AmpliTaq®

Applied Biosystems

DNA Polymerase

with GeneAmp® 10X PCR Buffer or GeneAmp® 10X PCR Buffer II & $MgCl_2$ Solution

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES
See notice to purchaser

Enzyme	Enzyme with Buffer	
	GeneAmp 10X PCR Buffer	GeneAmp 10X PCR Buffer II & $MgCl_2$ Solution
AmpliTaq DNA Polymerase 250 Units (U), 5 U/µL, 200 rxns	N808-0160	N808-0161
AmpliTaq DNA Polymerase 1000 Units (U), 5 U/µL, 800 rxns	N808-0171	N808-0172
Six Paq, AmpliTaq DNA Polymerase 6 x 250 U, 5 U/µL, 1200 rxns	N808-0166	N808-0167
Twelve Paq, AmpliTaq DNA Polymerase 12 x 250 U, 5 U/µL, 2400 rxns	N808-0152	N808-0153
AmpliTaq DNA Polymerase, 5000 U 5 x 1000 U, 5 U/µL, 4000 rxns	N808-0155	N808-0156
AmpliTaq DNA Polymerase, 25,000 U 25 x 1000 U, 5 U/µL, 20,000 rxns	N808-0185	N808-0186

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STORAGE AND STABILITY

Upon receipt, store the AmpliTaq DNA Polymerase and reagents at -20°C ± 10°C in a constant temperature freezer. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

INTRODUCTION

Applied Biosystems AmpliTaq DNA Polymerase is a recombinant, thermostable, 94 kDa DNA polymerase encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene which has been inserted into an *Escherichia coli* host.[1] This ultra-pure, stable polymerase is designed for performing the Polymerase Chain Reaction (PCR) process and is available either by itself or as a component of the GeneAmp PCR Reagent Kit (**Part No. N801-0055**) and the GeneAmp PCR Core Reagents (**Part No. N808-0009**). For manual cycle sequencing of DNA, use the AmpliCycle™ Sequencing Kit (**N808-0175**) featuring AmpliTaq DNA Polymerase, CS.

The PCR process is a simple and powerful method,[5] invented by K. Mullis, which allows amplification of DNA segments (including cDNA)[6] *in vitro* through a succession of incubation steps at different temperatures.[2,4,7,8,9] Typically, the double-stranded DNA is heat-denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle. The PCR process is based on the repetition of this cycle and can amplify DNA segments by at least 10^5 fold, and potentially as high as 10^9 fold under the conditions described below.[2]

AmpliTaq DNA Polymerase gives optimal results under reagent conditions provided by GeneAmp 10X PCR Buffer and GeneAmp 10X PCR Buffer II. These buffers provide preferred pH and ionic strength for PCR amplification reactions (see below for exact formulations). The magnesium ion concentration required to achieve optimal PCR amplification is dependent on the specific set of primers and template used. GeneAmp 10X PCR Buffer contains 15 mM $MgCl_2$ which would give a 1.5 mM final concentration when diluted to 1X. The 25 mM $MgCl_2$ solution supplied with GeneAmp 10X PCR Buffer II can be used to adjust magnesium ion concentration for any set of primer-template pairs.

LIST OF COMPONENTS

Reagent	Volume	Description
AmpliTaq DNA Polymerase	50 µL	1 tube, 250 U of 5 U/µL AmpliTaq DNA Polymerase.
	or	
	200 µL	1 tube, 1000 U of 5 U/µL AmpliTaq DNA Polymerase.
GeneAmp 10X PCR Buffer **(N808-0006)** **(N808-0129)**	1.5 mL 6 x 1.5 mL	Tube(s) containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin.
or		
GeneAmp 10X PCR Buffer II **(N808-0010)** **(N808-0130)**	1.5 mL 6 x 1.5 mL	Tube(s) containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl.
and	and	and
25 mM $MgCl_2$ Solution	1.5 mL 6 x 1.5 mL	Tube(s) containing 25 mM $MgCl_2$.
Product Insert	–	Containing AmpliTaq DNA Polymerase protocols.

ENZYME CHARACTERISTICS

The key component is AmpliTaq DNA Polymerase (Deoxynucleosidetriphosphate: DNA Deoxynucleotidyltransferase, EC 2.7.7.7).

Concentration: 5 Units/µL

Unit Definition: One unit of enzyme is defined as the amount that will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 minutes in a 10 minute incubation at 74°C under the analysis conditions below.

Analysis Conditions:[1] 25 mM TAPS (tris-[hydroxymethyl]-methyl-amino-propanesulfonic acid, sodium salt), pH 9.3 (at room temperature); 50 mM KCl; 2 mM $MgCl_2$; 1 mM ß-mercaptoethanol; 200 µM each dATP, dGTP, dTTP; 100 µM [α-^{32}P]-dCTP (0.05-0.1 Ci/mmole); salmon sperm DNA, activated by a modification of methods;[10] mixed in a final volume of 50 µL and incubated at 74°C for 10 minutes.

Storage Buffer: 20 mM Tris-HCl, pH 8.0 (at room temperature), 100 mM KCl, 0.1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dithiothreitol), 50% glycerol, 0.5% Tween® 20, 0.5% Nonidet® P 40.

Storage Temperature: Store AmpliTaq DNA Polymerase at -20°C, in a constant temperature freezer. If stored under proper conditions, the enzyme will remain active through the control date printed on the label.

Associated Activities: Endonuclease and exonuclease activities were not detectable after one hour incubation of 600 ng of supercoiled pBR322 (dam⁻, dcm⁻) or 600 ng of *Msp*I-digested pBR322 DNA, respectively, at 74°C, in the presence of 8 units of AmpliTaq DNA Polymerase. The enzyme has a fork-like-structure dependent, polymerization enhanced 5' to 3' nuclease activity but lacks a 3' to 5' exonuclease activity.[3,11,12]

MATERIALS REQUIRED BUT NOT SUPPLIED

Unless otherwise noted, the items listed are available from major laboratory suppliers (MLS) such as Baxter Scientific Products (McGaw Park, IL), Fisher Scientific (Pittsburgh, PA) or VWR Scientific (So. Plainfield, NJ).

Reagents	**Source**
Autoclaved deionized, ultrafiltered or glass distilled water, referred to as "water" in this product insert.	MLS

Equipment

Electrophoresis power supply	MLS
Gel electrophoresis equipment	MLS
GeneAmp® PCR Instrument System	**Applied Biosystems** (See **page 16** of this product insert).
Pipettors adjustable	MLS

Supplies

PCR Reaction Tubes (see **Table 1** for tube/thermal cycler combination)

GeneAmp Thin-Walled Reaction Tubes (0.5 mL, polypropylene)	**Applied Biosystems, (Part Nos. N801-0737, N801-0611, N801-0537).**
MicroAmp® Reaction Tubes (0.2 mL, polypropylene)	**Applied Biosystems, (Part Nos. N801-0533, N801-0540, N801-0612).**
Pipet tips with hydrophobic filters used with adjustable pipettors	MLS

PROTOCOLS FOR DNA AMPLIFICATION

1.0 GENERAL ADVICE

Due to the enormous amplification possible with the PCR process, small levels of DNA contamination, especially from previous PCR amplification reactions, samples with high DNA levels, and positive control templates, can result in product formation even in the absence of purposefully added template DNA.[13] If possible, all reactions should be set up in an area separate from PCR product analysis. The use of dedicated or disposable vessels, solutions and pipettes (preferably positive displacement pipettes or tips with hydrophobic filters) for DNA preparation, reaction mixing, and sample analysis will minimize cross contamination.[14] Use of the GeneAmp PCR Carry-over Prevention Kit (**Part No. N808-0068**) will prevent the reamplification of PCR products from previous amplifications.

2.0 REACTION MIX PREPARATION

2.1 A Master Mix of reagents (water, buffer, dNTPs, primers and enzyme) for all samples can be prepared first, then aliquoted to individual tubes. Magnesium chloride and the template DNA are then added. Using such mixes will minimize reagent pipetting losses, increase accuracy, and reduce the number of reagent transfers. Perform amplifications in the Applied Biosystems PCR reaction tube appropriate for your GeneAmp PCR Instrument System (see table in **Section 4.1**, and the instrument manual). Applied Biosystems PCR reaction tubes provide the best heat transfer because of their uniform fit in the wells of the corresponding instrument.

2.2 Since DNA may stick to plastic and since nucleases are often found on surfaces, it may be preferable to use sterile, siliconized tubes and pipette tips.

2.3 Reaction Mix

	Component	Addition Order	Volume per Reaction	Final Concentration
Master Mix	Autoclaved deionized, ultrafiltered or glass distilled water	1	*	1X
Master Mix	10X PCR Buffer II (Part No. N808-0010)	2	5 µl	10 mM Tris-HCl, pH 8.3, 50 mM KCl
Master Mix	dATP, dCTP, dGTP, dTTP (10 mM each dNTP)	3	1 µL, 1 µL, 1 µL, 1 µL	200 µM, 200 µM, 200 µM, 200 µM (or make daily as a mix of 1.25 mM of each dNTP; add 16 µL per reaction)
Master Mix	Primer 1	4	0.5-2.5 µL	0.2-1.0 µM
Master Mix	Primer 2	5	0.5-2.5 µL	0.2-1.0 µM
Master Mix	AmpliTaq DNA Polymerase	6	0.25 µL	1.25 Units/50 µL
Master Mix	25 mM $MgCl_2$ Solution	7	2-8 µL	1.0-4.0 mM**
	Experimental template		*	500 ng/50 µL
	Total Mix		50 µL	

* Any combination of water and experimental template volumes can be used as long as the total volume of the reaction (including buffer, dNTP primers, enzyme and $MgCl_2$ solution) equals 50 µL.

** The optimal magnesium chloride concentration may vary, depending on the primer and templates used and must be determined empirically. In most cases a final concentration of magnesium chloride in the range of 1.0 to 4.0 mM in the reaction mix will work well.

3.0 AMPLIFICATION OF DNA TEMPLATES

3.1 Gently mix (avoid generating bubbles) the AmpliTaq DNA Polymerase as well as other recently thawed reagents, then spin down in a microcentrifuge before pipetting. Pipette enzyme carefully and slowly; the viscosity of the 50% glycerol in the enzyme storage buffer can lead to errors. If possible use a positive displacement pipette. Using Master Mixes (see **Section 2.0**) will increase accuracy, reduce reagent loss on tips, and reduce tube-to-tube variability.

3.2 Except for amplification in the GeneAmp PCR System 9600, 9700 and 2400, control evaporation and refluxing by overlaying the mix with 50 to 100 µL of mineral oil (Sigma Chemical Co., St. Louis, MO. Product No. M5904). The oil should not interfere when withdrawing samples. If the entire volume is to be recovered, 50 µL of high purity chloroform should be added after amplification. The aqueous phase containing the DNA will then float on the chloroform-oil mixture, allowing easy collection. The use of AmpliWax® PCR Gem 50 (**Part No. N808-0150;** or **N808-0100** for 100 µL reaction volumes) as an alternative to oil may also ease sampling and increase reproducibility in amplification when used for hot starts at low target number or with high background DNA concentrations.[15]

3.3 The reagent concentration ranges in the Reaction Mix recipe in **Section 2.3** are a useful starting place for amplification of different DNA targets using primers designed by the user. Optimization of reactions for each primer-template pair may be necessary and can be achieved by varying magnesium chloride concentration, primer concentration, dNTP concentration, and cycle conditions. The effect of these variations can be monitored by examining the intensity and distribution of bands after electrophoresis on agarose followed by visualization with ethidium bromide staining of the gel.

3.4 The DNA segment to be amplified from the template can be up to 10 kb long,[16] although 100 to 1000 bases are more typical and easier to amplify. Start with enough copies of the template to be sure of obtaining a signal after 25 or 30 cycles: preferably > 10^4 copies but less than 500 ng total sample DNA per 50 µL. Low concentrations of target DNA may require up to 35 or more cycles to produce sufficient product for analysis.

3.5 If proteases are present in the sample DNA (such as impure genomic DNA), inactivate the proteases by heating samples to 95ºC for 5 minutes before adding AmpliTaq DNA Polymerase. The step can be carried out automatically with any of the GeneAmp PCR Instrument Systems.

3.6 The single-strand DNA primers should typically be 15 to 30 bases in length. The % G+C of primers should be near 50%, to maximize specificity. To avoid potential problems, primers should be purified by gel electrophoresis or HPLC ion-exchange chromatography. The optimal primer concentrations need to be determined empirically, by testing concentrations in the range of 0.1 to 1 µM. Primer concentrations that are too low will result in little or no PCR product, while concentrations that are too high may result in amplification of non-target sequences. Primer concentrations in the range of 0.2 to 0.5 µM should work for most PCR amplifications. Primer sequences should not complement within themselves or to each other, particularly at the 3' ends.

3.7 The optimal magnesium chloride concentration needs to be determined empirically, by testing concentrations up to 4 mM $MgCl_2$ for each primer set. Too little or too much $MgCl_2$ could reduce amplification efficiency or result in non-specific products. If the samples contain EDTA or other chelators, raise the $MgCl_2$ concentration in the reaction mix proportionately. Magnesium chloride concentrations should also be adjusted in parallel with significant changes in the concentration (higher or lower) of sample DNA and dNTPs.

3.8 Keep concentrations of dNTPs in the reaction mix balanced; if the concentration of any one dNTP is significantly different from the rest, AmpliTaq DNA Polymerase may tend to misincorporate them, slow down, and terminate prematurely.[3]

3.9 A range of volumes, dependent on the GeneAmp PCR Instrument System, will permit good amplification (see **Table 1** in **Section 4.1**). If variable volumes of reagents or template are used in the Master Mix, adjust the volume of water in the Master Mix by an equivalent amount to keep the concentrations of other reactants constant.

3.10 AmpliTaq DNA Polymerase gives optimal results under reagent conditions provided by GeneAmp 10X PCR Buffer and GeneAmp 10X Buffer II. If other PCR buffers are used, or sample preparation reagents are added that change the pH or ionic strength of the PCR, then pH and ionic strength must be optimized.

4.0 TEMPERATURE CYCLING AND CYCLE OPTIMIZATION

4.1 AmpliTaq DNA Polymerase is optimized and quality control tested for performance on GeneAmp PCR Instrument Systems. Optimal performance of the PCR process is influenced by choice of temperature, time at temperature, and length of time between temperatures for each step in the cycle.

A typical cycle consists of a melting step (94º-96ºC), to separate the complementary strands of DNA; a primer annealing step (37º-65ºC), depending on the target), to allow hybridization of the primers to the ssDNA and initiation of polymerization; and a primer extension step (72ºC), to complete the copy initiated during annealing. Begin with a template melting step (at least 1 minute at 94º-96ºC), then start cycling. Table 1 shows typical profile times for various GeneAmp PCR Instrument Systems. See their instruction manuals for details.

Samples can be stored at or below 4ºC (with the GeneAmp Carryover Prevention Kit [**Part No. N808-0068**] use 72ºC). The following steps 4.2 to 4.4 discuss the effect of changing various conditions.

Table 1. Applied Biosystems GeneAmp PCR Instrument Systems' Cycling Profile Times and Temperatures for AmpliTaq DNA Polymerase.

GeneAmp PCR Instrument System	Tubes and Volumes		Examples of Times and Temperatures			
	Tube Type	Volume Range in µL/Tube	Initial Step	Each of 25 or more Cycles		Final Step**
				Melt	Anneal/Extend	
DNA Thermal Cycler 480	GeneAmp Thin-walled Reaction	10-100	STEP CYCLE	STEP CYCLE		TIME DELAY
			1 min 45 sec 94°-96°C 1 cycle	45 sec 94°-96°C	45 sec-1 min 37°-72°C	6 min 72°C
GeneAmp PCR System 9700* System 9600 System 2400	MicroAmp Reaction	5-100	HOLD	CYCL (CYCLE)		HOLD
			1 min 94°-96°C 1 cycle	15 sec 94°-96°C	15-30 sec 37°-72°C	6 min** 72°C

* GeneAmp PCR System 9700 depends on mode.
** After the final step, store amplified samples in refrigerator or freezer until ready to use.

4.2 High G+C content DNA needs very high annealing (> 65ºC) and melting temperatures or the use of 7-deaza-2'-deoxy-GTP mixed with dGTP, to overcome secondary structures.[17] The half life of AmpliTaq DNA Polymerase (< 35 minutes at > 95ºC)[18] suggests 95º -96ºC as the maximum practical melting temperature. It is very important in the early cycles to be sure to melt completely the template DNA. When genomic DNA is used as the starting template, melting at 97ºC for the first few cycles will ensure single-stranded template for the PCR reaction. The melting temperature can be reduced for the later cycles because the smaller PCR product usually melts completely at a lower temperature (unless the PCR product is excessively G+C rich) than the starting genomic DNA.

4.3 Higher annealing temperatures (> 45ºC) generally result in much more specific product.[2,19] The optimum annealing temperature can be determined empirically by testing at 5ºC or smaller increments until the maximum in specificity is reached. At these temperatures AmpliTaq DNA Polymerase has significant activity so extension of primed templates is occurring. Two temperature rather than three temperature cycles may work where the anneal temperature is above 50ºC; the extension is completed at the anneal temperature. The slower extension rate at lower temperatures may require optimization of the time for such combined anneal-extend steps.

4.4 The length of the target sequence will affect the required extension time. Typically, AmpliTaq DNA Polymerase has an extension rate of 2,000 to 4,000 bases per minute at 70º-80ºC. Polymerization rates are significant even below 55ºC and with some templates, up to 85ºC.[3] As the amount of DNA increases in later cycles, the number of AmpliTaq DNA Polymerase molecules may become limiting for the extension time allotted. Increasing the extend times in later cycles may be needed to maintain efficiency of amplification. Use the autosegment extension feature for the DNA Thermal Cycler 480, or the AUTO program for the GeneAmp PCR System 9600, and AUTO X for the GeneAmp PCR System 9700/2400.

TROUBLESHOOTING

Observation	Possible Cause	Recommended Action
Reduce or no product band is observed.	Experimental sample concentration too low.	Increase experimental sample concentration.
	Experimental sample DNA damaged or degraded.	Use experimental sample that has been processed to minimize nicking and shearing.
	Enzyme concentration too low.	Increase enzyme concentration in increments of 0.25 Unit per 50 µL reaction.
	Magnesium concentration too low.	Increase magnesium concentration in increments of 0.2 mM.
	Denaturation time too long or too short.	Adjust denaturation time in increments of 5 seconds.
	Denaturation temperature too high or too low.	Adjust denaturation temperature in increments of 1 degree.
	Anneal/extend temperature too high.	Lower temperature in increments of 2°C.
	Anneal/extend time too short.	Lengthen time in increments of 30 seconds.
	Cycle number too low.	Increase cycle number in increments of 3.
	pH or ionic strength not optimal.	See **Section 3.10**.
	Primer design not optimal.	Review primer design and composition.

Observation	Possible Cause	Recommended Action
Product is multi-banded.	Anneal/extend temperature too low.	Raise temperature in increments of 2 degrees.
	Primer design not optimal.	Review primer design and composition.
	Reaction Not Hot Started.	Try Manual Hot Start or AmpliTaq Gold® DNA Polymerase.
Product band is smeared.	Carry-over contamination.	Set up PCR reactions in a separate area.
	Enzyme concentration too high.	Decrease enzyme concentration in increments of 0.25 Unit per 50 µL reaction.
	Magnesium concentration too high.	Decrease magnesium concentration in increments of 0.1 mM.
	Denaturation time too short.	Lengthen denaturation time in increments of 5 seconds.
	Denaturation temperature too low.	Raise denaturation temperature in increments of 1 degree.
	Anneal/extend time too long.	Shorten time in increments of 30 seconds.
	Cycle number too high.	Reduce cycle number in increments of 3 cycles.
	Reaction Not Hot Started.	Try Manual Hot Start or AmpliTaq Gold DNA Polymerase.
Partial or complete loss of product band.	Carry-over contamination.	Set up PCR reactions in a separate area.
	Experimental sample DNA degraded.	Test a new aliquot of sample.

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ORDERING INFORMATION
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N801-0002 GeneAmp® PCR System 9600, 240 V, 50 Hz
N801-0003 GeneAmp® PCR System 9600, 100 V, 50/60 Hz

N805-0001 GeneAmp® PCR System 9700, Multi-voltage

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