

PCR Core Systems



Technical Bulletin No. 254

INSTRUCTIONS FOR USE OF PRODUCTS M7660 AND M7665.

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I. Description

The PCR Core Systems I and II^(a) are designed for the exponential amplification of specific regions of DNA using the polymerase chain reaction^(a) (PCR; 1) with *Taq* DNA Polymerase^(b), a thermostable enzyme isolated from *Thermus aquaticus*. PCR Core System I provides the enzymes and buffers necessary for amplification; PCR Core System II provides the same reagents as System I but also includes positive control primers and template. Please see Section V for a discussion of using controls in PCR.

Although PCR is simple in theory, it can benefit from optimization of several parameters. This technical bulletin provides important and convenient information about performing PCR and includes protocols and troubleshooting tips for successful PCR amplifications.

PCR allows a DNA or cDNA template to be quickly and reliably amplified to greater than one million copies. Figure 1 illustrates the doubling of target DNA that occurs with each cycle of PCR amplification. Starting with minute amounts of DNA, the PCR process generates sufficient material for subsequent experimental analysis such as cloning, restriction digestion, electrophoresis and sequencing. The entire amplification process is performed in just a few hours.

PCR has aided in answering many diverse biological questions. Many variations of the original PCR method now exist, and numerous critical factors for successful amplification under a variety of conditions have been delineated. Although a detailed discussion of different types of PCR methods and applications is beyond the scope of this publication, a protocol for routine PCR, which is often sufficient for many applications, is provided. An extensive bibliography is provided in Section VIII.C for those desiring more comprehensive information on PCR and related applications.

II. Product Components

Product	Cat.#
PCR Core System I	M7660

For Laboratory Use. Each System I contains sufficient reagents for 200 amplification reactions (50µl each). Includes:

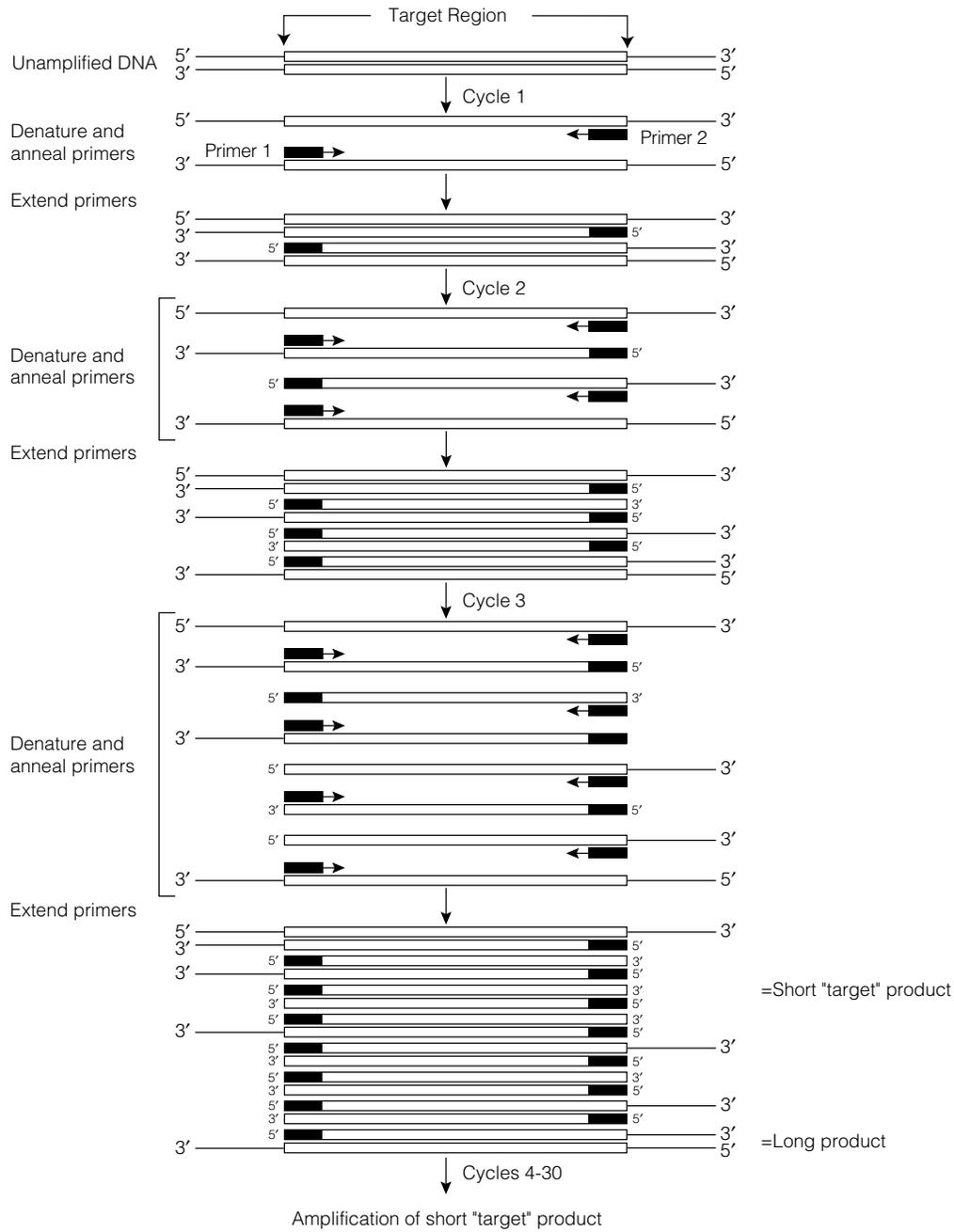
- 250u *Taq* DNA Polymerase
- 1.2ml Thermophilic DNA Polymerase 10X Reaction Buffer, MgCl₂-Free
- 1.2ml MgCl₂, 25mM Solution
- 1.2ml *Taq* DNA Polymerase 10X Buffer, with 15mM MgCl₂
- 200µl PCR Nucleotide Mix, 10mM
- 1 Protocol

Product	Cat.#
PCR Core System II	M7665

For Laboratory Use. Each System II contains sufficient reagents for 200 amplification reactions (50µl each). Includes:

- 250u *Taq* DNA Polymerase
- 1.2ml Thermophilic DNA Polymerase 10X Reaction Buffer, MgCl₂-Free
- 1.2ml MgCl₂, 25mM Solution
- 1.2ml *Taq* DNA Polymerase 10X Buffer, with 15mM MgCl₂
- 200µl PCR Nucleotide Mix, 10mM
- 100ng Positive Control Plasmid DNA
- 100µl Upstream Control Primer, 15µM
- 100µl Downstream Control Primer, 15µM
- 1 Protocol

Storage Conditions: Store all components at -20°C. See the label for expiration date.



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Figure 1. Diagram of the PCR amplification process.

III. PCR Protocol

To facilitate optimization, troubleshooting and validation of PCR, we strongly recommend performing concurrent positive and negative control reactions as discussed in Section V.

If working with multiple samples, assemble a master mix of water, MgCl₂, 10X buffer, primers, PCR Nucleotide Mix and *Taq* DNA Polymerase. Combine the appropriate multiples of these components (except template) and make aliquots of the appropriate volume, minus the specific volume to be occupied by the template DNA, to each reaction tube. Initiate the reaction by adding the template. Use individual pipette tips for all additions, being careful not to cross-contaminate the samples.

If possible, start with >10⁴ copies of the target sequence to obtain a signal in 25–30 cycles. Excess template is not beneficial to the reaction. Always ensure that the final DNA concentration is ≤10ng/μl. Less than 10 copies of a target can be amplified (2), but more cycles may be required to detect a signal by gel electrophoresis. Additional cycles may increase nonspecific amplification, evidenced by multiple bands when analyzed by gel electrophoresis.

Materials to Be Supplied by the User

- Mineral Oil (Cat.# DY1151)
- Nuclease-Free Water (Cat.# P1193)
- upstream primer
- downstream primer
- template DNA

A. Protocol

1. Combine the following components, as listed in Table 1, in sterile, 0.5–0.6ml microcentrifuge tubes. Amplification reactions may be scaled up or down as necessary. Use of a master mix, as described above, greatly facilitates the reaction setup and decreases tube-to-tube variability of the reaction components. See Section V for guidelines on selecting the optimum amounts of each component (e.g., magnesium concentration) to include in the reaction.

Table 1. Recommended Reaction Volumes and Final Concentrations of the PCR Core System Components.

Component	Component Volume	Final Concentration
MgCl ₂ , 25mM Solution	3μl	1.5mM
Thermophilic DNA Polymerase		
10X Reaction Buffer, MgCl ₂ -Free	5μl	1.0X
PCR Nucleotide Mix, 10mM each	1μl	200μM each
Upstream Control Primer	5–50pmol	0.1–1.0μM
Downstream Control Primer	5–50pmol	0.1–1.0μM
<i>Taq</i> DNA Polymerase, 5u/μl	0.25μl	1.25u/50μl
template DNA	variable	<0.5μg/50μl
Nuclease-Free Water to a final volume of	50μl	

2. If using a thermal cycler **without a heated lid**, overlay the reaction mix with 1–2 drops (approximately 50μl) of Mineral Oil to prevent evaporation during thermal cycling. Centrifuge the reaction mix in a microcentrifuge for 5 seconds.

Note: An Experienced User's Protocol can be found at the end of this Technical Bulletin.



Thaw

the 25mM MgCl₂ solution completely and vortex thoroughly before use. A separate buffer containing MgCl₂ is supplied for convenience. However, we strongly recommend optimizing the MgCl₂ concentration.

Step	Temperature	Time	Number of Cycles
Initial Denaturation:	95°C	2 minutes	1 cycle
	↓		
Denaturation:	95°C	0.5–1 minute	25–35 cycles
Annealing:	42–65°C	1 minute	
Extension:	72–74°C	1–2 minutes	
	↓		
Final Extension:	72–74°C	5 minutes	1 cycle
	↓		
Soak:	4°C	indefinite	1 cycle

Note: The annealing temperature for a specific amplification reaction will depend upon the sequences of the two primers. See Section IV.H for discussions on determining optimal annealing temperatures for PCR amplification.

Figure 2. Recommended thermal cycling conditions for PCR amplification. These guidelines apply to target sequences between 200 and 2,000bp and are optimal for the Perkin-Elmer thermal cycler model 480 or comparable thermal cyclers.

- Place the reactions in a thermal cycler that has been preheated to 95°C. We recommend heating the samples at 95°C for 2 minutes to ensure that the target DNA is completely denatured. Incubation longer than 2 minutes at 95°C is unnecessary and may reduce the yield.
- Start the thermal cycling program. The cycling profile given in Figure 2 may be used as a guideline. Optimize the amplification profile for each primer/target combination (Section V).

B. Analysis

- Analyze PCR products by agarose gel electrophoresis. The products should be readily visible in an ethidium bromide-stained gel under UV light.
- Store PCR products at –20°C until needed. The PCR products can be further purified using a number of procedures including the Wizard® PCR Preps DNA Purification System^(c) (Cat.# A7170) or AgarACE® Agarose-Digesting Enzyme^(d) (Cat.# M1741).

IV. Optimization of PCR

A. Routine PCR

As originally developed, the PCR process amplifies short (approximately 100–500bp) segments of a longer DNA molecule (1). A typical amplification reaction includes the target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer, magnesium and optional additives. The components of the reaction are mixed and placed in a thermal cycler, an automated instrument that ‘cycles’ the reaction through a predetermined series of specific temperatures and times. One cycle of amplification is defined by the series of temperature and time adjustments. Each cycle of PCR after the first cycle theoretically doubles the amount of targeted template sequence (amplimer). Therefore, ten cycles theoretically multiplies the amplimer by a factor of about one thousand; 20 cycles, by a factor of more than one million (Figure 1). PCR amplification can be completed in as little as 2 hours.

Each cycle of PCR amplification consists of a defined number of reaction steps. The steps are designed using temperature and duration time to denature the template, anneal the two oligonucleotide primers and extend the new complementary DNA strands by polymerization. These steps can be optimized for each template and primer pair combination. The target DNA is denatured by heating to 95°C or higher for 15–120 seconds. During denaturation, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA (ssDNA) template for primer annealing and polymerization (extension) by a thermostable polymerase. To anneal the oligonucleotide primers, the temperature of the next step in the cycle is reduced to approximately 40–60°C. At this temperature, the oligonucleotide primers can anneal to the ssDNA strands and serve as primers for DNA synthesis by the polymerase. This step requires approximately 30–60 seconds. Finally, to extend from the primer-bound template DNA, the reaction temperature is raised to the optimum for most thermostable DNA polymerases, which is approximately 72–74°C. Extension of the primer by the thermostable polymerase requires approximately 1 minute per kb to be amplified. Extension completes one cycle, and the next cycle begins by returning the reaction to 95°C for denaturation. After 20–40 cycles, the amplified nucleic acid may then be analyzed (e.g., for size, quantity or sequence) or it may be used in further experimental procedures (e.g., cloning or mutagenesis).

B. Magnesium Concentration

Magnesium concentration is a crucial factor affecting the performance of *Taq* DNA Polymerase. Reaction components, including template DNA, chelating agents present in the sample (e.g., EDTA or citrate), dNTPs and proteins, can affect the amount of free magnesium. In the absence of adequate free magnesium, *Taq* DNA Polymerase is inactive. Conversely, excess free magnesium reduces enzyme fidelity (3) and may increase the level of nonspecific amplification (4,5). For these reasons, it is important to empirically determine the optimal $MgCl_2$ concentration for each reaction. This is accomplished by preparing a series of reactions containing 1.5–3.0mM Mg^{2+} , in increments of 0.5mM, by adding 3, 4, 5 and 6 μ l of a 25mM $MgCl_2$ stock to 50 μ l reactions. Figure 3 shows a representative Mg^{2+} titration experiment.

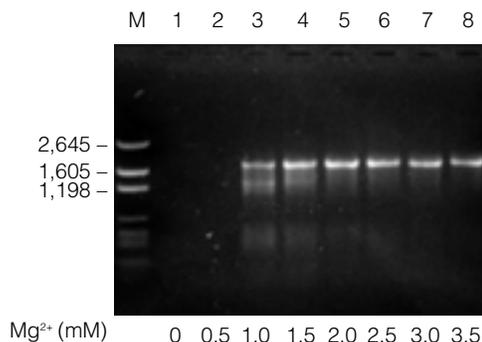


Figure 3. Effects of magnesium concentration on PCR amplification. Amplification of a 1.8kb fragment of the luciferase gene was performed using various concentrations of Mg^{2+} . PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Lane M, pGEM® DNA Markers (Cat.# G1741); lane 1, no Mg^{2+} ; lanes 2–8, increasing concentrations of Mg^{2+} in intervals of 0.5mM as indicated.

Promega's PCR Core Systems include Thermophilic DNA Polymerase 10X Buffer without $MgCl_2$, a solution of 25mM $MgCl_2$ and *Taq* DNA Polymerase 10X Buffer containing 15mM $MgCl_2$. The 10X buffer without $MgCl_2$ allows the researcher to adjust the Mg^{2+} concentration to the level that is optimal for each reaction using the 25mM $MgCl_2$ Solution. The 10X buffer containing 15mM $MgCl_2$ gives the researcher a convenient buffer resulting in a final $MgCl_2$ concentration of 1.5mM, which is optimal for many PCR methods.

Two important steps will ensure the reaction contains the appropriate amount of Mg^{2+} : **Magnesium solutions must be thawed completely and vortexed for several seconds prior to use** because magnesium chloride solutions can form concentration gradients when frozen. Thawing and vortexing is required to render the solution uniform with respect to magnesium salts. These two simple steps can eliminate a major source of many failed experiments.

Moreover, $MgCl_2$ provided in the *Taq* DNA Polymerase 10X Buffer could precipitate after multiple freeze-thaw cycles. Heating the reaction buffer to 90°C for 10 minutes restores the homogeneity of the solution (6).

C. Buffer Considerations

The PCR Core Systems are provided with *Taq* DNA Polymerase in Storage Buffer B, which is compatible with all available reaction buffers. When using Promega *Taq* DNA Polymerase in Storage Buffer A (not provided in these systems), consult the accompanying product information for reaction buffer compatibility.

D. Enzyme Choice

Several factors determine the correct enzyme(s) to use in PCR. *Taq* DNA polymerase was the first thermostable enzyme used for PCR and it is still the most popular. This polymerase exhibits relatively high processivity and is the least expensive of the commercially available polymerases. *Taq* DNA polymerase commonly generates PCR products with single deoxyadenosine (A) overhangs on the 3'-ends. These overhangs allow easy cloning into T-tailed vectors, such as the pGEM[®]-T^(e,f) Vector (Cat.# A3600 and A3610) and pGEM[®]-T Easy Vector (Cat.# A1360 and A1380) Systems^(e,f), which are linearized and modified to contain a single thymidine (T) at each 3'-end. Enzymes that exhibit 3'→5' exonuclease or proofreading activity are sometimes recommended to ensure accurate amplification of the PCR product. These enzymes (such as *Tli* and *Pfu* DNA Polymerases^(b); Cat.# M7101) normally generate blunt-end PCR products. Table 2 compares some enzymatic properties of the more common thermostable enzymes used in PCR.

Table 2. Common Thermostable Polymerases Used in PCR Amplification (7).

	<i>Taq/</i> Ampli <i>Taq</i> ®	<i>Tfi</i>	Stoffel Fragment	<i>Tth</i> (b)	VentR®/ (<i>Tli</i>)	Deep Vent™	<i>Pfu</i>	<i>Pwo</i>	UITma®
5'→3' Exonuclease Activity	Yes	Yes	No	Yes	No	No	No	No	No
3'→5' Exonuclease Activity	No	No	No	No	Yes	Yes	Yes	Yes	Yes
Reverse Transcriptase Activity	Weak	Yes	Weak	Yes	No	NA	NA	NA	Weak
Resulting DNA Ends	3' A	3' A	3' A	3' A	70% Blunt; 30% Single- Base Overhangs	>95% Blunt	Blunt	NA	>95% Blunt

NA = not available.

E. Enzyme Concentration

Promega recommends that 1.25 units of *Taq* DNA Polymerase be used per 50µl amplification reaction. For most applications, enzyme will be in excess; the inclusion of more enzyme will not significantly increase product yield. Increased amounts of enzyme and excessively long extension times increase the likelihood of generating artifacts due to the intrinsic 5'→3' exonuclease activity of *Taq* DNA Polymerase. Artifacts generally can be seen as smearing of bands in ethidium bromide-stained agarose gels (8–10).

The most frequent cause of excessive enzyme levels is pipetting error. Accurate dispensing of submicroliter (<1µl) volumes of enzyme solutions in 50% glycerol is nearly impossible. We strongly recommend the use of reaction master mixes sufficient for the number of reactions being performed to obviate this problem. A master mix increases the volumes of pipetted reagents and reduces pipetting errors.

F. Primer Design

PCR primers generally range in length from 15–30 bases and are designed to flank the region of interest. Primers should contain 40–60% (G + C) and care should be taken to avoid sequences that might produce internal secondary structure. The 3'-ends of the primers should not be complementary to avoid the production of primer-dimers. Primer-dimers unnecessarily delete primers from the reaction and result in an unwanted polymerase reaction that competes with the desired reaction. Avoid three G or C nucleotides in a row near the 3'-end of the primer as this may result in nonspecific primer annealing, increasing the synthesis of undesirable reaction products. Ideally, both primers should have nearly identical melting temperatures (T_m); in this manner, the two primers should anneal roughly at the same temperature. In any case, the annealing temperature of the reaction is dependent upon the primer with the lowest T_m .

The sequence of the primers can also include regions at the 5'-ends that may prove useful for downstream applications. For example, restriction enzyme sites can be designed into the primer pair for ease in downstream manipulations such as cloning. Regardless of primer choice, the final concentration of the primer in the reaction must be optimized. We recommend adding 50pmol of each primer (1 μ M final concentration in a 50 μ l reaction) as a starting point for optimization. Generally, nanograms of primer DNA equivalent to 50pmol is: 16.3ng \times *b*, where *b* is the number of bases in the primer.

G. Template Considerations

Successful amplification of the region of interest is dependent upon the amount and quality of the template DNA. Reagents commonly used to purify nucleic acids (e.g., salts, guanidine, proteases, organic solvents and SDS) are potent inhibitors of DNA polymerases. A final ethanol precipitation of the nucleic acid sample will eliminate most of the inhibitory agents. Spiking a control DNA fragment and the appropriate primer pair into the DNA preparation may be useful in verifying the purity of the DNA sample.

The amount of template required for successful amplification depends upon the complexity of the DNA sample. For example, whereas a 4kb plasmid containing a 1kb insert equates to 25% of the DNA being the target of interest, a 1kb gene in human genomic DNA (genome of 3.3 \times 10⁹bp) represents approximately 0.00003% of the input DNA. Therefore, approximately 1,000,000-fold more human genomic DNA is required to maintain the same number of target copies per reaction.

Two common mistakes encountered when trying to amplify target DNA are using too much plasmid DNA and too little genomic DNA. Table 3 lists the correlation of molecules per microgram of nucleic acids from some common RNA and DNA targets. Table 4 shows typical yields of genomic DNA from a variety of source materials. As a general guide for how much template DNA to use, start with a minimum of 10⁴ copies of the target sequence to obtain a signal in 25–30 cycles, but keep the final DNA concentration of the reaction \leq 10ng/ μ l.

Table 3. Conversion of Nucleic Acids from Microgram Amount to Number of Molecules.

Nucleic Acid	Amount	# of Molecules
1kb RNA	1 μ g	1.8 \times 10 ¹²
1kb dsDNA	1 μ g	9.18 \times 10 ¹¹
pGEM® Vector DNA	1 μ g	2.9 \times 10 ¹¹
lambda (λ) DNA	1 μ g	1.9 \times 10 ¹⁰
<i>E. coli</i> genomic DNA	1 μ g	2 \times 10 ⁸
human genomic DNA	1 μ g	3.0 \times 10 ⁵

Table 4. DNA Yields from Different Human Tissue Sources.

Source of DNA	Amount of Material Typically Used	Typical Yield
Purified genomic DNA	10–500ng	10–500ng
Whole blood	30µl	0.5–1µg
Blood spot	1/2 of a 5mm spot	1–3µg
Cell suspension	5 × 10 ⁵ cells	2–5µg
Buccal cells	Single mouth rinse	0.1–1µg
Chorionic villus biopsy	Small frond	1–3µg
Semen	30µl	5–10µg
Hair root	Single root	10–200ng
Tissue block	50mg	0–10µg

H. Primer Annealing Temperature

The sequences of the primers are a major consideration in determining the optimal temperature of the PCR amplification cycles. For primers with a high T_m , it may be advantageous to increase the annealing temperature. Higher temperatures minimize nonspecific primer annealing, increase the amount of specific product produced and reduce the amount of primer-dimer formation.

Numerous formulas exist to determine the theoretical T_m of nucleic acids (11,12), and these may serve as a starting point for annealing conditions. However, it is best to optimize the annealing conditions by performing the reaction at several temperatures starting approximately 5°C below the calculated T_m . The formula below can be used to estimate the melting temperature for any oligonucleotide:

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

where $[\text{Na}^+]$ is the molar concentration of monovalent cations and n = number of bases in the oligonucleotide.

As an example, to calculate the melting temperature of a 22mer oligonucleotide with 60% G+C in 50mM KCl:

$$\begin{aligned} T_m &= 81.5 + 16.6 \cdot (\log_{10}[0.05]) + 0.41 \cdot (60) - 675/22 \\ &= 81.5 + 16.6 \cdot (-1.30) + 24.60 - 30.68 \\ &= 53.84^\circ\text{C} \end{aligned}$$

I. Extension Temperature

During the extension step, allow approximately 1 minute for every 1kb to be amplified (minimum extension time of 1 minute). Generally, 25–40 cycles are sufficient for most reactions.

J. Hot Start PCR

Certain unwanted amplifications can occur in PCR, and these usually begin at room temperature once all reaction components are mixed. These unwanted reactions, such as nonspecific amplification and primer-dimer formation, can be avoided or reduced by “hot start” amplification. In general, hot start techniques limit the availability of one necessary reaction component until a temperature >60°C is reached. This can be done manually by the addition of the critical component once the reaction mixture reaches the higher temperature. However, this is tedious and can increase the chances of introducing contaminants into the reaction. Other approaches include using an antibody to inactivate the polymerase at lower temperatures. At higher temperatures, the antibody is denatured and binding is reversed, releasing a functional polymerase (13).

A convenient method for hot start PCR uses a wax bead to sequester the critical substance until the higher temperature is reached, at which point the missing component is released (14,15). Promega’s *TaqBead*[™] Hot Start Polymerase^(a) (Cat.# M5661) is ideal for hot start methods due to the retention of *Taq* DNA Polymerase in a wax bead until the amplification reaction reaches at least 60°C (16).

K. Nucleic Acid Cross-Contamination

It is important to minimize cross-contamination between samples and to prevent carryover of RNA and DNA from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipettes or aerosol-resistant tips to reduce cross-contamination during pipetting. Wear gloves and change them often. Consider using a contamination control technique (17) to prevent DNA carryover to subsequent reactions.

V. Using Controls in PCR

The Positive Control Plasmid DNA template included with the PCR Core System II can be used in several formats to ensure PCR performance. The control template can be used at a defined concentration or it may be titrated into reactions to ensure the sensitivity of the system under reaction conditions of the user’s choice. Using the reaction conditions and parameters as suggested in the system and the cycling profile indicated in Figure 2, the expected 323bp amplicon can be detected following amplification of as few as 1×10^2 copies of the control template. Using the control template in this manner, one may deduce how alternative reaction conditions or cycling profile changes might affect amplification efficiency.

An alternative use of the Positive Control is to test the compatibility of a DNA sample with PCR conditions. In this manner, the control template is titrated into reactions that contain a fixed amount of test or sample DNA. Successful amplification of the control template will indicate that the genomic DNA sample is compatible with the reaction conditions used (i.e., inhibitory agents are not present). This control is especially useful when attempting to amplify DNA isolated in the presence of high concentrations of denaturants. A decrease in the PCR amplification of the control template in the presence of sample DNA more than likely suggests that inhibitory agents are in the sample and that further purification is required.

Table 5 lists component volumes for setting up a control reaction. These conditions should serve as an excellent starting point. For a complete discussion on using internal controls in PCR, see reference 18.



Completely thaw and thoroughly vortex the MgCl₂, 25mM Solution, the buffers and any frozen solutions containing DNA.

Table 5. Recommended Reaction Volumes and Final Concentrations When Using the Controls of the PCR Core System II.

Component	Final Volume	Component Concentration
MgCl ₂ , 25mM Solution	3µl	1.5mM
Thermophilic DNA Polymerase 10X Buffer, MgCl ₂ -Free	5µl	1.0X
PCR Nucleotide Mix, 10mM	1µl	0.2mM
Upstream Control Primer, 15µM	3.3µl	1.0µM
Downstream Control Primer, 15µM	3.3µl	1.0µM
Taq DNA Polymerase, 5u/µl	0.25µl	1.25u/50µl
Positive Control Plasmid DNA, 1ng/µl	*1µl	1ng/50µl
Nuclease-Free Water to a final volume of	50µl	

*One nanogram of the Positive Control Plasmid is equivalent to 2.2 × 10⁸ molecules. The 323bp control amplification product is routinely observed when starting with <10⁵ molecules.

Step	Temperature	Time	Number of Cycles
Initial Denaturation:	95°C	2 minutes	1 cycle
Denaturation:	95°C	0.5 minute	35 cycles
Annealing:	60°C	1 minute	
Extension:	72°C	2 minutes	
Final Extension:	72°C	5 minutes	1 cycle
Soak:	4°C	indefinite	1 cycle

Figure 4. Recommended thermal cycling conditions for the Controls of the PCR Core System II.

VI. Troubleshooting PCR

Symptoms	Possible Causes	Comments
Low yield or no amplification	Insufficient number of cycles	Return reactions to thermal cycler for 5 or more cycles.
	Template degraded	Verify the integrity of the DNA by electrophoresis after incubation in the presence of Mg ²⁺ .
	Thermal cycler programmed incorrectly	Verify that times and temperatures are correct. Use step cycles, not hold segments.
	Temperature too low in some positions of thermal cycler	Perform a set of control reactions to determine whether certain positions in the thermal cycler give low yields.
	Top of thermal cycler is open	The top must be closed for correct heating and cooling.

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com.

E-mail: techserv@promega.com

VI. Troubleshooting PCR (continued)

Symptoms	Possible Causes	Comments
Low yield or no amplification (continued)	Inhibitor present	Reduce the volume of sample in the reaction. Ethanol precipitate to remove inhibitors.
	Improper reaction conditions	Reduce the annealing temperature and/or allow longer extension times for longer amplimers. Optimize Mg ²⁺ concentration.
	Missing reaction component	Check the reaction components and repeat the reaction.
	Mineral oil problem	The reaction must be overlaid with high-quality, nuclease-free light mineral oil. Do not use autoclaved mineral oil.
	Reaction tubes not autoclaved	Autoclaving tubes eliminates some contaminants that inhibit amplification.
	Poor primer design	Make sure primers are not self-complementary or complementary to each other. Try a longer primer.
	Incorrect primer specificity	Verify that the primers are complementary to the appropriate strands.
	Primer concentration too low	Verify primer concentration in the reaction. Increase primer concentration in the reaction.
	Suboptimal reaction conditions	Optimize Mg ²⁺ concentration, annealing temperature and extension time. Always vortex the Mg ²⁺ . Verify that primers are present in equal concentrations.
	Nucleotides or primers	Keep nucleotides and primers frozen in degraded aliquots, thaw quickly and keep on ice once thawed. Avoid multiple freeze-thaw cycles.
Multiple, nonspecific amplification products	Target sequence not present in target DNA	Redesign experiment or try other sources of target DNA.
	Suboptimal reaction conditions	Optimize MgCl ₂ concentration, annealing temperature, size extension time and cycle number to minimize nonspecific priming.
	Poor primer design	Make sure primers are not self-complementary or complementary to each other, especially near the 3'-ends. Try a longer primer. Avoid using three consecutive G or C nucleotides at the 3'-end of the primers.

VI. Troubleshooting PCR (continued)

Symptoms	Possible Causes	Comments
Multiple, nonspecific amplification products (continued)	Primer concentration too high	Verify primer concentration in the reaction. Try a lower concentration in the reaction.
	Contamination by another target 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 post-amplification. Wear gloves and change them often. Use UNG ^(f) (14) or another contamination control technique to prevent DNA carryover to subsequent reactions.
	Multiple target sequences exist in target DNA	Design new primers with higher specificity to target sequence.

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VIII. Appendix

A. Composition of Buffers and Solutions

All of the following reagents are provided in PCR Core System II. The Positive Control Primers, Upstream and Downstream, and Positive Control Plasmid DNA are provided only in PCR Core System II.

Taq DNA Polymerase 10X Buffer with 15mM MgCl₂

500mM KCl
 100mM Tris-HCl (pH 9.0 at 25°C)
 1.0% Triton® X-100
 15mM MgCl₂

Thermophilic DNA Polymerase 10X Reaction Buffer, MgCl₂-Free

500mM KCl
 100mM Tris-HCl (pH 9.0 at 25°C)
 1.0% Triton® X-100

PCR Nucleotide Mix, 10mM

Composed of 10mM each of dATP, dCTP, dGTP and dTTP in water.

Positive Control Plasmid DNA

Positive Control Plasmid DNA is supplied at 1ng/μl in TE buffer (pH 7.4).

Positive Control Primers, Upstream and Downstream

Upstream: 5´-(GCC ATT CTC ACC GGA TTC AGT CGT C)-3´

Downstream: 5´-(AGC CGC CGT CCC GTC AAG TCA G)-3´

The Control Primers, 15μM each, are provided in Nuclease-Free Water.

Taq DNA Polymerase Storage Buffer B

20mM Tris-HCl (pH 8.0)
 100mM KCl
 0.1mM EDTA
 1mM DTT
 50% glycerol
 0.5% Nonidet®-P40
 0.5% Tween®-20

B. Related Products

Thermostable DNA Polymerases

Product	Conc. (u/μl)	Size	Cat.#
<i>Taq</i> ^(b) DNA Polymerase in Storage Buffer B	5	100u	M1661
	5	500u	M1665
	5	2,500u	M1668

For Laboratory Use. The above items include 10X Reaction Buffer without MgCl₂ as well as MgCl₂, 25mM Solution.

Product	Conc. (u/μl)	Size	Cat.#
<i>Taq</i> ^(b) DNA Polymerase in Storage Buffer B	5	100u	M2661
	5	500u	M2665
	5	2,500u	M2668

For Laboratory Use. The above items include 10X Reaction Buffer with 15mM MgCl₂.

Product	Size	Cat.#
<i>Taq</i> Bead™ Hot Start Polymerase ^(a) , 1.25 units/bead, Nonbarrier	100 reactions	M5661

Product	Conc. (u/μl)	Size	Cat.#
<i>Tfi</i> ^(b) DNA Polymerase	5	100u	M1941
	5	1,000u	M1945
<i>Tli</i> DNA Polymerase	3	50u	M7101
<i>Tth</i> ^(b) DNA Polymerase	5	100u	M2101
	5	500u	M2105
<i>Pfu</i> DNA Polymerase*	2–3	100u	M7741
	2–3	500u	M7745

*For Research Use Only.
For Laboratory Use.

Markers

Product	Size	Cat.#
100bp DNA Ladder	250μl	G2101
1kb DNA Ladder	500μl	G5711

For Laboratory Use.

Reverse Transcription and RT-PCR

Product	Size	Cat.#
Access RT-PCR System ^(a,h)	100 reactions	A1250
Access RT-PCR Introductory System ^(a,h)	20 reactions	A1260
Reverse Transcription System ^(h,i)	100 reactions	A3500
AMV Reverse Transcriptase	300u	M5101
M-MLV Reverse Transcriptase	10,000u	M1701
M-MLV Reverse Transcriptase, RNase H Minus	10,000u	M5301
M-MLV Reverse Transcriptase, RNase H Minus Point Mutation	10,000	M3682
	50,000	M3683
Recombinant RNasin® Ribonuclease Inhibitor ^(h,i)	2,500u	N2511
RQ1 RNase-Free DNase	1,000u	M6101

For Laboratory Use.

C. Supplementary Bibliography

The following references provide additional information on various types of PCR to supplement the basic information presented in this technical bulletin.

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PCR Core Systems: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Sections III.A–B) the first time you use the PCR Core Systems.

PCR Protocol (Section III.A)	<ol style="list-style-type: none"> 1. Combine the following components in a sterile 0.5–0.6ml microcentrifuge tube. The reaction volume can be scaled as long as the final concentrations remain constant. <table border="1" style="width: 100%; border-collapse: collapse; margin: 10px 0;"> <thead> <tr style="background-color: #e0e0e0;"> <th style="text-align: left; padding: 5px;">Component</th> <th style="text-align: center; padding: 5px;">Component Volume</th> <th style="text-align: center; padding: 5px;">Final Concentration</th> </tr> </thead> <tbody> <tr> <td style="padding: 5px;">MgCl₂, 25mM Solution</td> <td style="text-align: center; padding: 5px;">3μl</td> <td style="text-align: center; padding: 5px;">1.5mM</td> </tr> <tr> <td style="padding: 5px;">Thermophilic DNA Polymerase 10X Reaction Buffer, MgCl₂-Free</td> <td style="text-align: center; padding: 5px;">5μl</td> <td style="text-align: center; padding: 5px;">1.0X</td> </tr> <tr> <td style="padding: 5px;">PCR Nucleotide Mix, 10mM each</td> <td style="text-align: center; padding: 5px;">1μl</td> <td style="text-align: center; padding: 5px;">200μM each</td> </tr> <tr> <td style="padding: 5px;">Upstream Control Primer</td> <td style="text-align: center; padding: 5px;">5–50pmol</td> <td style="text-align: center; padding: 5px;">0.1–1.0μM</td> </tr> <tr> <td style="padding: 5px;">Downstream Control Primer</td> <td style="text-align: center; padding: 5px;">5–50pmol</td> <td style="text-align: center; padding: 5px;">0.1–1.0μM</td> </tr> <tr> <td style="padding: 5px;"><i>Taq</i> DNA Polymerase, 5u/μl</td> <td style="text-align: center; padding: 5px;">0.25μl</td> <td style="text-align: center; padding: 5px;">1.25u/50μl</td> </tr> <tr> <td style="padding: 5px;">Template DNA</td> <td style="text-align: center; padding: 5px;"><u>variable</u></td> <td style="text-align: center; padding: 5px;"><0.5μg/50μl</td> </tr> <tr> <td style="padding: 5px;">Nuclease-Free Water to a final volume of</td> <td style="text-align: center; padding: 5px;">50μl</td> <td></td> </tr> </tbody> </table> <ol style="list-style-type: none"> 2. If using a thermal cycler without a heated lid, overlay the reactions with 1–2 drops of mineral oil and centrifuge briefly. 3. Place the reactions in a thermal cycler that has been preheated to 95°C and incubate for 2 minutes. 4. Start the thermal cycling program. The cycling profile given below may be used as a guideline. We recommend optimizing the cycling profile for each primer:target combination. <table border="1" style="width: 100%; border-collapse: collapse; margin: 10px 0;"> <thead> <tr> <th style="text-align: left; padding: 5px;">Step</th> <th style="text-align: center; padding: 5px;">Temperature</th> <th style="text-align: center; padding: 5px;">Time</th> <th style="text-align: center; padding: 5px;">Number of Cycles</th> </tr> </thead> <tbody> <tr> <td style="padding: 5px;">Initial Denaturation:</td> <td style="text-align: center; padding: 5px;">95°C</td> <td style="text-align: center; padding: 5px;">2 minutes</td> <td style="text-align: center; padding: 5px;">1 cycle</td> </tr> <tr> <td style="padding: 5px;">Denaturation:</td> <td style="text-align: center; padding: 5px;">95°C</td> <td style="text-align: center; padding: 5px;">0.5–1 minute</td> <td rowspan="3" style="text-align: center; vertical-align: middle; padding: 5px;">25–35 cycles</td> </tr> <tr> <td style="padding: 5px;">Annealing:</td> <td style="text-align: center; padding: 5px;">42–65°C</td> <td style="text-align: center; padding: 5px;">1 minute</td> </tr> <tr> <td style="padding: 5px;">Extension:</td> <td style="text-align: center; padding: 5px;">72–74°C</td> <td style="text-align: center; padding: 5px;">1–2 minutes</td> </tr> <tr> <td style="padding: 5px;">Final Extension:</td> <td style="text-align: center; padding: 5px;">72–74°C</td> <td style="text-align: center; padding: 5px;">5 minutes</td> <td style="text-align: center; padding: 5px;">1 cycle</td> </tr> <tr> <td style="padding: 5px;">Soak:</td> <td style="text-align: center; padding: 5px;">4°C</td> <td style="text-align: center; padding: 5px;">indefinite</td> <td style="text-align: center; padding: 5px;">1 cycle</td> </tr> </tbody> </table>	Component	Component Volume	Final Concentration	MgCl ₂ , 25mM Solution	3μl	1.5mM	Thermophilic DNA Polymerase 10X Reaction Buffer, MgCl ₂ -Free	5μl	1.0X	PCR Nucleotide Mix, 10mM each	1μl	200μM each	Upstream Control Primer	5–50pmol	0.1–1.0μM	Downstream Control Primer	5–50pmol	0.1–1.0μM	<i>Taq</i> DNA Polymerase, 5u/μl	0.25μl	1.25u/50μl	Template DNA	<u>variable</u>	<0.5μg/50μl	Nuclease-Free Water to a final volume of	50μl		Step	Temperature	Time	Number of Cycles	Initial Denaturation:	95°C	2 minutes	1 cycle	Denaturation:	95°C	0.5–1 minute	25–35 cycles	Annealing:	42–65°C	1 minute	Extension:	72–74°C	1–2 minutes	Final Extension:	72–74°C	5 minutes	1 cycle	Soak:	4°C	indefinite	1 cycle
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Analysis (Section III.B)	<ol style="list-style-type: none"> 1. Analyze PCR products by agarose gel electrophoresis. The products should be readily visible in an ethidium bromide-stained gel illuminated with UV light. 2. Store PCR products at –20°C. The PCR products can be further purified using the Wizard® PCR Preps DNA Purification System or AgarACE® Agarose-Digesting Enzyme, among others. 																																																					