

PCR Optimization -- Reaction Conditions and Components

The GeneAmp PCR process is widely employed in a tremendous variety of experimental applications to produce high yields of specific DNA target sequences. Since no single set of conditions can be applied to all PCR amplifications, individual reaction component concentrations (and time and temperature parameters) must be adjusted within suggested ranges for efficient amplification of specific targets. While there are a number of possible concentration parameters, logical titrations of interrelated reaction components can be readily defined. In addition, the time and temperature optima can often be determined within a few experiments.

Sample Volume and Reaction Tubes

Most PCR protocols are performed at the 25 μ L-100 μ L scale in 0.2 mL or 0.5 mL microcentrifuge tubes; however, 5 μ L reactions have been successful. Larger (> 100 μ L) volumes are usually not recommended, but, if they are used, longer incubation times may be needed to assure adequate thermal equilibration of the reaction mixture. Although generally the tubes do not need to be sterilized or siliconized, it is sound advice to autoclave tubes when dealing with small quantities of starting nucleic acid template. GeneAmp® PCR Reaction Tubes are designed for optimal fit in the DNA Thermal Cycler or DNA Thermal Cycler 480 sample block. The 0.5 mL GeneAmp® Thin-Walled Reaction Tubes are designed for use with the DNA Thermal Cycler 480, allowing the user to program shorter hold times (45 seconds or more) at each temperature in the PCR cycle. These tubes can also be used with the DNA Thermal Cycler, but hold times of at least 60 seconds are recommended.

The GeneAmp® PCR System 2400, 9600 and 9700 take advantage of the faster thermal equilibration properties of very thin-walled 0.2 mL MicroAmp® Reaction Tubes. MicroAmp® Reaction Tubes are used in conjunction with the heated covers on the 2400, 9600 and 9700 for oil-free PCR. These optimized PCR vessels are available with attached caps, multi-cap strips or full plate covers. Reaction volumes (5 μ L-100 μ L) are selected on the cycler at runtime so that sample temperature control is precise.

Vapor Barrier and Thermal Transfer Fluid

With the GeneAmp PCR System 9700, 9600, and 2400, the need for mineral oil as a vapor barrier, and mineral oil or glycerol as a thermal transfer fluid in the wells, is eliminated due to the tight fit of the tubes in the block, the thin walls of the MicroAmp Reaction Tubes and the heated cover of each instrument.

For the DNA Thermal Cycler 480 and DNA Thermal Cycler, the recommended mineral oil overlay acts as a vapor barrier to prevent evaporation and internal condensation that may reduce cycling efficiency. This vapor barrier can also be replaced with AmpliWax® PCR Gem 100 or AmpliWax® PCR Gem 50. AmpliWax PCR Gems not only replace mineral oil as a vapor barrier, but also automate Hot Start PCR, a technique that allows an increase in sensitivity and specificity. Mineral oil or glycerol is also sometimes used as a thermal transfer fluid in the deep wells of the sample block of the DNA Thermal Cycler 480 or DNA Thermal Cycler.

Template DNA or RNA

The PCR sample may be single- or double-stranded DNA or RNA. If the starting sample is RNA, total RNA, poly(A+) RNA, viral RNA or rRNA can be used. A reverse transcriptase (for example MuLV or *rTth* DNA Polymerase) is used to prepare first-strand cDNA prior to conventional PCR amplification. For RNA templates with high G+C content or complex secondary structure, the high-temperature reverse transcriptase activity of thermostable *rTth* DNA Polymerase is effective. When using DNA as the starting template, nanogram amounts of cloned template, up to microgram amounts of genomic DNA, or up to 20,000 target copies are chosen to start optimization trials. Even very low levels of sample (i.e., mRNA from tens of cells, DNA from single cells or individual viral genomes), however, may be sufficient for PCR amplification.

Primers

PCR primers are oligonucleotides, typically 15-30 bases long, hybridizing to opposite strands and flanking the region of interest in the target DNA. When choosing two PCR primers, it is important that they not contain bases complementary to themselves or with each other. Complementarity at the 3' ends should especially be avoided to minimize the formation of an artifactual product, often called "primer-dimer" or "primer-oligomer." A 40%-60% G+C content is recommended for both primers, avoiding internal secondary structure and long stretches of any one base. Also, primers should not sit on regions of secondary structure (within the target) having a higher melting point than the primer. Non-template, complementary 5' extensions may be added to primers to allow a variety of useful post-amplification manipulations of the PCR product without significant effect on the amplification itself. These 5' extensions can be restriction sites, promoter sequences, etc.

Optimal annealing temperatures and primer concentrations must be determined empirically. *Taq* DNA Polymerase has activity in the 25 °C-72 °C range. Therefore, primer extension will occur during the annealing step and the hybrid will be stabilized. Primers are always present at an excess and equal concentration in conventional (symmetric) PCR amplification and, typically, are within the range of 0.1 μ M to 1 μ M. It is generally advisable to use purified oligomers of the highest chemical integrity.

Deoxynucleoside Triphosphates

In the standard GeneAmp PCR protocol, each deoxynucleoside triphosphate (dNTP) concentration is 200 μ M. It is important to keep the four dNTP concentrations above the estimated K_m of each dNTP (10 μ M-15 μ M) and balanced for best base incorporation. However, deviations (from these standard recommendations) may be beneficial in certain applications. For example, when random mutagenesis of a specific target is desired, unbalanced dNTP concentrations promote a higher degree of misincorporation by *Taq* DNA Polymerase. Lowering the dNTP and magnesium ion by an equal molar concentration can improve fidelity. Modified dNTPs (dig-11-dUTP, 5-bromo-dUTP, inosine, biotin-11-dUTP, biotin-16-dUTP and 7-deaza dGTP) and dUTP are useful substrates for AmpliTaq® DNA Polymerase.

GeneAmp® PCR Buffers

Standard buffers for PCR amplification with AmpliTaq® DNA Polymerase are the GeneAmp® 10X PCR Buffer and the GeneAmp® PCR Buffer II. GeneAmp PCR Buffer II is the recommended buffer for PCR amplification with AmpliTaq Gold™ DNA Polymerase.

The GeneAmp 10X PCR Buffer is composed of 500 mM potassium chloride, 100 mM Tris-HCl (pH 8.3 at room temperature), 15 mM magnesium chloride and 0.01% (w/v) gelatin.

The GeneAmp 10X PCR Buffer II is composed of 500 mM potassium chloride and 100 mM Tris-HCl (pH 8.3 at room temperature). It is supplied with a separate solution of 25 mM magnesium chloride for optimization titrations. The addition of co-solvents to these standard buffers such as formamide or glycerol may be useful when trying to amplify G+C-rich target or through regions of strong secondary structure.

The recommended dilution buffer for AmpliTaq® DNA Polymerase and AmpliTaq Gold DNA Polymerase is 0.15% NP-40, 0.15% Tween 20, 0.1 mM EDTA and 25 mM Tris-HCl (pH 8.3 at room temperature).

Special buffer formulations are recommended for use with Stoffel Fragment, *rTth* DNA Polymerase and *rTth* DNA Polymerase, XL. The addition of cosolvents to differing salt concentrations of buffers will have an effect on T_m of primers, thus optimization of annealing temperatures will be necessary.

Magnesium Ion

In the presence of 0.8 mM total dNTP concentration, a magnesium chloride titration series in 0.5

mM increments over the 1 mM-4 mM range will locate the magnesium ion concentration, producing the highest yield of a specific PCR product. When using AmpliTaq® DNA Polymerase, too little free magnesium ion will result in little or no PCR product, and too much free magnesium ion may produce a variety of unwanted products and promote misincorporation.

AmpliTaq Gold™ DNA Polymerase

AmpliTaq Gold DNA Polymerase is an ultra-pure, modified form of AmpliTaq® DNA Polymerase designed to make the GeneAmp® PCR process easy. AmpliTaq Gold DNA Polymerase is provided as an inactive enzyme, requiring heat activation to regenerate polymerase activity. The necessity for thermal activation provides highly specific Hot Start PCR conditions resulting in lower background and increased yield of specific product (see Hot Start Section below). The improvements in specificity and product yield, along with the increased sensitivity for low copy detection and easy room temperature set-up makes AmpliTaq Gold enzyme the enzyme of choice for the majority of PCR applications. AmpliTaq Gold DNA Polymerase can be substituted for AmpliTaq® DNA Polymerase in most PCR reactions simply by adding a 10 minute 95 °C pre-PCR incubation step, providing an efficient Hot Start PCR, and delivering the appropriate amount of enzyme activity throughout the amplification process. Alternatively, the pre-PCR activation step can be omitted, and a minimum of 10 additional cycles can be added, allowing the enzyme to activate slowly during cycling. This provides a simple method of Time Release PCR, where polymerase activity builds more slowly with product accumulation, improving specificity.

For most PCR applications with a 50 µL reaction volume, 1.25-1.5 units of AmpliTaq Gold DNA Polymerase are recommended. The enzyme can be added conveniently to a master mix prepared at room temperature without risk of nonspecific product or primer artifact formation, thereby avoiding the tedium and possible accuracy problems associated with adding individual 0.25 µL enzyme aliquots to each tube. GeneAmp 10X PCR Buffer II is provided with the enzyme, and offers the necessary pH and ionic strength required for PCR amplification reactions performed with AmpliTaq Gold.

AmpliTaq® DNA Polymerase

AmpliTaq® DNA Polymerase is the recombinant form of *Taq* DNA Polymerase. It is obtained by expressing the *Taq* DNA polymerase gene in an *E. coli* host. Like native *Taq* DNA polymerase, it lacks endonuclease and 3'-5' exonuclease activities, but has a 5'-3' exonuclease activity. AmpliTaq® DNA Polymerase is the most thoroughly characterized enzyme for performing the GeneAmp PCR process.

AmpliTaq® DNA Polymerase, Stoffel Fragment

Stoffel Fragment is a modified form of AmpliTaq® DNA Polymerase from which the N-terminal 289 amino acids are deleted. Stoffel Fragment differs from AmpliTaq® DNA Polymerase in that it is more thermostable (by approximately 2-fold), exhibits optimal activity over a broader range of magnesium ion concentration (2 mM-10 mM) and lacks intrinsic 5' to 3' exonuclease activity. The unique properties of Stoffel Fragment make it especially useful for Arbitrarily Primed PCR (AP-PCR) or Random Amplified Polymorphic DNA (RAPD) amplification methods, whereby genomic DNA is amplified with a set of short primers of arbitrary sequence.

r*Tth* DNA Polymerase

Recombinant *Thermus thermophilus* (r*Tth*) DNA Polymerase is an ultrapure, thermostable recombinant DNA polymerase, suitable for amplification of RNA targets (RNA PCR). r*Tth* DNA Polymerase is able to reverse transcribe RNA to cDNA in the presence of Mn⁺² ion, and to also act as a DNA polymerase for PCR amplification. High temperature reverse transcription with r*Tth* DNA Polymerase allows for efficient cDNA synthesis from RNA templates that contain complex secondary structure. PCR amplification can be performed either after chelation of the Mn⁺² and subsequent addition of MgCl₂, as in the GeneAmp® Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit, or in a unique single buffer system, as in the GeneAmp® EZ r*Tth* RNA PCR Kit.

r*Tth* DNA Polymerase, XL

Recombinant *Thermus thermophilus* (r*Tth*) DNA Polymerase, XL, is an ultra-pure, thermostable recombinant DNA polymerase blend, designed for amplification of DNA or RNA targets >5 kb [XL (eXtra Long) PCR]. It is uniquely formulated to include the optimal amount of 3'-5' exonuclease activity. "Proofreading" activity, along with Hot Start and XL Buffer conditions, have been proposed as one of the key parameters in successful amplification of long target molecules. For DNA amplification, the optimized reaction buffer (XL Buffer) contains DMSO and glycerol, and is included with both the component product enzyme and complete GeneAmp® XL PCR Kit.

PCR Thermal Profiles

Using the GeneAmp® PCR System 9700 (or GeneAmp PCR System 9600 or 2400) with the two-temperature PCR protocol and GeneAmp® PCR Reagents, amplification of the Lambda control target DNA is guaranteed with a 15-second, 94 °C denaturation step and a 1-minute, 68 °C primer annealing/extension step. This will amplify a 500 bp product at least 10⁵-fold in 25 cycles, taking about 2.3 minutes per cycle.

Using the DNA Thermal Cycler 480, with the two-temperature PCR protocol and GeneAmp® PCR Reagents, amplification of the Lambda control target DNA is guaranteed with a 1-minute, 94 °C denaturation step and a 2-minute, 68 °C primer annealing/ extension step. This will amplify a 500 bp product at least 10⁵-fold in 25 cycles, taking about 4.25 minutes per cycle.

DNA denaturation is the critical step in the GeneAmp® PCR process and is often the focus of attention if PCR experiments fail. The practical range of denaturation temperatures for most samples is 94 °C-96 °C. On the GeneAmp PCR System 9700 (or GeneAmp PCR System 9600 or 2400), the computed sample temperature is used to time the incubation periods and 15-second denaturation times are routinely used.

On the DNA Thermal Cycler 480 and the DNA Thermal Cycler, the average block temperature is used to time the incubation periods. At least 1 minute must be specified for sample temperature equilibration for the most reliable amplification in standard 0.5 mL GeneAmp® PCR Reaction Tubes. Denaturation can often occur in the final seconds of the 1-minute incubation segment. (Shorter hold times of 158; 45 seconds may be specified only when using GeneAmp® Thin-Walled Reaction Tubes on the DNA Thermal Cycler 480.)

Annealing temperature is based on the T_m (melting temperature) of the oligonucleotides chosen for PCR amplification. If unwanted bands are observed, the annealing temperature is raised in 2 °C-5 °C increments in subsequent optimization runs. While the primer annealing temperature range is often 37 °C-55 °C, it may be raised as high as the extension temperature in some cases. In fact, high-temperature annealing should result in enhanced specificity. The merging of the primer annealing and primer extension steps results in a two-step GeneAmp PCR process, which has been successful in many applications, including those using the GeneAmp® PCR Reagent Kit bacteriophage Lambda Control DNA.

Primer extension, in most applications, occurs effectively at a temperature of 72 °C and seldom needs optimization. In the two-temperature GeneAmp PCR process, this temperature range may be 60 °C-70 °C. All GeneAmp® PCR Instrument Systems are able to automatically increase the extension time linearly with cycle number. This technique may enhance yield, especially in situations where the enzyme concentration limits amplification in late cycles. Typically, 25-45 cycles are required for extensive amplification (i.e., 10⁶-fold) of a specific target.

Hot Start PCR

The major obstacle to routine, sensitive and specific PCR amplification appears to be competing side reactions such as the amplification of non-target sequences in background DNA (mispriming) and primer-oligomerization. This mispriming and primer-oligomerization occurs mainly during pre-PCR setup when all reactants have been mixed, usually at room temperature, before thermal cycling is started.

In the Hot Start technique, the reaction is designed so that all reactants are not active until reaching a temperature high enough to suppress primer annealing to non-target sequences. Typically, in manual Hot Start, all reactants except *Taq* DNA Polymerase are mixed at room temperature below the mineral oil cap. Then, after all tubes have been loaded into a GeneAmp PCR Instrument System, and the temperature has been raised to 70 °C-80 °C, enzyme is added separately to each reaction. Although manual Hot Start can increase amplification specificity and yield, it is inconvenient and can cause reproducibility and contamination problems.

AmpliTaq Gold™ DNA Polymerase is a modified version of AmpliTaq® DNA Polymerase provided in an inactive form which is then heat activated. Hence, PCR setup on many samples can be performed at room temperature without concern for extension at misprimed sites. AmpliTaq Gold DNA Polymerase can be completely or partially activated in a pre-PCR heat step (conventional Hot Start) or can be allowed to activate slowly during thermal cycling (Hot Start and Time Release PCR). By increasing the amount of AmpliTaq Gold DNA Polymerase in the reaction slowly with cycle number, specific product yield is increased without buildup of misprimed products. The use of AmpliTaq Gold DNA Polymerase in the GeneAmp PCR process increases amplification specificity and sensitivity, improving product yield. This increased sensitivity makes AmpliTaq Gold DNA Polymerase ideal for the majority of PCR applications, and is especially suited for low copy pathogen detection and the amplification of degraded samples, as well as for multiplex PCR or allelic discrimination.

For specific applications (such as GeneAmp® XL PCR) where *Taq* DNA polymerase is not the enzyme of choice, or when a vapor barrier is desired, Hot Start PCR can be accomplished through the use of AmpliWax® PCR Gems, precisely aliquoted beads of specially cleaned and formulated wax.

Suggestions for Successful PCR Product Analysis

PCR products are usually analyzed by ethidium bromide-stained agarose gel electrophoresis, Southern blotting/probe hybridization, or fluorescence assay.

If there is no yield of the desired PCR product, reproducible addition of the enzyme should be confirmed, preferably in a master mix. Complete DNA denaturation should be ensured in each cycle by using appropriate GeneAmp® or MicroAmp®

Reaction Tubes and by allowing sufficient time at the denaturation plateau temperature. Slightly higher denaturation temperatures should be checked and the chemical integrity of the primers should be considered. Preincubation at 95 °C for 5 to 10 minutes in the absence of enzyme to inactivate harmful proteases, or nucleases in the sample, is often helpful. This preincubation also ensures complete denaturation of complex starting templates. Consider performing the Hot Start technique.