GeneAmp® Gold RNA PCR Reagent Kit

Protocol

Applied Biosystems

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Contents

Introduction 1
- RNA-PCR Kit Features 1
- RT-PCR Schematic 4

Materials and Equipment 5
- GeneAmp Gold RNA PCR Reagent Kit 5
- Kit Components 5
- Control RNA and Primers 7
- Storage and Stability 7
- Enzyme Characteristics 8
- Materials Required But Not Supplied 9

Safety Information 10
- Document User Attention Words 10
- Ordering Kits and Reagents 10
- About MSDSs 10
- Ordering MSDSs 10
- Safety 11

Preventing Contamination 12
- Introduction 12
- General PCR Practices 12

Reagent Optimization Guidelines 13
- General Guidelines 13
- Preparing RT and PCR Reactions 13
- Reaction Tubes 13
- Magnesium Chloride Concentration 14
- Primer Guidelines 14
- RNA Template 15
- Primers for RT 16

Cycle Optimization Guidelines 17
RT Thermal Cycling Conditions 17
PCR Amplification Conditions 17
Protocol for One-Step RNA PCR 19
Overview 19
Preparation of Reagents 19
Reaction Mix Preparation 19
One-Step Reaction Mix Preparation 20
Thermal Cycling Parameters for One-Step RNA PCR 21
Protocol for Two-Step RNA PCR 22
Overview 22
Preparation of Reagents 22
Reaction Mix Preparation 22
Two-Step Reaction Mix Preparation 22
RT Reaction Mix Preparation 23
RT Thermal Cycling Parameters for Two-Step RNA PCR 24
PCR Reaction Mix Preparation 25
PCR Thermal Cycling Parameters for Two-Step RNA PCR 27
Appendix A. Additional Primer and PCR Product Information 28
Additional Primers and PCR Products from pAW109 28
pAW109 Transcribed RNA 29
Performance Characteristics 29
Appendix B. References 30
Appendix C. Technical Support 32
Contacting Technical Support 32
To Contact Technical Support by E-mail 32
Hours for Telephone Technical Support 32
To Contact Technical Support by Telephone or Fax 33
To Reach TechnicalSupport Through the Internet 36
To Obtain Documents on Demand 37

Introduction

RNA-PCR Kit Features

The GeneAmp® Gold RNA PCR Reagent Kit (P/N 4308206) is designed for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from total RNA, messenger RNA (mRNA), or viral RNA. The kit has the following features:

- Incorporation of the "Hot Start" technique by using AmpliTaq Gold® DNA Polymerase, which results in an increase in sensitivity, specificity, and yield of PCR products
- The capability to perform a unique, coupled one-step RNA PCR amplification, with reverse transcription followed by amplification in a single tube with a single buffer
- The capability to perform a more traditional two-step RNA PCR amplification, with reverse transcription followed by a separate PCR amplification step

The kit includes all the essential reagents to perform the reverse transcription of RNA to complementary DNA (cDNA) and the subsequent cDNA amplification using a single reaction tube and a single reaction buffer. This one-step process combines the versatility of MultiScribe™ Reverse Transcriptase, derived from Murine Leukemia Virus (MuLV), with the unique properties of AmpliTaq Gold® DNA Polymerase.

The provided RT-PCR Reaction Buffer has been optimized for both efficient reverse transcription by MultiScribe Reverse Transcriptase and efficient PCR amplification by AmpliTaq Gold DNA Polymerase, enabling it to be used in either the coupled one-step procedure or the traditional two-step procedure.

Hot Start Technique

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

Because the unintended annealing occurs at the beginning of the PCR reaction, the non-specific products are efficiently amplified throughout the remaining PCR cycles. This unintended amplification results in poor yield of the desired product reducing the sensitivity of the experiment both by decreasing the desired amplification signal and by obscuring the signal with a high background.

Conversely, when the Hot Start PCR method is used, primers bind only to their specific target and the polymerase activity is directed to only the desired target. This results in increased sensitivity and yield, and decreased non-specific background amplification.

AmpliTaq Gold DNA Polymerase is a chemically modified form of *Thermus aquaticus* (Taq) DNA polymerase that requires high temperature activation. The modified enzyme is provided in an inactive state and remains so throughout the reverse transcription step. When the sample is heated to 95 °C, the modifier is permanently released, regenerating the active enzyme and initiating PCR amplification of the cDNA generated in the RT reaction. This thermal activation or "Hot Start" property of AmpliTaq Gold DNA Polymerase simplifies the procedure and provides increased sensitivity, specificity, and yield over conventional PCR techniques.

One-Step RNA-PCR

The coupled one-step RNA-PCR procedure eliminates the requirement for multiple manipulations and reagent additions following the reverse transcription reaction. This one-step procedure reduces the risk of contamination.

The one-step reaction can utilize any of these primers for the reverse transcription reaction: a sequence-specific primer, random hexamers, or oligo $d(T)_{16}$. In the one-step procedure, use sequence-specific primers (downstream or reverse PCR primers) to achieve the highest specificity.

Two-Step RNA-PCR

The two-step RNA-PCR procedure, in which an initial reverse transcription reaction is followed by an independent PCR amplification, is useful when detecting multiple transcripts from a single cDNA reaction, or when storing a portion of the cDNA for later use.

The two-step reaction can utilize any of these primers for the reverse transcription reaction: a sequence-specific primer, random hexamers, or oligo $d(T)_{16}$. In the two-step procedure, use either random hexamers

or oligo $d(T)_{16}$ as the cDNA synthesis primers to achieve the highest specificity in an RNA PCR amplification.

The table below compares the one-step and two-step features.

Comparison of RNA-PCR methods

Method	Primers for cDNA synthesis	Features
One-step	Sequence-specific primer, oligo $d(T)_{16}$ or random hexamers	♦ Requires single Reaction Mix ♦ Reduces risk of contamination
Two-step	Sequence-specific primer, oligo $d(T)_{16}$ or random hexamers	♦ Requires two Reaction Mixes ♦ cDNA can be stored or used for multiple amplifications

GeneAmp RNA PCR Control Kit

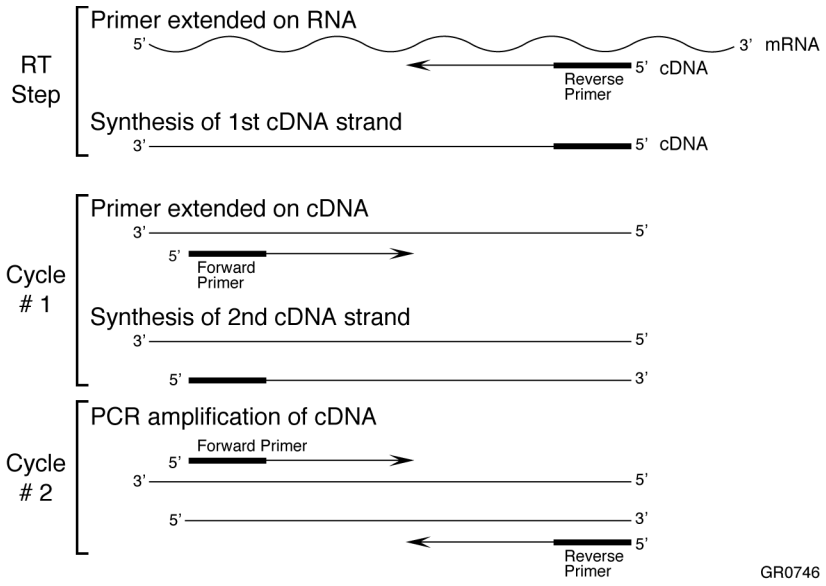
As a positive control, the kit contains a pAW109 complementary RNA (cRNA) template transcribed from the plasmid pAW109. The plasmid pAW109 contains an insert of a synthetic linear array of primer sequences for multiple target genes constructed such that “upstream” primers are followed by complementary sequences to their “downstream” primers in the same order.

The insert in pAW109 is identical to the insert previously described for the plasmid pAW108 (Wang *et al*, 1989) and is depicted in “A schematic diagram of the general arrangement of the RNA transcribed from plasmid pAW109.” on page 29.

The primers included in this kit flank an IL-1α site and can be used to amplify a 308-bp sequence within the target. There is sufficient positive control pAW109 RNA and primers for at least 30 control RNA PCR amplifications.

RT-PCR Schematic

As shown in the figure below, one-step RNA-PCR uses a single buffer that enables RT and PCR amplification to occur without interruption.



Schematic representation of RNA-PCR using the GeneAmp Gold RNA PCR Reagent Kit.

Materials and Equipment

GeneAmp Gold RNA PCR Reagent Kit

The GeneAmp Gold RNA PCR Reagent Kit (P/N 4308206) is the complete kit providing materials to perform the reverse transcription of RNA and subsequent PCR amplification of samples plus control material (RNA and primers).

Kit Components

The GeneAmp Gold RNA PCR Reagent Kit is comprised of two module kits that can be purchased separately:

Kit	P/N	Contents
GeneAmp Gold RNA PCR Reagent Kit	4308206	♦ GeneAmp® Gold RNA PCR Core Kit ♦ GeneAmp® Gold RNA PCR Control Kit ♦ Protocol (P/N 4310253)
GeneAmp Gold RNA PCR Core Kit	4312765	♦ RNA PCR Reagents ♦ Protocol (P/N 4310253)
GeneAmp Gold RNA PCR Control Kit	4308238	♦ pAW109 Control Reagents ♦ Protocol (P/N 4310253)

The contents of the modules are described in the tables on pages 6–7.

The GeneAmp Gold RNA PCR Reagent Kit and Core Kit each have a set of reagents sufficient to perform 100 RNA PCR amplifications of 50 µL each. The GeneAmp Gold RNA PCR Control Kit (included in the Reagent Kit) has sufficient template and primers to perform at least 30 control reactions.

The following reagents are supplied with the GeneAmp Gold RNA PCR Core Kit:

The GeneAmp RNA PCR Core Kit reagents (P/N 4312765)

Reagent	Quantity (µL)	Description
MultiScribe™ Reverse Transcriptase	100	One tube containing 50 U/µL, MultiScribe (Murine Leukemia Virus) Reverse Transcriptase in DEPC water with 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.01% Nonidet P40, 50% (v/v) glycerol.
RNase Inhibitor	100	One tube containing 20 U/µL RNase Inhibitor in DEPC water with 20 mM HEPES-KOH, pH 7.6, 50 mM KCL, 8 mM DTT, 50% (v/v) glycerol.
AmpliTaq Gold DNA Polymerase	50	One tube containing 5 U/µL of AmpliTaq Gold DNA Polymerase in 100 mM KCl, 20 mM Tris-HC, pH 9.0, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween® 20, and 50% (v/v) glycerol.
dNTP Blend	1000	One tube containing 10 mM Deoxyribonucleotide triphosphates (2.5 mM each dATP, dCTP, dGTP, and dTTP) dissolved in DI water, titrated to pH 7.0 with NaOH.
100 mM DTT	500	One tube containing 100 mM Dithiothreitol in RNase free water.
5X RT-PCR Buffer	1500	One tube containing 150 mM Tris-HCl and 100 mM KCl, pH 8.3.
$MgCl_2$ solution	1500	One tube containing 25 mM $MgCl_2$.
Random Hexamers	100	One tube containing 50 µM random primers for reverse transcription of RNA in 10 mM Tris-HCl, pH 8.3.
Oligo $d(T)_{16}$	100	One tube containing 50 µM poly dT tailed primer for reverse transcription of RNA, in 10 mM Tris-HCL, pH 8.3. The sequence is: 5´-TTTTTTTTTTTTTTTT-3´.

The following reagents are supplied with the GeneAmp RNA PCR Control Kit.

The GeneAmp RNA PCR Control Kit reagents (P/N 4308238)

Reagent	Quantity (µL)	Description
pAW109 Control RNA	50	One tube containing 10^6 copies/µL of RNA transcribed from pAW109 in 30 µg/mL *E. coli* rRNA, dissolved in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, and 100 mM NaCl.
Primer DM151	50	One tube containing 15 µM "upstream" IL-1α primer for amplification of pAW109 RNA, supplied in 10 mM Tris-HCl, pH 8.3. The sequence is: 5´–GTCTCTGAATCAGAAATCCTTCTATC–3´.
Primer DM152	50	One tube containing 15 µM "downstream" IL-1α primer for reverse transcription and amplification of pAW109 RNA, supplied in 10 mM Tris-HCl, pH 8.3. The sequence is: 5´–CATGTCAAATTTCACTGCTTCATCC–3´.

Control RNA and Primers

The DM151 and DM152 primer pair amplifies a 308-bp product from the control pAW109 provided in the kit. The primer set amplifies a 421-bp product from authentic IL-1α mRNA. Refer to "Additional Primer and PCR Product Information" on page 28 for more information.

Storage and Stability

Upon receipt, store the GeneAmp Gold RNA PCR Kit at –15 to –25 °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.

Enzyme Characteristics

AmpliTaq Gold DNA Polymerase

AmpliTaq Gold DNA Polymerase 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).

Characteristics of AmpliTaq Gold DNA Polymerase

Item	Description
Unit Definition	The enzyme is provided at 5 Units/μL. One unit of enzyme will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 min in a 10 min incubation at 74 °C under conditions listed under Analysis Conditions. **Note** The enzyme is shipped in an inactive form. Before analyzing, the enzyme is activated by heating for three hours at 80 °C.
Analysis Conditions	The analysis conditions are in the presence of 25 mM TAPS (tris-hydroxymethyl-methyl-amino-propanesulfonic) pH 9.3, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ß-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	The storage buffer contains 20 mM Tris-HCl, pH 9.0 (room temperature), 100 mM KCl, 0.1 mM EDTA, 1.0 mM DTT (dithiothreitol), 0.5% Tween® 20, 50% (v/v) glycerol.
Storage Conditions	Store at –20 °C. If stored under proper conditions, the enzyme will remain active through the control date on the label.
Associated Activities	Non-specific endonuclease and exonuclease activities were not detectable after a 1 hour incubation of 600 ng of supercoiled pBR322 (dam$^-$ dcm$^-$) or 600 ng of *Msp* I–digested pBR322 DNA. The incubations were performed 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. The enzyme lacks a 3´ to 5´ exonuclease activity.

MultiScribe Reverse Transcriptase

MultiScribe Reverse Transcriptase, a recombinant form of the Murine Leukemia Virus (MuLV), is an RNA-dependent DNA polymerase that uses single-stranded RNA as a template in the presence of a primer to synthesize a complementary DNA strand. This enzyme is optimal for first strand cDNA synthesis. It is used in this kit for its robust reverse

transcription capability and its compatibility with random hexamers, oligo (dT), and gene specific primers.

Characteristics of MultiScribe Reverse Transcriptase

Item	Description
MultiScribe Reverse Transcriptase	The enzyme is provided at 50 Units/µL. One unit of enzyme will incorporate 1 nmol of dTTP into acid-precipitable material in 10 min at 37 °C using poly rA•oligo $d(T)_{12-18}$ as a template primer. The ratio of oligo d(T) to poly rA is 3:1.
Source	The enzyme is purified from *E.coli* expressing the *pol* gene of MuLV on a plasmid.
Storage Buffer	The buffer contains 20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.01% (w/v) Nonidet® P40, 50% (v/v) glycerol, in diethylpyrocarbonate (DEPC) treated deionized (DI) water.

Materials Required But Not Supplied

The items listed below are required in addition to the reagents supplied in the GeneAmp Gold RNA PCR Reagent Kit.

User-supplied reagents

Equipment Item	Source
Centrifuge	Major laboratory suppliers (MLS)
Disposable gloves	MLS
MicroAmp Disposables	Applied Biosystems
Microcentrifuge	MLS
Pipettors, positive-displacement or air-displacement	MLS
Pipette tips, with filter plugs	MLS
Vortexer	MLS
RNase-free water	MLS
GeneAmp PCR Instrument System	Applied Biosystems

Safety Information

Document User Attention Words

Four user words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as follows.

Note This word is used to call attention to information.

IMPORTANT This word calls attention to information that is necessary for correct operation of the instrument.

CAUTION This word informs the user that damage to the instrument could occur if the user does not comply with the information. It also indicates a potentially hazardous situation that could result in minor or moderate injury to the user.

! WARNING ! This word informs the user that serious physical injury to the user or other persons could occur if these required precautions are not taken.

Ordering Kits and Reagents

To order additional kits and reagents, please contact Applied Biosystems at one of the regional offices listed in this protocol. Have the part number of the kit or reagent you are ordering available when ordering.

About MSDSs

Material and Safety Data Sheets (MSDSs) provide information about physical characteristics, health hazards, safety precautions, first aid, spill clean-up, and disposal procedures. Read the MSDSs before handling reagents or interacting with the instrument.

Ordering MSDSs

Material Safety Data Sheets (MSDSs) for hazardous chemicals manufactured by Applied Biosystems will accompany your first shipment. To receive additional copies of MSDSs at no extra cost, call Applied Biosystems at (800) 327-3002.

! WARNING ! Some of the chemicals used in this manual may be hazardous. Follow the safety precautions stated in the specific chemical warnings that appear throughout the protocol.

! WARNING ! **Some of the chemicals referred to in this manual may not have been provided with your kit. If the chemicals are not provided, they are not manufactured or sold by Applied Biosystems. Please obtain the Material Safety Data Sheets from their manufacturers.**

! WARNING ! **Some of the chemicals in this kit are sensitizers. While sensitizers are not hazardous in nature, some individuals may be sensitive to individual compounds.**

Safety

! WARNING ! **CHEMICAL HAZARD. Certain chemicals in this kit may be hazardous and therefore require special handling. Do not store, handle, or work with any chemicals or hazardous materials unless you have received appropriate safety training and have read and understood the Material Safety Data Sheet. Comply with all federal, state, and local laws related to chemical storage, handling, and disposal.**

Preventing Contamination

Introduction

Due to the high throughput and repetitive nature of PCR, special laboratory practices are necessary in order to avoid false positive amplifications (Kwok and Higuchi, 1989). This is because of the capability for single DNA molecule amplification provided by the PCR process (Saiki *et al*, 1985; Mullis *et al*, 1987).

General PCR Practices

Please follow these recommended procedures:

- 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, for PCR setup, and for PCR amplification and 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 10% bleach solution.

Reagent Optimization Guidelines

General Guidelines

These guidelines have been developed from the experience obtained at Applied Biosystems in the design of RT and PCR reactions.

Preparing RT and PCR Reactions

Please follow these guidelines:

- Gently mix (avoid generating bubbles), Multiscribe Reverse Transcriptase, RNase inhibitor, AmpliTaq Gold DNA Polymerase and other thawed reagents, prior to pipetting.
- Spin the reagents down before pipetting.
- Pipette the enzymes carefully and slowly as they are in a viscous, 50%-glycerol solution.
- Prepare Master Mixes containing water, buffers, dNTPs, magnesium chloride, and enzyme, for both reverse transcription and PCR amplification.
- Use any combination of water, primer, and experimental volumes as long as the final combined volume is 50 μL.
- Refrain from adding additional EDTA, citrate, or other chelators with the primers or experimental sample. If the final concentration of chelator exceeds 0.11 mM, additional $MgCl_2$ may need to be added.

Reaction Tubes

The reaction tube guidelines are listed below.

- Use MicroAmp® 96 well Reaction Plates or MicroAmp® Reaction Tubes for your GeneAmp® Applied Biosystems tubes provide the best heat transfer because they fit uniformly into the wells of GeneAmp PCR Systems.
- Use sterile reaction tubes and pipette tips to avoid nuclease contaminated plastic.

Magnesium Chloride Concentration

Too little or too much magnesium chloride can reduce reverse transcriptase and amplification efficiency or result in nonspecific products.The optimal magnesium chloride concentration must be determined empirically. The guidelines for determining the optimum magnesium chloride concentration for each primer set are listed below.

- Test concentrations of magnesium chloride in the range of 1.25–5 mM in 0.5-mM increments.
- Raise the magnesium chloride concentration in the reaction mix proportionately if the samples contain EDTA, citrate, or other chelators.
- Adjust the magnesium chloride concentration in parallel with significant changes in the concentration (higher or lower) of sample RNA or dNTPs. This will keep the free magnesium ion concentration constant.

Primer Guidelines

Guidelines for designing and using primers are listed below.

- Sequence-specific primers for RNA and cDNA should be 15–30 bases in length.
- The % G +C should be near 50%.
- Purify primers by gel electrophoresis or HPLC ion-exchange chromatography.
- Determine the optimum primer concentration empirically by testing concentrations ranging 0.1–1.0 μM primer. In general concentrations ranging 0.15–0.5 μM will work for most amplifications.
  - Too low a concentration will result in little or no PCR product
  - Too high a concentration will result in amplification of non-target sequences.
- Primer sequences should not complement within themselves or to each other, particularly at the 3´ ends.

RNA Template

This kit is designed to reverse transcribe and amplify RNA targets from mRNA or total RNA. The detection sensitivity of this kit is 500 copies of RNA.

A cDNA synthesis of up to 5 kb in length can be achieved with a subsequent amplification of up to 4 kb in length.

The guidelines for RT/PCR amplification of an RNA template are listed below.

- Start with at least 500 copies, but less than 1 μg total of the RNA template, to ensure a signal after 43 cycles. The cycle number can be reduced if you start with more than 500 copies. For example, 10,000 copies of RNA typically can be amplified with 35 cycles.
- Amplify the positive control, pAW109, using a range of 500–1,000,000 copies diluted in the presence of 0.1 μg/μL of carrier RNA. Always make fresh working solutions from stock preparations of pAW109.

Primers for RT

The following primers can be used for cDNA synthesis:

- Random hexamers
- Oligo $d(T)_{16}$
- Sequence-specific reverse primers

The choice of primers for reverse transcription is best made after experimentally evaluating all three priming systems. For short RNA sequences containing no hairpin loops, any of the three priming systems should work equally well. For longer RNA transcripts or sequences containing hairpin loops, consider the following guidelines:

Primers for two-step RT-PCR

Primers	Selection Guidelines
Random hexamers	Try first for use with long reverse transcripts or reverse transcripts containing hairpin loops Use to transcribe all RNA (rRNA, mRNA, and tRNA)
Sequence-specific reverse primer	Use to reverse transcribe RNA-containing complementary sequences only
Oligo $d(T)_{16}$	Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails Avoid long mRNA transcripts or amplicons greater than two kilobases upstream

Cycle Optimization Guidelines

RT Thermal Cycling Conditions

The reverse transcriptase incubation condition guidelines are listed below.

- Incubate reactions for 12 minutes at 42 °C when reverse transcribing nonstructured RNA that is less than 2 kb in length.
- Raise the incubation temperature to 45 °C for 12 minutes for highly-structured RNA.
- If trying to achieve long transcripts, keep the temperature at 42 °C and increase the incubation time up to 20 minutes.

Note The half-life of MultiScribe reverse transcriptase is 10 minutes at 42 °C.

PCR Amplification Conditions

Anneal-Extend Temperature

The selection of 60 °C for a combined anneal-extend temperature is optimal for amplification of the pAW109 cDNA. Optimal conditions for other primer-template pairs may require raising or lowering the annealing temperature in the range of 37–65 °C, followed by an extension temperature of 70–72 °C. In general, higher annealing temperatures result in a more specific product (Saiki *et al*, 1988 and Rychlik *et al*, 1990). The following are recommended guidelines.

- Determine the optimal annealing temperature empirically by testing increments of 5 °C or less until reaching the maximum in specificity and product.
- Amplification extension temperatures are typically 70–72 °C. When the anneal temperature is above 60 °C, it may be optimal to use two temperatures rather than three temperatures. In this case, complete the extension at the anneal temperature. The extension at lower temperatures may require time optimization for such combined anneal-extend steps.

Note Computer programs designed to calculate primer melting temperatures (Tm) such as Primer Express Applications-Based Primer Design Software (P/N 402809) can assist you in narrowing the suggested range of annealing temperatures for empirical determination.

Anneal-Extend Time

The length of the target sequence will affect the required extension time. AmpliTaq Gold DNA Polymerase has an extension rate of 2000–4000 bases per minute at 70–72 °C.

Note Polymerase activity is significant between 55–85 °C (Innis *et al*, 1988).

Typically, for products < 1 kb in length, the annealing times are 15–30 seconds and the extension times are 30–60 seconds. Using a combined anneal-extend step, 1 minute is sufficient for both steps.

As the amount of DNA increases in later cycles, the number of AmpliTaq Gold DNA Polymerase molecules may become limiting for the extension time allotted. To compensate for this, we recommend the following guidelines:

- Increase the extend times in later cycles to maintain the efficiency of amplification.
- Use the AUTO program for the GeneAmp PCR Systems 2400, 9600, and 9700.

Protocol for One-Step RNA PCR

Overview

Note The following procedure is optimized for one-step RNA PCR using the GeneAmp Gold RNA PCR Reagent Kit pAW109 control reagents to generate the 308 bp amplification product.

To design RNA PCR experiments for your samples and primers, refer to “Reagent Optimization Guidelines” on page 13 and “Cycle Optimization Guidelines” on page 17.

Preparation of Reagents

The kit is stored at –15 to –25 °C. Prior to use, thaw all reagents except the enzymes. When the reagents are thawed, keep them on ice. Keep the enzymes (AmpliTaq Gold DNA Polymerase, MultiScribe Reverse Transcriptase, and RNase Inhibitor) in a freezer until immediately prior to use. Mix kit components by vortexing (or gentle mixing for enzymes), and using a microcentrifuge, briefly spin down the tube contents. When finished with the kit return it to the –15 to –25 °C freezer.

Reaction Mix Preparation

Preparing a Reaction Mix of RNA PCR components is recommended in order to increase the precision of the results. The use of a Reaction Mix reduces the number of reagent transfers and minimizes volume loss due to pipetting.

General steps for Reaction Mix preparation

Step	Action
1	Prepare Reaction Mix by combining all the nonenzymatic components listed in the appropriate table.
2	Mix the components by pipetting up and down.
3	Vortex briefly.
4	Add the enzymatic components listed for the appropriate Reaction Mix.
5	Mix the components by inverting the microcentrifuge tube or by vortexing gently.

One-Step Reaction Mix Preparation

The ingredients of a 50-µL reaction, one-step reaction mix are listed in the table below. To make the one-step Reaction Mix, follow the instructions described in “Reaction Mix Preparation” on page 19.

IMPORTANT MultiScribe Reverse Transcriptase and RNase Inhibitor are sensitive to air oxidation. Keep these reagents at –20 °C until just prior to use.

One-Step Reaction Mix Components

Component	Volume/Tube (µL)	Final Concentration
pAW109 RNA[a]	1.0	10^6 copies
RNase-free water	26.7	–
5X RT-PCR Buffer	10.0	1X
25 mM Magnesium Chloride	3.5	1.75 mM
10 mM dNTP Blend	4.0	0.8 mM (200 µM of each dNTP)
RNase Inhibitor (20 Units/µL)	0.5	10 Units/50 µL
100 mM DTT	2.5	5.0 mM
15 µM Gene Specific Upstream Primer DM151	0.5	0.15 µM
15 µM Gene Specific Downstream Primer DM152	0.5	0.15 µM
AmpliTaq Gold DNA Polymerase (5.0 Units/µL)	0.5	2.5 Units/50 µL
MultiScribe Reverse Transcriptase (50 Units/µL)	0.3	15 Units/50 µL
Total	50.0	

a. The sensitivity of the Control Reagent Kit reagents will allow successful amplification with as little as 1,000 copies of pAW109 RNA (refer to “Performance Characteristics” on page 29). When testing the kit at low copy number, make dilutions in the presence of 0.1 µg/µL of carrier (such as *E. coli* ribosomal or transfer) RNA.

Thermal Cycling Parameters for One-Step RNA PCR

The thermal cycling parameters are optimized for the GeneAmp Gold RNA PCR Reagent Kit pAW109 control reagents to generate a 308-bp amplification product. The parameters described here are only for GeneAmp PCR Systems 2400, 9600, and 9700. See thermal cycler manuals for details on operation.

Some thermal cyclers may require longer (~60 sec) denaturation times. Refer to the manufacturers operating manuals.

Note All GeneAmp PCR Systems display the "Calculated Sample Temperature."

Cycle parameters for one-step RNA PCR with pAW109 control reagents

Step	**Reverse Transcription**[a]	**AmpliTaq Gold Activation**	**PCR** CYCLE (43 cycles)[b]		**PCR** (Final Step)
	HOLD	HOLD			HOLD
			Denature	Anneal/ Extend	Anneal/ Extend
Time	12 min	10 min	20 sec	60 sec	7 min
Temperature	42 °C	95 °C	94 °C	62 °C	72 °C

a. When using random hexamers or oligo $d(T)_{16}$ for the RT priming, perform a 10 min, 25 °C HOLD prior to the 12 min, 42 °C reverse transcription for primer annealing. For more information, refer to "Reagent Optimization Guidelines" on page 13 and "Cycle Optimization Guidelines" on page 17.

b. The cycle number can be reduced when amplifying more than 10,000 copies of target RNA. For more information refer to "Cycle Optimization Guidelines" on page 17.

Protocol for Two-Step RNA PCR

Overview

Note The following procedure is optimized for two-step RNA PCR using the pAW109 control reagents to generate the 308-bp amplification product.

To design RNA PCR experiments for your samples and primers, refer to “Reagent Optimization Guidelines” on page 13 and “Cycle Optimization Guidelines” on page 17.

Preparation of Reagents

The kit is stored at –15 to –25 °C. Prior to use, thaw all reagents except the enzymes. When the reagents are thawed, keep them on ice. Keep the enzymes (AmpliTaq Gold DNA Polymerase, MultiScribe Reverse Transcriptase, and RNase Inhibitor) in a freezer until immediately prior to use. Mix kit components by vortexing (or gentle mixing for enzymes), and using a microcentrifuge, briefly spin down the tube contents. When finished with the kit, return it to the –15 to –25 °C freezer.

Reaction Mix Preparation

Preparing a reaction mix of RT and PCR components for multiple samples is recommended in order to increase the precision of the results. The use of a reaction mix reduces the number of reagent transfers and minimizes volume loss due to pipetting.

General steps for Reaction Mix preparation

Step	Action
1	Prepare reaction mix by combining all the nonenzymatic components listed in the appropriate table.
2	Mix the components by pipetting up and down.
3	Vortex briefly.
4	Add the enzymatic components listed for the appropriate Reaction Mix.
5	Mix the components by inverting the microcentrifuge tube or by vortexing gently.

Two-Step Reaction Mix Preparation

The two-step RT-PCR reaction requires two reaction mixes:

- RT Reaction Mix
- PCR Reaction Mix

RT Reaction Mix Preparation

The ingredients of a 20 µL, RT Reaction Mix are listed in the table below. To make the RT Reaction Mix, follow the instructions described in "Reaction Mix Preparation" on page 22.

The reaction volume for the RT step can be varied from 10–100 µL. A 10-µL reaction volume is sufficient to reverse transcribe up to 200 ng of total RNA. Increase the reaction volume when >200 ng of total RNA is reverse transcribed. For quantitation, a larger RT reaction volume can improve accuracy. When changing the reaction volume, make sure the final concentration is consistent with that described in the table below.

IMPORTANT MultiScribe Reverse Transcriptase and RNase Inhibitor are sensitive to air oxidation. Keep these reagents at –20˚C until just prior to use.

Two-Step RT Reaction Mix

Component	Volume/Tube µL)	Final Concentration
pAW109 RNA[a]	1.0	10^6 copies
RNase-free water	7.7	–
5X RT-PCR Buffer	4.0	1X
25 mM Magnesium Chloride	2.0	2.5 mM
10 mM dNTP Blend	2.0	1 mM (250 µM of each dNTP)
RNase Inhibitor	0.5	10 Units/20 µL
100 mM DTT	2.0	10 mM
Random Hexamer[b]	0.5	1.25 µM
MultiScribe Reverse Transcriptase (50 Units/µL)	0.3	15 Units/20 µL
Total	20.0	

a. The sensitivity of the Control Reagent Kit reagents allows amplification with as little as 1,000 copies of pAW109 RNA (refer to "Performance Characteristics" on page 29). When testing the kit at low copy number, make dilutions in the presence of 0.1 µg/µL of carrier (such as *E.coli* ribosomal or transfer) RNA.

b. Random hexamers, oligo $d(T)_{16}$, or sequence-specific DM 152 downstream primer can be used for primers of cDNA synthesis. When using an oligo $d(T)_{16}$ primer, use 0.5 µL of primer in a 20 µL reaction. This ensures that the final concentration of oligo $d(T)_{16}$ in the Reaction Mix is 1.25 µM. When using DM 152 primer use 0.5 µL in a 20 µL reaction (0.375 µM final concentration).

RT Thermal Cycling Parameters for Two-Step RNA PCR

The thermal cycler parameters described here are optimized for the GeneAmp Gold RNA PCR Reagent Kit pAW109 control reagents to generate a 308-bp amplification product.

The parameters described here are only for the GeneAmp PCR Systems 2400, 9600, and 9700. See thermal cycler manuals for details on operation.

Note All GeneAmp PCR Systems display the Calculated Sample Temperature.

Cycling parameters for the RT step of the two-step process

Step	Hybridization[a]	Reverse Transcription[b]
	HOLD	HOLD
Time	10 min	12 min
Temperature	25 °C	42 °C

a. If using random hexamers or oligo $d(T)_{16}$ primers for first-strand cDNA synthesis, incubate the primers at 25 °C for ten minutes. If using a sequence-specific reverse primer, this step is not necessary.

b. For information on optimizing reverse transcriptase reactions refer to “Cycle Optimization Guidelines” on page 17.

PCR Reaction Mix Preparation

There are two possible reaction mixes for the second cDNA amplification step, reaction mix one and reaction mix two. Use reaction mix one if you do not need to perform multiple PCRs or to archive cDNA. Use reaction mix two if you have multiple targets for PCR or if you need to archive a portion of the cDNA.

Reaction Mix One

Use all 20 µL of the first step (RT) and add the following for a 50 µL reaction:

Two-Step PCR Reaction Mix

Component	Volume/Tube (µL)	Final Concentration
RT reaction	20.0	–
RNase-free water	19.0	–
5X RT-PCR Buffer	6.0	1X
25 mM Magnesium Chloride	1.5	1.75 mM
10 mM dNTP Blend	2.0	0.8 mM (200 µM of each dNTP)
15 µM Gene Specific Upstream Primer DM151	0.5	0.15 µM
15 µM Gene Specific Downstream Primer DM152[a]	0.5	0.15 µM
AmpliTaq Gold DNA Polymerase (5.0 Units/µL)	0.5	2.5 Units/50 µL
Total	50.0	

a. Omit primer DM152 if it was included in the RT step as the reverse transcription primer and add 19.5 µL of RNase-free water instead of 19.0 µL.

Reaction Mix Two

Use an aliquot (3 µL) of the first step (RT) and add the following for a 50 µL reaction:

Two-Step PCR Reaction Mix (two)

Component	Volume/Tube (µL)	Final Concentration
RT reaction	3.0	–
RNase-free water	29.2	–
5X RT-PCR Buffer	9.4	1X
25 mM Magnesium Chloride	3.2	1.75 mM
10 mM dNTP Blend	3.7	0.8 mM (200 µM of each dNTP)
15 µM Gene Specific Upstream Primer DM151	0.5	0.15 µM
15 µM Gene Specific Downstream Primer DM152	0.5	0.15 µM
AmpliTaq Gold DNA Polymerase (5.0 Units/µL)	0.5	2.5 Units/50 µL
Total	50.0	

PCR Thermal Cycling Parameters for Two-Step RNA PCR

The thermal cycler parameters described here are optimized for the GeneAmp Gold RNA PCR Reagent Kit pAW109 control reagents to generate a 308-bp amplification product.

Some thermal cyclers may require longer (~60 sec) denaturation times. Refer to the manufacturers operating manuals.

The parameters described here are only for the GeneAmp PCR Systems 2400, 9600, and 9700. See thermal cycler manuals for details on operation.

Note All GeneAmp PCR Systems display the Calculated Sample Temperature.

Cycling parameters for the PCR step of the two-step process

Step	**AmpliTaq Gold Activation**	**PCR** CYCLE (43 cycles)[a]		**PCR** (FInal Step)
	HOLD			HOLD
		Denature	Anneal/ Extend	Anneal/Extend
Time	10 min	20 sec	1 min	7 min
Temperature	95 °C	94 °C	62 °C	72 °C

a. The cycle number can be reduced when amplifying more than 5,000 copies of target RNA. For more information refer to “Cycle Optimization Guidelines” on page 17.

Appendix A. Additional Primer and PCR Product Information

Additional Primers and PCR Products from pAW109

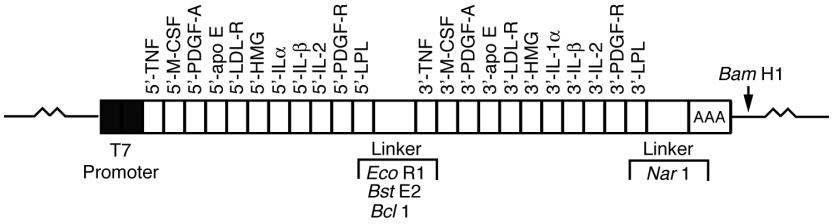
The DNA sequences for the other GeneAmplimer® primers in the control pAW109 sequences are shown below. Refer to “pAW109 Transcribed RNA” on page 29 to see a map of the pAW109 polylinker.

GeneAmplimer primers for amplicons in pAW109 RNA

Gene Name	Primer Information		PCR Product Size (bp) From	
	Name	Sequence	pAW109	mRNA
TNF	AW112 AW113	5′–CAGAGGGAAGAGTTCCCCAG–3′ 5′–CCTTGGTCTGGTAGGAGACG–3′	301	325
M–CSF	AW111 GM20	5′–GAACAGTTGAAAGATCCAGTG–3′ 5′–TCGGACGCAGGCCTTGTCATG–3′	302	192
PDGF-A	AW116 AW117	5′–CCTGCCCATTCGGAGGAAGAG–3′ 5′–TTGGCCACCTTGACGCTGCG–3′	301	225
PDGF-B	AW118 AW119	5′–GAAGGAGCCTGGGTTCCCTG–3′ 5′–TTTCTCACCTGGACAGGTCG–3′	300	226
LDL-R	AW125 AW126	5′–CAATGTCTCACCAAGCTCTG–3′ 5′–TCTGTCTCGAGGGGTAGCTG–3′	301	258
HMG	AW102 AW104	5′–TACCATGTCAGGGGTACGTC–3′ 5′–CAAGCCTAGAGACATAATCATC–3′	303	247
IL-1α	DM151 DM152	5′–GTCTCTGAATCAGAAATCCTTCTATC–3′ 5′–CATGTCAAATTTCACTGCTTCATCC–3′	308	421
IL-1β	DM155 DM156	5′–AAACAGATGAAGTGCTCCTTCCAGG–3′ 5′–TGGAGAACACCACTTGTTGCTCCA–3′	306	391
IL-2	DM135 DM136	5′–GAATGGAATTAATAATTACAAGAATCCC–3′ 5′–TGTTTCAGATCCCTTTAGTTCCAG–3′	305	229
PDGF-R	AW158 AW159	5′–TGACCACCCAGCCATCCTTC–3′ 5′–GAGGAGGTGTTGACTTCATTC–3′	300	228
LPL	AW155 AW157	5′–GAGATTTCTCTGTATGGCACC–3′ 5′–CTGCAAATGAGACACTTTCTC–3′	301	276

pAW109 Transcribed RNA

A schematic map of transcribed pAW109 RNA is shown below.



A schematic diagram of the general arrangement of the RNA transcribed from plasmid pAW109.

Performance Characteristics

Each lot of the GeneAmp Gold RNA PCR Reagent Kit has been shown, under the conditions stated for One-Step RNA PCR (page 19), to yield a visible 308-bp band on an agarose gel stained with ethidium bromide starting with 1000 copies of pAW109 RNA.

Appendix B. References

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Appendix C. Technical Support

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Hours for Telephone Technical Support

In the United States and Canada, technical support is available at the following times:

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