AmpliTaq Gold® DNA Polymerase, LD

Hot Start, Strong Finish™

Protocol

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

Information in this document is subject to change without notice. Applied Biosystems assumes no responsibility for any errors that may appear in this document.

APPLIED BIOSYSTEMS DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. IN NO EVENT SHALL APPLIED BIOSYSTEMS BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

Notice to Purchaser: Limited License

Use of this product is covered by US patent claims and corresponding patent claims outside the US. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims) and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

TRADEMARKS:

Applera, Applied Biosystems, AB (Design), GeneAmp, Hot Start Strong Finish, and MicroAmp are registered trademarks of Applied Biosystems or its subsidiaries in the U.S. and/or certain other countries.

AmpliTaq Gold is a registered trademark of Roche Molecular Systems, Inc.

All other trademarks are the sole property of their respective owners.

Part Number 4338858 Rev. D
6/2010

Contents

About AmpliTaq Gold DNA Polymerase, LD

1

In This Chapter

Product Overview

AmpliTaq Gold DNA Polymerase, LD

AmpliTaq Gold® DNA Polymerase, LD (low DNA) 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 (Lawyer *et al.,* 1989). This enzyme is identical to AmpliTaq Gold® DNA Polymerase but is further purified through a proprietary process to reduce bacterial DNA introduced from the host.

AmpliTaq Gold DNA Polymerase, LD is recommended for PCR applications that require low background levels of bacterial DNA. The very low DNA levels in the preparation make this product especially useful for PCR amplification of low copy number bacterial target sequences. The purification process ensures that non-specific, false positive DNA products due to DNA contamination are minimized during the Polymerase Chain Reaction (PCR).

IMPORTANT! Due to the special care needed for very low background DNA amplifications with AmpliTaq Gold DNA Polymerase, LD, carefully read "Preventing DNA Contamination" on page 2-2 before proceeding.

Performance Characteristics

AmpliTaq Gold DNA Polymerase, LD is QC-tested to verify that less than or equal to 10 copies of bacterial 16S ribosomal RNA gene sequences are present in a standard 5.0-unit aliquot.

Each lot of AmpliTaq Gold DNA Polymerase, LD yields a specifically expressed visible band on an ethidium bromide-stained agarose gel. This visible band represents a 142 base pair product created with 10 copies of HIV-1 positive control DNA per reaction.

Hot Start, Strong Finish™

AmpliTaq Gold DNA Polymerase, LD is a chemically modified enzyme that automates the Hot Start technique and creates a strong finish in your PCR experiment.

The Hot Start technique for performing PCR (Faloona *et al*., 1990; Chou *et al*., 1992) is a simple modification of the original PCR method whereby the amplification reaction is initiated above the optimal primer-annealing temperature. In conventional PCR methods, active reaction components are exposed to suboptimal annealing temperatures resulting in unintended priming and subsequent formation of nonspecific products.

Because nonspecific product formation occurs at the beginning of the PCR, these nonspecific products can be efficiently amplified throughout the remaining PCR cycles. This unintended amplification of nonspecific products can result in poor yield of the desired product. The sensitivity of the experiment is reduced, both by decreasing the desired amplification signal and by obscuring it with high background signal.

Conversely, when the Hot Start PCR method is used, primers bind only to their specific target and the polymerase activity is directed only to that target. This process results in increased sensitivity and yield, and decreased nonspecific product amplification.

Enzyme Provided In the Inactive State

The AmpliTaq Gold DNA Polymerase, LD enzyme is provided in an inactive state. When the chemical moiety is attached to the enzyme, the enzyme is inactive. This inactive enzyme allows flexibility in the reaction setup, including pre-mixing of PCR reagents at room temperature. Because the enzyme is inactive during set-up and during the first ramp of PCR, when the reaction goes through sub-optimal primer annealing temperatures, mis-primed primers are not extended.

AmpliTaq Gold DNA Polymerase, LD activation requires a heat activation step, well above optimal annealing. This heat activation step provides an automated chemical hot start. AmpliTaq Gold DNA Polymerase, LD can be completely or partially activated in a pre-PCR heat step, or it can be allowed to activate slowly during thermal cycling. Slow activation can provide a hot start and a "time release" of active enzyme, where polymerase activity builds as PCR product accumulates.

Enzyme Activation

Activation of AmpliTaq Gold DNA Polymerase, LD depends on three factors (Birch *et al*., 1996; Bost *et al*., 1997):

- Incubation temperature
- Incubation time
- Reaction pH

The activation is performed by heating the enzyme reaction mix for 1 to 10 minutes at 95°C. Upon activation, the modifier is permanently released, regenerating the active enzyme and initiating PCR amplification.

Note: For more information about AmpliTaq Gold DNA Polymerase, LD see Appendix A, "Enzyme Characteristics."

Multiplexing

Multiplexing is an amplification technique in which multiple primer sets are used to amplify multiple specific targets simultaneously. The use of AmpliTaq Gold DNA Polymerase, LD significantly reduces non-specific product amplification, allowing specific multiplex amplification to occur. For guidelines on performing multiplex PCR see Appendix B, "Multiplex PCR Amplification."

GeneAmp 10X PCR Gold Buffer

GeneAmp® 10X PCR Gold Buffer is formulated to activate AmpliTaq Gold DNA Polymerase, LD for highly specific and robust PCR amplification. The ionic strength and pH of the GeneAmp 10X PCR Gold Buffer is optimized for use with AmpliTaq Gold DNA Polymerase, LD.

GeneAmp dNTPs

We recommend that you use Applied Biosystem's GeneAmp® dNTPs to ensure low background bacterial DNA.

Materials and Equipment

Two Kits Available

AmpliTaq Gold DNA Polymerase, LD is available in two kit configurations.

- The AmpliTaq Gold DNA Polymerase, LD, 250 Units, (PN 4338856) reagent kit
- The AmpliTaq Gold DNA Polymerase, LD, 1000 Units, (PN 4338857) reagent kit

Kit Components

The following reagents are supplied with each AmpliTaq Gold DNA Polymerase, LD kit:

Table 1-1 AmpliTaq Gold DNA Polymerase, LD 250 unit and 1000 unit kit contents

	250 Unit Kit	**1000 Unit Kit**
Part Number	4338856	4338857
AmpliTaq Gold DNA Polymerase, LD 5 Units/µL	1 x 50 µL tube containing 250 Units	1 x 200 µL tube containing 1000 Units
GeneAmp 10X PCR Gold Buffer	1 x 1.5 mL tube containing 150 mM Tris-HCl, 500 mM KCl, pH 8.0 (at room temperature)	4 x 1.5 mL tubes containing 150 mM Tris-HCl, 500 mM KCl, pH 8.0 (at room temperature)
25 mM $MgCl_2$ Solution	1 x 1.5 mL tube containing 25 mM $MgCl_2$	4 x 1.5 mLtubes containing 25 mM $MgCl_2$

Materials Required but Not Supplied

In addition to the reagents supplied in the box, the items listed in the following table are required:

Item	Source
dNTP	Applied Biosystems
GeneAmp® PCR System 9700 and 2700 Thermal Cyclers	Applied Biosystems
MicroAmp® disposables	Applied Biosystems
Agarose	Major laboratory supplier (MLS)
Disposable gloves	MLS
Electrophoresis apparatus	MLS
Microcentrifuge	MLS
Pipets, positive-displacement or air-displacement	MLS
Pipet tips with filter plugs	MLS
Polypropylene tubes	MLS
TE buffer	MLS
Vortex	MLS

Storage and Stability

Store the AmpliTaq Gold DNA Polymerase, LD reagents at –20 °C in a constant-temperature freezer. If stored under the recommended conditions, the enzyme will remain active through the control date printed on the label.

General Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note: Calls attention to useful information.

IMPORTANT! Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

Indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

Chemical Safety

Chemical Hazard Warning

CHEMICAL HAZARD. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the MSDSs provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. See "About MSDSs."
- Minimize contact with chemicals. When handling chemicals, wear appropriate personal protective equipment such as safety glasses, gloves, and protective clothing. For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, a fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the cleanup procedures recommended in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.

After you enter the required information, click **View/Deliver Selected Documents Now**.

Protocol for PCR Set Up

2

In This Chapter

Preventing DNA Contamination

About DNA Contamination in PCR Reactions

Due to the enormous amplification that occurs during PCR, small levels of DNA contamination can result in product formation even in the absence of purposefully added template DNA (Kwok and Higuchi, 1989). Using AmpliTaq Gold DNA Polymerase, LD is only one key to attaining very low background levels of bacterial DNA. To fully exploit the high purity of this product, you must take care to ensure that all PCR reagents and supplies are free of background bacterial DNA sequences and that PCR amplifications occur in a DNA-free environment.

DNA contamination can come from:

- Previous PCR amplifications
- Samples with high DNA levels
- Cross contamination
- Positive control templates

General PCR Practices

Follow these recommendations to prevent DNA contamination in PCR reactions:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products.
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.

- Clean lab benches and equipment periodically with a 10% bleach solution.
- Use dedicated or disposable sterile vessels, solutions, and pipets (preferably positive-displacement pipets or tips with hydrophobic filters) to minimize cross-contamination during DNA preparation, reaction mixing, and sample analysis (Kwok, 1990).

Protocol for Setting Up PCR

Prepare a Master Mix

In order to increase accuracy and reduce the number of reagent transfers in the PCR setup, prepare a master mix of reagents (water, buffer, dNTPs, primers, and enzyme) for all samples and aliquot into individual tubes.

Recipe for the Master Mix

Use the following steps to prepare a master mix:

1.	Thaw and gently mix each of the reagents. **Note:** Avoid generating bubbles when mixing the enzyme.  **CHEMICAL HAZARD. AmpliTaq Gold DNA Polymerase, LD** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Spin the enzyme solution down in a microcentrifuge.
3.	Pipet the enzyme and buffers carefully and slowly. **Note:** The viscosity of the glycerol in the enzyme storage buffer can lead to pipetting errors.
4.	Prepare a master mix by adding the reagents in the order and the proportions shown in Table 2-1 on page 2-5.

Combine the following reagents to prepare the master mix.

Table 2-1 Master mix reagents

Component	Volume per Reaction (µL)	Concentration in Master Mix
Water	*	—
10X PCR Gold Buffer	5	1X
25 mM $MgCl_2$	2—8	1.0—4.0 mM†
10 mM dATP	1	200 µM
10 mM dCTP	1	200 µM
10 mM dGTP	1	200 µM
10 mM dTTP‡	1	200 µM
User-provided Primer 1	1—5	0.2—1.0 µM
User-provided Primer 2	1—5	0.2—1.0 µM
Template	*	<1 µg/reaction§
AmpliTaq Gold DNA Polymerase, LD	0.25	1.25 Units/reaction
Total Volume	50 µL	—

*Any combination of water and template can be used as long as the total volume of the master mix, sample and primers equal 50 µL.

†The optimal magnesium chloride concentration may vary, depending on the primer and template 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.

‡dUTP substitution typically requires a concentration twice that of any other dNTP for optimal amplification.

§Preferably > 10^4 copies of template but < 1 µg DNA/reaction.

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.

Reagent Optimization Guidelines for Custom Applications

About Reagent Optimization

Optimizing reactions for each primer-template pair may be necessary. Achieve optimization by following the suggested guidelines for designing the primers and by varying the concentration of the following reagents:

- Template
- Primer
- $MgCl_2$
- dNTPs
- Enzyme

The effect of these variations can be monitored by examining the intensity and distribution of amplification products after electrophoresis on agarose followed by visualization with ethidium bromide staining of the gel.

Optimizing the Template Concentration

Use the following guidelines to optimize the template concentration:

- The DNA segment to be amplified from the template can be 5 to 10 kb long (Jeffreys *et al*., 1988), although 100 to 1000 bases are more typical and easier to amplify.
- Start with enough copies of the template to obtain a signal after 25 to 30 cycles: preferably more than 10^4 copies, but less than 1 µg of human genomic DNA per 50 µL reaction.
- If the target DNA concentration is low, more than 35 cycles may be required to produce sufficient product for analysis. As few as 1 to 10 target copies can be amplified (Saiki, Gelfand, Stoffel, 1988; Chou *et al*., 1992). Validation for low copy number amplifications is best done for an average of 5 to 10 target molecules per sample to avoid statistically arising dropouts (false negatives).

Designing the Primers

Use the following guidelines when designing your primers:

- The single-stranded DNA primers should be 15 to 30 bases in length.
- To avoid potential problems, primers should be purified by gel electrophoresis or HPLC ion-exchange chromatography.
- Primer sequences should not complement within themselves or to each other, particularly at the 3′ ends, to avoid template-independent amplification of primer sequences (or "primer dimer"). Primer dimer can lead to other, larger primer artifacts. Primer dimer may occur to some extent even without an apparent overlap.

Optimizing the Primer Concentration

Use the following guidelines to optimize the primer concentration:

- Determine ptimal primer concentrations empirically by testing concentrations in the range of 0.1 to 1.0 µM.
  - Primer concentrations that are too low result in little or no PCR product.
  - Primer concentrations that are too high may result in amplification of nontarget sequences.
- Primer concentrations in the range of 0.2 to 0.5 µM work for most PCR amplifications.
- Reducing each primer concentration (for example, to 0.2 µM) helps reduce amplification of nonspecific products.

Optimizing the $MgCl_2$ Concentration

The magnesium ion concentration required for optimal PCR amplification depends on the specific set of primers and template.

Too much or too little $MgCl_2$ reduces amplification efficiency or results in amplification of non-target sequences. The optimal $MgCl_2$ concentration must be determined empirically.

Follow these guidelines to determine the optimum $MgCl_2$ concentration for each primer set:

- Use the 25 mM $MgCl_2$ supplied to adjust the magnesium ion concentration.
- Vary the concentration of $MgCl_2$ around a midpoint of 2.5 mM. A typical range is 1.0 to 4.0 mM.

- Raise the $MgCl_2$ concentration in the reaction mix proportionately if the samples contain EDTA, citrate, or other chelators.
- Adjust the $MgCl_2$ concentration in parallel with significant changes in the concentration (higher or lower) of sample DNA or dNTPs to keep the free magnesium ion constant. For example, reduce the concentration of dNTP from 200 µM each to 40 µM each, and reduce the $MgCl_2$ concentration to 640 µM.

Optimizing the dNTP Concentration

The dNTP concentration provided for the Reaction Mix is balanced. If the blend is altered and the concentration of any one dNTP is significantly different from the rest, then AmpliTaq Gold DNA Polymerase, LD will tend to misincorporate, slow down and/or terminate prematurely (Innis *et al*., 1988). Lower concentrations of dNTPs (40 µM) favor increased polymerase fidelity (Eckert and Kunkel, 1992).

Optimizing the Enzyme Concentration

Increasing the AmpliTaq Gold DNA Polymerase, LD concentration up to 2X the recommended amount may improve the yield of amplification product.

Thermal Cycling 3

In This Chapter

Specific Considerations for Thermal Cycling with AmpliTaq Gold DNA Polymerase, LD

Heat Activation Required

AmpliTaq Gold DNA Polymerase, LD must be activated by heat, either before or during PCR cycling. For more information refer to "Adjusting the Hold Period for Activation" on page 3-6.

Product Yield Can Be Optimized

AmpliTaq Gold DNA Polymerase, LD enhances the specificity of PCR, so that both polymerase concentration and cycle number can be increased to yield greater product. If a pre-PCR incubation is not used, Time Release PCR can be implemented by increasing the cycle number. It may take up to five additional PCR cycles to give equivalent product yield. Additionally, a combination or a shorter pre-PCR heat step and extra cycles can improve results.

Optimizing Thermal Cycling

Selecting Two- or Three-Temperature Thermal Cycling

Optimize thermal cycling conditions for each DNA template by using one of the following PCR temperature conditions:

- Two-temperature PCR — Use the two-temperature PCR when primer annealing temperatures are more than 60 °C. (See "Two-Temperature Thermal Cycling" on page 3-4.)
- Three-temperature PCR — Use the three-temperature PCR when the templates have high G+C content and/or secondary structure, or desired primer annealing temperatures are below 60 °C. (See "Three-Temperature Thermal Cycling" on page 3-5.)

Two-Temperature Thermal Cycling

Two-temperature PCR consolidates the annealing and extension steps into one. The extension is completed at the annealing temperature. Two-temperature PCR consists of:

- Denaturation of DNA template
- Annealing and extension of primers

Note: Fifteen seconds for denaturation and annealing is adequate when using GeneAmp PCR System thermal cyclers, which display a calculated sample temperature. Some models of thermal cyclers may require longer times.

Table 3-1 Two-temperature thermal cycling on the GeneAmp PCR System 9700 or 2700

Step	AmpliTaq Gold DNA Polymerase, LD Enzyme Activation	PCR		PCR (Final Step)
	HOLD	CYCLE (30 cycles)		HOLD
		Denature	Anneal/ Extend	
Temp	95 °C	95 °C	60 to 70 °C*	72 °C
Time	5 min†	15 sec	60 sec/kb	7 min

*Adjust the temperature according to the primer melting temperature.
†Adjust the time according to the desired initial enzyme activation (refer to "Thermal Activation Profile" on page A-3). Start with an initial activation of 95 °C for 5 minutes and adjust as required (refer to "Adjusting the Hold Period for Activation" on page 3-6 for more details).

Three-Temperature Thermal Cycling

Three-temperature PCR consists of:

- Denaturation of DNA template
- Annealing of the primers to the template
- Extension of the primers

Note: Fifteen seconds for denaturation and annealing is adequate when using GeneAmp PCR System thermal cyclers, which display a calculated sample temperature. Some models of thermal cyclers may require longer times.

Table 3-2 Three-temperature thermal cycling on the GeneAmp PCR System 9700 or 2700

Step	AmpliTaq Gold DNA Polymerase, LD Enzyme Activation	PCR			PCR (Final Step)
	HOLD	CYCLE (30 cycles)			HOLD
		Denature	Anneal	Extend	
Temp	95 °C	95 °C	37 to 65 °C*	72 °C	72 °C
Time	5 min†	15 sec	15 sec	60 sec/kb	7 min

*Adjust the temperature according to the primer melting temperature.
†Adjust the time according to the desired initial enzyme activation (refer to "Thermal Activation Profile" on page A-3). Start with an initial activation of 95 °C for 5 minutes and adjust as required (refer to ""Adjusting the Hold Period for Activation" on page 3-6" for more details).

Optimization Guidelines

Adjusting the Hold Period for Activation

For general PCR runs, we recommend a pre-PCR activation setup of 95 °C for 5 minutes. Perform a titration of pre-PCR activation times (2 to 10 minutes in 1 minute intervals) to find the best up-front enzyme activity for your reaction. Activation of AmpliTaq Gold DNA Polymerase, LD can also be modulated to release active enzyme slowly over time (time release), allowing enzyme activity to increase with cycle number as the amount of template increases. This type of PCR can be accomplished as follows:

- With no activation during the pre-PCR hold period
- With limited activation during the pre-PCR hold period

In a "Time Release" protocol, the enzyme is released slowly to match the template concentration, which further increases the specificity. When a no or limited (1 to 2 minute) pre-PCR activation is used, the enzyme is released gradually during the denaturation step (95 °C for 15 seconds) of each cycle. Because the enzyme is released slowly, up to 5 additional cycles may be required.

Limiting the amount of active enzyme at the beginning of the amplification reaction when low amounts of substrate molecules are present enhances high specificity in the early PCR cycles. See page A-3 for the thermal activation profile for AmpliTaq Gold DNA Polymerase, LD.

Adjusting the Denaturation Conditions

The following are guidelines for adjusting the denaturation conditions:

- It is very important in the early cycles to make sure that your DNA template is completely melted.
- The maximum denaturation temperature should not exceed 95 to 96 °C (Gelfand and White, 1990).
- 15 seconds is adequate when using GeneAmp PCR System thermal cyclers with a calculated in-tube temperature. Some models of thermal cyclers may require longer denaturation times.

Adjusting the Annealing Conditions

The following are guidelines for adjusting annealing conditions:

- For increased product specificity, use annealing temperatures greater than 45 °C (Saiki, Gelfand, and Stoffel, 1988; Rychlik, Spencer, and Rhoads, 1990).
- Use two-temperature PCR for annealing temperatures greater than 60 °C.
- Determine the optimum annealing temperature empirically by testing at 5 °C or smaller increments, until the maximum specificity is reached. At annealing temperatures, activated AmpliTaq Gold DNA Polymerase, LD has significant activity that results in the extension of primed templates.
- Computer programs designed to calculate primer melting temperatures (T_m) can assist you in narrowing the range of annealing temperatures for empirical determination. A T_m calculator can be found on the Applied Biosystems Web site at **http://www.appliedbiosystems.com**. Click **Services & Support > Technical Tools > Tm Calculator**. In addition, the GeneAmp PCR System 9700 Thermal Cycler also contains a T_m calculator.
- 15 seconds is adequate when using GeneAmp PCR System thermal cyclers with a calculated in tube temperature. Some models of thermal cyclers may require longer annealing times.

Adjusting the Extension Conditions

The following are guidelines for adjusting the extension conditions:

- The length of the target sequence affects the required extension time. Longer targets require increased extension times. As a general rule, allow an extension time of approximately 60 seconds per 1000 bases at 72 °C.
- As the amount of DNA increases, the number of DNA polymerase molecules may become limiting. Compensate for this limitation by increasing the extension time in later cycles.
- For two-temperature PCR, extension temperatures typically lower than 72 °C; extension times may need to be lengthened accordingly.

Storing Sample Reactions

Following thermal cycling, samples can be stored at 4 °C until subsequent analysis.

Analyzing the Reaction

Analysis of the Reaction

After thermocyling is complete, you can create a visual representation of the amplification product by running an agarose gel. Perform the electrophoresis steps in the following table.

Table 3-3 To run an agarose gel:

1.	Electrophorese 5 µL of the control reaction through a 2% NuSieve, 0.5% SeaKem, or similar composition agarose gel.
2.	Electrophorese the samples at 70 to 80V/10 cm for 1.25 to 1.5 hours.
3.	Stain the gel with 0.5 µg/mL of ethidium bromide solution.  **WARNING** **CHEMICAL HAZARD**. Ethidium bromide causes eye, skin, and respiratory tract irritation and is a known mutagen (i.e., it can change genetic material in a living cell and has the potential to cause cancer). Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Enzyme Characteristics

A

AmpliTaq Gold DNA Polymerase, LD

Unique Characteristics

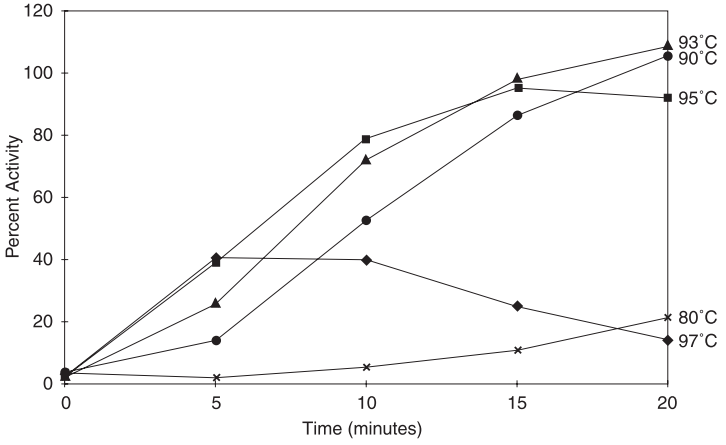
AmpliTaq Gold DNA Polymerase, LD is a chemically modified form of AmpliTaq DNA Polymerase. This thermostable 94-kDa protein is encoded by a modified form of a *Thermus aquaticus* DNA polymerase gene and expressed in an *Escherichia coli* host (Lawyer *et al*., 1989). See Table A-1, "Characteristics of AmpliTaq Gold DNA Polymerase, LD," on page A-2.

Table A-1 Characteristics of AmpliTaq Gold DNA Polymerase, LD

Item	Description
Unit definition	The enzyme is provided at 5 U/µL. One unit of enzyme is defined as the amount that will incorporate 10 nmol of dNTPs into acid-insoluble material per 30 minutes in a 10-minute incubation at 74 °C under the conditions listed in this table under "Analysis conditions." **Note:** The enzyme is shipped in an inactive form. Before analyzing, the enzyme is activated by heating for 3 hours at 80 °C.
Analysis conditions	The analysis conditions are in the presence of the following: • 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 β-mercaptoethanol • 200 µM each of dATP, dGTP, dTTP • 100 µM [α-^{32}P]•dCTP (0.05-0.1 Ci/mmol) • salmon sperm DNA, activated by a modification of methods in Richardson *et al.*, 1966; mixed in a final volume of 50 µL and incubated at 74 °C for 10 min.
Storage buffer	Amplitaq Gold DNA Polymerase, LD is stored in a buffer containing the following: • 20 mM Tris-HCl, pH 9.0 (at room temperature) • 100 mM KCl, 0.1 mM EDTA (ethylenediaminetetraacetic acid) • 1.0 mM DTT (dithiothreitol) • 50% v/v glycerol • 0.5% w/v Tween 20®. (See "Recipe for the Master Mix" on page 2-4 and "Chemical Safety" on page 1-8 for instructions.)
Associated activities	Endonuclease and exonuclease activities were not detectable after 1 hour incubation of 600 ng of supercoiled pBR322 (dam$^-$,dcm$^-$) or 600 ng of *Msp*1-digested pBR322 DNA, respectively, at 74 °C, in the presence of 8 units of AmpliTaq Gold DNA Polymerase. The enzyme has a fork-like, structure-dependent, polymerization-enhanced, 5´-to-3´ nuclease activity. It lacks a 3´-to-5´ exonuclease activity (Innis *et al*., 1988; Holland *et al*., 1991).

Thermal Activation Profile

The activation profile of AmpliTaq Gold DNA Polymerase, LD in Gold buffer at different temperatures is shown in the graph below.



Multiplex PCR Amplification B

Designing Multiplex PCR Amplification

About Multiplex PCR

Multiplex PCR is an amplification technique in which multiple primer sets amplify specific targets in a single reaction. Using AmpliTaq Gold DNA Polymerase, LD simplifies the multiplexing process by reducing non-specific product amplification and "primer dimer" formation.

Designing Multiplex PCR

When designing multiplex PCR, optimize the following factors:

- Primer selections
- Reagent concentrations
- PCR conditions

To optimize the multiplex PCR factors:

1.	Select primers that have similar melting temperatures and amplified products of distinguishable lengths. **Note:** Primers can be optimized with a specialized primer program.
2.	Optimize the following conditions separately for each product: • Annealing temperature • Primer concentration • $MgCl_2$ concentration
3.	Combine reactions and reoptimize combined components as in step 2 if necessary.

Troubleshooting

C

Troubleshooting Guide:

Observation	Possible Cause	Recommended Action
Low levels or no product band visible	Template concentration too low	Increase sample concentration.
	Experimental sample DNA damaged or degraded	Use sample that has been processed to minimize shearing and nicking.
	Denaturation time too short or too long	Adjust time in increments of 5 seconds.
	Denaturation temperature too low or too high	Adjust temperature in increments of 1 °C.
	Annealing/extension temperature too high	Lower temperature in increments of 2 °C.
	Annealing/extension time too short	Lengthen time in increments of 15 seconds.
	Cycle number too low	Increase cycle number in increments of three cycles.
	Primer design not optimal	Review primer design and composition.
	Preincubation/activation time not sufficient	Increase pre-PCR heat step in increments of 1 minute, or use “Time Release” protocol (refer to “Adjusting the Hold Period for Activation” on page 3-6).

Observation	Possible Cause	Recommended Action
Product band is smeared	Carryover contamination	See Chapter 2, "Protocol for PCR Set Up."
	Denaturation time too short or too long	Adjust time in increments of 5 seconds.
	Denaturation temperature too low	Increase temperature in increments of 1 °C.
	Annealing/extension time too long	Shorten time in increments of 15 seconds.
	Cycle number too high	Shorten cycle number in increments of three cycles.
	Experimental sample DNA degraded	Test a new aliquot of sample.
Nonspecific amplification with or without a product band	Carryover contamination	See "About DNA Contamination in PCR Reactions" on page 2-2.
	Nonspecific priming	Increase the anneal temperature in 1 to 2 °C increments.
	Too much initial enzyme activity	Reduce pre-PCR activation time or use a "Time Release" protocol. (See "Adjusting the Hold Period for Activation" on page 3-6.)
	Primer design not optimal	Review primer design and composition.

Bibliography

Birch, D. E., Kolomodin, L., Laird, W. J., McKinney, N., Wong, J., Young, K. K. Y., Zangenberg, G. A., and Zoccoli, M. A. 1996. Simplified Hot Start PCR. Product review in *Nature* 381: 445-446.

Bost, D. A., Zalloua, P., and Abramson, R. D. 1997. AmpliTaq Gold: Biochemical Characterization and PCR Optimization. *The FASEB Journal.* 11: A1370.

Chou, Q., Russell, M., Birch, D.E., Raymond, J., and Bloch, W. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low- copy-number amplifications. *Nucleic Acids Res*. 20:1717–1723.

Eckert, K.A. and Kunkel, T.A. 1992. The fidelity of DNA polymerases used in the polymerase chain reactions. In: *PCR: A Practical Approach*. McPherson, M.J., Quirke, P., and Taylor, G.R., eds. New York: Oxford University Press. 225–244.

Faloona, F., Weiss, S., Ferre, F., and Mullis, K. 1990. Direct detection of HIV sequences in blood high-gain polymerase chain reaction [abstract]. In: *6th International Conference on AIDS*, University of California, San Francisco: San Francisco (CA). Abstract 1019.

Gelfand, D.H. and White, T.J. 1990. Thermostable DNA polymerases. In: *PCR Protocols: A Guide to Methods and Applications.* Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds. San Diego: Academic Press. 129–141.

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5′→3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* 88:7276–7280.

Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl. Acad. Sci. USA* 85:9436–9440.

Jeffreys, A.J., Wilson, V., Neumann, R., and Keyte, J., 1988. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res.* 16:10953-10971.

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Kwok, S. 1990. Procedures to minimize PCR-product carry-over. In: *PCR Protocols: A Guide to Methods and Applications.* Innis, M.A., Gefland, D.H., Sninsky, J.J., and White, T.J., eds. San Diego: Academic Press. 142–145.

Lawyer, F.C., Stoffel, S., Saiki, R.K., Myambo, K., Drummond, R., and Gelfand, D.H. 1989. Isolation, characterization, and expression in *E. coli* of the DNA polymerase gene from the extreme thermophile, *Thermus aquaticus*, J. Biol. Chem. 264:6427-6437.

Richardson, C.C. 1966. DNA polymerase from *Escherichia coli*. In: *Procedures in Nucleic Acid Research.* Cantoni, G.L. and Davies, D.R., eds. New York: Harper & Row. 263–276.

Rychlik, W., Spencer, W.J., and Rhoads, R.E. 1990. Optimization of the annealing temperature for DNA amplification *in vitro* [published erratum appears in *Nucleic Acids Res* 1991 Feb 11;19(3):698]. *Nucleic Acids Res*. 18:6409–6412.

Saiki, R.K., Gelfand, D.H., Stoffel, S., *etbal*. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491.

Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

Worldwide Sales and Support
Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at www.appliedbiosystems.com.

www.appliedbiosystems.com

Applied Biosystems

Applied Biosystems is committed to providing the world's leading technology and information for life scientists.

6/2010
Part Number 4338858, Rev. D