TaqMan® Ribosomal RNA Control Reagents

VIC™ Probe

Protocol

Applied Biosystems

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1 Introduction

1 Introduction

Overview

About This Chapter

This chapter describes the TaqMan® Ribosomal RNA Control Reagents and provides important information about safety and preventing contamination.

In This Chapter

The following topics are discussed in this chapter:

Purpose of the Kit

About the Kit

The TaqMan Ribosomal RNA Control Reagents are designed to detect the 18S ribosomal RNA (rRNA) gene. The amplicon generated from the 18S gene for both human RNA and DNA is 187 bp in length. The primer and probe sequences contained in this kit are conserved among a diverse group of eukaryotes including man, rat, mouse, *Xenopus*, *Saccharomyces*, *Giardia*, Maize, and *Arabidopsis*.

Ribosomal RNA levels provide an endogenous control for PCR quantitation studies. Refer to the *ABI PRISM 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression (*P/N 4303859). For additional information on the use of rRNA as an endogenous control, refer to Bhatia, *et al.*, 1994; deLeeuw, *et al.*, 1989; Duhl, *et al.*, 1992.

Basics of the 5´ Nuclease Assay

The RT-PCR reaction exploits the 5´ nuclease activity of the AmpliTaq Gold® DNA Polymerase 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, the reporter dye and quencher dye become separated, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

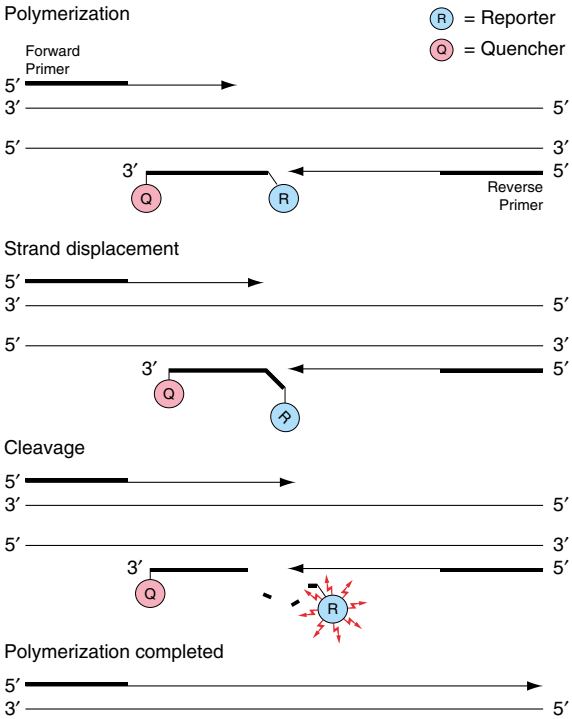


Figure 1-1 The fork-like-structure-dependent, polymerization associated, 5´ to 3´ nuclease activity of AmpliTaq Gold DNA Polymerase during PCR.

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 DNA Polymerase 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.

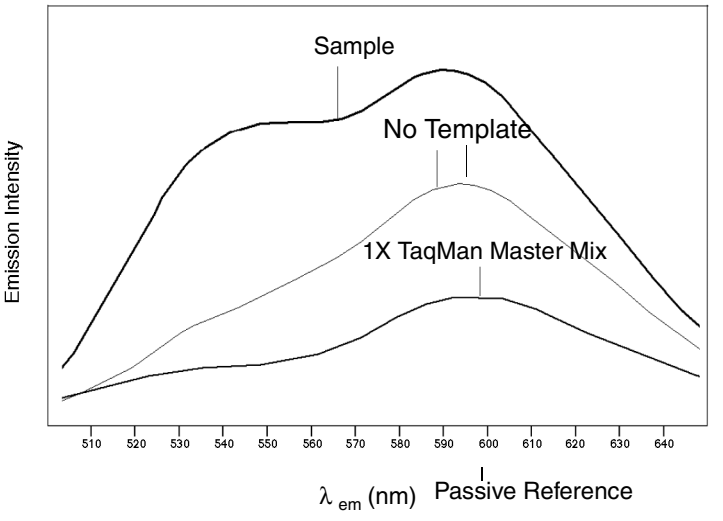


Figure 1-2 An overlay of three emission scans, post-PCR.

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, nonspecific amplification is not detected.

TaqMan Probe

The TaqMan 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 and VIC™ dye have also been used as reporter dyes. Each of the reporters is quenched by TAMRA™ dye, or non-fluorescent quencher attached via a linker arm that is usually located at the 3´end.

About This Protocol

This protocol describes how to:

- Perform PCR using:
  - TaqMan® PCR Core Reagents
  - TaqMan® Universal PCR Master Mix
- Perform one-step RT-PCR using:
  - TaqMan® EZ RT-PCR Core Reagents
  - TaqMan® Gold RT-PCR Reagents

- Perform two-step RT-PCR using TaqMan Gold RT-PCR Reagents and:
  - TaqMan PCR Core Reagents
  - TaqMan Universal PCR Master Mix
- Analyze and interpret data

Amplicon Size

The amplicon generated from the 18S gene for both human RNA and DNA is 187 bp in length.

Materials and Equipment

Kit Components

The TaqMan Ribosomal RNA Control Reagents (P/N 4308329) contain the probe and primers sufficient to perform one thousand 50-µL reactions. The kit includes the following components:

Component	Description
Ribosomal RNA Probe (VIC™ dye)	One tube containing 250 µL of 40-µM probe in TE buffer
Ribosomal RNA Forward Primer	One tube containing 250 µL of 10-µM primer in TE buffer
Ribosomal RNA Reverse Primer	One tube containing 250 µL of 10-µM primer in TE buffer
Control RNA (Human)	One tube containing 100 µL of 50-ng/µL of total human Raji RNA in 10-mM Tris-HCl, pH 7.0, 100-mM NaCl, and 1-mM EDTA

IMPORTANT The TaqMan® VIC™ dye must be configured as a Pure Dye on the Sequence Detection Systems. Refer to the appropreate SDS User's Manual for generating new spectra components.

Performance Specifications

The following performance specifications apply to the TaqMan Ribosomal RNA Control Reagents.

Description	Performance Specification
Detection limit of human Raji cell line	100 femtograms of total RNA per 50-µL reaction in two-step RT-PCR
Dynamic range of rRNA on the Sequence Detection Systems	Five orders of magnitude on the Sequence Detection Systems

Core Kits Supplied by the User

One of the TaqMan Core Reagent Kits listed in the following table is required in addition to the reagents supplied in the TaqMan Ribosomal RNA Control Reagents.

Application	TaqMan Core Reagents[a]	Source
PCR	TaqMan® PCR Core Reagents	Applied Biosystems (P/N N808-0228)
	TaqMan® Universal PCR Master Mix	Applied Biosystems (P/N 4304437)
RT-PCR	TaqMan® Gold RT-PCR Kit	Applied Biosystems (P/N N808-0232)
	TaqMan® EZ RT-PCR Core Reagents	Applied Biosystems (P/N N808-0236)
	TaqMan® One-Step RT-PCR Master Mix Reagent Kit	Applied Biosystems (P/N 4309169)
RT	TaqMan® Reverse Transcription Reagents	Applied Biosystems (P/N N808-0234)

a. See your local Applied Biosystems representative for a current listing of available reagents.

Materials Required but Not Supplied

The following items are required when using TaqMan Ribosomal RNA Control Reagents, but are not supplied. See the table for source information.

User-Supplied Materials

Item	Source
7900HT Sequence Detection System 7700 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)	

User-Supplied Materials *(continued)*

Item	Source
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
TaqMan® TAMRA Probe ♦ 5000–6000 pmoles ♦ 15,000–25,000 pmoles ♦ 50,000–100,000 pmoles	Applied Biosystems ♦ P/N 450025 ♦ P/N 450024 ♦ P/N 450003
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	
MicroAmp® Optical Caps	Applied Biosystems (P/N 4323032)
MicroAmp® 96-well Tray/Retainer Set (10 sets)	Applied Biosystems (P/N 403081)

User-Supplied Materials *(continued)*

Item	Source
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 7700 instrument only)	Applied Biosystems (P/N 4305822)
Sequence Detection Systems 384-Well Spectral Calibration Kit	Applied Biosystems (P/N 4323977)
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 Ribosomal RNA Control Reagents at –15 to –25 °C in a constant-temperature freezer. Store the product away from light. This product is light sensitive. If stored under the recommended conditions, the product will maintain performance for one year from time of receipt.

Safety

Documentation User Attention Words

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

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

⚠ 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

Overview

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).

Hot Start PCR

To improve PCR specificity and sensitivity by controlling mispriming events, the Hot Start technique was introduced (Faloona *et al.*, 1990). Hot Start PCR is a simple modification of the original PCR process in which the amplification reaction is started at an elevated temperature. This was initially performed manually, by adding an essential component of the reaction to the reaction mixture only after that mixture had been heated to an elevated temperature. However, this approach was often cumbersome and time consuming, especially when using large numbers of samples.

False Positives

Special laboratory practices are necessary in order to avoid false positive amplifications (Higuchi, *et al.*, 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; Saiki *et al.*, 1988). Because of the enormous amplification possible with PCR, amplicon carryover can result in sample contamination. Other sources of contamination could be from samples with high DNA levels or from positive control templates.

When dUTP replaces dTTP as a dNTP substrate in PCR and the method described below is used, AmpErase UNG treatment can prevent the reamplification of carryover PCR products in subsequent experiments Sninsky and Gelfand, pers. comm.) This method uses enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to degrade specifically PCR products from previous PCR amplifications or to degrade mis-primed, non-specific products produced prior to specific amplifications, but not degrade native nucleic acid templates.

The method used to make PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR mix and treating subsequent PCR mixes with the enzyme uracil N-glycosylase (UNG, EC 3.2.2-) prior to amplification (Longo *et al.*, 1990).

The AmpErase UNG provided in this product is a pure, nuclease-free, 26-kDa enzyme encoded by the *Escherichia coli* uracil N-glycosylase

gene which has been inserted into an *E. coli* host to direct the expression of the native form of the enzyme (Higuchi *et al.*, 1989).

Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried over PCR products, we encourage users to continue using the specific devices and suggestions described in this protocol booklet and in Kwok (1990) and Higuchi(1989) to minimize cross-contamination from non-dU-containing PCR products or other samples.

AmpErase UNG Inactivation

A ten minute hold cycle at 95 °C is necessary to cleave the dU-containing PCR products that are carried over from an earlier PCR. Because UNG is not completely deactivated during the 95 °C incubation, it is important to keep the reaction temperatures greater than 55 °C, to prevent amplicon degradation.

Prevention of PCR Product Carryover

Use primers that contain dA nucleotides near the 3´ ends so that any primer-dimer generated is efficiently degraded by AmpErase UNG at least as well as any dU-containing PCR products. The further a dA nucleotide is from the 3´ end, the more likely that partially degraded primer-dimer molecules may serve as templates for a subsequent PCR amplification.

Production of primer dimer could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3´ terminal dU-nucleotides should be considered. Single-stranded DNA with terminal dU nucleotides are not substrates for AmpErase UNG (Delort *et al.*, 1985) and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for AmpErase UNG.

The concentration of AmpErase UNG and the time of the incubation step necessary to prevent amplification of contaminating dU-containing PCR product depends on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination expected. In most cases, using AmpErase UNG at 1 U/100 µL reaction and incubation at 50 °C for two minutes is sufficient.

Do not attempt to use AmpErase UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR product, preventing further amplification.

UNG in Two-Step Reactions

When two-step RT-PCR is performed with the TaqMan® Gold RT-PCR Kit or the TaqMan® Universal Master Mix kit, AmpErase UNG treatment can prevent the reamplification of carry-over PCR products. When dUTP replaces dTTP during PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of the carry-over rRNA amplicon per 50-µL reaction.

UNG in One-Step Reactions

AmpErase UNG can be used to remove amplicon contamination in one-step RT-PCR when using the TaqMan EZ RT-PCR Core Reagents. The rT*th* DNA Polymerase contained in the kit is thermally stable and is used at temperatures at which Amperase UNG is inactive. Because one-step EZ RT-PCR utilizes dUTP, amplicons generated during this reaction contain uridine residues.

UNG cannot be used when one-step RT-PCR is performed using the TaqMan Gold RT-PCR Kit or the TaqMan® One-Step RT-PCR Master Mix Reagents Kit. UNG is active at the temperatures for reverse transcription. The active UNG enzyme would remove uracil bases that are incorporated into the newly synthesized complementary DNA (cDNA) strand.

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 PCR reactions with and without UNG. A positive signal in the reaction without UNG indicates contamination of reaction components.

General PCR Practices

Certain laboratory practices are necessary in order to avoid false-positive amplifications (Kwok and Higuchi, 1989). This is because the PCR process is capable of amplifying single DNA molecules (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

- Wear a clean lab coat (one never worn while handling amplified PCR products or doing sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever contamination is possible.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification and detection

- 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 regularly 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.

2 Performing PCR

Overview

About This Chapter

This chapter describes two methods for performing PCR:

- Using TaqMan® PCR Core Reagents
- Using TaqMan® Universal PCR Master Mix

In This Chapter

The following topics are discussed in this chapter:

Preparing Reaction Mix Components for PCR

Preparation of Reagents

Prior to use, thaw all of the reagents at room temperature except the enzymes. After the reagents are thawed, place them on ice. Keep the enzymes in a freezer until immediately prior to use.

After thawing, mix the kit components, except the enzymes, by vortexing and using a microcentrifuge to briefly spin down the tube contents. Mix the enzymes by gentle inversion of the tube. Protect the fluorescent dye-labeled probe from excessive exposure to light.

Template

The template used in PCR reactions is DNA or cDNA.

Reaction Mix Preparation

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

Step	Action
1	Prepare Reaction Mix by combining all the nonenzymatic components listed in the appropriate table. **⚠ 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	Mix the components by pipetting up and down.
3	Vortex briefly.
4	Add the enzymatic components (for example: AmpliTaq Gold® DNA Polymerase) 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.
5	Mix the components by inverting the microcentrifuge tube.

PCR Using the TaqMan PCR Core Reagents

Overview

PCR can be performed using DNA templates with the TaqMan PCR Core Reagents (P/N N808-0228). Follow the instructions described in the *TaqMan PCR Reagent Kit Protocol* (P/N 402823).

PCR Reaction Mix

The ingredients of a 50-µL reaction, PCR Reaction Mix are listed in the table below. To make the PCR Reaction Mix, follow the instructions described in “Reaction Mix Preparation” on page 2-2.

⚠ CAUTION **CHEMICAL HAZARD**. **TaqMan PCR Core Reagents** may cause eye and skin irritation. They may cause discomfort if swallowed or inhaled. Always use adequate ventilation such as that provided by a fume hood. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

PCR Reaction Mix

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
10X TaqMan® PCR Buffer A	5.0	1X
25 mM Magnesium Chloride	11.0	5.5 mM
10 mM deoxyATP	1.0	200 µM
10 mM deoxyCTP	1.0	200 µM
10 mM deoxyGTP	1.0	200 µM
20 mM deoxyUTP	1.0	400 µM
10 µM Ribosomal RNA Forward Primer	0.25	50 nM
10 µM Ribosomal RNA Reverse Primer	0.25	50 nM
40 µM Ribosomal RNA Probe (VIC™ dye)	0.25	200 nM
AmpErase® UNG (1 U/µL)	0.5	0.01 U/µL
AmpliTaq Gold® DNA Polymerase (5.0 U/µL)	0.25	0.025 U/µL
Total	21.5	—

a. The volume of RNase-free water will be (28.5 µL – DNA sample volume).

Thermal Cycling Parameters for PCR

Use the following thermal cycling parameters for PCR:

Step	UNG Activation	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

IMPORTANT The 2-min, 50 °C step is required for optimal UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

Performing PCR Using the TaqMan Universal PCR Master Mix

Overview

PCR can be performed using DNA templates with the TaqMan Universal PCR Master Mix (P/N 4304437). Follow the instructions described in the *TaqMan Universal PCR Master Mix Protocol* (P/N 4304449).

Using TaqMan Universal PCR Master Mix

The ingredients of a 50-µL reaction, PCR Reaction Mix using the TaqMan Universal PCR Master Mix are listed in the table below.

⚠ CAUTION CHEMICAL HAZARD. **TaqMan Universal PCR Master Mix** may cause eye and skin irritation. It may cause discomfort if swallowed or inhaled. Always use adequate ventilation such as that provided by a fume hood. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
TaqMan® Universal PCR Master Mix	25.0	1X
10 µM Ribosomal RNA Forward Primer	0.25	50 nM
10 µM Ribosomal RNA Reverse Primer	0.25	50 nM
40 µM Ribosomal RNA Probe (VIC™)	0.25	200 nM
Total	25.75	—

a. The volume of RNase-free water will be (24.25 µL – DNA sample volume).

Thermal Cycling Parameters for PCR

Use the following thermal cycling parameters for PCR:

Step	UNG Activation	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

IMPORTANT The 2-min, 50 °C step is required for optimal AmpErase UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

3 RT-PCR

Overview

About This Chapter

This chapter describes several methods for performing RT-PCR:

- One-step
  - Using TaqMan® EZ RT-PCR Core Reagents
  - Using TaqMan® Gold RT-PCR Reagents
- TaqMan One-Step RT-PCR Master Mix Reagents Kit
- Two-step using TaqMan® Gold RT-PCR Kit and
  - TaqMan® PCR Core Reagents
  - TaqMan® Universal PCR Master Mix

If you are performing PCR instead, see Chapter 2, “Performing PCR.”

In This Chapter

The following topics are discussed in this chapter:

Preparing Reaction Mix Components for RT-PCR

Quantity of Total RNA

The number of reactions depends upon the plate set up by the user. Between 100 fg and 10 ng of total RNA may be used for a one-step RT-PCR reaction. The No Template Control reaction is the complete RT-PCR formulation without the target RNA.

Preparation of Reagents

Prior to use, thaw all or the reagents at room temperature except the enzymes and the RNase Inhibitor. After the reagents are thawed, place them on ice. Keep the enzymes in a freezer until immediately prior to use.

After thawing, mix the kit components, except the enzymes and RNase Inhibitor, by vortexing and using a microcentrifuge to briefly spin down the tube contents. Mix the enzymes and RNase Inhibitor by gentle inversion of the tube. Protect the fluorescent dye-labeled probe from excessive exposure to light.

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.

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 and RNase Inhibitor (for example: AmpliTaq Gold® DNA Polymerase, MultiScribe™ Reverse Transcriptase, RNase Inhibitor, or r*Tth* DNA Polymerase for EZ RT-PCR) listed for the appropriate reaction mix.
5	Mix the components by inverting the microcentrifuge tube.

One-Step RT-PCR Using the TaqMan EZ RT-PCR Reagents

Overview

RT-PCR can be performed using RNA templates with the TaqMan EZ RT-PCR Core Reagents (P/N N808-0236). Follow the instructions described in the *TaqMan EZ RT-PCR Kit Protocol* (P/N 402877).

Description of One-Step EZ RT-PCR

One-step EZ RT-PCR is RT as well as PCR in a single buffer system using the r*Tth* DNA polymerase enzyme for both steps. 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. The carryover prevention enzyme, AmpErase® uracil-N-glycosylase (UNG), can be used with one-step RT-PCR using the TaqMan EZ RT-PCR Core Reagents.

EZ Reaction Mix

The ingredients used for a 50-µL RT-PCR reaction mix.

EZ Reaction Mix

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
5X TaqMan® EZ Buffer	10.0	1X
25 mM Manganese Acetate	11.0	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 Ribosomal RNA Forward Primer	0.25	50 nM
10 µM Ribosomal RNA Reverse Primer	0.25	50 nM
40 µM Ribosomal RNA Probe (VIC™)	0.25	200 nM
AmpErase® UNG (1 U/µL)	0.5	0.01 U/µL
r*Tth* DNA Polymerase (2.5 U/µL)	2.0	0.1 U/µL
Total	30.25	—

a. The volume of RNase-free water will be (19.75 µL – RNA sample volume).

Note To run a control sample, dilute the Control RNA (Human) 1:50 using RNase-free water. Use 1 µL of the diluted RNA in the reaction.

Reaction Preparation

For a description of how to prepare the reaction, refer to the *TaqMan EZ RT-PCR Kit Protocol* (P/N 402877).

Thermal Cycling for One-Step EZ RT-PCR

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

Step	UNG Activation	Reverse Transcription	UNG Deactivation	PCR	
	HOLD	HOLD	HOLD	CYCLE (40 cycles)	
				Denature	Anneal/ Extend
Temp	50 °C	60 °C	95 °C	94 °C	60 °C
Time	2 min	30 min	5 min	15 sec	1 min
Volume	50 µL				

IMPORTANT The 2-min, 50 °C step is required for optimal AmpErase UNG enzyme activity.

One-Step RT-PCR Using the TaqMan Gold RT-PCR Kit

Overview

RT-PCR can be performed using RNA templates with the TaqMan Gold RT-PCR Kit (P/N N808-0232). Follow the instructions in the *TaqMan Gold RT-PCR Kit Protocol* (P/N 402876).

The TaqMan Gold RT-PCR Kit can be used to perform one-step or two-step RT-PCR.

Description of One-Step Gold RT-PCR

One-step Gold RT-PCR is 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 cannot be used with one-step RT-PCR when using the TaqMan Gold RT-PCR Kit.

Reducing Nonspecific Interactions

This protocol requires an initial incubation of the reaction mixture for 30 minutes at 48 °C (see page 3-7). This RT step co-incubates the PCR primers and TaqMan® probes at a temperature well below their annealing temperatures. This incubation will lead to non-specific interactions between the primers, probe, and template for this target.

To minimize the level of these non-specific interactions in one-step RT-PCR using the TaqMan Gold RT-PCR kit, use the Ribosomal RNA TaqMan probe at a concentration of 50 nM. This probe concentration allows accurate determination of C_T values.

Note that for multiplex applications in which a second target is run in the same well, a robust amplification for this second target can affect the multicomponenting accuracy around the ribosomal RNA reaction end-point. This will have no effect on accurate determination of the C_T values for either target.

One-Step Reaction Mix

The ingredients for a 50-µL RT-PCR reaction mix using the TaqMan Gold RT-PCR Kit are listed in the table below.

One-Step Reaction Mix

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
10X TaqMan® Buffer A	5.0	1X
25 mM Magnesium Chloride	11.0	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 Ribosomal RNA Forward Primer	0.25	50 nM
10 µM Ribosomal RNA Reverse Primer	0.25	50 nM
40 µM Ribosomal RNA Probe (VIC™)	0.0625[b]	50 nM
RNase Inhibitor (20 U/µL)	1.0	0.4 U/µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	0.25	0.25 U/µL
AmpliTaq Gold® DNA Polymerase (5 U/µL)	0.25	0.025 U/µL
Total	24.0625	—

a. The volume of RNase-free water will be (25.94 µL – RNA sample volume).

b. For reaction mixtures using this probe reagent it is recommended to start from a 1:10 dilution of the stock probe solution.

Note To run a control sample, dilute the Control RNA (human) 1:50 using RNase-free water. Use 1 µL of the diluted RNA in the reaction.

Reaction Preparation

For a description of how to prepare the reaction, refer to the *TaqMan Gold RT-PCR Kit Protocol* (P/N 402876).

Thermal Cycling for One-Step Gold RT-PCR

The following thermal cycling parameters are optimized for the one-step RT-PCR for the Ribosomal RNA system. See thermal cycler manuals for details on operation.

Step	RT	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	48 °C	95 °C	95 °C	60 °C
Time	30 min	10 min	15 sec	1 min
Volume	50 µL			

IMPORTANT The 10-min, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

One-Step RT-PCR Using the TaqMan One-Step RT-PCR Master Mix Reagents Kit

One-Step RT-PCR Protocol for 18S rRNA

The protocol for one-step 18S rRNA is listed below.

Reaction Component	Volume per Reaction (µL)	Volume per 100 Reactions (µL)	Final Value
2X Master Mix without UNG	25	2500	1X
40X MultiScribe and RNase Inhibitor Mix	1.25	125	0.25 U/µL 0.4 U/µL
Forward primer	Variable	Variable	50 to 900 nM
Reverse primer	Variable	Variable	50 to 900 nM
TaqMan probe, 25 µM	Variable	Variable	250nM
RNA sample, 50 ng	Variable	Variable	10 pg to 10 ng
Water	Variable	Variable	—
Total	50	5000	—

Thermal Cycling Parameters for Use with One-Step RT-PCR Master Mix Reagent Kit

Step	RT	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/ Extend
Temp	48 °C	95 °C	95 °C	60 °C
Time	30 min	10 min	15 sec	1 min
Volume	50 µL			

Two-Step RT-PCR Using the TaqMan Gold RT-PCR Kit

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 (P/N N808-0234) prime total RNA samples for reverse transcription using MultiScribe Reverse Transcriptase.

Incompatible Template

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 synthesis of 18S rRNA. 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 reverse transcription. The table below lists the recommended range of initial template quantities for the reverse transcription (RT) step.

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

Guidelines

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.
- Use only random hexamers to reverse transcribe the total RNA samples for gene expression assays.

Preparing the Reactions

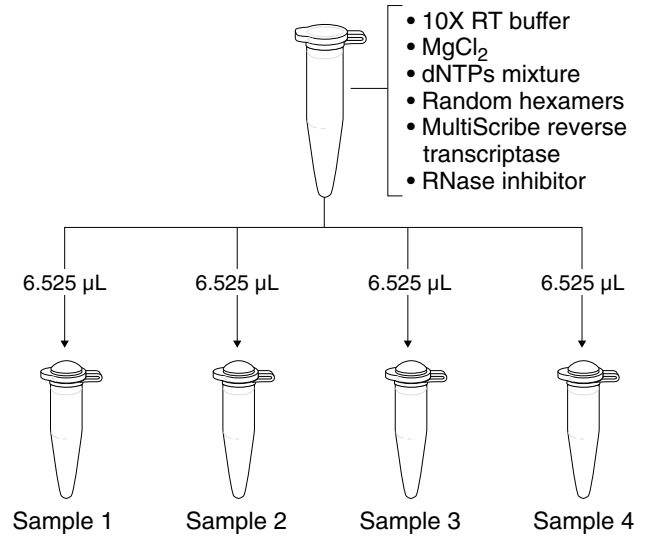
The following procedure describes the preparation of four different test samples for reverse transcription. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents (P/N N808-0234).

⚠ CAUTION CHEMICAL HAZARD. **TaqMan Reverse Transcription Reagents** may cause eye and skin irritation. They may cause discomfort if swallowed or inhaled. Always use adequate ventilation such as that provided by a fume hood. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To prepare the reverse transcription reactions:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 0.2-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed. If preparing four samples, follow the recommended volumes shown below.
<table>
<tr><th rowspan="2">Component</th><th colspan="2">Volume (µL)</th><th rowspan="2">Final Concentration</th></tr>
<tr><th>Per Sample</th><th>Reaction Mix (x4)</th></tr>
<tr><td>RNase-free water</td><td>See below[a]</td><td>See below[a]</td><td>—</td></tr>
<tr><td>10X RT Buffer</td><td>1.0</td><td>4.0</td><td>1X</td></tr>
<tr><td>25 mM $MgCl_2$</td><td>2.2</td><td>8.8</td><td>5.5 mM</td></tr>
<tr><td>deoxyNTPs Mixture</td><td>2.0</td><td>8.0</td><td>500 µM per dNTP</td></tr>
<tr><td>Random hexamers (50 µM)</td><td>0.5</td><td>2.0</td><td>2.5 µM</td></tr>
<tr><td>RNase Inhibitor (20 U/µL)</td><td>0.2</td><td>0.8</td><td>0.4 U/µL</td></tr>
<tr><td>MultiScribe™ Reverse Transcriptase (50 U/µL)</td><td>0.625</td><td>2.5</td><td>3.125 U/µL</td></tr>
<tr><td>Total[b]</td><td>6.525</td><td>26.1</td><td>—</td></tr>
</table>
a. The volume of RNase-free water (µL) will be 3.475–RNA sample volume in a 10-µL reaction.
b. If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.
Note RT volume can vary from 10 µL to 100 µL. Increasing the RT volume will reduce the total number of reactions.</td></tr>
<tr><td>2</td><td>Label four 0.2-mL microcentrifuge tubes for the four test samples.</td></tr>
<tr><td>3</td><td>Transfer 60 ng to 2 µg (up to 3.475 µL) of each total RNA sample to the corresponding microcentrifuge tube.</td></tr>
<tr><td>4</td><td>If necessary, dilute each total RNA sample to a volume of 3.475 µL with RNase-free, deionized water.</td></tr>
<tr><td>5</td><td>Cap the tubes and gently tap each to mix the diluted samples.</td></tr>
<tr><td>6</td><td>Briefly centrifuge the tubes to eliminate air bubbles in the mixture.</td></tr>
</table>

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

Step	Action
7	Label four 0.2-mL MicroAmp® Reaction Tubes for the four total RNA test samples.
8	Pipet 6.525 µL of the reaction mix (from step 1) to each MicroAmp Reaction Tube (from step 7). 
9	Transfer 3.475 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	Briefly centrifuge the tubes to force the solution to the bottom and to eliminate air bubbles from the mixture.
12	Transfer each reaction to: ♦ MicroAmp® Optical Tubes or ♦ Wells of a MicroAmp® Optical 96-well or 384-well Reaction Plate
13	Cap the tubes or plate with MicroAmp® Optical Caps.
14	Centrifuge the tubes or plate to spin down the contents and eliminate air bubbles from the solutions.

Thermal Cycling

To perform reverse transcription thermal cycling:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Load the reactions into a thermal cycler.</td></tr>
<tr><td>2</td><td>Program your thermal cycler with the following conditions:
<table>
<tr><th rowspan="2">Step</th><th>Hexamer Incubation[a]</th><th>RT</th><th>Reverse Transcriptase Inactivation</th></tr>
<tr><td>HOLD</td><td>HOLD</td><td>HOLD</td></tr>
<tr><td>Temp</td><td>25 °C</td><td>37 °C</td><td>95 °C</td></tr>
<tr><td>Time</td><td>10 min</td><td>60 min</td><td>5 min</td></tr>
<tr><td>Volume</td><td colspan="3">10 µL</td></tr>
</table>
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.</td></tr>
<tr><td>3</td><td>Begin reverse transcription.

IMPORTANT After thermal cycling, store all cDNA samples at –15 to –25 °C.</td></tr>
</table>

PCR Reaction Mix Using the TaqMan PCR Core Reagents

The ingredients of a 50-µL PCR reaction are listed below.

⚠ CAUTION **CHEMICAL HAZARD**. **TaqMan PCR Core Reagents** may cause eye and skin irritation. They may cause discomfort if swallowed or inhaled. Always use adequate ventilation such as that provided by a fume hood. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
10X TaqMan® Buffer A	5.0	1X
25 mM Magnesium Chloride	11.0	5.5 mM
10 mM deoxyATP	1.0	200 µM
10 mM deoxyCTP	1.0	200 µM
10 mM deoxyGTP	1.0	200 µM
20 mM deoxyUTP	1.0	400 µM
10 µM Ribosomal RNA Forward Primer	0.25	50 nM
10 µM Ribosomal RNA Reverse Primer	0.25	50 nM
40 µM Ribosomal RNA Probe (VIC™)	0.25	200 nM
AmpErase® UNG (1U/µL)	0.5	0.01 U/µL
AmpliTaq Gold® DNA Polymerase (5.0 U/µL)	0.25	0.025 U/µL
Total	21.5	—

a. The volume of RNase-free water will be (28.5 µL – cDNA sample volume).

Note To run a control sample, use 5 µL of the completed RT-reaction from the previous step.

PCR Reaction Preparation

For a description of how to prepare the PCR reaction, refer to the *TaqMan Gold RT-PCR Kit Protocol* (P/N 402876), or *TaqMan Universal PCR Master Mix Protocol (*P/N 4304449).

PCR Reaction Thermal Cycling Parameters

The cycling parameters for the PCR step (step two) of a 50-µL, two-step RT-PCR reaction using the TaqMan Gold RT-PCR Kit are listed below.

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

IMPORTANT The 2-min, 50 °C step is required for optimal AmpErase UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

PCR Reaction Mix Using TaqMan Universal PCR Master Mix

The ingredients of a 50-µL reaction, PCR Reaction Mix using the TaqMan Universal PCR Master Mix are listed in the table below.

⚠ CAUTION CHEMICAL HAZARD. **TaqMan Universal PCR Master Mix** may cause eye and skin irritation. It may cause discomfort if swallowed or inhaled. Always use adequate ventilation such as that provided by a fume hood. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
TaqMan® Universal PCR Master Mix	25	1X
10 µM Ribosomal RNA Forward Primer	0.25	50 nM
10 µM Ribosomal RNA Reverse Primer	0.25	50 nM
40 µM Ribosomal RNA Probe (VIC™)	0.25	200 nM
Total	25.75	—

a. The volume of RNase-free water will be (24.25 µL – DNA sample volume).

PCR Reaction Preparation

For a description of how to prepare the PCR reaction, refer to the *TaqMan Gold RT-PCR Kit Protocol* or *TaqMan Universal PCR Master Mix Protocol* (P/N 4304449).

PCR Reaction Thermal Cycling Parameters

The cycling parameters for the PCR step (step two) of a 50-µL, two-step RT-PCR reaction using the TaqMan Gold RT-PCR Kit are listed below.

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

IMPORTANT The 2-min, 50 °C step is required for optimal AmpErase UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold DNA Polymerase.

4 Data Analysis

Overview

About This Chapter

This chapter describes how to analyze your data by:

- Changing the baseline for the amplification plot
- Interpreting the results

In This Chapter

The following topics are discussed in this chapter:

Changing the Baseline on the ABI PRISM Sequence Detection Systems

Because of the abundance of rRNA, low C_T values are obtained in TaqMan® RT-PCR applications. During the data analysis step, if the amplification plot appears as shown in Figure 4-1 (linear ordinate) or Figure 4-2 (logarithmic ordinate), then it is necessary to change the baseline numbers.

The baseline should be adjusted such that the amplification curve growth begins at a cycle number that is beyond the highest baseline number.

When the baseline is adjusted correctly, the amplification plot will appear as shown in Figure 4-3 (linear ordinate) or Figure 4-4 (logarithmic ordinate).

Note Refer to http://www.appliedbiosystems.com/support/tutorials/baseline for more information on this subject.

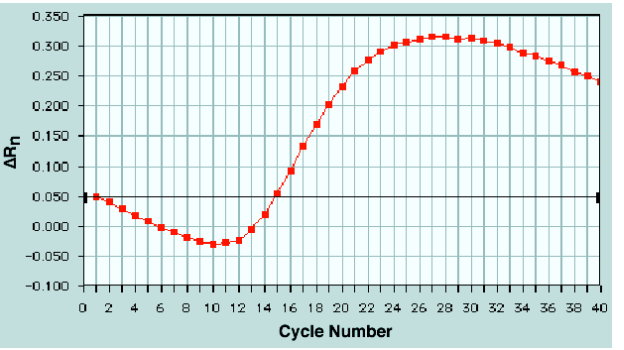


Figure 4-1 Amplification plot with incorrect baseline (linear ordinate)

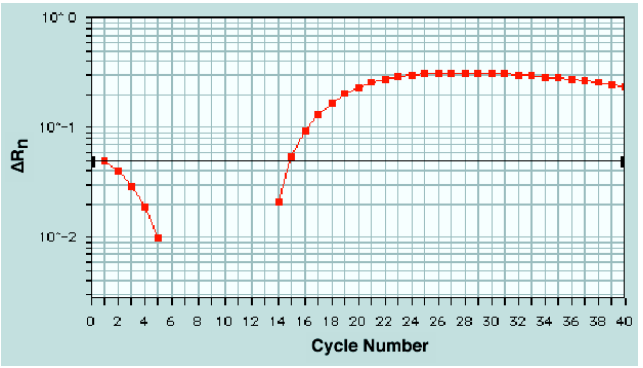


Figure 4-2 Amplification plot with incorrect baseline (logarithmic ordinate)

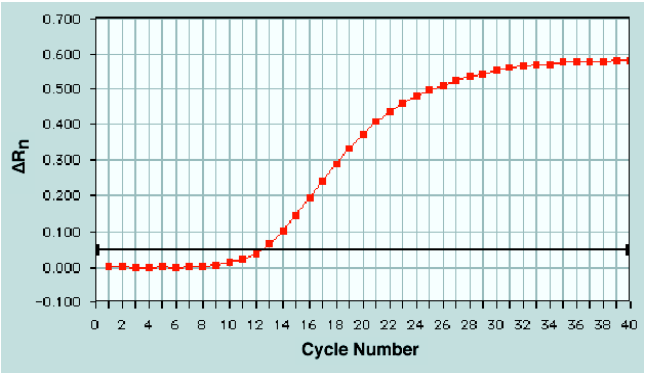


Figure 4-3 Amplification plot with correct baseline (linear ordinate)

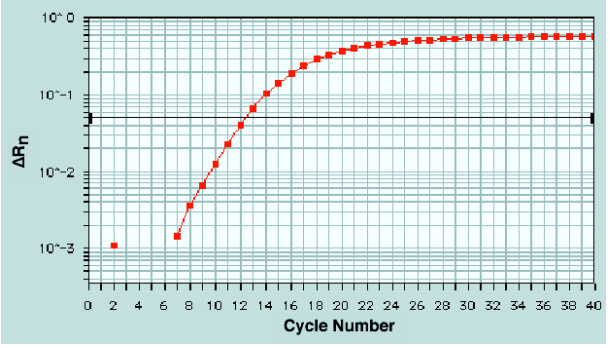


Figure 4-4 Amplification plot with correct baseline (logarithmic ordinate)

Low C_T Values in No Template Control

The universal ribosomal sequences utilized in this module are found throughout the environment. Because of the ubiquitous nature of these sequences and prevalence, care must be taken to minimize environmental contamination (see “Preventing Contamination” on page 1-14).

If the No Template Control tubes yield C_T values less than 40 cycles, then consideration must be given to the impact the background will have on quantitation in the test samples.

For example, if the No Template Control (NTC) yields a C_T value of 35 cycles and the test samples yield C_T values in the range of 25 cycles then the ΔC_T value between the NTC and the test sample is 10 cycles.

In this example, if 100% amplification efficiency is assumed, then a ten-fold difference in template concentration results in a ΔC_T value of 3.3 cycles. A ΔC_T equal to 10 cycles results in a 0.1% difference in quantitation of template copy number. This has an insignificant impact on relative quantitation.

If the ΔC_T value is <7 to 9 cycles, then the C_T value for the NTC and its impact on relative quantitation will be have to be evaluated on a case-by-case basis.

Interpreting the Results

Normalization

The Passive Reference is a dye included in the 10X TaqMan® Buffer A, in the TaqMan® Universal Master Mix, and in the 5X TaqMan® EZ Buffer, 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 pure dye components generate the composite spectrum. This spectrum represents one fluorescent reading from one well. Dyes available for multicomponent analysis are:

Types of Dyes	Dyes
Reporters	FAM™, TET™, JOE™, VIC™
Quenchers	TAMRA™, NON-FLUORESCENT QUENCHER
Passive Reference	ROX™

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:

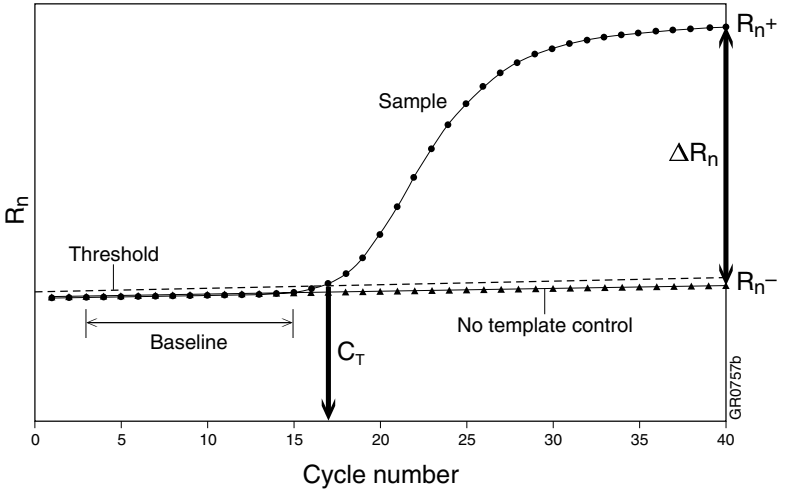
$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$ PCR with template

$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$ 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 graph of R_n versus cycle number shown, the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.



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 (cycles 0–5)	Fluorescent emissions have not stabilized to new buffer conditions of reaction mix. This does not affect PCR, or the final results.	Reset lower 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 as seen in Figure 4-1 on page 4-2 and Figure 4-2 on page 4-3.	C_T value <15, amplification signal detected in early cycles	Refer to “Preparing for Data Analysis” on page 4-2.

Troubleshooting *(continued)*

Observation	Possible Cause	Recommended Action
Multicomponent signal for ROX™ is not linear	Pure dye component's spectra are incorrect	Recalibrate the instrument with pure dye standards.
	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	Review "General PCR Practices" on page 1-16.
Standard deviation of C_T value >0.16	Inaccurate pipetting	Prepare a Reagent Mix. Refer to "Reaction Mix Preparation" on page 2-2 or 3-2. Use positive-displacement pipettors.

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C

Technical Support

Services & Support

Applied Biosystems Web Site

To access the Applied Biosystems Web site, go to:

http://www.appliedbiosystems.com

At the Applied Biosystems Web site, you can:

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