TaqMan® Gold RT-PCR Kit

Protocol

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Contents

1 Introduction

2 Control Reactions for RT-PCR

3 Reverse Transcription for the 18S Amplicon

4 Data Analysis

5 *Reagent Optimization*

6 *Cycle Optimization*

1 Introduction

Overview

About This Chapter

This chapter describes the TaqMan® Gold RT-PCR Kit and provides important information about safety.

In This Chapter

The following topics are discussed in this chapter:

Purpose of the Kit

RNA Detection

The TaqMan Gold RT-PCR Kit (P/N N808-0233) is designed for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from either total RNA or mRNA. The reagents in this kit can be used for one-step or two-step RT-PCR with ABI PRISM® Sequence Detection Systems.

One-step RT-PCR performs RT as well as PCR in a single buffer system. The reaction proceeds without the addition of reagents between the RT and PCR steps. This offers the convenience of a single-tube preparation for RT and PCR amplification. However, the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase, UNG), cannot be used with one-step RT-PCR.

Two-step RT-PCR is performed in two separate reactions. This is useful when detecting multiple transcripts from a single cDNA reaction, or when storing a portion of the cDNA for later use. The advantage of the two-step RT-PCR amplification is that the UNG enzyme can be used to prevent carryover contamination.

Comparison of RT-PCR Methods

Method	Primers for cDNA Synthesis	Features
One-step	Sequence-specific reverse primer	♦ Requires single Reaction Mix ♦ UNG cannot be used
Two-step for all amplicons except 18S	Random hexamers Oligo $d(T)_{16}$ Sequence-specific reverse primer	♦ cDNA can be stored for later use ♦ UNG can be used ♦ Requires two Reaction Mixes
Two-step for the 18S amplicon	Random hexamers	♦ cDNA can be stored for later use ♦ UNG can be used ♦ Requires two Reaction Mixes

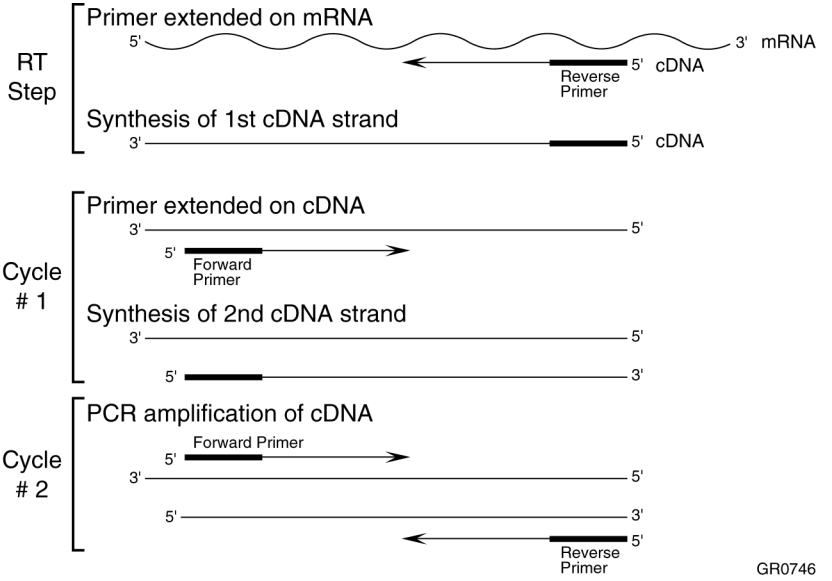
The TaqMan Gold RT-PCR Kit contains reagents to conduct a 5´ nuclease assay. Detection of RT-PCR product with no downstream

processing is accomplished within minutes of PCR completion by monitoring the increase in fluorescence of a dye-labeled probe.

The TaqMan Gold RT-PCR Kit contains MultiScribe™ Reverse Transcriptase for first strand cDNA synthesis, and thermal stable AmpliTaq Gold® DNA Polymerase for second strand cDNA synthesis and DNA amplification.

One-Step RT-PCR

One-step RT-PCR uses a single buffer that enables RT and PCR amplification to occur without interruption. Below is a schematic representation of RT-PCR using the TaqMan Gold RT-PCR Kit. Hybridization of the TaqMan® probe is not shown.



Primers for Two-Step RT-PCR for All Amplicons Except 18S

For two-step RT-PCR, 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 work equally well. For longer RNA transcripts or sequences containing hairpin loops, consider the following guidelines below.

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

Primers for Two-Step RT-PCR for 18S Amplicons

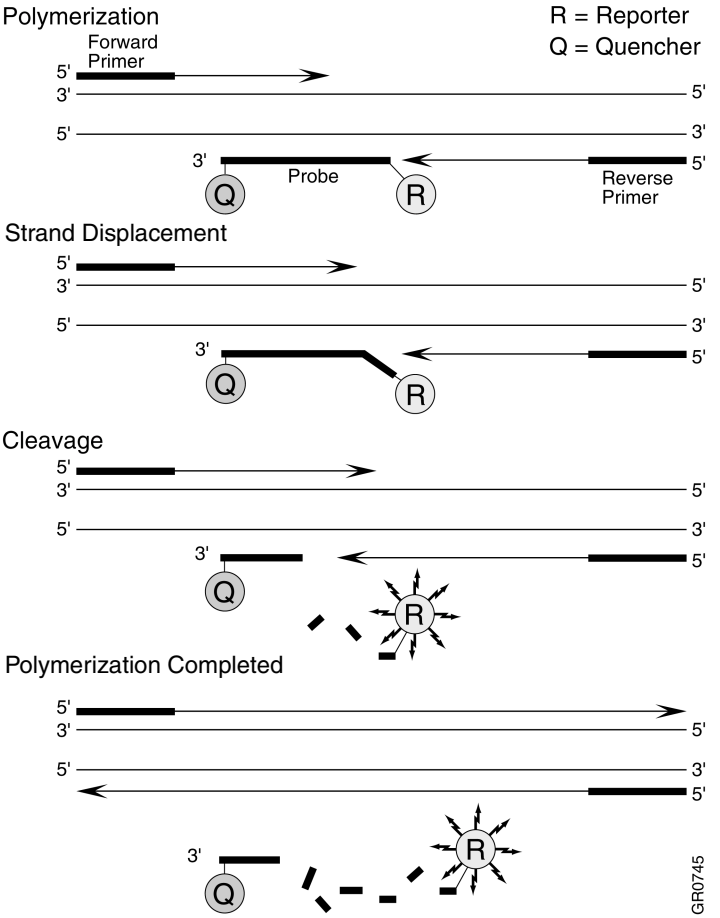
For two-step RT-PCR, random hexamer primers can be used for cDNA synthesis:

- Use with long reverse transcripts or reverse transcripts containing hairpin loops
- Use to transcribe all RNA (rRNA, mRNA, and tRNA)

Basics of the 5´ Nuclease Assay

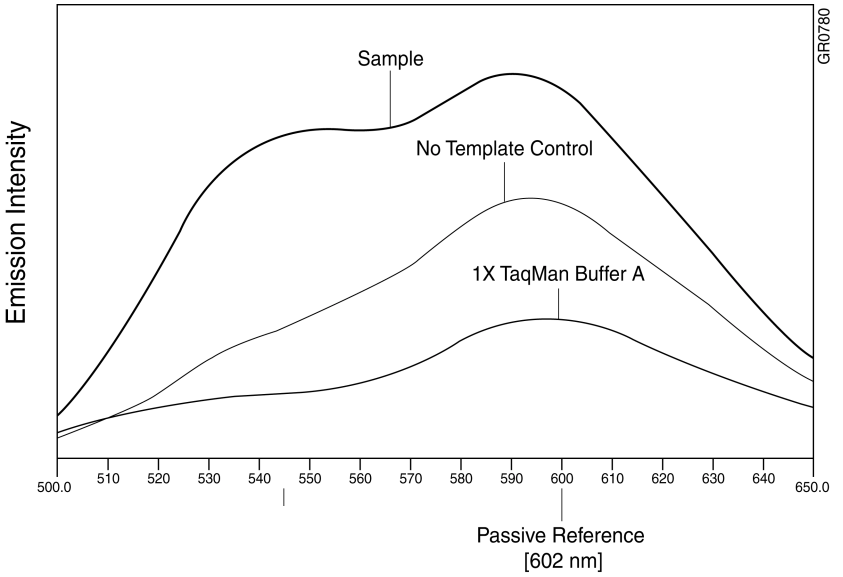
The RT-PCR reaction exploits the 5´ nuclease activity of AmpliTaq Gold enzyme to cleave a TaqMan probe during PCR. The TaqMan probe contains a reporter dye at the 5´ end of the probe and a quencher dye at the 3´ end of the probe.

During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The figure below shows the forklike-structure-dependent, polymerization-associated, 5´ to 3´ nuclease activity of AmpliTaq Gold DNA Polymerase during PCR.



When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites.

The 5´ to 3´ nucleolytic activity of the AmpliTaq Gold enzyme cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The figure below shows an overlay of three emission scans measured on a Sequence Detection System, post-PCR.



The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any nonspecific amplification is not detected.

TaqMan Probe

The probe consists of an oligonucleotide with a 5´-reporter dye and a 3´-quencher dye. A fluorescent reporter dye, such as FAM™ dye, is covalently linked to the 5´ end of the oligonucleotide. TET™ dye, JOE™ dye, and VIC™ dye have also been used as reporter dyes. Each of the reporters is quenched by TAMRA™ dye attached via a linker arm that is usually located at the 3´ end.

MultiScribe Reverse Transcriptase

MultiScribe Reverse Transcriptase (P/N N808-0018) is a recombinant Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase. MultiScribe Reverse Transcriptase is similar to MuLV Reverse Transcriptase, but differs in its recommended usage.

AmpliTaq Gold DNA Polymerase

AmpliTaq Gold is a thermal stable DNA polymerase. The enzyme has a 5´ to 3´ nuclease activity, but lacks a 3´ to 5´ exonuclease activity (Innis *et al.*, 1988; Holland *et al.*,1991). With AmpliTaq Gold enzyme, Hot Start PCR™ and Time Release PCR can be introduced into existing amplification systems with little or no modification of cycling parameters or reaction conditions. These techniques improve amplification of most templates by lowering background and increasing amplification of specific products.

TaqMan Gold RT-PCR Kit

The TaqMan Gold RT-PCR Kit consists of three modules: the TaqMan® PCR Core Reagents, the TaqMan® Reverse Transcription Reagents and the TaqMan® GAPDH Control Reagents. The kit can be purchased with or without the TaqMan GAPDH Control Reagents module. Each module is also available individually.

The TaqMan Gold RT-PCR Kit (P/N N808-0233) provides reagents to perform the reverse transcription of RNA to cDNA and subsequent PCR amplification. This kit can be used in either one-step or two-step RT-PCR, depending upon the application. The TaqMan GAPDH Control Reagents (P/N 402869) contain the primers, probe, and Control RNA template for fluorogenic detection of the human GAPDH transcript.

Using two enzymes, the TaqMan Gold RT-PCR kit provides a check for the presence of contaminating genomic DNA in the RNA sample. Genomic DNA could be amplified by AmpliTaq Gold DNA Polymerase and lead to a false positive result. By including a No RT Control reaction, *i.e.*, reaction that lacks reverse transcriptase, interfering genomic DNA amplification can be detected.

The TaqMan Gold RT-PCR Kit may be used for Real Time or Plate Read (endpoint) Detection of an RNA transcript. Analysis is performed using a Sequence Detection System available from Applied Biosystems.

AmpErase UNG (P/N N808-0096), a component in the PCR Carry-Over Prevention Kit (P/N N808-0068) and the TaqMan PCR Core Reagents (P/N N808-0228), can be used with two-step RT-PCR. However, UNG cannot be used with one-step RT-PCR. UNG is active at the same temperatures as those required for the reverse transcription reaction in one-step RT-PCR. Therefore, UNG would act to remove any uracil incorporated into the cDNA (AmpErase UNG Product Insert, 1993).

GAPDH Fluorogenic Probe

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe supplied with the TaqMan Gold RT-PCR Kit (P/N N808-0233) uses a JOE reporter dye to detect amplification of the Control RNA template included in the kit. The TaqMan GAPDH Control Reagents detect the RNA transcript of the human GAPDH gene (Ercolani *et al.*, 1988), a constitutively expressed housekeeping gene.

For more information concerning the GAPDH probe, see Appendix B.

Materials and Equipment

Kit Components

The TaqMan Gold RT-PCR Kit can be purchased with two or three modules:

Kit	Part Number	Contents[a]
TaqMan Gold RT-PCR Kit with controls	N808-0233	♦ TaqMan PCR Core Reagent Kit (P/N N808-0228) ♦ TaqMan Reverse Transcription Reagents (P/N N808-0234) ♦ TaqMan GAPDH Control Reagents (P/N 402869) ♦ Protocol (P/N 402876)
TaqMan Gold RT-PCR Kit without controls	N808-0232	♦ TaqMan PCR Core Reagent Kit (P/N N808-0228) ♦ TaqMan Reverse Transcription Reagents (P/N N808-0234) ♦ Protocol (P/N 402876)

a. The contents of the modules are described in the three tables beginning on page 1-10.

The number of reactions that can be performed depends on whether you are performing a one-step or two-step reaction.

- One-step: 200 reactions (50 µL each)
- Two-step: 200 RT reactions (10 µL each) and 200 PCR reactions (50 µL each)
- GAPDH Control Reagents: 100 reactions (50 µL each)

TaqMan PCR Core Reagent Kit (P/N N808-0228)

Reagent	Quantity	Description
AmpliTaq Gold DNA Polymerase	250 U	One tube containing 5 U/µL of AmpliTaq Gold DNA Polymerase in 20 mM Tris-HCl, pH 9.0, 100 mM KCl, 0.1 mM EDTA, 1.0 mM DTT, 50% glycerol, and 0.5% (w/v) Tween® 20
AmpErase UNG	100 U	One tube containing 1 U/µL uracil-N-glycosylase in 30 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 5% (v/v) glycerol, and 0.05% (w/v)Tween 20
deoxyATP	320 µL	One tube containing 10 mM deoxyadenosine triphosphate
deoxyCTP	320 µL	One tube containing 10 mM deoxycytidine triphosphate
deoxyGTP	320 µL	One tube containing 10 mM deoxyguanosine triphosphate
deoxyUTP	320 µL	One tube containing 20 mM deoxyuridine triphosphate
10X TaqMan® Buffer A	1.2 mL	One tube containing 500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl, pH 8.3, and 600 nM Passive Reference
$MgCl_2$ solution	3 mL	Two tubes containing 25 mM $MgCl_2$

TaqMan Reverse Transcription Reagents (P/N N808-0234)

Reagent	Quantity	Description
MultiScribe Reverse Transcriptase	5000 U	One tube containing 50 U/µL of recombinant Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase in 20 mM Tris-HCl, pH 7.5, 0.1 mM Na_2EDTA, 1 mM DTT, 0.01% (v/v) NP-40, and 50% (v/v) glycerol
RNase Inhibitor	4000 U	Two tubes containing 20 U/µL of RNase Inhibitor in 20 mM HEPES-KOH, pH 7.6, 50 mM KCl, 8 mM DTT, and 50% (v/v) glycerol
dNTP Mixture	1 mL	One tube containing 2.5 mM deoxyadenosine triphosphate, 2.5 mM deoxycytidine triphosphate, 2.5 mM deoxyguanosine triphosphate, and 2.5 mM deoxythymidine triphosphate
Oligo $d(T)_{16}$	100 µL	One tube containing 50 µM oligodeoxynucleotide of sequence $d(T)_{16}$ in 10 mM Tris-HCl, pH 8.3
Random Hexamers	100 µL	One tube containing 50 µM short oligodeoxyribonucleotides of random sequence $[d(N)_6]$ in 10 mM Tris-HCl, pH 8.3
10X RT Buffer	1.5 mL	One tube containing 500 mM KCl, 100 mM Tris-HCl, pH 8.3
$MgCl_2$ solution	1.5 mL	One tube containing 25 mM $MgCl_2$

The TaqMan GAPDH Control Reagents (P/N 402869)

Reagent	Volume (µL)	Description
GAPDH Forward Primer	100	One tube containing a 10 µM solution of primer in TE buffer
GAPDH Reverse Primer	100	One tube containing a 10 µM solution of primer in TE buffer
GAPDH Probe	100	One tube containing a 5 µM solution of probe in TE buffer
Control RNA (Human)	100	One tube containing 50 ng/µL of total human RNA in 10 mM Tris-HCl, pH 7.0, 100 mM NaCl, and 1 mM EDTA

Materials Required but Not Supplied

The following items are required when using the TaqMan Gold RT-PCR Kit, but are not supplied. See the table for source information.

User-Supplied Materials

Item	Source
7900HT Sequence Detection System 7000 Sequence Detection System	See your local Applied Biosystems representative for the instrument or software best suited to meet your needs.
Primer Express™ software (single-use license)	Applied Biosystems
Sequence Detection primers ♦ Min 4000 pmol purified for sequence detection ♦ Min 40,000 pmol purified for sequence detection ♦ Min 130,000 pmol purified for sequence detection	Applied Biosystems ♦ P/N 4304970 ♦ P/N 4304971 ♦ P/N 4304972
TaqMan® MGB Probe ♦ 5000–6000 pmoles ♦ 15,000–25,000 pmoles ♦ 50,000–100,000 pmoles	Applied Biosystems ♦ P/N 4316034 ♦ P/N 4316033 ♦ P/N 4316032

User-Supplied Materials *(continued)*

Item	Source
TaqMan® TAMRA Probe ♦ 50,000–100,000 pmoles ♦ 15,000–25,000 pmoles ♦ 5000–6000 pmoles	Applied Biosystems ♦ P/N 450003 ♦ P/N 450024 ♦ P/N 450025
MicroAmp® Optical 96-Well Reaction Plate and Optical Caps	Applied Biosystems (P/N 403012)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems (P/N N801-0560)
ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode	Applied Biosystems (P/N 4309849)
Note The MicroAmp Optical 96-Well Reaction Plate may be sealed with: ♦ MicroAmp® Optical Caps or ♦ ABI PRISM™ Optical Adhesive Cover The Optical Adhesive Cover must be used with a compression pad and applicator, which are included in the starter pack.	
MicroAmp Optical Caps	Applied Biosystems (P/N 4323032)
ABI PRISM Optical Adhesive Cover Starter Pack containing 20 optical adhesive covers, one applicator, and one compression pad. **Note** The MicroAmp Optical 96-well Reaction Plate may be sealed with MicroAmp Optical caps or ABI PRISM Optical Adhesive Cover	Applied Biosystems (P/N 4313663)
MicroAmp® Optical Tubes	Applied Biosystems (P/N N801-0933)
Sequence Detection Systems Spectral Calibration Kit (for the 7700 instrument only)	Applied Biosystems (P/N 4305822)
Sequence Detection Systems 384-Well Spectral Calibration Kit	Applied Biosystems (P/N 4323977)

User-Supplied Materials *(continued)*

Item	Source
ABI PRISM® 7900 Sequence Detection Systems 96-Well Spectral Calibration Kit	Applied Biosystems (P/N 4328639)
ABI PRISM® 7000 Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (P/N 4328895)
Centrifuge with adapter for 96-well plate	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS
NuSieve 4% (3:1) agarose gels, for DNA <1 kb	FMC BioProducts (P/N 54928)
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement or air-displacement	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Storage and Stability

Upon receipt, store the TaqMan Gold RT-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.

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.

⚠ CAUTION 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.

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

⚠ DANGER 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.

Chemical Hazard Warning

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

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* 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 (*e.g.*, 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.

Chemical Waste Hazard Warning

⚠ WARNING CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

- 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.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* 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 (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- 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.

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.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current material safety data sheet (MSDS) before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ WARNING CHEMICAL HAZARD. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below

To order documents by automated telephone service:

Step	Action
1	From the U.S. or Canada, dial **1.800.487.6809**.
2	Follow the voice instructions to order documents (for delivery by fax). **Note** There is a limit of five documents per fax request.

To order documents by telephone:

In the U.S.	Dial **1.800.345.5224**, and press **1**.
In Canada	Dial **1.800.668.6913**, and press **1** for English or **2** for French.

To view, download, or order documents through the Applied Biosystems web site:

Step	Action
1	Go to **http://www.appliedbiosystems.com**
2	Click **SERVICES & SUPPORT** at the top of the page, click **Documents on Demand**, then click **MSDS.**
3	Click **MSDS Index**, search through the list for the chemical of interest to you, then click on the MSDS document number for that chemical to open a PDF version of the MSDS.

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

Preventing Contamination

Introduction

Due to the high throughput and repetitive nature of the 5´ nuclease assay, 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).

UNG

UNG is a pure nuclease-free, 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. This gene has been inserted into an *E. coli* host to direct expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UNG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA.

UNG in Two-Step Reactions

When two-step RT-PCR is performed with the TaqMan Gold RT-PCR Kit, UNG treatment can prevent the reamplification of carryover PCR products. When dUTP replaces dTTP in PCR amplification, UNG treatment can remove up to 200,000 copies of the GAPDH amplicon per 50-µL reaction.

UNG in One-Step Reactions

UNG cannot be used when one-step RT-PCR is performed with reagents from the TaqMan Gold RT-PCR Kit. Because UNG is active at temperatures required to complete reverse transcription, the active UNG enzyme would remove uracil bases incorporated into the newly synthesized cDNA strand (AmpErase UNG Product Insert, 1993).

UNG can be used to detect reagent contamination in one-step RT-PCR. Because one-step RT-PCR utilizes dUTP, amplicons generated during this step contain uridine residues. If contamination is suspected from previous PCR runs, performing PCR with and without AmpErase UNG will help to identify the source of contamination. To do this, set up parallel No Template Control (NTC) PCR reactions with and without UNG. A positive signal in the reaction without UNG indicates contamination of reaction components.

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.

Fluorescent Contaminants

Since fluorescent contaminants may interfere with this assay and give false positive results, it may be necessary to include a No Amplification Control tube that contains sample and no enzyme. If the absolute fluorescence of the No Amplification Control is greater than that of the No Template Control after PCR, fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler.

Control Reactions for RT-PCR 2

Overview

About This Chapter

This chapter describes the preparation of GAPDH control reactions for RT-PCR.

In This Chapter

The following topics are discussed in this chapter:

Preparing GAPDH Control Reactions for One-Step RT-PCR

Overview

This procedure is optimized for the GAPDH Control Reagents. If you are using another set of primers and probe (except 18S rRNA), make sure to optimize your reaction conditions. The number of reactions depends upon the plate setup by the user. Between 2 pg and 1 µg of total RNA may be used per reaction. The No Template Control reaction is the complete RT-PCR formulation without the target RNA.

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, except enzymes and RNase Inhibitor, by vortexing and using a microcentrifuge to briefly spin down the tube contents. Protect the fluorescent dye labeled probe from excessive exposure to light. When finished with the kit return it to the –15 to –25 °C freezer.

Reaction Mix Preparation

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

⚠ CAUTION **CHEMICAL HAZARD**. **TaqMan PCR Core Reagents** may cause eye and skin irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare Reaction Mix (in general):

Step	Action
1	Prepare Reaction Mix by combining all the nonenzymatic components listed in the appropriate table.
2	Vortex briefly.
3	Add the enzymatic components (*e.g.*, polymerase, reverse transcriptase, RNase Inhibitor) listed for the appropriate Reaction Mix.
4	Mix the components by inverting the microcentrifuge tube.

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 2-2.

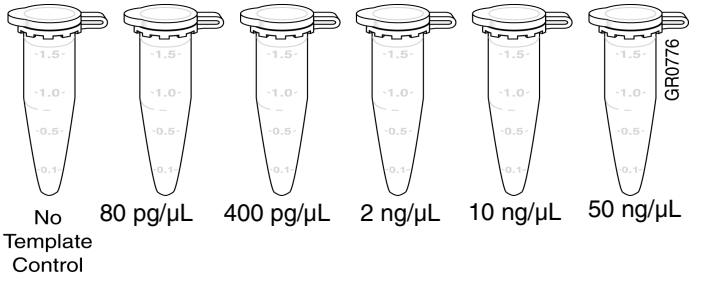
Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
10X TaqMan Buffer A	5	1X
25 mM Magnesium Chloride	11	5.5 mM
10 mM deoxyATP	1.5	300 µM
10 mM deoxyCTP	1.5	300 µM
10 mM deoxyGTP	1.5	300 µM
20 mM deoxyUTP	1.5	600 µM
10 µM GAPDH Forward Primer	1.0	200 nM
10 µM GAPDH Reverse Primer	1.0	200 nM
5 µM GAPDH Probe	1.0	100 nM
AmpliTaq Gold DNA Polymerase (5.0 U/µL)	0.25	0.025 U/µL
MultiScribe Reverse Transcriptase (50 U/µL)	0.25	0.25 U/µL
RNase Inhibitor	1.0	0.4 U/µL
Total	26.5 µL	

a. Volume of RNase-free water (µL) = 23.5–RNA sample volume.

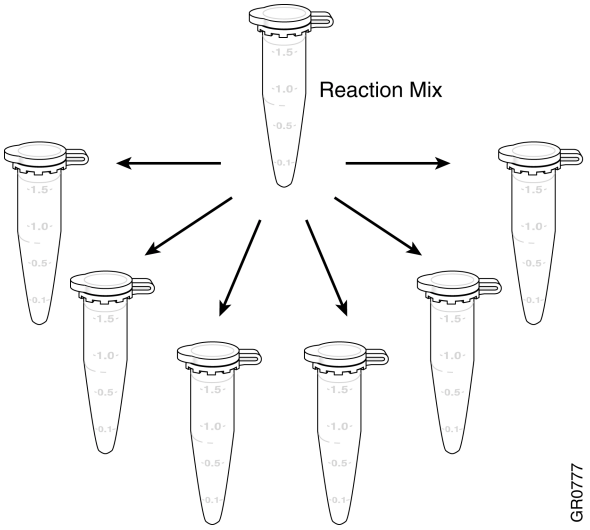
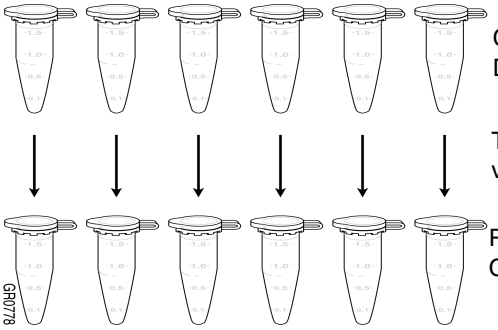
One-Step RT-PCR

The following example demonstrates the dynamic range of the GAPDH Control RT-PCR using triplicate reactions of five RNA concentrations.

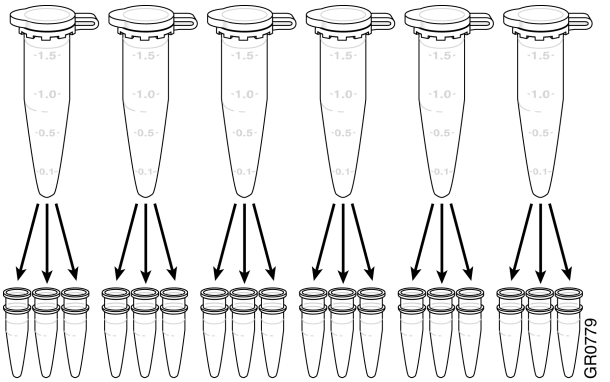
To perform one-step RT-PCR:

Step	Action
1	a. Prepare several dilutions of Human Control RNA within the range of 80 pg to 50 ng. b. Make dilutions with 1X TE buffer. Include a No Template Control reaction containing no template, only 1X TE buffer.  For example, prepare six concentrations of Control RNA: 0 ng/µL (No Template Control), 80 pg/µL, 400 pg/µL, 2 ng/µL, 10 ng/µL, and 50 ng/µL.
2	Prepare the Reaction Mix as described in “One-Step Reaction Mix Preparation” on page 2-3. Example: This brings the final volume of the Reaction Mix to 49 µL for one reaction. Prepare enough Reaction Mix for 26 reactions (49 µL × 26 reactions = 1274 µL). This provides excess volume for loss occurring during multiple reagent transfers.

To perform one-step RT-PCR: *(continued)*

Step	Action
3	For each dilution of Control RNA, label one corresponding 1.5-mL microcentrifuge tube. Example: Aliquot 196 µL (49 µL × 4 reaction volumes) of Reaction Mix into each 1.5-mL microcentrifuge tube. 
4	Transfer the same volume from each of the RNA dilution tubes to the corresponding microcentrifuge tube containing the Reaction Mix. Example: Transfer 4 µL (1 µL per reaction x 4 reactions) of each RNA dilution to the corresponding microcentrifuge tube. For the No Template Control tube, transfer 4 µL of 1X TE buffer. 

To perform one-step RT-PCR: *(continued)*

Step	Action
5	Mix the components gently by inverting the tube.
6	For each dilution of Control RNA, transfer 50 µL of the Reaction Mix + RNA from the microcentrifuge tube into the replicate number of MicroAmp® Optical Tubes or multiple wells of a MicroAmp® Optical 96-Well Reaction Plate. 
7	a. Using MicroAmp® Optical Caps, cap the tubes and briefly centrifuge to remove air bubbles and collect the liquid at the bottom of the tube. b. Transfer the plates to the thermal cycler block. Use the appropriate system for PCR amplification (7700 SDS, 7900HT SDS, 7000 SDS, or GeneAmp® 5700 Sequence Detection System).
8	Perform RT-PCR as described in “Thermal Cycling Parameters for One-Step RT-PCR” on page 2-7.

Prepare Five-Fold Dilutions of Control RNA

Final Concentration of Control RNA	Dilution of Reagents
50 ng/µL	Control RNA (50 ng/µL) only
10 ng/µL	5 µL of Control RNA + 20 µL of 1X TE buffer
2 ng/µL	5 µL of 10-ng/µL RNA dilution + 20 µL of 1X TE buffer
400 pg/µL	5 µL of 2-ng/µL RNA dilution + 20 µL of 1X TE buffer

Prepare Five-Fold Dilutions of Control RNA *(continued)*

80 pg/µL	5 µL of 400-pg/µL RNA dilution + 20 µL of 1X TE buffer
0 ng/µL	1X TE buffer only

Thermal Cycling Parameters for One-Step RT-PCR

The following thermal cycling parameters are optimized for the Human GAPDH system. See thermal cycler manuals for details on operation.

Step	Reverse Transcription	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	48 °C	95 °C	95 °C	60 °C
Time	30 min	10 min	15 sec	1 min

Run Types

The TaqMan® Gold RT-PCR Kit is designed for two run types: Real Time Detection and Plate Read Detection. Real Time Detection monitors fluorescence during each PCR cycle. Plate Read (endpoint) Detection collects one fluorescence scan per tube after PCR is completed. The features of these detection systems are listed below.

Run Type	Real Time	Plate Read Only
Fluorescence Detection	During each PCR cycle	Only endpoint
Analysis Results	Quantitation of initial template amount	Detection of final amplified product

Preparing GAPDH Control Reactions for Two-Step RT-PCR

Overview

This procedure is optimized for the GAPDH Control Reagents. The number of reactions depends upon the plate setup by the user. Between 20 pg and 2 µg of total RNA may be used per 100 µL reaction. The NTC reaction is the complete RT and PCR formulation without the target RNA.

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 AmpliTaq Gold DNA Polymerase, MultiScribe Reverse Transcriptase, and RNase Inhibitor in a freezer until immediately prior to use. Mix kit components, except enzymes, by vortexing and using a microcentrifuge to briefly spin down the tube contents. Protect the fluorescent dye-labeled probe from excessive exposure to light. 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 accuracy of the results. The use of a Reaction Mix reduces the number of reagent transfers and minimizes volume loss due to pipetting.

⚠ CAUTION CHEMICAL HAZARD. TaqManReverse Transcription Reagents may cause eye and skin irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ CAUTION **CHEMICAL HAZARD. TaqMan PCR Core Reagents** may cause eye and skin irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare Reaction Mix (in general):

Step	Action
1	Prepare Reaction Mix by combining all the nonenzymatic components listed in the appropriate table. **⚠ WARNING** **CHEMICAL HAZARD. Magnesium chloride** may cause eye, skin, and respiratory tract irritation, central nervous system depression, and kidney damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **⚠ CAUTION** **CHEMICAL HAZARD. AmpErase(R) uracil N-glycosylase** may cause eye and skin irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2	Vortex briefly.
3	Add the enzymatic components (*e.g.*, polymerase, reverse transcriptase, RNase Inhibitor) listed for the appropriate Reaction Mix. **⚠ CAUTION** **CHEMICAL HAZARD. AmpliTaq Gold DNA Polymerase** 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.
4	Mix the components by inverting the microcentrifuge tube.

Two-Step Reaction Mix Preparation

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

- RT Reaction Mix
- PCR Reaction Mix

The preparation for these mixes is described below.

RT Reaction Mix Preparation

Reaction Volume

The reaction volume for the RT step can be varied from 10 to 100 µL. For quantitation, a larger RT reaction volume can improve accuracy. A 100-µL RT reaction will efficiently convert a maximum of 2 µg total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more

than 2 µg total RNA in a 100-µL reaction. When changing the reaction volume, make sure the final concentration is consistent with that described in the table on page 2-11.

Preparing the RT Mix

The ingredients of a 10-µL and 100-µL, two-step RT Reaction Mixes are listed in the table on page 2-11. To make the RT Reaction Mix, follow the instructions described in “Reaction Mix Preparation” on page 2-8.

Component	Volume/Tube (µL)		Final Concentration
	10-µL Reaction	100-µL Reaction	
RNase-free water	see below[a]	see below[a]	–
10X TaqMan RT Buffer	1.0	10.0	1X
25 mM Magnesium Chloride	2.2	22.0	5.5 mM
deoxyNTPs Mixture	2.0	20.0	500 µM of each dNTP
Random Hexamers[b]	0.5	5.0	2.5 µM
RNase Inhibitor	0.2	2.0	0.4 U/µL
MultiScribe Reverse Transcriptase (50 U/µL)	0.25	2.5	1.25 U/µL
Total	6.15	61.5	—

a. The volume of RNase-free water (µL) will be 3.85–RNA sample volume in a 10-µL reaction or 38.5–RNA sample in a 100-µL reaction.

b. Random hexamers, oligo $d(T)_{16}$, or sequence-specific reverse primers can be used for primers of cDNA synthesis. If using an oligo $d(T)_{16}$ primer, use 0.5 µL of primer in a 10-µL reaction. This ensures that the final concentration of oligo $d(T)_{16}$ in the Reaction Mix is 2.5 µM. If using a sequence-specific reverse primer, the final concentration should be ≈ 200 nM.

PCR Reaction Mix Preparation

The ingredients of a 50-µL, two-step PCR Reaction Mix are listed in the table below.

PCR Reaction Mix Preparation

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
10X TaqMan Buffer A	5	1X
25 mM Magnesium Chloride	11	5.5 mM
10 mM deoxyATP	1	200 µM
10 mM deoxyCTP	1	200 µM
10 mM deoxyGTP	1	200 µM
20 mM deoxyUTP	1	400 µM
GAPDH Probe	1	100 nM
GAPDH Forward Primer	1	200 nM

PCR Reaction Mix Preparation *(continued)*

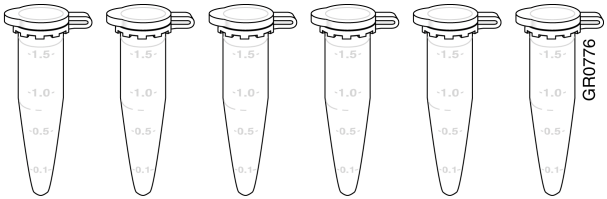
Component	Volume/Tube (µL)	Final Concentration
GAPDH Reverse Primer	1	200 nM
AmpErase UNG	0.5	0.01 U/µL
AmpliTaq Gold DNA Polymerase (5.0 U/µL)	0.25	0.025 U/µL
Total	23.75 µL	

a. Volume of RNase-free water (µL) = 26.25–cDNA sample volume.

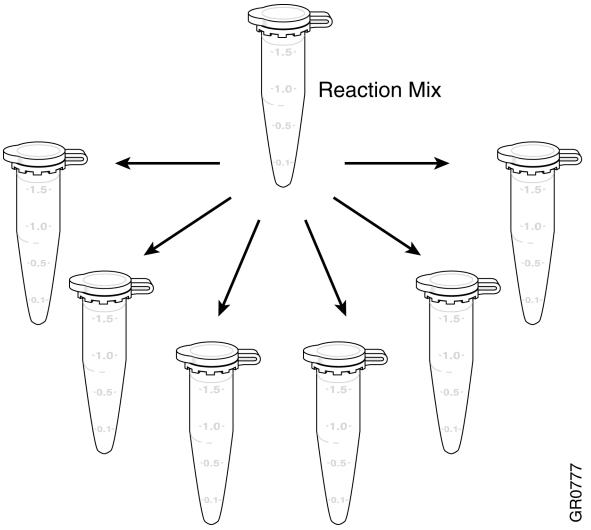
Two-Step RT-PCR

The following example demonstrates the dynamic range of the two-step GAPDH Control RT-PCR using triplicate cDNA synthesis reactions of five different RNA concentrations. The reaction volume for cDNA synthesis is 10 µL, the reaction volume of PCR is 50 µL. The reaction volume for cDNA synthesis can be varied between 10 to 100 µL. A 10 µL reaction volume is sufficient to reverse transcribe up to 200 ng of total RNA.

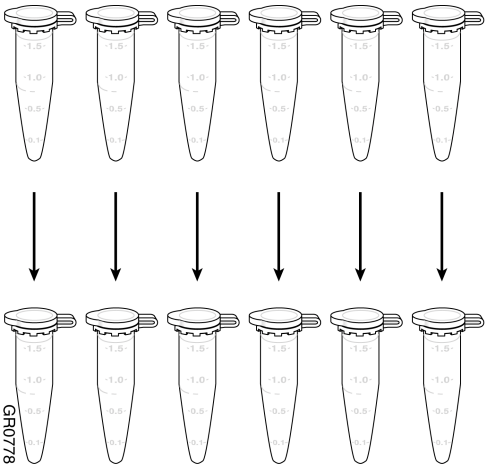
To perform two-step RT-PCR:

Step	Action
1	a. Prepare several dilutions of Human Control RNA within the range of 80 pg to 50 ng. b. Make dilutions with 1X TE buffer. c. Include a NTC reaction containing no template, only 1X TE buffer.  No Template Control For example, prepare six concentrations of Control RNA: 0 ng/µL (No Template Control), 80 pg/µL, 400 pg/µL, 2 ng/µL, 10 ng/µL, and 50 ng/µL.
2	Prepare the RT Reaction Mix as described in “RT Reaction Mix Preparation” on page 2-9. Example: This brings the final volume of the RT Reaction Mix to 9 µL for one reaction. Prepare enough RT Reaction Mix for 30 reactions (9 µL x 30 reactions = 270 µL).

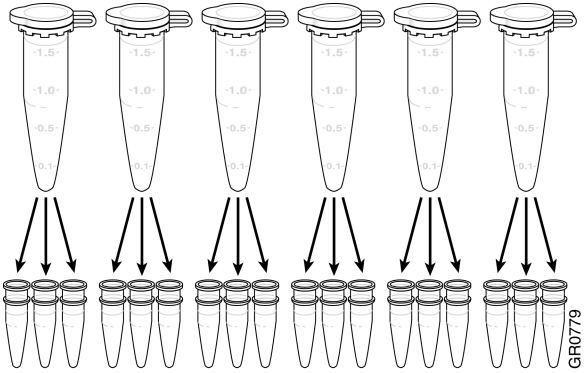
To perform two-step RT-PCR: *(continued)*

Step	Action
3	For each dilution of Control RNA, label one corresponding 1.5-mL microcentrifuge tube. Example: Aliquot 36 µL (9 µL x 4 reaction volumes) of RT Reaction Mix into each 1.5-mL microcentrifuge tube. 

To perform two-step RT-PCR: *(continued)*

Step	Action
4	Transfer the same volume from each of the RNA dilution tubes to the corresponding microcentrifuge tube containing the Reaction Mix. Example: Transfer 4 µL (1 µL per reaction x 4 reactions) of each RNA dilution to the corresponding microcentrifuge tube. For the No Template Control tube, transfer 4 µL of 1X TE buffer. 
5	Mix the components gently by inverting the tube.

To perform two-step RT-PCR: *(continued)*

Step	Action
6	For each dilution of Control RNA, transfer 10 µL of the Reaction Mix + RNA from the microcentrifuge tube into the replicate number of MicroAmp Optical Tubes or multiple wells of a MicroAmp Optical 96-Well Reaction Plate 
7	a. Using MicroAmp Optical Caps, cap the tubes and briefly centrifuge to remove air bubbles and collect the liquid at the bottom of the tube. b. Transfer the plates to the thermal cycler block. Use the appropriate system for PCR amplification (7700 SDS, 7000 SDS, 7900HT SDS or GeneAmp 5700 Sequence Detection System).
8	Perform RT as described in “Reverse Transcription” on page 2-17.
9	Prepare the PCR Reaction Mix as described in “PCR Reaction Mix Preparation” on page 2-11. Example: The final volume of the PCR Reaction Mix is 50 µL for one reaction (40 µL x 22 reactions = 880 µL).
10	Remove the 96-Well Reaction Plate after RT is finished. Open the MicroAmp Optical Caps carefully. Example: Transfer 40 µL of PCR Reaction Mix into each tube.

To perform two-step RT-PCR: *(continued)*

Step	Action
11	a. Using fresh MicroAmp Optical Caps, cap the tubes and briefly centrifuge to remove air bubbles and collect the liquid at the bottom of the tube. b. Transfer the plates to the thermal cycler block. Use the appropriate system for PCR amplification (7700 SDS, 7900HT SDS, 7000 SDS or GeneAmp 5700 SDS).
12	Perform PCR as described below.

Thermal Cycling Parameters for Two-Step PCR

Reverse Transcription

Step	Incubation[a]	RT	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temperature	25 °C	48 °C	95 °C
Time	10 min	30 min	5 min

a. When using random hexamers or oligo d(T)16 primers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 min) is necessary to maximize primer-RNA template binding. During incubation the hexameric or oligo d(T)16 primers are extended by reverse transcriptase. If using a sequence specific reverse primer, the incubation step is not necessary.

PCR

Step	UNG Incubation	AmpliTaq Gold Activation	PCR[a]	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min

a. It is important to keep the reaction temperatures >55 °C to prevent amplicon degradation.

Run Types

The TaqMan Gold RT-PCR Kit is designed for two run types, Real Time Detection and Plate Read Detection. Real Time Detection monitors fluorescence during each PCR cycle. Plate Read (endpoint) Detection collects one fluorescence scan per tube after PCR is completed.The features of these detection systems are listed in the table below.

Run Type	Real Time	Plate Read Only
Fluorescence Detection	During each PCR cycle	Only endpoint
Analysis Results	Quantitation of initial template amount	Detection of final amplified product

Reverse Transcription for the 18S Amplicon 3

Overview

About This Chapter

This chapter describes reverse transcription for the 18S amplicon.

In This Chapter

The following topics are discussed in this chapter:

Guidelines for Reverse Transcription of the 18S Amplicon

Overview

Synthesis of cDNA from total RNA samples is the first step in the two-step RT-PCR gene expression quantification experiment. In this step, random hexamers from the TaqMan® Reverse Transcription Reagents prime total RNA samples for reverse transcription (RT) using MultiScribe™ Reverse Transcriptase.

Recommended Template

Use only total RNA samples to generate cDNA for the 18S amplicon.

The following table lists the known template incompatibilities.

Template	Explanation
Poly A^+	The 18S rRNA endogenous control assay cannot accurately evaluate cDNA generated from poly A^+ RNA samples because most of the rRNA has been removed from them.

Template Quality

The quality of your results is directly related to the purity of your RNA template. Therefore, use only well-purified samples for 18S. Because ribonuclease and genomic DNA contamination are common problems in gene expression studies, purify your samples accordingly to ensure the best results.

Template Quantity

If possible, use spectrophotometric analysis to determine the concentrations of purified total RNA samples before RT. The table below lists the recommended range of initial template quantities for the RT step.

Initial Template	Quantity of Total RNA (per 100-µL RT reaction)
Total RNA	60 ng to 2 µg

Reaction Volume

Follow the guidelines below to ensure optimal RT performance.

- A 100-µL RT reaction will efficiently convert a maximum of 2 µg total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more than 2 µg total RNA.

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 work equally well. For longer RNA transcripts or sequences containing hairpin loops, consider the following guidelines:

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)

Preparing the Reactions

Overview

The following procedure describes the preparation of four different test samples for reverse transcription. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents (P/N N808-0234).

Preparing the Reactions

⚠ CAUTION CHEMICAL HAZARD. TaqMan Reverse Transcription Reagents may cause eye and skin irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the reverse transcription reactions:

Step	Action
1	In a 1.5-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed. If preparing four samples, follow the recommended volumes below.

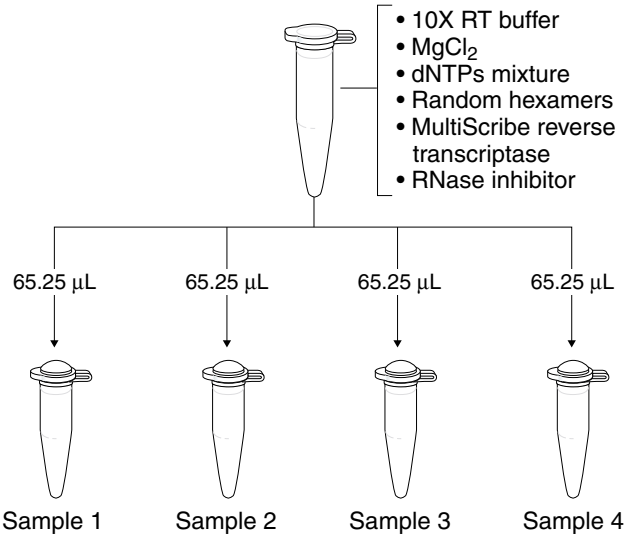
Component	Volume (µL)		Final Conc.
	Per Sample	Reaction Mix (x4)	
RNase-free water	see below[a]	see below[a]	—
10X RT Buffer	10.0	40.0	1X
25 mM $MgCl_2$	22.0	88.0	5.5 mM
deoxyNTPs Mixture	20.0	80.0	500 µM per dNTP
Random Hexamers	5.0	20.0	2.5 µM
RNase Inhibitor	2.0	8.0	0.4 U/µL
MultiScribe Reverse Transcriptase (50 U/µL)	6.25	25.0	3.125 U/µL[b]
Total[c]	65.25	261.0	—

a. The volume of RNase-free water (µL) will be 34.75–RNA sample volume in a 100-µL reaction.

b. To generate 18S cDNA, a higher concentration of MultiScribe Reverse Transcriptase is needed.

c. If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.

To prepare the reverse transcription reactions: *(continued)*

Step	Action
2	Label four 1.5-mL microcentrifuge tubes for the four test samples.
3	Transfer 60 ng to 2 µg (up to 34.75 µL) of each total RNA sample to the corresponding microcentrifuge tube.
4	If necessary, dilute each total RNA sample to a volume of 34.75 µL with RNase-free, deionized water.
5	Cap the tubes and gently tap each to mix the diluted samples.
6	Centrifuge the tubes briefly to eliminate air bubbles in the mixture.
7	Label four 0.2-mL MicroAmp® Reaction Tubes for the four total RNA test samples.
8	Pipet 65.25 µL of the reaction mix (from step 1) to each MicroAmp Reaction Tube (from step 7). 
9	Transfer 34.75 µL of each dilute total RNA sample to the corresponding MicroAmp Reaction Tube.
10	Cap the reaction tubes and gently tap each to mix the reactions.
11	Centrifuge the tubes briefly to force the solution to the bottom and to eliminate air bubbles from the mixture.

Thermal Cycling Conditions

Reverse Transcription Conditions for the 18S Amplicon

To conduct RT thermal cycling:

Step	Action
1	Load the reactions into a thermal cycler.
2	Program your thermal cycler with the following conditions:

Step	Hexamer Incubation[a]	RT	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temp.	25 °C	37 °C	95 °C
Time	10 min	60 min	5 min
Volume	100 µL		

a. When using random hexamers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 min) is necessary to maximize primer-RNA template binding.

3	Begin reverse transcription. **IMPORTANT** After thermal cycling, store all cDNA samples at –15 to –25 °C.

4 Data Analysis

Overview

About This Chapter

This chapter describes how to analyze the data generated in your experiment.

In This Chapter

The following topics are discussed in this chapter:

Preparing for Data Analysis

Interpreting the Results

The Passive Reference is a dye included in the 10X TaqMan® Buffer A and does not participate in the 5´ nuclease assay. The Passive Reference provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

Multicomponenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the three pure dye components generate the composite spectrum. This spectrum represents one fluorescent reading from one well. For the GAPDH Control System the three dyes used for Multicomponent Analysis are:

- TAMRA™ Quencher
- ROX™ Passive Reference
- JOE™ Reporter

R_n and ΔR_n Values

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube.

R_n^+ is the R_n value of a reaction containing all components including the template.

R_n^- is the R_n value of an unreacted sample. This value may be obtained from the early cycles of a Real Time run, those cycles prior to a detectable increase in fluorescence. This value may also be obtained from a reaction not containing template.

ΔR_n is the difference between the R_n^+ value and the R_n^- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

$\Delta R_n = (R_n^+) - (R_n^-)$

where:

$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$ PCR with template

$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$ PCR without template or early cycles of a Real Time reaction

Real-Time Detection

The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor.

On the amplification plot of R_n versus cycle number shown below, the C_T occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.

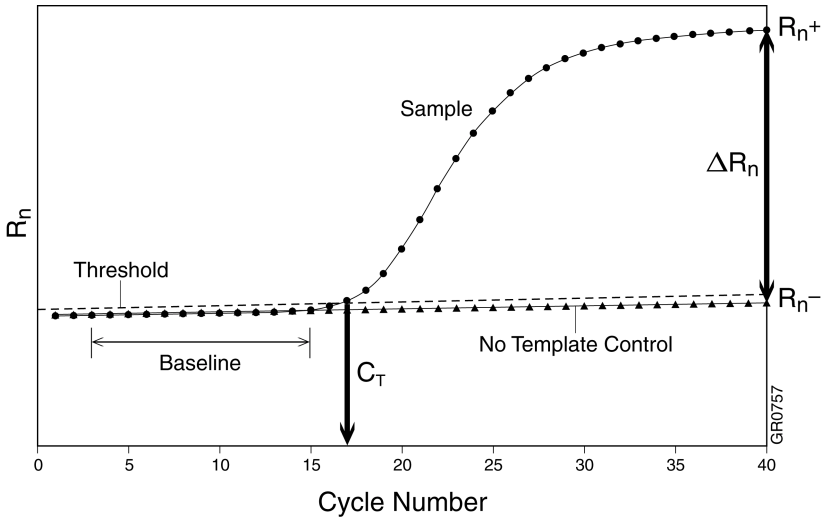


Plate Read Analysis

Plate Read analysis relies on endpoint data to calculate ΔR_n. To ensure statistically high confidence levels using either the GAPDH probe or a custom probe, run the protocol with at least three No Template Controls per microplate.

A positive result is defined as ΔR_n values greater than the threshold ΔR_n. The threshold ΔR_n is calculated by multiplying the standard deviation of three R_n^- values by an appropriate multiplier. The value for the appropriate multiplier is selected from a table of *t*-distribution values and depends upon the desired confidence level (Beyer, 1984). For 99% confidence levels, the multiplier is 6.965.

When more than three No Template Controls are run, the multiplier for the standard deviation decreases. Refer to a table of *t*-distribution values for the appropriate multiplier (Beyer, 1984).

To evaluate reproducibility, calculate the coefficient of variation on replicate samples. Inconsistent results (coefficients of variation exceeding 10%) may be caused by pipetting errors and incomplete mixing.

Determination of Threshold ΔR_n Value

To determine threshold ΔR_n value:

- On one microplate, measure both reporter and passive reference fluorescence, and determine the normalized reporter for each No Template Control tube (R_n^-).
- Determine the mean and standard deviation of R_n^-.
- When running three No Template Controls, multiply the R_n^- standard deviation by 6.965 to determine the threshold ΔR_n for the system.

For example, if the mean R_n^- for a system is 0.5 with a standard deviation of 0.02, the threshold ΔR_n is 6.965×0.02 or 0.14. Any $\Delta R_n > 0.14$ is a positive result and indicates that the sample contains target.

Performance Guarantees

System Performance Guarantee

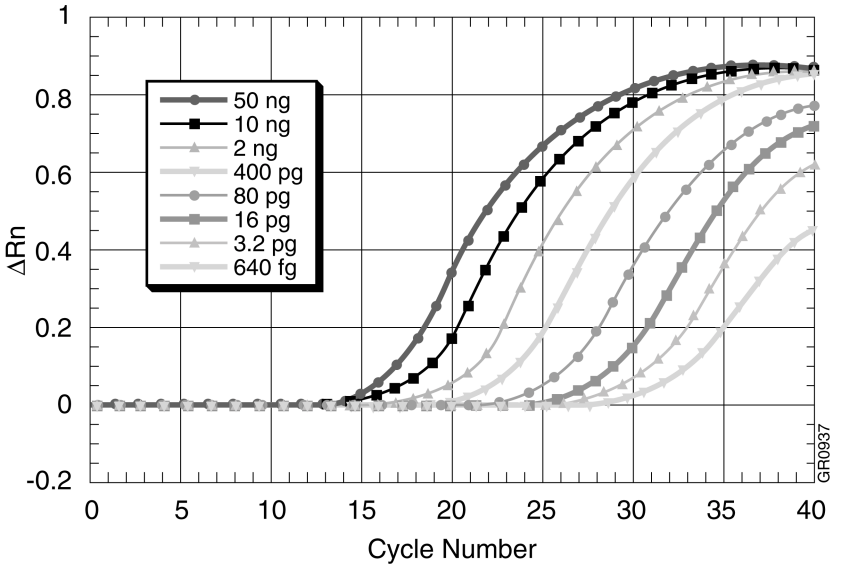
For a 50-µL reaction using 50 ng of Control RNA (Human) a positive result will be achieved using the reagents in the kit, the instructions in the protocol, and a Sequence Detection System instrument.

A positive result on a Real-Time instrument is defined as a series of 36 replicates which gives a mean C_T value with a standard deviation $\leq$0.16.

A positive result on an endpoint instrument is defined as any value greater than the threshold ΔR_n determined by analysis of three or more No Template Controls for a defined set of system conditions.

System Demonstrated Performance

The detection range has been demonstrated between 2 pg and 200 ng of total RNA control. This is equal to approximately 200–2 $\times$ 10^6 GAPDH transcript copies. Below is an example of Human GAPDH one-step RT-PCR.



Reagent Optimization 5

Overview

About This Chapter

This chapter describes reagent optimization guidelines.

In This Chapter

The following topics are discussed in this chapter:

Designing Probes and Primers

Probes

The following guidelines are for designing probes:

- Using Primer Express™ software, design probes with a T_m of 68 to 70 °C.
- Try to keep the G+C content in the range of 20 to 80%.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- No G on the 5´end.
- The TaqMan® probe is target sequence specific and the sequence can be chosen either from the upper stand or the lower strand. Select the strand that gives the probe with more Cs than Gs.
- To maximize the signal, vary the probe concentration in the range of 50 to 250 nM.

For details see the Applied Biosystems *Research News* article, "Guidelines for Designing TaqMan Fluorogenic Probes for 5´ Nuclease Assays."

Primers

These guidelines have been developed from the experience obtained at Applied Biosystems in the design of primers:

- Choose primers after designing the probe.
- Using Primer Express software, design primers with a melting temperature (T_m) of 58 to 60 °C.
- The T_m of the forward and reverse primers, as estimated by the nearest neighbor method (Rychlik, 1990), should be approximately equal.
- Primers should be 15 to 30 bases in length.
- Try to keep the G+C content in the range of 20 to 80%.
- Try to avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- The total number of Gs and Cs in the last five nucleotides at the 3´ end of the primer should not exceed two.
- The optimum primer concentration must be determined empirically by testing concentrations in the 50 to 900 µM range.

- Primer sequences should not complement within themselves or to each other, particularly at the 3´ ends. Overlaps result in the formation of primer artifacts called primer dimers. Primer dimers may occur to some extent even without such an overlap. Maintaining low primer concentration minimizes dimer products.
- Place the forward and reverse primers as close as possible to the probe without overlapping the probe. Preferable amplicon size is 50 to 150 bp.

Reagent Optimization

Overview

To detect a transcript of the target gene by fluorescence, a set of primers and a custom fluorescent probe must be designed. Using a set of custom primers and fluorescent probe, it is necessary to optimize the concentration of reaction components. This can be achieved by varying the concentrations of the following system components:

- RNA Template
- Random hexamers/oligo $d(T)_{16}$
- Magnesium chloride
- dNTPs
- AmpliTaq Gold® DNA Polymerase

The effect of these variations can be evaluated by comparing the magnitude of ΔR_n for Plate Read (endpoint) analysis or the C_T for Real-Time Detection after each change.

RNA Template

Start with enough copies of the RNA template to be sure of obtaining a signal by 25 to 30 cycles. Preferably, begin with 10,000 copies of template and less than 100 ng of total RNA per 50 µL. Low concentrations of target RNA may require up to 35 or more cycles to produce sufficient product for analysis.

The Control RNA provided in the TaqMan® GAPDH Control Reagents is formulated at 50 ng/µL. The copy number for the GAPDH transcript has been found to be ~100 copies/pg. The GAPDH control RNA contains contaminating genomic DNA that should not interfere with dilutions at 10^{-6} or greater.

Amplification of high G+C content cDNA requires one or more of the following modifications to overcome secondary structure (McConlogue *et al.*, 1988; Smith *et al.*, 1990; Sarkar *et al.*, 1990):

- High annealing (~60 °C) and melting temperatures
- Cosolvents such as glycerol, DMSO, etc.
- Use of 7-deaza-dGTP

Random Hexamers/ Oligo d(T)$_{16}$

The optimum random hexamers or oligo d(T)$_{16}$ concentration depends on the amount of RNA template. Excess random hexamers may reduce reverse transcriptase efficiency, depending on the template.

- When <200 ng of total RNA is used for reverse transcription, the final concentration of random hexamers or oligo d(T)$_{16}$ is 2.5 µM.
- When >200 ng of total RNA is used for reverse transcription, the final concentration of random hexamers or oligo d(T)$_{16}$ is 2.5 to 10 µM.

Magnesium Chloride Concentration

The optimum magnesium chloride concentration for both reverse transcription and PCR amplification is determined by empirical testing. Magnesium chloride concentrations can be optimized in increments of 0.5 mM within the 4.0 to 7.0 mM range. Suboptimal magnesium chloride concentrations reduce reverse transcription and PCR amplification efficiency or result in the production of nonspecific products. Samples containing EDTA, citrate, or other chelators, require a proportional increase in the magnesium chloride concentration in the reaction mix. The magnesium ion concentration should remain constant in solution and be adjusted in parallel with significant changes in the concentrations of sample RNA, cDNA, and dNTPs.

dNTP Concentration

Concentrations of dNTPs in the reaction mix should remain balanced. If the concentration of any one of these is significantly different from the rest, the AmpliTaq Gold DNA Polymerase may misincorporate, slow down, and/or terminate prematurely (Innis *et al.*, 1988). Substitution of dUTP for dTTP for control of PCR product carryover, however, may require higher concentrations of dUTP (typically twice that of any other dNTP) for the best amplification (Kwok, 1990; Orrego, 1990).

When oligo d(T)$_{16}$ is used for the first strand cDNA synthesis, it may be necessary to increase dNTP concentration. Determine the optimal concentration in the 500 µM to 1 mM range of each dNTP.

AmpliTaq Gold DNA Polymerase

AmpliTaq Gold DNA Polymerase is provided in an inactive state and is activated by heat. Because of this feature, the concentration of polymerase required for a typical PCR amplification depends on cycling parameters. Start with a concentration of 1.25 U/50 µL reaction volume, then increase the amount of the AmpliTaq Gold DNA Polymerase in the Reaction Mix by 0.25-U increments. Optimal results are obtained with a 9- to 12-minute pre-PCR heat step at 92 to 95 °C.

Because of the enhanced specificity of AmpliTaq Gold PCR, increasing the concentration of AmpliTaq Gold DNA Polymerase may result in significantly higher yields of specific product, without increasing background.

6 Cycle Optimization

Overview

About This Chapter

This chapter describes cycle optimization guidelines.

In This Chapter

The following topics are discussed in this chapter:

One-Step Cycle Optimization Guidelines

Thermal Cycling Conditions

The thermal cycling conditions for the one-step reaction consists of the following:

- Reverse transcription
- AmpliTaq Gold® DNA Polymerase activation
- PCR denaturation, annealing, and extension

Reverse Transcription

The time and temperature conditions for the reverse transcription reaction should be optimized empirically. This is accomplished by adjusting reaction temperatures in the range of 42 to 52 °C. Reaction time adjustments should be made in 10-minute increments and range from 20 to 60 minutes. The efficiency of the reverse transcription step depends upon the length and secondary structure of the RNA template.

AmpliTaq Gold DNA Polymerase Activation

AmpliTaq Gold DNA Polymerase is a chemically modified form of AmpliTaq® DNA Polymerase. The modification renders the enzyme inactive.

Upon thermal activation, the modifier is released, resulting in active enzyme. The high-temperature incubation step required for activation ensures that active enzyme is generated only at temperatures where the DNA is fully denatured.

PCR Denaturation

The purpose of the denaturation step (92 to 95 °C) is to separate complementary strands of nucleic acid.

The half-life of AmpliTaq Gold enzyme activity (approximately 40 minutes at 95 °C) suggests 95 °C as the maximum practical denaturation temperature. Glycerol effectively lowers the T_m of primers and the required denaturation temperature of PCR product by 2.5 to 3.0 °C (Gelfand and White, 1990). At 95 °C in the presence of 10% glycerol, the effective denaturation temperature is 97.5 to 98.0 °C.

PCR Annealing and Extension

The primer annealing/extension step (55 to 70 °C, depending upon the primers) allows hybridization of the primers to the single-stranded DNA, initiation of polymerization, and completion of primer extension. The optimum anneal temperature can be determined empirically by testing

at increments of 5 °C or smaller, until the maximum fluorescent signal is reached.

The optimum extension time can be determined within a 1- to 2-minute range. As the amount of DNA increases, the number of AmpliTaq Gold DNA Polymerase molecules may become limiting for the extension time slotted. Increasing the extension time in later cycles may be necessary to maintain the efficiency of amplification.

Two-Step Cycle Optimization Guidelines for All Amplicons Except 18S

Thermal Cycling Conditions

The thermal cycling conditions for the two-step reaction consists of the following:

Step One: Reverse Transcription

- Primer incubation
- Reverse transcription
- Reverse transcriptase inactivation

Step Two: PCR

- UNG incubation
- AmpliTaq Gold enzyme activation
- PCR denaturation, annealing and extension

Primer Incubation

When random hexamers or oligo $d(T)_{16}$ primers are used for first-strand cDNA synthesis, a primer incubation step is necessary at 25 °C for 10 minutes. During incubation this lower temperature allows the hexameric or oligo $d(T)_{16}$ primers to hybridize and extend by the reverse transcriptase. If using a sequence specific reverse primer, the incubation step is not necessary.

Reverse Transcription

The time and temperature conditions for the reverse transcription reaction should be optimized empirically. This is accomplished by adjusting reaction temperatures in the range of 42 to 52 °C. Reaction time adjustments should be made in 10-minute increments and range from 20 to 60 minutes. The efficiency of the reverse transcription step depends upon the length and secondary structure of the RNA template.

PCR Denaturation

The purpose of the denaturation step (92 to 95 °C) is to separate complementary strands of nucleic acid.

The half-life of AmpliTaq Gold DNA Polymerase activity (approximately 40 minutes at 95 °C) suggests 95 °C as the maximum practical denaturation temperature. Glycerol effectively lowers the T_m of primers and the required denaturation temperature of PCR product by 2.5 to 3.0 °C (Gelfand and White, 1990). At 95 °C in the presence of 10% glycerol, the effective denaturation temperature is 97.5 to 98.0 °C.

PCR Annealing and Extension

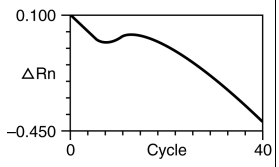
The primer annealing/extension step (55 to 70 °C, depending upon the primers) allows hybridization of the primers to the single-stranded DNA, initiation of polymerization, and completion of primer extension. The optimum anneal temperature can be determined empirically by testing at increments of 5 °C or smaller, until the maximum fluorescent signal is reached.

The optimum extension time can be determined empirically to within 1 to 2 minutes. As the amount of DNA increases, the number of AmpliTaq Gold DNA Polymerase molecules may become limiting for the extension time slotted. Increasing the extension time in later cycles may be necessary to maintain the efficiency of amplification.

Troubleshooting

A

Troubleshooting

Observation	Possible Cause	Recommended Action
$\Delta R_n \leq$ No template Control ΔR_n, and no amplification plot	Inappropriate reaction conditions	Troubleshoot RT-PCR optimization.
	Incorrect dye components chosen	Check dye component prior to data analysis.
	Reaction component omitted	Check that all the correct reagents were added.
	Incorrect primer or probe sequence	Resynthesize with appropriate sequence.
	Degraded template or no template added	Repeat with fresh template.
	Reaction inhibitor present	Repeat with purified template.
$\Delta R_n \leq$ No Template Control ΔR_n, and both reactions show an amplification plot	Amplicon contamination of reagents Template contamination of reagents	Check technique and equipment to confine contamination. Use fresh reagents.
Shifting R_n value during the early cycles of PCR (cycle 0–5)	Fluorescent emissions have not stabilized to new buffer conditions of reaction mix. This does not affect PCR, or the final results.	Reset upper value of baseline range. Add probe to the buffer component and allow it to equilibrate at room temperature prior to Reagent Mix formulation.
Abnormal amplification plot: 	C_T value <15, amplification signal detected in early cycles	Reset upper value of baseline range. Dilute the sample to increase the C_T value.

Troubleshooting *(continued)*

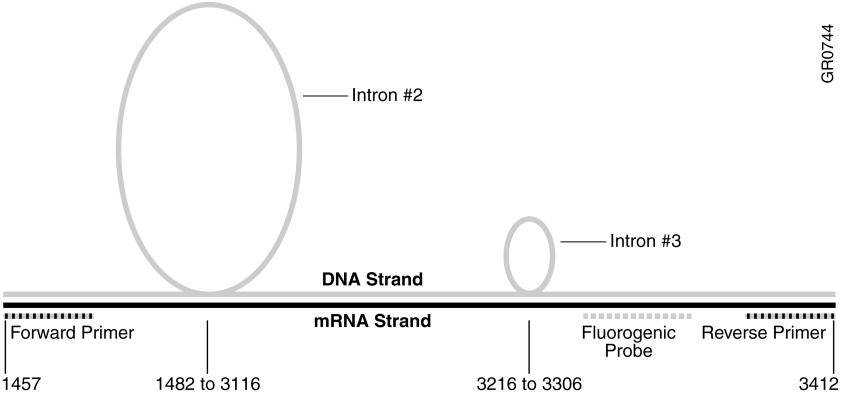
Observation	Possible Cause	Recommended Action
Multicomponent signal for ROX™ is not linear	Pure dye component's spectra are incorrect	Rerun pure dye spectra.
	Incorrect dye components choosen	Choose correct dyes for data analysis.
Small ΔR_n	PCR efficiency is poor	Reoptimize reaction conditions.
	Low copy number of target	Increase starting copy number.
C_T value is higher than expected	Less template added than expected	Increase sample amount.
	Sample is degraded	Evaluate sample integrity.
C_T value is lower than expected	More sample added than expected	Reduce sample amount.
	Template or amplicon contamination	
Standard deviation of C_T value >0.16	Inaccurate pipetting	Prepare a Reagent Mix. Use positive-displacement pipettors.

GAPDH Probe Characteristics

B

System Design

The mRNA target chosen was the transcript of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The mRNA amplicon target starts at base 1457 of the genomic sequence (HUMGAPDHG, J04038, in GenBank), spans two introns (intron number two from base 1482 to 3116, intron number three from base 3216 to 3306 inclusive) and ends at base 3412. The mRNA amplicon size is 226 bases. Below is a graphic representation of the genomic DNA sequence from which the GAPDH mRNA target was generated.



C

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Technical Support

D

Services & Support

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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

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