GeneAmp® Fast PCR Master Mix (2X)

Protocol

Applied Biosystems

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Trademarks

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Contents

Preface

This preface contains:

Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–**IMPORTANT, CAUTION, WARNING, DANGER**–implies a particular level of observation or action, as defined below:

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

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

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

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 Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" on page vi.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or 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, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on 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.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Waste Hazard

CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or 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, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.

- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).

Additional information about biohazard guidelines is available at: **http://www.cdc.gov**

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Overview

Purpose

Use the GeneAmp® Fast PCR Master Mix (2✕) (PN 4362070) for fast DNA amplification on the Applied Biosystems 9800 Fast Thermal Cyclerwith the 96-Well Aluminum Sample Block Module.

Chemistry Overview

The GeneAmp® Fast PCR Master Mix (2✕) contains a hot start polymerase system that has been optimized to decrease the overall PCR amplification time and eliminate an activation step. The master mix is a premix of all components except the primers necessary to amplify your DNA target. Performance is similar to that of the AmpliTaq Gold® DNA Polymerase.

About This Protocol

This protocol provides:

- Background information about DNA amplification on the 9800 Fast thermal cycler
- A list of equipment and materials for using the GeneAmp® Fast PCR Master Mix (2✕)
- Procedures for using the GeneAmp® Fast PCR Master Mix (2✕)

Materials and Equipment

Master Mix Contents

The GeneAmp® Fast PCR Master Mix (2✕) (PN 4362070) contains two 1.25 mL bottles of master mix, sufficient for 250 20-µL PCR reactions.

Master Mix Contents

Each bottle of GeneAmp Fast PCR Master Mix is 2✕ the recommended usage concentration. Each bottle contains the following:

- AmpliTaq DNA Polymerase
- GeneAmp PCR Buffer
- dNTP
- $MgCl_2$
- Stabilizers

Note: The GeneAmp® Fast PCR Master Mix (2✕) is also available without this protocol (PN 4359187).

Storage and Stability

The GeneAmp® Fast PCR Master Mix (2✕) should be stored at 2 to 8 °C for up to one year.

Materials and Equipment Not Included

The table below includes user supplied equipment and materials for performing the GeneAmp® Fast PCR Master Mix (2✕). Unless otherwise noted, many items listed are available from major laboratory suppliers (MLS).

Materials and Equipment	Source
Adhesive Seal Applicator	Applied Biosystems (PN 4333183)
Applied Biosystems 9800 Fast Thermal Cycler With 96-Well Aluminum Sample Block Module	Applied Biosystems (PN 4352604)
GeneAmp® Fast PCR Master Mix (2✕)	Applied Biosystems
	(PN 4362070) with protocol
	(PN 4359187) without protocol
MicroAmp® Clear Adhesive Film	Applied Biosystems (PN 4306311)

Materials and Equipment	Source
Optical 96-Well Fast Thermal Cycling Plates with Barcode (code 128), 20 plates	Applied Biosystems (PN 4346906)
Reagent Tubes With Caps, 10-mL	Applied Biosystems (PN 4305932)
Centrifuge with plate holders	MLS
Disposable gloves	MLS
Gel electrophoresis and UV equipment	MLS
Microcentrifuge	MLS
Pipette tips, aerosol resistant, nuclease-free: 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range	MLS
Pipettors (positive-displacement, air-displacement, or multichannel): 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range	MLS
Polypropylene tubes	MLS
RNase-free, sterile-filtered water	MLS
Vortexer	MLS

Documents	Part Number
Applied Biosystems 9800 Fast Thermal Cycler With 96-Well Aluminum Sample Block Base Module User Guide	4350088
ABI PRISM™ 6100 Nucleic Acid PrepStation User Guide	4326242
ABI PRISM™ 6700 Automated Nucleic Acid Workstation User Guide	4304309
DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol	4343586

Preventing Contamination

Overview

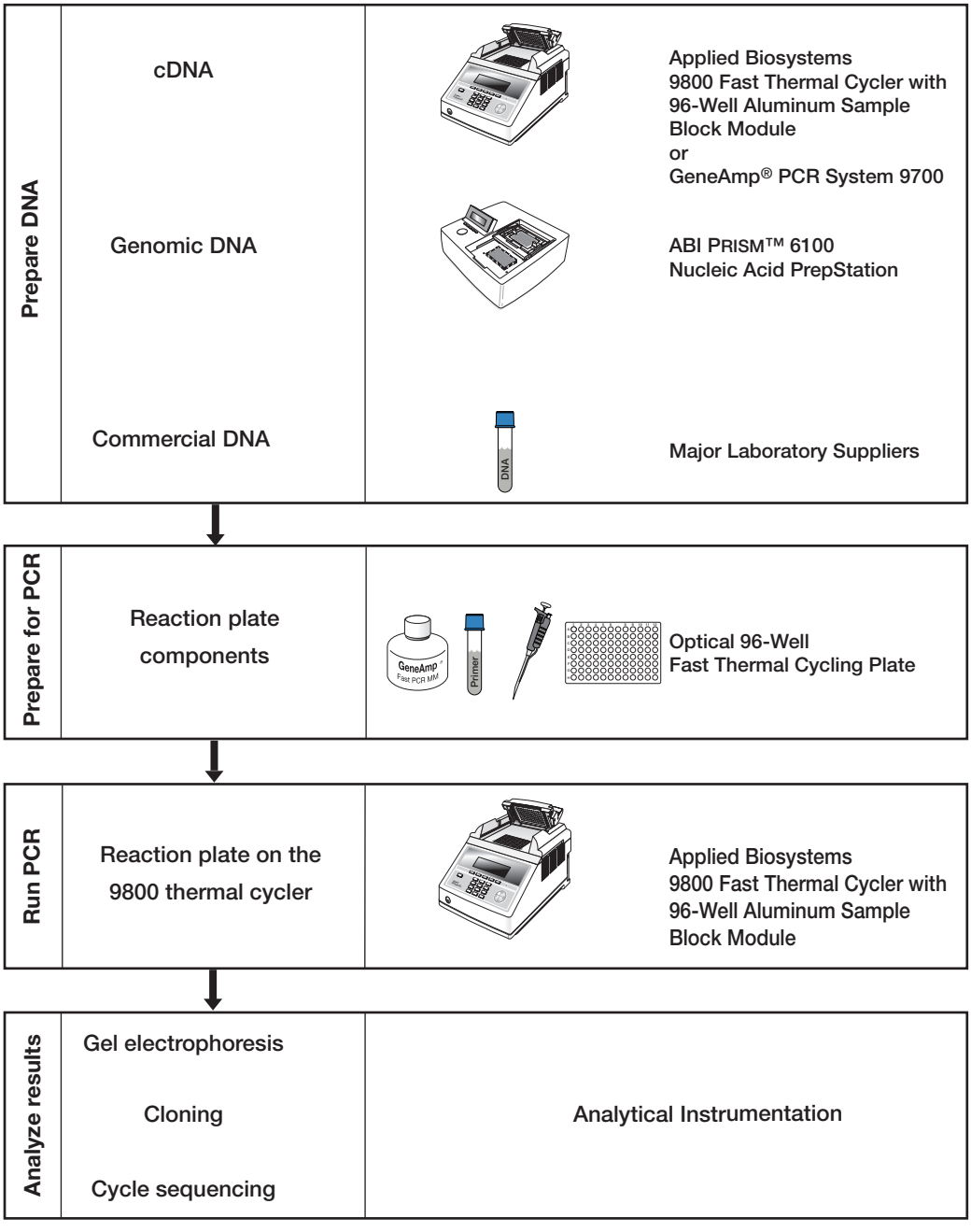
The repetitive nature of PCR assays requires special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The PCR process allows for the amplification of a single DNA molecule (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

General PCR Practices

General PCR practices to prevent contamination:

- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Do not bring amplified PCR products into the PCR setup area.
- 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.
- Open and close all sample tubes and reaction plates carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use positive-displacement or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.

Procedural Overview



Preparing DNA Samples

You can use either complementary DNA (cDNA) or genomic DNA (gDNA) samples with the GeneAmp Fast PCR Master Mix (2✕) for fast DNA amplification.

cDNA Samples

Use an Applied Biosystems thermal cycler to synthesize cDNA from RNA using one of the following Applied Biosystems reverse transcription (RT) kits.

- GeneAmp® Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (PN N8080069)
- GeneAmp® EZ r*Tth* RNA PCR Kit (PN N8080179, N8080178)
- TaqMan® EZ RT-PCR Kit (PN N808-0235)
- High-Capacity cDNA Archive Kit (PN 4322171)

The general procedure is as follows:

1. Obtain an RNase-free RNA sample
2. Prepare RT Master Mix
3. Set up the Applied Biosystems thermal cycler and software
4. Perform reverse transcription

gDNA Samples

Use the Applied Biosystems 6100 Nucleic Acid PrepStation and one of the following Applied Biosystems chemistry sets to purify gDNA.

- NucPrep® Chemistry System
- BloodPrep™ Chemistry System
- ABI PRISM™ TransPrep System

Refer to the *ABI PRISM™ 6100 Nucleic Acid PrepStation User Guide*, PN 4326242, and the *DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol*, PN 4343586, for procedures to purify gDNA from various sample types. A general procedure is shown on page 7.

The general procedure is as follows:

1. Digest sample with digestion buffer
2. Incubate
3. Add purification solution
4. Set up the ABI PRISM™ 6100 Nucleic Acid PrepStation and software
5. Pass lysates across a 96-well purification tray
6. Wash lysates
7. Elute purified DNA
8. Purify gDNA

Performing PCR Amplification

Overview

To perform PCR amplification using the GeneAmp® Fast PCR Master Mix (2✕), you can run one of the protocols listed in the table below.

Protocol	Page Number
Fast PCR Protocol	page 9
Optimizing the Fast PCR Protocol	page 14
Converting a Standard Three-Step Protocol to a Fast Protocol	page 21

About Fast PCR

During PCR amplification, the polymerase in the Fast PCR Master Mix amplifies target DNA using sequence-specific primers. Customize the protocol for your specific DNA sample.

GeneAmp Fast PCR Master Mix is provided at a convenient 2✕ concentration. Only the addition of DNA template and primers is required.

Reagent Preparation Guidelines

Follow these guidelines to ensure optimal PCR performance:

- Prior to use:
  - Mix the GeneAmp® Fast PCR Master Mix (2✕) thoroughly by swirling the bottle.
  - Vortex the primers, then centrifuge the tube briefly.
  - Place frozen DNA samples on ice to thaw. After the samples are thawed, vortex them, then centrifuge the tubes briefly.
- Prepare the PCR reaction mix before transferring it to the reaction plate for thermal cycling. See "Preparing the Reaction Mix" on page 10.
- Prepare the reaction plates for Fast DNA amplification using GeneAmp® Fast PCR Master Mix (2✕) no more than 2 hours before starting the instrument run.

Fast PCR Protocol

Overview

Applied Biosystems recommends performing four replicates of each reaction. For one reaction, follow the steps listed in "Preparing the Reaction Mix" on page 10. The recommended reaction volume is 20 µL for the Optical 96-Well Fast Thermal Cycling Plate.

Reaction Components

The table below lists the components, volumes, and concentrations that comprise the reaction mix.

Component	Volume per Reaction[a]	Final Conc.
Deionized water	see below[b]	—
User-provided Primer 1	_ µL	0.2–1.0 µM
User-provided Primer 2	_ µL	0.2–1.0 µM
User-provided experimental template	see below[b]	<1 µg/reaction[c]
GeneAmp® Fast PCR Master Mix (2X)	10 µL	1X
Total volume	20 µL	—

a. Reaction volume may be adjusted to your experimental design, keeping the concentrations of reactants constant.

b. Use any combination of water and template as long as the total volume of the PCR Master Mix, sample, and primers equals 20 µL.

c. Preferably >10^2 copies of template but <0.2 µg g DNA per reaction.

Preparing the Reaction Mix

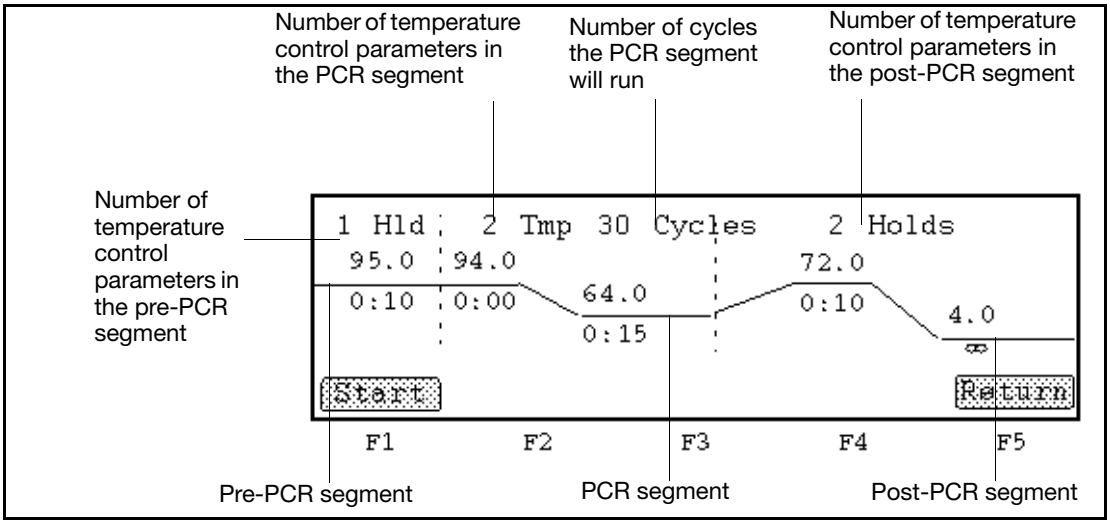
To prepare the reaction mix:

1.	Thaw reagents on ice. See "Reaction Components" on page 14.
2.	Calculate the amount of template, primers, and deionized water needed to make up a total volume of 20 µL when added to the master mix. **Note:** The amount of each component depends on your specific primer and template concentrations. See "Reaction Components" on page 14.
3.	Pipette the calculated amount of template, primers, and deionized water into replicate wells on an optical 96-Well Fast thermal cycling plate. **Note:** Applied Biosystems recommends running four replicates of each sample. For reaction replicates, multiply the amount of each component volume by the number of reactions needed.
4.	Gently swirl the bottle of GeneAmp Fast PCR Master Mix (2X) to evenly mix the contents. **IMPORTANT!** Do not introduce bubbles into the master mix solution.
5.	Pipette 10 µL of the GeneAmp Fast PCR Master Mix (2X) into each reaction well.
6.	Seal the plate with MicroAmp Clear Adhesive Film. **Note:** Ensure good contact between the film and the plate by using the Adhesive Seal Applicator.
7.	Place the plate in a plate centrifuge and spin for 30–60 sec to ensure the liquid collects at the bottom of each well.
8.	Perform thermal cycling using the parameters described in "Thermal Cycling" on page 11.

Note: For reaction replicates, prepare the reaction mix by multiplying the amount of each component volume by the number of reactions needed.

Thermal Cycling

The 9800 instrument comes with a default PCR thermal cycling method. The Create screen displays this default method. The default PCR thermal profile uses two-step PCR. You can run the default method, or use it as a template to create a customized method.



9800 Fast Thermal Cycling Profile

Program the 9800 Fast thermal cycler using a combination of the parameters in the table below for your specific target sequence.

IMPORTANT! The GeneAmp Fast PCR Master Mix (2X) has been optimized for annealing/extension temperatures at or above 62 °C. The recommended upper limit is 72 °C.

Step	Fast Start of Enzyme	PCR		PCR (Final Step)	
	HOLD	Cycle (35 Cycles)		HOLD	HOLD
		Denature	Anneal/Extend		
Temp	95 °C	94 °C	62–72 °C[a]	72 °C	4 °C
Time	10 sec	0 sec	25 sec/Kb[b]	10–60 sec	∞

a. Empirically determine the annealing/extension temperature. Adjust the temperature according to the primer's melting temperature (T_m).

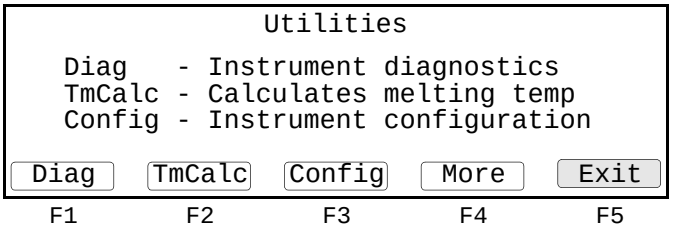
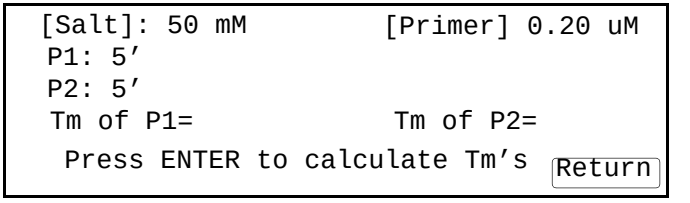
b. The length of the target sequence determines the annealing/extension time. The time of 25 sec/Kb is a starting point. Use this starting point to optimize the time for your target sequence.

Using the T_m Calculator

The Applied Biosystems Web site, www.appliedbiosystems.com, provides a T_m calculator for your use. Go to:

/Support/Application Tools & Tutorials/PCR/T_m Calculator.

The procedure below shows you how to use the T_m calculator to determine the annealing/extension temperature of a primer set of known sequence.

1.	In the Main menu, press **Util** to open the Utilities screen. 
2.	Press **TmCalc** to open the Tm Calculator. 
3.	Enter the salt concentration using values of 5 to 1000. **Note:** The default is 50.
4.	Enter the primer concentration using values of 0.01 to 10.00. **Note:** The default it 0.20.
5.	Enter the primer sequence in P1.

6.	Enter the primer sequence in P2, then press **Enter** to calculate the T_ms. **Note:** The melting points are displayed. Use this information to program a run. For more information about creating methods and runs, refer to the *Applied Biosystems 9800 Fast Thermal Cycler with 96-Well Aluminum Sample Block Base Module User Guide* (PN 4350088).
7.	Press **Return** to go back to the Utilities screen.

Optimizing the Fast PCR Protocol

Overview

Follow the guidelines described below to optimize the Fast protocol for your target sequence. For creating a new method on the 9800 Fast thermal cycler, refer to the *Applied Biosystems 9800 Fast Thermal Cycler with 96-Well Aluminum Sample Block Base Module User Guide*.

Reaction Components

The table below lists the components, volumes, and concentrations that comprise the reaction mix.

Component	Volume per Reaction[a]	Final Conc.
Deionized water	see below[b]	—
User-provided Primer 1	_ µL	0.2–1.0 µM
User-provided Primer 2	_ µL	0.2–1.0 µM
User-provided template	see below[b]	<1 µg/reaction[c]
GeneAmp® Fast PCR Master Mix (2X)	10 µL	1X
Total volume	20 µL	—

a. You may adjust the reaction volume to your experimental design, keeping the concentrations of reactants constant.

b. Use any combination of water and template as long as the total volume of the PCR Master Mix, sample, and primers equals 20 µL.

c. Preferably $>10^2$ copies of template but <0.2 µg DNA per reaction.

Optimizing the Template Concentration

Use the following guidelines to optimize the template concentration:

- Start with enough copies of the template to obtain a signal after 30–40 cycles; preferably more than 10^2 copies, but less than 0.2 µg of genomic DNA per 20 µL reaction.
- If the target DNA concentration is low, more than 35 cycles may be required to produce sufficient product for analysis. You can amplify as few as 1 to 10 target copies (Saiki *et al*., 1988; Chou *et al*., 1992). To avoid statistically arising dropouts (false negatives), validate for low copy number amplifications for an average of 5–10 target molecules per sample.

Note: Proteases may be present in the sample DNA. Proteases may degrade the GeneAmp Fast PCR polymerase, resulting in little or no yield of DNA product. You can inactivate proteases by heating the DNA samples to 95 °C for 5 minutes before adding PCR Master Mix. You can automate this step with any of the Applied Biosystems GeneAmp PCR instrument systems.

Designing the Primers

Use the following guidelines to design your primers:

- The single-stranded DNA primers should be 15–30 bases in length.
- The %G+C of primers should be near 50%, to maximize specificity.
- Calculate the melting temperature of your primer(s) with the T_m calculator. Use this number as a starting point to determine the annealing/extension temperatures.
- To avoid potential problems, purify primers by gel electrophoresis or HPLC ion-exchange chromatography.
- Primer sequences should not complement within themselves or to each other, particularly at the 3′ ends. This restriction avoids template-independent amplification of primer sequences (or "primer dimer"), which can lead to other, larger primer artifacts. Primer-dimer may occur to some extent even without an apparent overlap.
- Use primer design software to assist in primer selection.
- Do not use an annealing/extension temperature below 62 °C.

Optimizing the Primer Concentration

Use the following guidelines to optimize the primer concentration:

- Determine optimal primer concentrations empirically by testing concentrations in the range of 0.1–1.0 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 non-target sequences, which are evidenced by secondary bands and/or smearing when viewed on a gel.
- Primer concentrations in the range of 0.2–0.5 µM work for most PCR amplifications.
- Reducing each primer concentration (*e.g.*, to 0.2 µM) helps reduce amplification of non-specific products.

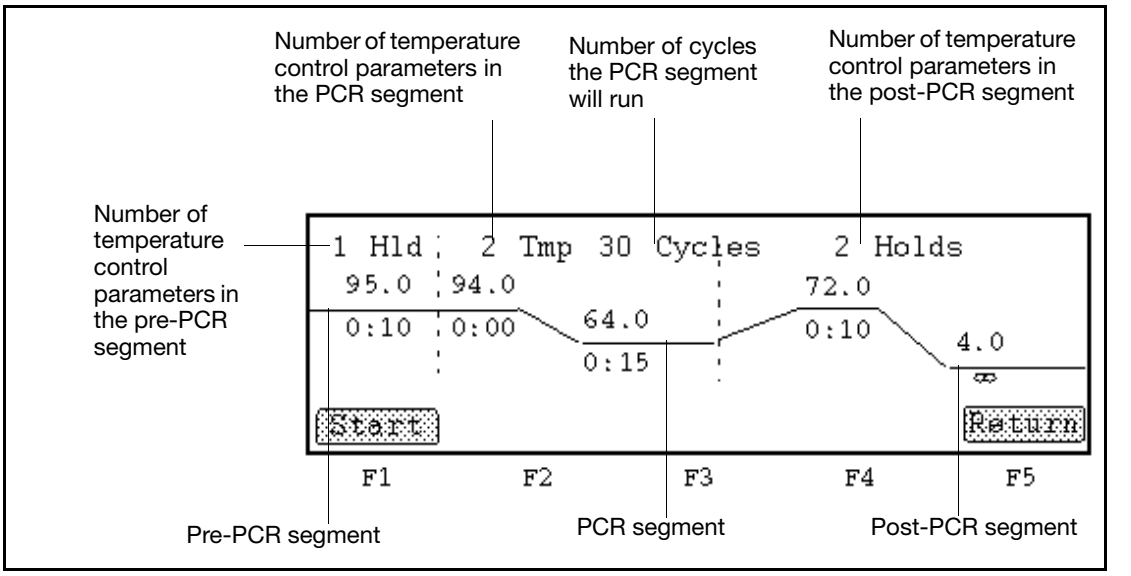
Preparing the Reaction Mix

To prepare the reaction mix:

1.	Thaw reagents on ice. See "Reaction Components" on page 14.
2.	Calculate the amount of template, primers, and deionized water needed to make up a total volume of 20 µL when added to the master mix. **Note:** The amount of each depends on your specific primer and template concentrations. See "Reaction Components" on page 14.
3.	Pipette the calculated amount of template, primers, and deionized water into replicate wells on an optical 96-Well Fast thermal cycling plate. **Note:** Applied Biosystems recommends running four replicates of each sample.
4.	Gently swirl the bottle of GeneAmp Fast PCR Master Mix (2X) to evenly mix the contents. **IMPORTANT!** Do not introduce bubbles into the master mix solution.
5.	Pipette 10 µL of the GeneAmp Fast PCR Master Mix (2X) into each reaction well.
6.	Seal the plate with MicroAmp Clear Adhesive Film. **Note:** Ensure good contact between the film and the plate by using the Adhesive Seal Applicator.
7.	Place the plate in a plate centrifuge and spin for 30–60 sec to ensure the liquid collects at the bottom of each well.
8.	Perform thermal cycling using the parameters described in "Thermal Cycling" on page 18.

Thermal Cycling

The 9800 instrument comes with a default PCR thermal cycling profile called a method. The Create screen displays this default method. The default PCR thermal profile uses two-step PCR. You can run the default method, or use it as a template to create a customized method.



9800 Fast Thermal Cycling Profile

Program the 9800 thermal cycler using a combination of the following parameters for your specific target sequence. For creating a new method on the 9800 Fast thermal cycler, refer to the *Applied Biosystems 9800 Fast Thermal Cycler with 96-Well Aluminum Sample Block Base Module User Guide*.

IMPORTANT! The GeneAmp Fast PCR Master Mix has been optimized for annealing/extension temperatures at or above 62 °C. The recommended upper limit is 72 °C.

Step	Fast Start of Enzyme	PCR		PCR (Final Step)	
	HOLD	Cycle (35 Cycles)		HOLD	HOLD
		Denature	Anneal/Extend		
Temp	95 °C	94 °C	62–72 °C[a]	72 °C	4 °C
Time	10 sec	0 sec	25 sec/Kb[b]	10–60 sec	∞

a. Optimize the temperature starting from the primer's melting temperature (T_m).
b. The length of the target sequence determines the annealing/extension time.

Using the T_m Calculator

The Applied Biosystems Web site, www.appliedbiosystems.com, provides a T_m calculator for your use. Go to:

/Support/Application Tools & Tutorials/PCR/T_m Calculator.

See "Using the T_m Calculator" described on page 12 for a procedure on how to use the T_m calculator to determine the annealing temperature.

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 denatured.
- The maximum denaturation temperature should not exceed 95–96 °C (Gelfand and White, 1990).
- Ten seconds is adequate when using the 9800 Fast thermal cycler, which displays a calculated sample temperature. Some models of thermal cyclers may require longer denaturation times.

Adjusting the Annealing/ Extension Conditions

The following are guidelines for adjusting annealing/extension conditions:

- Use two-step PCR for annealing/extension temperatures >62 °C.
- The optimum annealing/extension temperature can be determined empirically by testing at 1–2 °C increments, until the maximum specificity is reached.
- Computer programs designed to calculate primer melting temperatures (T_m) can assist you in narrowing the range of annealing/extension temperatures for empirical determination.

  Note: A T_m calculator can be found on the Applied Biosystems Web site, *www.appliedbiosystems.com*. Then go to: */Support/Application Tools & Tutorials/PCR/T_m Calculator.*

  In addition, the Applied Biosystems 9800 Fast thermal cycler also contains a T_m calculator. See "Using the T_m Calculator" described on page 12 for a procedure to determine the starting point for optimizing the annealing/extension temperature.
- The length of the target sequence affects the required annealing/extension time. Longer targets require increased times. As a general rule, allow an annealing/extension time of approximately 25 seconds per 1000 bases (1 Kb).
- As the amount of DNA increases, the number of DNA polymerase molecules may become limiting. This limitation can be compensated for by increasing the annealing/extension time in later cycles using the 9800 Fast thermal cycler's Auto X function.

For creating a new method on the 9800 Fast thermal cycler, refer to the *Applied Biosystems 9800 Fast Thermal Cycler with 96-Well Aluminum Sample Block Base Module User Guide*.

Converting a Standard Three-Step Protocol to a Fast Protocol

Overview

If you are currently using a standard three-step thermal cycling protocol, you can convert your protocol into Fast using new parameters for your thermal cycling profile. Follow the procedures listed in this section to determine reaction components and perform the protocol, then follow the steps in "Converting the Thermal Cycling Profile" on page 22.

Reaction Components

The table below lists the components, volumes, and concentrations that comprise the reaction mix.

Component	Volume per Reaction[a]	Final Conc.
Deionized water	see below[b]	—
User-provided Primer 1	_ µL	0.2–1.0 µM
User-provided Primer 2	_ µL	0.2–1.0 µM
User-provided experimental template	see below[b]	<1 µg/reaction[c]
GeneAmp Fast PCR Master Mix (2X)	10 µL	1X
Total volume	20 µL	—

a. You may adjust the reaction volume to your experimental design, keeping the concentrations of reactants constant.

b. Use any combination of water and template as long as the total volume of the PCR Master Mix, sample, and primers equals 20 µL.

c. Preferably >10^2 copies of template but <0.2 µg DNA per reaction.

Preparing the Reaction Mix

To prepare the reaction mix:

1.	Thaw reagents on ice if necessary. See "Reaction Components" on page 21.
2.	Calculate the amount of template, primers, and deionized water needed to make up a total volume of 20 µL when added to the master mix. **Note:** The amount of each depends on your specific primer and template concentrations (see "Reaction Components" on page 21).
3.	Pipette the calculated amounts of template, primers, and deionized water into replicate wells on an optical 96-Well Fast thermal cycling plates. **Note:** Applied Biosystems recommends running four replicates of each sample.
4.	Gently swirl the bottle of GeneAmp Fast PCR Master Mix (2X) to evenly mix the contents. **IMPORTANT!** Do not introduce bubbles into the master mix solution.
5.	Pipette 10 µL of GeneAmp Fast PCR Master Mix (2X) into each reaction well.
6.	Seal the plate with MicroAmp Clear Adhesive Film. **Note:** Ensure good contact between the film and the plate by using the Adhesive Seal Applicator.
7.	Place the plate in a plate centrifuge and spin for 30–60 sec to ensure the liquid collects at the bottom of each well and not on the sides.
8.	Perform thermal cycling using the parameters supplied below.

Converting the Thermal Cycling Profile

Convert your standard three-step thermal cycling profile into a two-step profile; then follow the 9800 default profile on page 23. Two-step PCR consolidates the annealing and extension steps into one step. The primer extension is completed immediately after annealing.

Two-step PCR consists of:

- Denaturing the DNA template
- Annealing and extension of primers

Use the following steps to convert your three-step profile.

1. Shorten the initial denaturation step to 10 sec at 95 °C. The GeneAmp Fast PCR Master Mix polymerase enzyme does not require activation.
2. Use the annealing temperature and the extension temperature from your three-step program to determine the average temperature. Then apply a time of 25 sec/Kb.

   IMPORTANT! The minimum temperature must be 62 °C.
3. Shorten the in-cycle denaturation step to 0 sec at 94–95 °C.
4. Shorten the final hold step to 10–60 sec at 72 °C.

9800 Fast Thermal Cycling Profile

Program the 9800 Fast thermal cycler using a combination of the parameters in the table below for your specific target sequence. For creating a new method on the 9800 Fast thermal cycler, refer to the *Applied Biosystems 9800 Fast Thermal Cycler with 96-Well Aluminum Sample Block Base Module User Guide*.

IMPORTANT! The GeneAmp Fast PCR Master Mix has been optimized for annealing/extension temperatures at or above 62 °C. The recommended upper limit is 72 °C.

Step	Fast Start of Enzyme	PCR		PCR (Final Step)	
	HOLD	Cycle (35 Cycles)		HOLD	HOLD
		Denature	Anneal/Extend		
Temp	95 °C	94 °C	62–72 °C[a]	72 °C	4 °C
Time	10–60 sec	0 sec	25 sec/Kb[b]	10–60 sec	∞

a. Empirically determine the annealing/extension temperature. Adjust the temperature according to the primer's melting temperature (T_m).

b. The annealing/extension time is dependent on the length of the target sequence. The time of 25 sec/Kb is a starting point. Use this starting point to optimize the time for your target sequence.

Using the T_m Calculator

The Applied Biosystems Web site, www.appliedbiosystems.com, provides a T_m calculator for your use. Go to:

/Support/Application Tools & Tutorials/PCR/T_m Calculator.

See "Using the T_m Calculator" described on page 12 for a procedure on how to use the T_m calculator to determine the annealing temperature.

Analyzing Results

Data analysis depends on the type of analysis you have selected. Use the GeneAmp Fast PCR Master Mix (2X) to amplify samples for gel electrophoresis, cloning, and cycle sequencing. Refer to documentation written for your specific sample analysis for information regarding how to analyze the data.

Troubleshooting

Observation	Possible Cause	Recommended Action
Reduced or no product band visible	Template concentration too low	Increase sample concentration.
	Experimental sample DNA damaged or degraded	Use sample that has been processed and stored properly 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 low or too high	Increase temperature in increments of 1 °C starting at 62 °C.
	Annealing/extension time too short	Lengthen time in increments of 10 seconds each.
	Cycle number too low	Increase cycle number in increments of three cycles.
	Primer design not optimal	Design primers with T_m of 62 °C or higher.
	Difficult templates	1. Increase annealing/extension time by increments of 10 seconds. 2. Increase annealing/extension temperature in increments of 1 °C starting at 62 °C. 3. Use a three-step protocol.
	Samples at room temperature for more than 2 hours.	Maintain samples at room temperature for less than 2 hours or keep on ice.
Product band is smeared	Carryover contamination	See “Preventing Contamination” on page 4.
	Denaturation temperature too low	Increase temperature in increments of 1 °C.
	Cycle number too high	Shorten cycle number in increments of three cycles.
	Experimental sample DNA degraded	Test a new aliquot of sample.
	Samples at room temperature for more than 2 hours.	Maintain samples at room temperature for less than 2 hours or keep on ice.

Observation	Possible Cause	Recommended Action
Non-specific amplification with or without a product band	Carryover contamination	See “Preventing Contamination” on page 4.
	Non-specific priming	Increase the anneal temperature in 1–2 °C increments.
	Assembled reaction mix has been sitting at room temperature for more than 2 hrs.	Place the assembled reaction mix on ice.
	Primer design not optimal	Review primer design and composition.

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Index

O

P

R

S

T

W

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