higher specificity to target sequence in template DNA or cDNA.
Low yield or no first-strand product (RT-PCR)	RNA was degraded. Verify RNA integrity by denaturing agarose gel electrophoresis. Ensure that reagents, tips and tubes are RNase-free. Isolate RNA in the presence of a ribonuclease inhibitor (e.g., Promega RNasin® Ribonuclease Inhibitor). Repurify the DNA or RNA template if the nucleic acid appears degraded. AMV reverse transcriptase was thermally inactivated. If an initial denaturation/annealing step is introduced into the protocol, be certain to add the enzyme mix containing AMV reverse transcriptase after denaturation and subsequent 45°C equilibration. Poor primer specificity. Verify that the reverse transcription primer is complementary to the downstream RNA sequence. Poor primer annealing. If oligo(dT) primers or random hexamers were used as the reverse transcription primer, verify that the annealing step was carried out at an appropriate temperature prior to reverse transcription. RNA template was impure. Carryover of reagents (e.g., SDS, NaCl, heparin, guanidine thiocyanate) from some RNA purification methods can interfere with RT-PCR. Reduce volume of target RNA, perform additional purification steps or change purification method. Target RNA was not present in the sample or was present at low levels. Use poly(A)+ RNA, rather than total RNA, as a template to increase mRNA target abundance. Alternatively, isolate RNA from different starting material with a higher abundance of the desired target RNA
Amplification product with a higher-than-expected molecular weight (RT-PCR)	Genomic DNA sequences related to the RNA template contaminated the RNA preparation. Treat the RNA sample with RQ1 RNase-Free DNase to degrade contaminating DNA.

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