

Enzymatic Amplification of DNA by PCR: Standard Procedures and Optimization

UNIT 15.1

**BASIC
PROTOCOL**

This unit describes a method for amplifying DNA enzymatically by the polymerase chain reaction (PCR), including procedures to quickly determine conditions for successful amplification of the sequence and primer sets of interest, and to optimize for specificity, sensitivity, and yield. The first step of PCR simply entails mixing template DNA, two appropriate oligonucleotide primers, *Taq* or other thermostable DNA polymerases, deoxyribonucleoside triphosphates (dNTPs), and a buffer. Once assembled, the mixture is cycled many times (usually 30) through temperatures that permit denaturation, annealing, and synthesis to exponentially amplify a product of specific size and sequence. The PCR products are then displayed on an appropriate gel and examined for yield and specificity.

Many important variables can influence the outcome of PCR. Careful titration of the MgCl_2 concentration is critical. Additives that promote polymerase stability and processivity or increase hybridization stringency, and strategies that reduce nonspecific primer-template interactions, especially prior to the critical first cycle, generally improve amplification efficiency. This protocol, using *Taq* DNA polymerase (see UNIT 3.5), is designed to optimize the reaction components and conditions in one or two stages. The first stage (steps 1 to 7) determines the optimal MgCl_2 concentration and screens several enhancing additives. Most suppliers (of which there are many) of *Taq* and other thermostable DNA polymerases provide a unique optimized MgCl_2 -free buffer with MgCl_2 in a separate vial for user titration. The second stage (steps 8 to 13) compares methods for preventing pre-PCR low-stringency primer extension, which can generate nonspecific products. This has come to be known as “hot start,” whether one omits an essential reaction component prior to the first denaturing-temperature step or adds a reversible inhibitor of polymerase. Hot-start methods can greatly improve specificity, sensitivity, and yield. Use of any one of the hot-start approaches is strongly recommended if primer-dimers or other nonspecific products are generated, or if relatively rare template DNA is contained in a complex mixture, such as viral nucleic acids in cell or tissue preparations. This protocol suggests some relatively inexpensive methods to achieve hot start, and lists several commercial hot-start options which may be more convenient, but of course more expensive.

NOTE: Use only molecular biology-grade water (i.e., DNase, RNase, and nucleic acid free) in all steps and solutions.

Materials

- 10× MgCl_2 -free PCR buffer (see recipe)
- 50 μM oligonucleotide primer 1: 50 pmol/ μl in sterile H_2O (store at -20°C)
- 50 μM oligonucleotide primer 2: 50 pmol/ μl in sterile H_2O (store at -20°C)
- Template DNA: 1 μg mammalian genomic DNA or 1.0 to 100.0 pg of plasmid DNA (UNIT 2.1-2.4)
- 25 mM 4dNTP mix (see recipe)
- 5 U/ μl *Taq* DNA polymerase (native or recombinant)
- Enhancer agents (optional; see recipe)
- 15 mM (L), 30 mM (M), and 45 mM (H) MgCl_2

Mineral oil

TaqStart Antibody (Clontech)

- Ficoll 400 (optional): prepare as 10× stock; store indefinitely at room temperature
- Tartrazine dye (optional): prepare as 10× stock; store indefinitely at room temperature

**The Polymerase
Chain Reaction**

15.1.1

Table 15.1.1 Master Mixes for Optimizing Reaction Components

Components	Final concentration	Per reaction	Master mix ^a (μl)			
			I	II	III	IV
10× MgCl ₂ -free PCR buffer	1×	10 μl	40.0	40.0	40.0	40.0
Primer 1	0.5 μM	1 μl	4.0	4.0	4.0	4.0
Primer 2	0.5 μM	1 μl	4.0	4.0	4.0	4.0
Template DNA ^b	Undiluted	1 vol	4 vol	4 vol	4 vol	4 vol
25 mM 4dNTP mix ^c	0.2 mM	0.8 μl	3.2	3.2	3.2	3.2
<i>Taq</i> polymerase	2.5 U	0.5 μl	2.0	2.0	2.0	2.0
DMSO ^d (20×)	5%	5 μl	—	20.0	—	—
Glycerol ^d (10×)	10%	10 μl	—	—	40.0	—
PMPE ^d (100×)	1%	1 μl	—	—	—	4.0
H ₂ O	—	To 90 μl	To 360	To 360	To 360	To 360

^aTotal volume = 360 μl (i.e., enough for $n + 1$ reactions).

^bTemplate DNA volume (“vol”) is generally 1 to 10 μl.

^cIf 2 mM 4dNTP mix is preferred, use 10 μl per reaction, or 40 μl for each master mix; adjust the volume of water accordingly.

^dSubstitute with other enhancer agents (see recipe in Reagents and Solutions) as available.

0.5 ml thin-walled PCR tubes

Automated thermal cycler

Additional reagents and equipment for DNA preparation (UNIT 2.1-2.4, agarose gel electrophoresis (UNIT 2.5A), nondenaturing PAGE (UNIT 2.7), or sieving agarose gel electrophoresis (UNIT 2.8), restriction endonuclease digestion (UNIT 3.1), and Southern blotting and hybridization (UNITS 2.9 & 6.4)

NOTE: Do not use DEPC to treat water, reagents, or glassware.

NOTE: Reagents should be prepared in sterile, disposable labware, taken directly from its packaging, or in glassware that has been soaked in 10% bleach, thoroughly rinsed in tap water followed by distilled water, and if available, exposed to UV irradiation for ~10 min. Multiple small volumes of each reagent should be stored in screw-cap tubes. This will then serve as the user’s own optimization “kit.” Thin-walled PCR tubes are recommended.

Optimize reaction components

1. Prepare four reaction master mixes according to the recipes given in Table 15.1.1.

Enhancing agents probably work by different mechanisms, such as protecting enzyme activity and decreasing nonspecific primer binding. However, their effects cannot be readily predicted—what improves amplification efficiency for one primer pair may decrease the amplification efficiency for another. Thus it is best to check a panel of enhancers during development of a new assay.

2. Aliquot 90 μl master mix I into each of three 0.5-ml thin-walled PCR tubes labeled I-L, I-M, and I-H. Similarly, aliquot mixes II through IV into appropriately labeled tubes. Add 10 μl of 15 mM MgCl₂ into one tube of each master mix (labeled L; 1.5 mM final). Similarly, aliquot 10 μl of 30 mM and 45 mM MgCl₂ to separate tubes of each master mix (labeled M and H, respectively; 3.0 and 4.5 mM final concentrations respectively).

It is helpful to set the tubes up in a three-by-four array to simplify aliquotting. Each of the three Mg²⁺ concentrations is combined with each of the four master mixes.

3. Overlay the reaction mixture with 50 to 100 µl mineral oil (2 to 3 drops).

To include hot start in the first step, overlay reaction mixes with oil before adding the MgCl₂, heat the samples to 95°C in the thermal cycler or other heating block, and add the MgCl₂ once the elevated temperature is reached. Once the MgCl₂ has been added, do not allow the samples to cool below the optimum annealing temperature prior to performing PCR.

Alternatives to mineral oil include silicone oil and paraffin beads. Additionally, certain cyclers feature heated lids that are designed to obviate the need for an oil overlay.

Choose cycling parameters

4. Using the following guidelines, program the automated thermal cycler according to the manufacturer's instructions.

30 cycles: 30 sec	94°C	(denaturation)
30 sec	55° (GC content ≤50%) or	
	60°C (GC content >50%)	(annealing)
~60 sec/kb		
product sequence	72°C	(extension)

Cycling parameters are dependent upon the sequence and length of the template DNA, the sequence and percent complementarity of the primers, and the ramp times of the thermal cycler used. Thoughtful primer design will reduce potential problems (see Commentary). Denaturation, annealing, and extension are each quite rapid at the optimal temperatures. The time it takes to achieve the desired temperature inside the reaction tube (i.e., the ramp time) is usually longer than either denaturation or primer annealing. Thus, ramp time is a crucial cycling parameter. Manufacturers of the various thermal cyclers on the market provide ramp time specifications for their instruments. Ramp times are lower with thin-walled reaction tubes. The optimal extension time also depends on the length of the target sequence. Allow ~1 min/kb for this step for target sequences >1 kb, and as little as a 2-sec pause for targets <100 bases in length.

The number of cycles depends on both the efficiency of the reaction and the amount of template DNA in the reaction. Starting with as little as 100 ng of mammalian genomic DNA (~10⁴ cell equivalents), after 30 cycles, 10% of the reaction should produce a band that is readily visible on an ethidium bromide-stained gel as a single predominant band. With more template, fewer cycles may suffice. With much less template, further optimization is recommended rather than increasing the cycle number. Greater cycle numbers (e.g., >40) can reduce the polymerase specific activity, increase nonspecific amplification, and deplete substrate (nucleotides). Many investigators lengthen the time for the last extension step—to 7 min, for example—to try to ensure that all the PCR products are full length.

These guidelines are appropriate for most commercially available thermal cyclers. For rapid cyclers, consult the manufacturers' protocols.

Analyze the product

5. Electrophorese 10 µl from each reaction on an agarose (UNIT 2.5A), nondenaturing polyacrylamide (UNIT 2.7), or sieving agarose gel (UNIT 2.8) appropriate for the PCR product size expected. Stain with ethidium bromide.

For resolution of PCR products between 100 and 1000 bp, an alternative to nondenaturing polyacrylamide gels or sieving agarose is a composite 3% (w/v) NuSieve (FMC Bioproducts) agarose/1% (w/v) SeaKem (FMC Bioproducts) agarose gel. SeaKem increases the mechanical strength of the gel without decreasing resolution.

An alternative to ethidium bromide, SYBR Gold Nucleic Acid Gel Stain (Molecular Probes), is 25 to 100 times more sensitive than ethidium bromide, is more convenient to use, and permits optimization of 10- to 100-fold lower starting template copy number.

Table 15.1.2 Master Mixes for Optimizing First-Cycle Reactions

Components	Final concentration	Master mix (μl)			
		A	B	C	D
10× PCR buffer	1×	10	10	10	10
MgCl ₂ (L, M, or H)	Optimal	10	10	10	10
Primer 1	0.5 μM	1.0	1.0	1.0	1.0
Primer 2	0.5 μM	1.0	1.0	1.0	1.0
Additive	Optimal	V ^a	V ^a	V ^a	V ^a
Template DNA	— ^b	V ^a	V ^a	V ^a	V ^a
25 mM 4dNTP mix ^b	0.2 mM	0.8	0.8	0.8	0.8
Taq polymerase	2.5 U	0.5	0.5	—	—
Taq pol + TaqStart	2.5 U	—	—	—	1.0
H ₂ O	To 100 μl	V ^a	V ^a	V ^a	V ^a
Preparation temperature		Room temperature	Ice slurry	Room temperature	Room temperature

^aV, variable amount (total volume should be 100 μl).

^bUse undiluted or diluted template DNA based on results obtained in step 6.

- Examine the stained gel to determine which condition resulted in the greatest amount of product.

Minor, nonspecific products may be present even under optimal conditions.

- To ensure that the major product is the correct one, digest an aliquot of the reaction with a restriction endonuclease known to cut within the PCR product. Check buffer compatibility for the restriction endonuclease of choice. If necessary, add Na⁺ or precipitate in ethanol (UNIT 2.1A) and resuspend in the appropriate buffer. Electrophore the digestion product on a gel to verify that the resulting fragments have the expected sizes.

Alternatively, transfer the PCR products to a nitrocellulose or nylon filter and hybridize with an oligonucleotide derived from the sequence internal to the primers (UNITS 2.9 & 6.4). With appropriately stringent hybridization and washing conditions, only the correct product (and possibly some minor related products) should hybridize.

Optimize the first cycle

These optional steps optimize initial hybridization and may improve efficiency and yield. They are used when primer-dimers and other nonspecific products are detected, when there is only a very small amount of starting template, or when a rare sequence is to be amplified from a complex mixture. For an optimal reaction, polymerization during the initial denaturation and annealing steps should be prevented. Taq DNA polymerase activity can be inhibited by temperature (reaction B), physical separation (reaction C), or reversible antibody binding (reaction D). PCR without hot start is performed for comparison (reaction A).

- Prepare four reaction mixtures using the optimal MgCl₂ concentration and additive requirement determined in step 6. Prepare the mixes according to the recipes in Table 15.1.2. Use the following variations for addition of Taq polymerase.

- Prepare reactions A and C at room temperature.
- Chill all components of reaction B in an ice slurry before they are combined.

- c. For reaction D, combine 1.0 μ l TaqStart antibody with 4.0 μ l of the dilution buffer provided with the antibody, add 1.0 μ l *Taq* DNA polymerase (for 1:4:1 mixture of these components), mix, and incubate 5 to 10 min at room temperature before adding to reaction mixture D (glycerol and PMPE are compatible with TaqStart antibody but DMSO will interfere with antibody binding).

To ensure that the reaction does not plateau and thereby obfuscate the results, use the smallest amount of template DNA necessary for visualization of the PCR product by ethidium bromide staining. Use the results from step 6 to decide how much template to use. If the desired product stains intensely, dilute the starting material as much as 1/100. If only a faint signal is apparent, use undiluted sample.

9. Overlay each reaction mixture with 50 to 100 μ l mineral oil.
10. Heat all reactions 5 min at 94°C.

It is most convenient to use the automated thermal cycler for this step and then initiate the cycling program directly.

11. Cool the reactions to the appropriate annealing temperature as determined in step 4. Add 0.5 μ l *Taq* DNA polymerase to reaction C, making sure the pipet tip is inserted through the layer of mineral oil into the reaction mix.

*Time is also an important factor in this step. If the temperature drops below the annealing temperature and is allowed to remain low, nonspecific annealing will occur. *Taq* DNA polymerase retains some activity even at room temperature.*

12. Begin amplification of all four reactions at once, using the same cycling parameters as before.
13. Analyze the PCR products on an agarose gel and evaluate the results as in steps 5 and 6.
14. Prepare a batch of the optimized reaction mixture, but omit *Taq* DNA polymerase, TaqStart antibody, PMPE, and 4dNTP mix—these ingredients should be added fresh just prior to use. If desired, add Ficoll 400 to a final concentration of 0.5% to 1% (v/v) and tartrazine to a final concentration of 1 mM.

Adding Ficoll 400 and tartrazine dye to the reaction mix precludes the need for a gel loading buffer and permits direct application of PCR products to agarose or acrylamide gels. At these concentrations, Ficoll 400 and tartrazine do not decrease PCR efficiency and do not interfere with PMPE or TaqStart antibodies. Other dyes, such as bromphenol blue and xylene cyanol, do inhibit PCR. Tartrazine is a yellow dye and is not as easily visualized as other dyes; this may make gel loading more difficult.

Ficoll 400 and tartrazine dye may be prepared as 10 \times stocks and stored indefinitely at room temperature.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Enhancer agents

For a discussion of how to select enhancer agents, see Commentary.

5 \times stocks:

25% acetamide (20 μ l/reaction; 5% final)

5 M *N,N,N*-trimethylglycine (betaine; 20 μ l/reaction; 1 M final)

40% polyethylene glycol (PEG) 8000 (20 μ l/reaction; 8% final)

10× stocks:

Glycerol (concentrated; 10 µl/reaction; 10% final)

20× stocks:

Dimethylsulfoxide (DMSO; concentrated; 5 µl/reaction; 5% final)

Formamide (concentrated; 5 µl/reaction; 5% final)

100× stocks:

1 U/µl Perfect Match Polymerase Enhancer (Stratagene; 1 µl/reaction; 1 U final)

10 mg/ml acetylated bovine serum albumin (BSA) or gelatin (1 µl/reaction; 10 µg/ml final)

1 to 5 U/µl thermostable pyrophosphatase (PPase; Roche Diagnostics; ; 1 µl/reaction; 1 to 5 U final)

5 M tetramethylammonium chloride (TMAC; betaine hydrochloride; 1 µl/reaction; 50 mM final)

0.5 mg/ml *E. coli* single-stranded DNA-binding protein (SSB; Sigma; 1 µl/reaction; 5 µg/ml final)

0.5 mg/ml Gene 32 protein (Amersham Pharmacia Biotech; 1 µl/reaction; 5 µg/ml final)

10% Tween 20, Triton X-100, or Nonidet P-40 (1 µl/reaction; 0.1% final)

1 M (NH₄)₂SO₄ (1 µl/reaction; 10 mM final; use with thermostable DNA polymerases other than *Taq*)

MgCl₂-free PCR buffer, 10×

500 mM KCl

100 mM Tris·Cl, pH 9.0 (at 25°C; see APPENDIX 2)

0.1% Triton X-100

Store indefinitely at −20°C

This buffer can be obtained from Promega; it is supplied with Taq DNA polymerase.

4dNTP mix

For 2 mM 4dNTP mix: Prepare 2 mM each dNTP in TE buffer, pH 7.5 (APPENDIX 2). Store up to 1 year at −20°C in 1-ml aliquots.

For 25 mM 4dNTP mix: Combine equal volumes of 100 mM dNTPs (Promega). Store indefinitely at −20°C in 1-ml aliquots.

COMMENTARY

Background Information

The theoretical basis of the polymerase chain reaction (PCR; see chapter introduction) was probably first described in a paper by Kleppe et al. (1971). However, this technique did not excite general interest until the mid-1980s, when Kary Mullis and co-workers at Cetus developed PCR into a technique that could be used to generate large amounts of single-copy genes from genomic DNA (Saiki et al., 1985, 1986; Mullis et al., 1986; Embury et al., 1987).

The initial procedure entailed adding a fresh aliquot of the Klenow fragment of *E. coli* DNA polymerase I during each cycle because this enzyme was inactivated during the subsequent denaturation step. The introduction of thermostable *Taq* DNA polymerase from *Thermus*

aquaticus (Saiki et al., 1988) alleviated this tedium and facilitated automation of the thermal cycling portion of the procedure. *Taq* DNA polymerase also permitted the use of higher temperatures for annealing and extension, which improved the stringency of primer–template hybridization and thus the specificity of the products. This also served to increase the yield of the desired product.

All applications of PCR depend upon an optimized PCR. The basic protocol in this unit optimizes PCR for several variables, including MgCl₂ concentration, enhancing additives—dimethyl sulfoxide (DMSO), glycerol, or Perfect Match Polymerase Enhancer (PMPE)—and prevention of pre-PCR mispriming. These and other parameters can be extremely impor-

tant, as every element of PCR can affect the outcome (see Critical Parameters and Troubleshooting for discussion of individual parameters).

There are several PCR optimization kits and proprietary enhancers on the market (Table 15.1.3). Optimization kits generally provide a panel of buffers in which the pH, buffer, non-ionic detergents, and addition of $(\text{NH}_4)_2\text{SO}_4$ are varied, MgCl_2 may be added at several concentrations, and enhancers (e.g., DMSO, glycerol, formamide, betaine, and/or proprietary compounds) may be chosen. The protocol presented here is aimed at keeping the costs low and the options broad.

Critical Parameters and Troubleshooting

MgCl₂ concentration

Determining the optimum MgCl_2 concentration, which can vary even for different primers from the same region of a given template (Saiki, 1989), can have an enormous influence on PCR success. In this protocol three concentrations are tested—1.5 mM (L), 3.0 mM (M), and 4.5 mM (H)—against three enhancers. Enhancers tend to broaden the MgCl_2 optimal range, contributing to the success of the PCR at one of these concentrations. A 10× buffer optimized for a given enzyme and a separate vial of MgCl_2 are typically provided with the polymerase, so that the user may titrate the MgCl_2 concentration for their unique primer-template set.

Reagent purity

For applications that amplify rare templates, reagent purity is the most important parameter, and avoiding contamination at every step is critical.

To maintain purity, store multiple small volumes of each reagent in screw-cap tubes.

For many applications, simply using high-quality reagents and avoiding nuclease contamination is sufficient; however, avoid one common reagent used to inactivate nucleases, diethylpyrocarbonate (DEPC). Even tiny amounts of chemical left after treatment of water by autoclaving are enough to ruin a PCR.

Primer selection

This is the factor that is least predictable and most difficult to troubleshoot. Simply put, some primers just do not work. To maximize the probability that a given primer pair will

work, pay attention to the following parameters.

General considerations. An optimal primer set should hybridize efficiently to the sequence of interest with negligible hybridization to other sequences present in the sample. If there are reasonable amounts of template available, hybridization specificity can be tested by performing oligonucleotide hybridization as described in UNIT 6.4. The distance between the primers is rather flexible, ranging up to 10 kb. There can be, however, a considerable drop-off in synthesis efficiency with distances >3 kb (Jeffreys et al., 1988). Small distances between primers, however, lessen the ability to obtain much sequence information or to reamplify with nested internal oligonucleotides, should that be necessary.

Design primers to allow demonstration of the specificity of the PCR product. Be sure that there are diagnostic restriction endonuclease sites between the primers or that an oligonucleotide can detect the PCR product specifically by hybridization.

Several computer programs can assist in primer design (see Internet Resources at end of unit). These are most useful for avoiding primer sets with intra- and intermolecular complementarity, which can dramatically raise the effective T_m . Given the abundance of primers relative to template, this can preclude template priming. Computer primer design is not foolproof. If possible, start with a primer or primer set known to efficiently prime extensions. In addition, manufacturers' Web sites offer technical help with primer design.

Complementarity to template. For many applications, primers are designed to be exactly complementary to the template. For others, however, such as engineering of mutations or new restriction endonuclease sites, or for efforts to clone or detect gene homologs where sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. It is best to have mismatches (e.g., in a restriction endonuclease linker) at the 5' end of the primer. The closer a mismatch is to the 3' end of the primer, the more likely it is to prevent extension.

The use of degenerate oligonucleotide primers to clone genes where only protein sequence is available, or to fish out gene homologs in other species, has sometimes been successful, but it has also failed an untold (and unpublished) number of times. When the reaction works it can be extremely valuable, but it can

Table 15.1.3 PCR Optimization Products

Optimization goal	Supplier	Product
Optimization support	Perkin-Elmer	Technical information in appendix to catalog
Optimization support	Promega	PCR troubleshooting program on the Internet: http://www.promega.com/amplification/assistant
Optimization kits	Boehringer-Mannheim, Invitrogen, Stratagene, Sigma, Epicentre Technologies, Life Technologies	Several buffers, Mg ²⁺ , and enhancers which may include DMSO, glycerol, formamide, (NH ₄) ₂ SO ₄ , and other unspecified or proprietary agents
Quick startup	Amersham Pharmacia Biotech	Ready-To-Go Beads “optimized for standard PCR” and Ready-To-Go RAPD Analysis Beads (buffer, nucleotides, <i>Taq</i> DNA polymerase)
Quick startup	Fisher	EasyStart PCR Mix-in-a-Tube—tubes prepackaged with wax beads containing buffer, MgCl ₂ , nucleotides, <i>Taq</i> DNA polymerase
Quick startup	Life Technologies	PCR SuperMix—1.1× conc.—premix containing buffer, MgCl ₂ , nucleotides, <i>Taq</i> DNA polymerase
Quick startup	Marsh Biomedical	Advanced Biochemicals Red Hot DNA Polymerase—a new rival for <i>Taq</i> polymerase with convenience features
Hot-start/physical barrier	Fisher, Life Technologies	Molecular Bio-Products HotStart Storage and Reaction Tubes—preadhered wax bead in each tube; requires manual addition of one component at high temperature
Hot-start/separate MgCl ₂	Invitrogen	HotWax Mg ²⁺ beads—wax beads contain preformulated MgCl ₂ which is released at first elevated-temperature step
Hot-start/separate MgCl ₂	Stratagene	StrataSphere Magnesium Wax Beads—wax beads containing preformulated Mg ²⁺
Hot Start/separate polymerase	Promega	TaqBead Hot Start Polymerase—wax beads encapsulating <i>Taq</i> DNA polymerase which is released at first elevated-temperature step
Hot-start/reversible inactivation of polymerase by antibody binding	Clontech	TaqStart Antibody, TthStart Antibody—reversibly inactivate <i>Taq</i> and <i>Tth</i> DNA polymerases until first denaturation at 95°C
Hot-start/antibody binding	Life Technologies	PlatinumTaq—contains PlatinumTaq antibody
Hot-start/antibody binding	Sigma	JumpStart Taq—contains TaqStart antibody
Hot-start/reversible chemical modification	Perkin-Elmer	AmpliTaq Gold—activated at high temperature
Hot-start/reversible chemical modification	Qiagen	HotStarTaq DNA Polymerase—activated at high temperature
Enhancer	Boehringer Mannheim, New England Biolabs	<i>Tth</i> pyrophosphatase, thermostable
Enhancer	Clontech	GC-Melt (in Advantage-GC Kits)—proprietary
Enhancer	CPG	Taq-FORCE Amplification System and MIGHTY Buffer—proprietary
Enhancer	Fisher	Eppendorf MasterTaq Kit with TaqMaster Enhancer—proprietary
Enhancer	Life Technologies	PCRx Enhancer System—proprietary
Enhancer	Promega	<i>E.coli</i> Single Stranded Binding Protein (SSB)
Enhancer	Qiagen	Q-Solution—proprietary
Enhancer	Stratagene	Perfect Match Polymerase Enhancer—proprietary
Enhancer	Stratagene	TaqExtender PCR Additive—proprietary

15.1.8

also generate seemingly specific products that require much labor to identify and yield no useful information. The less degenerate the oligonucleotides, especially at the 3' end, the better. Caveat emptor.

Primer length. A primer should be 20 to 30 bases in length. It is unlikely that longer primers will help increase specificity significantly.

Primer sequence. Design primers with a GC content similar to that of the template. Avoid primers with unusual sequence distributions, such as stretches of polypurines or polypyrimidines, as their secondary structure can be disastrous. It is worthwhile to check for potential secondary structure using one of the appropriate computer programs that are available.

"Primer-dimers." Primer-dimers are a common artifact most frequently observed when small amounts of template are taken through many amplification cycles. They form when the 3' end of one primer anneals to the 3' end of the other primer, and polymerase then extends each primer to the end of the other. The ensuing product can compete very effectively against the PCR product of interest. Primer-dimers can best be avoided by using primers without complementarity, especially in their 3' ends. Should they occur, optimizing the $MgCl_2$ concentration may minimize their abundance relative to that of the product of interest.

Template

Aside from standard methods for preparing DNA (UNIT 2.1-2.4), a number of simple and rapid procedures have been developed for particular tissues (Higuchi, 1989). Even relatively degraded DNA preparations can serve as useful templates for generation of moderate-sized PCR products. The two main concerns regarding template are purity and amount.

A number of contaminants found in DNA preparations can decrease the efficiency of PCR. These include urea, the detergent SDS (whose inhibitory action can be reversed by nonionic detergents), sodium acetate, and, sometimes, components carried over in purifying DNA from agarose gels (Gelfand, 1989; Gyllenstein, 1989; K. Hicks and D. Coen, unpub. observ.). Additional organic extractions, ethanol precipitation from 2.5 M ammonium acetate, and/or gel purification on polyacrylamide rather than agarose, can all be beneficial in minimizing such contamination if the simplest method (precipitating the sample with ethanol and repeatedly washing the pellet with 70% ethanol) is not sufficient.

Clearly the amount of template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100 ng of genomic DNA is sufficient to detect a PCR product from a single-copy mammalian gene. Using too much template is not advisable when optimizing for $MgCl_2$ or other parameters, as it may obscure differences in amplification efficiency. Moreover, too much template may decrease efficiency due to contaminants in the DNA preparation.

Amount of template, especially in terms of the amount of target sequence versus nonspecific sequences, can have a major effect on the yield of nonspecific products. With less target sequence, it is more likely that nonspecific products will be seen. For some applications, such as certain DNA sequencing protocols where it is important to have a single product, gel purification of the specific PCR product and reamplification are advisable.

Taq and other thermostable DNA polymerases

Among the advantages conferred by the thermostability of *Taq* DNA polymerase is its ability to withstand the repeated heating and cooling inherent in PCR and to synthesize DNA at high temperatures that melt out mismatched primers and regions of local secondary structure. The enzyme, however, is not infinitely resistant to heat, and for greatest efficiency it should not be put through unnecessary denaturation steps. Indeed, some protocols (e.g., UNIT 15.7 and the "hot start" method described here) recommend adding it after the first denaturation step.

Increasing the amount of *Taq* DNA polymerase beyond 2.5 U/reaction can sometimes increase PCR efficiency, but only up to a point. Adding more enzyme can sometimes increase the yield of nonspecific PCR products at the expense of the product of interest. Moreover, *Taq* DNA polymerase is not inexpensive.

A very important property of *Taq* DNA polymerase is its error rate, which was initially estimated at 2×10^{-4} nucleotides/cycle (Saiki et al., 1988). The purified enzyme supplied by manufacturers lacks a proofreading 3'→5' exonuclease activity, which lowers error rates of other polymerases such as the Klenow fragment of *E. coli* DNA polymerase I. For many applications, this does not present any difficulties. However, for sequencing clones derived from PCR, or when starting with very few templates, this can lead to major problems. Direct sequencing of PCR products (UNIT 15.2),

sequencing numerous PCR-generated clones, and/or the use of appropriate negative controls can help overcome these problems. Alternatively, changing reaction conditions (Eckert and Kunkel, 1990) or changing to a non-*Taq* DNA polymerase (with greater fidelity) may be useful.

Another important property of *Taq* DNA polymerase is its propensity for adding nontemplated nucleotides to the 3' ends of DNA chains. This can be especially problematic in cloning PCR products. It is frequently necessary to "polish" PCR products with enzymes such as other DNA polymerases before adding linkers or proceeding to blunt-end cloning. Conversely, addition of a nontemplated A by *Taq* DNA polymerase can be advantageous in cloning (UNIT 15.4).

Certain PCR protocols may work better with one thermostable polymerase rather than another. Table 15.1.4 lists currently available thermostable DNA polymerases by generic and trade names, the original source of native and recombinant enzymes, the supplier, the end generated (3'A addition versus blunt), and associated exonuclease activities. A 3' to 5' exonuclease activity is proofreading. Removal of the 5' to 3' exonuclease activity of *Taq* DNA polymerase (N-terminal deletion) is reported to produce a higher yield. A 5' to 3' exonuclease activity may degrade the primers somewhat. Proofreading enzymes synthesize DNA with higher fidelity and can generate longer products than *Taq*, but tend to generate low yields. Enzyme blends (Table 15.1.5) have been optimized for increased fidelity and length along with sensitivity and yield.

Hot start

What happens prior to thermal cycling is critical to the success of PCR. *Taq* DNA polymerase retains some activity even at room temperature. Therefore, under nonstringent annealing conditions, such as at room temperature, products can be generated from annealing of primers to target DNA at locations of low complementarity or having complementarity of just a few nucleotides at the 3' ends. The latter would in effect create new templates "tagged" with the primer sequences. Subsequent cycles amplify these tagged sequences in abundance, both generating nonspecific products and possibly reducing amplification efficiency of specific products by competition for substrates or polymerase. Thus conditions preventing polymerization prior to the first temperature-controlled steps are desirable. In this protocol, three

methods of inhibiting polymerization prior to the temperature-controlled step are compared. These include physical separation of an essential reaction component prior to the first denaturation step, cooling reagents to 0°C, and reversibly blocking enzymatic activity with an antibody.

Denaturation of the template before *Taq* polymerase or $MgCl_2$ is added to the reaction provides a dramatic improvement in specificity and sensitivity in many cases (Chou et al., 1992). The main drawback of this method is that it requires opening the reaction tubes a second time to add the essential missing component. This creates both an inconvenience and an increase in the risk of contamination, an important consideration when testing for the presence of a given sequence in experimental or clinical samples.

Cooling all components of the reaction mixture to 0°C prior to mixing is more convenient and the least expensive method but is also the least reliable. Transferring the PCR reaction tubes from the ice slurry to a 95°C preheated thermocycler block may improve the chance of success.

Reversible inhibition of *Taq* DNA polymerase by TaqStart antibody (Clontech) is the most convenient method and very effective (Kellogg et al., 1994). Complete reactions can be set up, overlaid with oil, and stored at 4°C for up to several hours prior to thermal cycling with no loss of sensitivity or specificity compared to the other hot start methods (M.F. Kramer and D.M. Coen, unpub. observ.). Cycling is initiated immediately following 5-min denaturation of the antibody at 94°C. DMSO inhibits antibody binding and should not be used with TaqStart.

Several hot-start products are now commercially available (Table 15.1.3). Success with each may depend on strict adherence to the manufacturer's protocols, even on a specific thermocycler. Wax barrier and reversible antibody binding methods are more forgiving, while chemical modifications have more stringent activation temperature requirements.

Deoxyribonucleoside triphosphates

In an effort to increase efficiency of PCR, it may be tempting to increase the concentration of dNTPs. Don't! When each dNTP is 200 mM, there is enough to synthesize 12.5 mg of DNA when half the dNTPs are incorporated. dNTPs chelate magnesium and thereby change the effective optimal magnesium concentration. Moreover, dNTP concentrations >200

Table 15.1.4 Thermostable DNA Polymerases

DNA polymerase		Biological source	Supplier	Product ends	Exonuclease activity
Generic name	Trade name				
<i>Pfu</i>	—	<i>Pyrococcus furiosus</i>	Stratagene, Promega	Blunt	3'-5' (proofreading)
<i>Pfu</i> (exo-)	—	<i>Pyrococcus furiosus</i>	Stratagene	Blunt	No
<i>Psp</i>	Deep Vent	<i>Pyrococcus</i> sp.GB-D	New England Biolabs	Blunt	3'-5' (proofreading)
<i>Psp</i> (exo-)	Deep Vent (exo-)	<i>Pyrococcus</i> sp.GB-D	New England Biolabs	Blunt	No
<i>Pwo</i>	—	<i>Pyrococcus woesei</i>	Boehringer Mannheim	Blunt	3'-5' (proofreading)
<i>Taq</i> (native and/or recombinant)	—	<i>Thermus aquaticus</i>	Ambion, Amersham Pharmacia Biotech, Boehringer Mannheim, Clontech, Fisher, Life Technologies, Marsh Biomedical, Perkin Elmer, Promega, Qiagen, Sigma, Stratagene	3'A	5'-3'
<i>Taq</i> , N-terminal deletion	Stoffel fragment Klen-Taq	<i>Thermus aquaticus</i>	Perkin-Elmer, Sigma	3'A	No
<i>Tbr</i>	DyNAzyme	<i>Thermus brocianus</i>	MJ Research	— ^a	5'-3'
<i>Tfl</i>	—	<i>Thermus flavus</i>	Promega, Epicentre Technologies	Blunt	— ^a
<i>Tli</i>	Vent	<i>Thermococcus litoralis</i>	New England Biolabs (Vent), Promega	Blunt	3'-5' (proofreading)
<i>Tli</i> (exo-)	Vent (exo-)	<i>Thermococcus litoralis</i>	New England Biolabs	Blunt	No
<i>Tma</i>	UITma	<i>Thermotoga maritima</i>	Perkin-Elmer	Blunt	3'-5' (proofreading)
<i>Tth</i>	—	<i>Thermus thermophilus</i>	Amersham Pharmacia Biotech, Boehringer Mannheim, Epicentre Technologies, Perkin Elmer, Promega	3' A	5'-3'

^aNo information at this time.

mM each increase the error rate of the polymerase. Millimolar concentrations of dNTPs actually inhibit *Taq* DNA polymerase (Gelfand, 1989).

The protocol in this unit calls for preparing 4dNTPs in 10 mM Tris-Cl/1 mM EDTA (TE buffer), pH 7.4 to 7.5. This is easier and less prone to disaster than neutralization with sodium hydroxide. However, EDTA also chelates magnesium, and this should be taken into account if stocks of dNTPs are changed. Alternatively, to lower the risk of contamination, a 4dNTP mix can be made by combining equal volumes of commercially prepared stocks.

Enhancers

Enhancers are used to increase yield and specificity and to overcome difficulties encountered with high GC content or long templates. Nonionic detergents (Triton X-100, Tween 20, or Nonidet P-40) neutralize charges of ionic detergents often used in template preparation, and should be used in the basic reaction mixture, rather than as optional enhancers. Higher yields can be achieved by stabilizing/enhancing the polymerase activity with enzyme-stabilizing proteins (BSA or gelatin), enzyme-stabilizing solutes such as betaine or betaine-HCl (TMAC), enzyme-stabilizing solvents (glycerol), solubility-enhancing sol-

Table 15.1.5 Thermostable DNA Polymerase Blends

Product (trade name)	Supplier	Thermostable DNA polymerases and other components
Expand High Fidelity, Expand Long Template, and Expand 20kb PCR Systems	Boehringer Mannheim	<i>Taq</i> + <i>Pwo</i>
KlenTaq LA Polymerase Mix	Clontech, Sigma	KlenTaq-1 (5'-exonuclease deficient <i>Taq</i>) + unspecified proofreading polymerase
Advantage-HF PCR Kit	Clontech	KlenTaq-1 + unspecified proofreading polymerase + TaqStart Antibody
Advantage-cDNA and Advantage-GC cDNA Polymerase Mixes and Kits	Clontech	KlenTaq-1 + unspecified proofreading polymerase + TaqStart Antibody; GC Kit contains GC Melt
Advantage Genomic and Advantage-GC Genomic Polymerase Mixes and Kits	Clontech	<i>Tth</i> + unspecified proofreading polymerase + TthStart Antibody; GC Kit contains GC Melt
eLONGase Enzyme Mix	Life Technologies	<i>Taq</i> + <i>Psp</i> + unspecified proofreading polymerase(s) + eLONGase Buffer
Platinum Taq DNA Polymerase	Life Technologies	<i>Taq</i> + <i>Psp</i> + Platinum <i>Taq</i> Antibody
Platinum High Fidelity DNA Polymerase	Life Technologies	<i>Taq</i> + <i>Psp</i> + <i>Taq</i> Antibody
DyNAzyme EXT Polymerase	MJ Research	<i>Tbr</i> with unspecified enhancer
GeneAmp XL PCR and XL RNA PCR Kits	Perkin-Elmer	<i>Tth</i> + <i>Tli</i>
OmniBase Sequencing Enzyme Mix	Promega	Unspecified proofreading polymerase(s) with thermostable pyrophosphatase
AccuTaq LA DNA Polymerase Mix	Sigma	<i>Taq</i> + unspecified proofreading polymerase
TaqPlus Long and TaqPlus Precision PCR Systems	Stratagene	<i>Pfu</i> + <i>Taq</i> ; TaqPlus Precision Reaction Buffer (proprietary)
Accurase Fidelity PCR Enzyme Mix; Calypso High Fidelity Single Tube RT-PCR System	Tetralink	<i>Thermus sp.</i> + <i>Thermococcus sp.</i> ; Calypso also contains AMV-RT

vents (DMSO or acetamide), molecular crowding solvents (PEG), and polymerase salt preferences [(NH₄)SO₄ is recommended for polymerases other than *Taq*]. Greater specificity can be achieved by lowering the T_M of dsDNA (using formamide), destabilizing mismatched-primer annealing (using PMPE or hot-start strategies), and stabilizing ssDNA (using *E. coli* SSB or T4 Gene 32 Protein). Amplification of high-GC-content templates can be improved by decreasing the base pair composition dependence of the T_M of dsDNA (with betaine; Rees et al., 1993). Betaine is an osmolyte widely distributed in plants and animals and is nontoxic, a feature that recommends it for convenience in handling, storage, and disposal. Betaine may be the proprietary ingredient in various commercial formulations. For long templates, a higher pH is recommended (pH 9.0). The pH of Tris buffer decreases at high temperatures, long-template PCR requires

more time at high temperatures, and increased time at lower pH may cause some depurination of the template, resulting in reduced yield of specific product. Inorganic phosphate (PPi), a product of DNA synthesis, may accumulate with amplification of long products to levels that may favor reversal of polymerization. Accumulation of PPi may be prevented by addition of thermostable PPase. When large numbers of samples are being analyzed, the convenience of adding PCR products directly to a gel represents a significant time savings. Some companies combine their thermostable polymerase with a red dye and a high density component to facilitate loading of reaction products onto gels without further addition of loading buffer.

Thermal cycling parameters

Each step in the cycle requires a minimal amount of time to be effective, while too much

time can be both wasteful and deleterious to the DNA polymerase. If the amount of time in each step can be reduced, so much the better.

Denaturation. It is critical that complete strand separation occur during the denaturation step. This is a unimolecular reaction which, in itself, is very fast. The suggested 30-sec denaturation used in the protocol ensures that the tube contents reach 94°C. If PCR is not working, it is well worth checking the temperature inside a control tube containing 100 µl water. If GC content is extremely high, higher denaturation temperatures may be necessary; however, *Taq* DNA polymerase activity falls off quickly at higher temperatures (Gelfand, 1989). To amplify a long sequence (>3 kb), minimize the denaturation time to protect the target DNA from possible effects, such as depurination, of lowered pH of the Tris buffer at elevated temperatures.

Annealing. It is critical that the primers anneal stably to the template. Primers with relatively low GC content (<50%) may require temperatures lower than 55°C for full annealing. On the other hand, this may also increase the quantity of nonspecific products. For primers with high GC content, higher annealing temperatures may be necessary. It can be worthwhile, although time-consuming, to experiment with this parameter. Some manufacturers have thermal cyclers on the market which are capable of forming a temperature gradient across the heating units, thus permitting annealing temperature optimization in one run. As with denaturation, the time for this step is based mainly on the time it takes to reach the proper temperature, because the primers are in such excess that the annealing reaction occurs very quickly.

Extension. The extension temperature of 72°C is close to the optimal temperature for *Taq* DNA polymerase (~75°C), yet prevents the primers from falling off. Indeed, primer extension begins during annealing, because *Taq* DNA polymerase is partially active at 55°C and even lower temperatures (Gelfand, 1989).

The duration of extension depends mainly on the length of the sequence to be amplified. A duration of 1 min per kb product length is usually sufficient.

Certain protocols, including others in this chapter, end the PCR with a long final extension time in an attempt to try to make products as complete as possible.

Ramp time. Ramp time refers to the time it takes to change from one temperature to another. Using water baths and moving samples

manually from temperature to temperature probably gives the shortest ramp times, which are mainly the time required for the tube's contents to change temperature. Different thermal cyclers have different ramp times; basically, the shorter the better.

The Stratagene Robocycler uses a robotic arm to move samples from one constant-temperature block to another, virtually eliminating block ramp time, but a ramp time for tube contents must be calculated (~1 sec/°C) and added to denaturation, annealing, and extension times. Rapid cyclers that utilize positive-displacement pipet tips or capillary tubes for the PCR reactions dramatically reduce the ramp times.

Generally, the more "high-performance" thermal cyclers with short ramp times are proportionally more costly. There are many new thermal cyclers on the market priced below \$5000, which perform quite well (Beck, 1998).

Anticipated Results

Starting with ≥100 ng mammalian DNA (≥10⁴ molecules), the basic protocol can be used to determine which MgCl₂ concentration, enhancing additive, and initial conditions will yield a predominant PCR product from a single-copy sequence that is readily visible on an ethidium bromide-stained gel. It is possible that other minor products will also be visible.

Time Considerations

The basic protocol can be completed in a single day. Assembly of the reaction mixtures should take ~1 hr. Cycling should take less than 3 hr. Preparing, running, and staining the gel should take another few hours. Further checks on specificity of the product such as restriction endonuclease digestion or Southern blot hybridization will take another few hours or days, respectively.

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Key Reference

Saiki et al., 1988. See above.

Demonstrates the ease and power of PCR using Taq DNA polymerase.

Internet Resources

<http://www.promega.com/amplification/ampotech.html>

Offers Amplification Assistant, a PCR troubleshooting program.

<http://www.genome.wi.mit.edu/>

Provides access to www Primer Picking (Primer 3); select experimental web-based software under Genome Center Software.

<http://www.alkami.com/primers/>

Contains free primer design tools and tips.

<http://bioinformatics.weizmann.ac.il/mb/bioguide/pcr/contents.html>

Contains useful tips and links.

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