SYBR® Green PCR Master Mix and RT-PCR

Protocol

Applied Biosystems

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1 Introduction

Overview

About This Chapter

This chapter describes the SYBR® Green PCR Master Mix and provides important safety information.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Purpose of the Kit	1-2
Materials and Equipment	1-3
Safety	1-6
Preventing Contamination and Nonspecific Amplification	1-9
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Purpose of the Kit

About the Kit

The SYBR Green PCR Master Mix is a convenient premix of all the components, except primers, template and water necessary to perform real-time PCR using SYBR® Green I Dye. Direct detection of polymerase chain reaction (PCR) product is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded (ds) DNA.

One-Step or Two-Step RT-PCR can be performed using the SYBR® Green RT-PCR Reagents Kit (refer to “Materials and Equipment” on page 1-3).

In RNA quantitation assays, the SYBR Green PCR Master Mix is used in the second step of a two-step reverse-transcription polymerase chain reaction (RT-PCR) protocol. In a One-Step RT-PCR protocol, MultiScribe™ Reverse Transcriptase and RNase Inhibitor are added to the SYBR Green PCR Master Mix.

The SYBR Green PCR Master Mix is designed for use with the ABI PRISM® 7700 Sequence Detection System (SDS), the ABI PRISM® 7900HT SDS, the ABI PRISM® 7000 SDS, or the GeneAmp® 5700 SDS.

For the best quantitation results, use the following:

- Primer Express™ software for primer design
- Applied Biosystems reagents
- Applied Biosystems universal thermal cycling conditions

About This Protocol

This protocol describes how to perform PCR and One-Step or Two-Step RT-PCR using SYBR Green PCR Master Mix.

Materials and Equipment

Description of Master Mix

The SYBR Green PCR Master Mix is supplied in a 2X concentration and contains sufficient reagents to perform 200 50-µL reactions. The mix is optimized for SYBR Green reactions and contains SYBR Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference, and optimized buffer components.

⚠ WARNING CHEMICAL HAZARD. **SYBR Green** may cause eye, skin, and respiratory tract irritation. It is readily absorbed through the skin and is a combustable liquid and vapor (keep away from heat and flame). This product contains material which may cause liver and blood damage. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

For SYBR Green PCR and One-Step or Two-Step RT-PCR, the following components are available:

Kit	P/N	Contents
SYBR Green PCR Master Mix	4309155	♦ SYBR Green PCR Master Mix (200-50 µL reactions)
SYBR Green RT-PCR Reagents Kit	4310179	♦ SYBR Green PCR Master Mix (200-50 µL reactions) ♦ TaqMan Reverse Transcription Reagents (200-10 µL reactions)
Protocol	4310251	—

Materials Required but Not Supplied

The items listed in the following tables are required in addition to the reagents supplied in the SYBR Green PCR Master Mix.

User-Supplied Materials

Item	Source
7900HT Sequence Detection System 7000 Sequence Detection System	See your local Applied Biosystems representative for the instrument best suited to meet your needs.
ABI PRISM® 7900 Sequence Detection Systems 96-well Spectral Calibration Kit	Applied Biosystems (P/N 4328639)

User-Supplied Materials *(continued)*

Item	Source
ABI PRISM® 7000 Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (P/N 4328895)
ABI PRISM™ Cap Installing Tool	Applied Biosystems (P/N 4330015)
ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode	Applied Biosystems (P/N 4309849)
ABI PRISM™ Optical Adhesive Cover Starter Pack containing 20 optical adhesive covers, one applicator, and one compression pad.	Applied Biosystems (P/N 4313663)
MicroAmp® Optical Caps	Applied Biosystems (P/N 4323032)
MicroAmp® Optical Tubes	Applied Biosystems (P/N N801-0933)
MicroAmp® Optical 96-well Reaction Plate	Applied Biosystems (P/N N801-0560)
Note The MicroAmp Optical 96-well Reaction Plate may be sealed with: ♦ MicroAmp Optical Caps or ♦ ABI PRISM™ Optical Adhesive Cover	
MicroAmp® Optical 96-well Reaction Plate and Optical Caps	Applied Biosystems (P/N 403012)
MicroAmp® 96-well Tray/Retainer Set (10 sets)	Applied Biosystems (P/N 403081)
Primer Express™ software (single-use license)	Applied Biosystems
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)
Centrifuge with adapter for 96-well plate	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS

User-Supplied Materials *(continued)*

Item	Source
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 SYBR Green PCR Master Mix at 2 to 8 °C and TaqMan Reverse Transcription Reagents at –20 °C. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

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 and Nonspecific Amplification

Overview

The DNA amplification capability of the PCR process makes special laboratory practices necessary. Potential contamination can be introduced by samples with high DNA concentrations, from the DNA Template Controls, or from PCR carryover contamination. In addition, due to the nonspecific nature of SYBR Green I Detection, any double stranded DNA will be detected. Therefore, it is recommended to check for nonspecific product formation by dissociation curve or gel analysis.

For more information on the polymerase chain reaction, refer to Kwok and Higuchi, 1989. For more information on the prevention of unintended products, refer to Mullis and Faloona, 1987.

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.

AmpliTaq Gold DNA Polymerase

Applied Biosystems introduced AmpliTaq Gold® DNA Polymerase to perform an automated, convenient, and efficient Hot Start. 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.

When AmpliTaq Gold DNA Polymerase is added to the reaction mixture at room temperature, the inactive enzyme is not capable of primer extension. Any low-stringency mispriming events that may have occurred will not be enzymatically extended and subsequently amplified.

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.

Optional Use of AmpErase UNG

AmpErase® uracil-N-glycosylase (UNG) treatment can be useful in preventing the reamplification of carryover PCR products. Although the SYBR Green PCR Master Mix does not contain UNG, dTTP has been replaced with dUTP, thus making the SYBR Green Master Mix compatible with the use of UNG. If PCR carryover contamination is suspected, UNG should be used to troubleshoot the problem. UNG can

be purchased individually (P/N N808-0096) or as part of the SYBR® Green Core Reagents Kit (P/N 4304886).

Fluorescent Contaminants

Since fluorescent contaminants can interfere with SYBR Green I assays and give false-positive results, it may be necessary to include a No Amplification Control tube that contains sample, but 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.

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/l00 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.

General PCR Practices

When preparing samples for PCR amplification:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves.

- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipette or aerosol-resistant pipette tips
- Clean lab benches and equipment periodically with 10% bleach solution.

Amplicon Independent Amplification (Including Primer-Dimer)

Introduction

This section discusses the use of dissociation curves to detect nonspecific amplification.

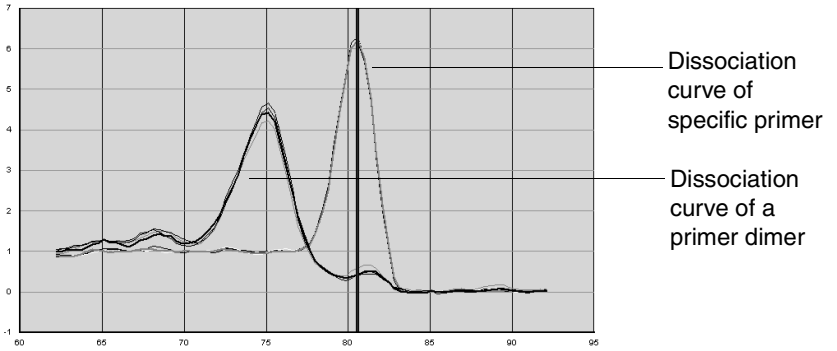
Dissociation Curve Defined

A dissociation curve is a graph that displays dissociation data from the amplicons of quantitative PCR runs. Change in fluorescence is plotted against temperature. The change in fluorescence is due to a dye or probe interacting with double-stranded DNA.

Using Dissociation Curves

General Information

The GeneAmp® 5700 Sequence Detection System (SDS), the ABI PRISM® 7900HT SDS, ABI PRISM® 7700 SDS, and the ABI PRISM® 7000 SDS enable dissociation curves to be run to detect nonspecific amplification through computer software. Nonspecific amplification, including primer-dimers, may affect the quality of amplification data.



The dissociation curves above show typical primer-dimer formation. The specific product is shown with a melting temperature (T_m) of 80.5 °C, while the primer-dimer has a characteristically lower T_m of 75 °C.

Primer-dimer will be most prevalent in No Template Control (NTC) wells and sample wells containing low concentrations of template.

When to Generate Dissociation Curves

The GeneAmp® 5700 SDS, ABI PRISM® 7900HT SDS, ABI PRISM® 7700 SDS, and ABI PRISM® 7000 SDS can be set up to generate a dissociation curve in either of these instances:

- Immediately after the real-time PCR run
- Independently of the real-time PCR run

Note In the presence of AmpErase UNG and dUTP, product degradation may occur from a previously run PCR plate due to residual AmpErase UNG activity.

Note Refer to the appropriate SDS User's Manual for further information on generating a dissociation curve.

Note The 7700 instrument uses a separate Dissociation Curve Analysis software that employs the multicomponent data exported from the SDS software v 1.7a or later to display the dissociation curves for each sample.

Using Agarose Gels to Check PCR Product Purity

The absence of nonspecific amplification can be confirmed by analyzing the PCR amplification products by agarose gel electrophoresis.

To check PCR product purity with agarose gels:

Step	Action
1	Load 12 to 15 µL of sample per well on an ethidium bromide-stained 4% NuSieve 3:1 agarose gel. **⚠ WARNING CHEMICAL HAZARD. Ethidium bromide** causes eye, skin, and respiratory tract irritation and is a known mutagen (*i.e.*, it can change genetic material in a living cell and has the potential to cause cancer). 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.
2	Run the gel: ♦ For PCR fragments <100 bp, run the gel at 80 to 100 V for 45 to 60 min. ♦ For PCR fragments 100 to 250 bp, run the gel at 100 to 115 V for 1 to 1.5 h.
3	Run samples 1/3 to 1/2 the length of the gel, without letting the dye run off the bottom of the gel. Use a UV lamp to check the migration of the samples.

2 PCR

Overview

About This Chapter

This chapter describes how to design and amplify custom target sequences for quantitation.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Designing Custom Target Sequences for Quantitation	2-2
Amplifying Custom Target Sequences for Quantitation	2-4

Designing Custom Target Sequences for Quantitation

Overview

We recommend the following steps to design custom primers and identify target sequences for amplification and quantitation:

Step	Action
1	Install Primer Express Software
2	Identify Target Sequence and Amplicon Size
3	Design Primers

Identifying Target Sequence and Amplicon Size

A target template is DNA, cDNA, total RNA, or a plasmid containing the nucleotide sequence of interest.

Design primers to amplify short segments of a target (RNA, DNA, or cDNA) within the target sequence. These short segments of DNA and cDNA are called amplicons. Shorter amplicons work most efficiently: the most consistent results are obtained for amplicon sizes in the 50 to 150 bp range.

Designing Primers

Design primers using Primer Express software as described in the *Primer Express User Bulletin* (P/N 4317594).

General Guidelines

Follow these general guidelines:

- Primers can be designed as close as possible to each other provided that they do not overlap.
- Keep the GC content in the 20 to 80% range.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- When using Primer Express software, the T_m should be 58 to 60 °C.
- The five nucleotides at the 3´ end should have no more than two G and/or C bases.

If the template is...	Then...
DNA	Design the primers as described above.
cDNA	
RNA	
plasmid DNA	
Genomic DNA	Design the primers as described above. Also see "Selecting an Amplicon Site for Genomic DNA" below.

Selecting an Amplicon Site for Genomic DNA

Overview

Selecting a good amplicon site ensures amplification of the target mRNA without co-amplifying the genomic sequence, pseudogenes, and related genes. SYBR Green chemistry can be useful for screening amplicon sites when using TaqMan chemistry for gene expression.

Guidelines

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair has to be specific to the target gene and does not amplify pseudogenes or other related genes.
- Primers must be designed following Primer Express guidelines.
- Test the amplicons and select ones that have the highest signal-to-noise ratio (*i.e.*, low C_T with cDNA and no amplification with no template control or genomic DNA).
- If no good sequence is found, it may be necessary to examine the sequence and redesign the amplicon or simply screen for more sites.

If the gene you are studying does not have introns, then you cannot design an amplicon that will amplify the mRNA sequence without amplifying the genomic sequence. In this case, it may be necessary to run RT minus controls.

Amplifying Custom Target Sequences for Quantitation

Overview

We recommend the following steps for the development of real-time quantitative PCR assays:

Step	Action	See Page
1	Order Reagents	2-4
2	Quantitate Primers	2-4
3	Optimize Primer Concentrations for:	
	♦ PCR	4-2
	♦ One-Step RT-PCR	4-5
	♦ Two-Step RT-PCR	4-9

Ordering Reagents

See “Materials Required but Not Supplied” on page 1-3 for a list of required reagents and equipment.

Quantitating Primers

Use a spectrophotometric method to determine the concentrations of the primers received:

- Measure the absorbance at 260 nm of a 1:100 dilution of each oligonucleotide in TE buffer.
- Calculate the oligonucleotide concentration (C) in μM using the method shown in the table on the next page.

Chromophore	Extinction Coefficient	Number	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
Total	—	—	167,950

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette pathlength × oligonucleotide concentration/100

$0.13 = 167{,}950\ M^{-1}cm^{-1} \times 0.3\ cm \times C/100$

$C = 258\ \mu M$

Reverse Transcription

3

Overview

About This Chapter

This chapter provides procedures for performing reverse transcription (RT).

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Reverse Transcription for All Amplicons Except 18S	3-2
Reverse Transcription for the 18S Amplicon	3-5

Reverse Transcription for All Amplicons Except 18S

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, oligo $d(T)_{16}$, or sequence specific reverse primers from the TaqMan Reverse Transcription Reagents (P/N N808-0234) prime total RNA samples for RT using Multiscribe Reverse Transcriptase.

Guidelines

Follow the guidelines below to ensure optimal RT performance.

- A 100-µL RT reaction efficiently converts a maximum of 2 µg total RNA to cDNA. Perform multiple RT reactions in multiple wells if you are using more than 2 µg of total RNA.
- Use random hexamers, oligo $d(T)_{16}$, or sequence specific reverse primers to reverse transcribe the total RNA samples for gene expression assays.

The choice of primers for RT 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)
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

Two-Step RT-PCR RT Reaction Mix

RT Reaction Mix

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	See below[a]	—
10X TaqMan RT Buffer	1.0	1X
25 mM $MgCl_2$	2.2	5.5 mM
deoxyNTPs Mixture (2.5 mM)	2.0	500 µM per dNTP
Random Hexamers[b] (50 µM)	0.5	2.5 µM
RNase Inhibitor (20 U/L)	0.2	0.4 U/µL
MultiScribe™ Reverse Transcriptase (50 U/µL)	0.25	1.25 U/µL
Total	6.15[c]	—

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

b. Random hexamers, oligo $d(T)_{16}$, or sequence-specific reverse primers can be used for primers of cDNA synthesis.

c. 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.

Thermal Cycling Parameters for RT Reactions

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

a. If 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.

Performing RT Reactions

The procedure for generating cDNA using the TaqMan Reverse Transcription Reagents is described below.

⚠ 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 perform RT reactions:

Step	Action
1	Prepare the RT Reaction Mix by combining all the nonenzymatic components.
2	Vortex briefly.
3	Add the enzymatic components (*e.g.*, MultiScribe Reverse Transcriptase, RNase Inhibitor) and the RNA.
4	Mix the components by inverting the microcentrifuge tube.
5	Transfer the contents to a MicroAmp® Optical Tube or multiple wells of a MicroAmp® Optical 96-Well Reaction Plate.
6	Cap the plate/tubes with MicroAmp® Optical Caps. **Note** Alternatively, you may seal the plate with a MicroAmp Optical Adhesive Cover. However, do not use the cover with MicroAmp Optical Tubes.
7	Centrifuge the plate/tubes briefly to remove air bubbles and collect the liquid at the bottom of the tube.
8	Transfer the plates to the thermal cycler block.
9	Perform RT.
10	Remove the 96-well reaction plate after thermal cycling is complete.

Reverse Transcription for 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 using MultiScribe Reverse Transcriptase.

Recommended Template

Use 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.
Non-human	Except for 18S rRNA, all assays are human-specific.

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 reverse transcription. 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)
Human Total RNA	60 ng to 2 µg

Guidelines

Follow the guidelines below to ensure optimal RT performance.

- Poly A^+ RNA samples are not recommended for 18S experiments because most rRNA has been removed from them.
- 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 RT. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents (N808-0234).

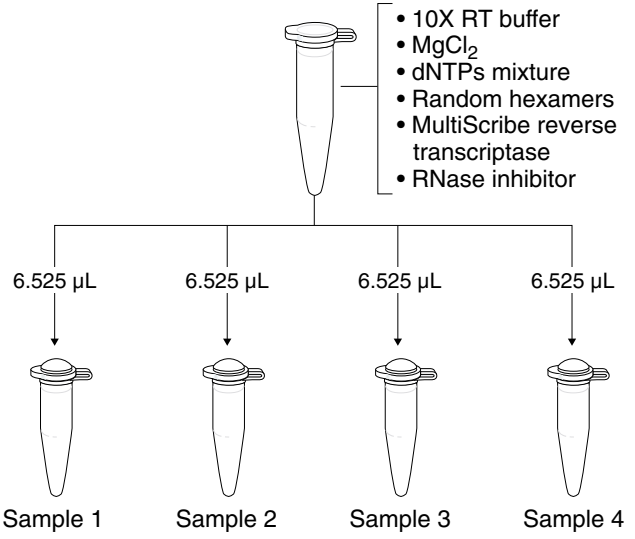
Note The kit contains sufficient quantities to perform 200 RT reactions with a reaction size of 10 µL.

⚠ 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 RT reactions for 18S amplicon:

<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 Conc.</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 (2.5 mM)</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.</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>
</table>

To prepare the RT reactions for 18S amplicon: *(continued)*

Step	Action
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 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 Optical Reaction Tube (see step 4).
10	Cap the reaction tubes and tap each gently to mix the reactions.
11	Centrifuge the tubes briefly to force the solution to the bottom and to eliminate air bubbles from the mixture.
12	Transfer each reaction to either ♦ MicroAmp Optical tubes, or, ♦ Wells of a MicroAmp Optical 96-well reaction plate.
13	Cap the MicroAmp Optical tubes or plate with MicroAmp Optical caps.
14	Centrifuge the plate or tubes to spin down the contents and eliminate air bubbles from the solutions.

Thermal Cycling

To conduct RT 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 RT.

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

Optimizing Primer Concentrations

4

Overview

About This Chapter

This chapter describes how to optimize primer concentrations for PCR, one-step RT-PCR, and two-step RT-PCR.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Optimizing Primer Concentrations for PCR	4-2
Optimizing Primer Concentrations for One-Step RT-PCR	4-5
Optimizing Primer Concentrations for Two-Step RT-PCR	4-9

Optimizing Primer Concentrations for PCR

Overview

The purpose of the procedure below is to determine the minimum primer concentrations giving the lowest threshold cycle (C_T) and maximum ΔR_n while minimizing nonspecific amplification. The reaction volumes are 50 µL. Use 10 to 100 ng of genomic DNA or 1 to 10 ng of cDNA template.

PCR Master Mix is used in the procedure on page 4-2 to run four replicates of each of the nine conditions shown in the table below. The master mix is described in "PCR Master Mix for Primer Optimization" on page 4-3.

Reverse Primer (nM)	Forward Primer (nM)		
	50	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Optimizing Primer Concentrations for PCR

To optimize primer concentrations for PCR:

Step	Action
1	Load the plate for both a template and a No Template Control (NTC) matrix, as shown in "Plate Configuration for Primer Optimization for PCR" on page 4-3.
2	Place the plate in the appropriate ABI PRISM® Sequence Detection System. Use the thermal cycling conditions in "Thermal Cycling Parameters for Primer Optimization" on page 4-4. **Note** SYBR Green must be calibrated on the instrument. Please refer to the appropriate instrument User's Manual to calibrate the instrument with SYBR Green.
3	At the end of the run: ♦ Tabulate the results for the yield. This analysis will identify the optimum concentrations of primers for PCR yield. ♦ Tabulate the results for the C_T value. This analysis will identify the optimum primer concentrations for C_T and for the absence of nonspecific amplification.

PCR Master Mix for Primer Optimization

⚠ WARNING CHEMICAL HAZARD. SYBR Green may cause eye, skin, and respiratory tract irritation. It is readily absorbed through the skin and is a combustable liquid and vapor (keep away from heat and flame). This product contains material which may cause liver and blood damage. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

PCR Master Mix for Primer Optimization

Component	Volume (µL) for One 50-µL Reaction	Volume (µL) for 100 50-µL Reactions	Final Concentration
2X SYBR Green PCR Master Mix	25	2500	1X
Forward Primer	Variable	Variable	50 to 900 nM
Reverse Primer	Variable	Variable	50 to 900 nM
Template	Variable	Variable	1 to 100 ng
Water	Variable	Variable	—
Total	50	5000	—

Plate Configuration for Primer Optimization for PCR

Wells	PCR Master Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
A1–A4	25	0.5	0.5	5.0	19.0	50
A5–A8	25	0.5	3.0	5.0	16.5	50
A9–A12	25	0.5	9.0	5.0	10.5	50
B1–B4	25	3.0	0.5	5.0	16.5	50
B5–B8	25	3.0	3.0	5.0	14.0	50
B9–B12	25	3.0	9.0	5.0	8.0	50
C1–C4	25	9.0	0.5	5.0	10.5	50
C5–C8	25	9.0	3.0	5.0	8.0	50
C9–C12	25	9.0	9.0	5.0	2.0	50
D1–D4	25	0.5	0.5	0	24.0	50
D5–D8	25	0.5	3.0	0	21.5	50
D9–D12	25	0.5	9.0	0	15.5	50

Plate Configuration for Primer Optimization for PCR *(continued)*

Wells	PCR Master Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
E1–E4	25	3.0	0.5	0	21.5	50
E5–E8	25	3.0	3.0	0	19.0	50
E9–E12	25	3.0	9.0	0	13.0	50
F1–F4	25	9.0	0.5	0	15.5	50
F5–F8	25	9.0	3.0	0	13.0	50
F9–F12	25	9.0	9.0	0	7.0	50

Thermal Cycling Parameters for Primer Optimization

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

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

Confirm the Absence of Nonspecific Amplification

To confirm the absence of nonspecific amplification:

Step	Action
1	Analyze the PCR products by agarose gel electrophoresis.
2	Generate a dissociation curve on the GeneAmp 5700 SDS and the ABI PRISM 7900 SDS, ABI PRISM 7700 SDS, and the ABI PRISM 7000 SDS.

Optimizing Primer Concentrations for One-Step RT-PCR

Overview

The procedure below is used to optimize one-step RT-PCR reactions.

One-Step RT-PCR Master Mix is used in the procedure on page 4-5 to run four replicates of each of the nine conditions shown in the table below. The master mix is described in "One-Step RT-PCR Master Mix for Primer Optimization" on page 4-6.

Reverse Primer (nM)	Forward Primer (nM)		
	50	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Reducing Nonspecific Amplification

For one-step RT-PCR, this protocol requires an initial incubation of the reaction mixture for 30 minutes at 48 °C (see "Thermal Cycling Parameters for Primer Optimization" on page 4-11). This RT step coincubates the PCR primers at a temperature below their annealing temperatures. AmpliTaq Gold enzyme will slowly activate at 48 °C and may lead to nonspecific amplification. To minimize the level of nonspecific amplification in one-step RT-PCR using SYBR Green PCR Master Mix, lower primer concentrations are recommended. If nonspecific amplification is still problematic, reverting to two-step RT-PCR is recommended.

Optimizing Primer Concentrations

To optimize primer concentrations for One-Step RT-PCR:

Step	Action
1	Load the plate for both a template and a No Template Control (NTC) matrix. Refer to "Plate Configuration for Primer Optimization for One-Step RT-PCR" on page 4-7.
2	Place the plate in the instrument. Use the thermal cycling conditions in "Thermal Cycling Parameters for Primer Optimization" on page 4-11. **Note** SYBR Green must be calibrated on the instrument. Please refer to the appropriate instrument User's Manual on how to calibrate the instrument for SYBR Green.

To optimize primer concentrations for One-Step RT-PCR: *(continued)*

Step	Action
3	At the end of the run: ♦ Tabulate the results for the yield. This analysis will identify the optimum concentrations of primers for PCR yield. ♦ Tabulate the results for the C_T value. This analysis will identify the optimum primer concentrations for C_T and for the absence of nonspecific amplification.

One-Step RT-PCR Master Mix for Primer Optimization

⚠ WARNING CHEMICAL HAZARD. SYBR Green may cause eye, skin, and respiratory tract irritation. It is readily absorbed through the skin and is a combustable liquid and vapor (keep away from heat and flame). This product contains material which may cause liver and blood damage. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

One-Step RT-PCR Master Mix for Primer Optimization

Component	Volume (µL) for One 50-µL Reaction	Volume (µL) for 100 50-µL Reactions	Final Conc.
2X SYBR Green PCR Master Mix	25	2500	1X
Reverse Transcription Reagents:			
♦ MultiScribe Reverse Transcriptase (50 U/µL)	0.25	25	0.25 U/mL
♦ RNase Inhibitor (20 U/µL)	1.0	100	0.4 U/mL
Forward Primer	Variable	Variable	50 to 900 nM
Reverse Primer	Variable	Variable	50 to 900 nM
Template	Variable	Variable	1 to 100 ng
Water	Variable	Variable	—
Total	50	5000	—

Plate Configuration for Primer Optimization for One-Step RT-PCR

Wells	PCR Master Mix + RT Reagents (µL)[a]	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
A1–A4	26.25	0.5	0.5	5.0	17.75	50
A5–A8	26.25	0.5	3.0	5.0	15.25	50
A9–A12	26.25	0.5	9.0	5.0	9.25	50
B1–B4	26.25	3.0	0.5	5.0	15.25	50
B5–B8	26.25	3.0	3.0	5.0	12.75	50
B9–B12	26.25	3.0	9.0	5.0	6.75	50
C1–C4	26.25	9.0	0.5	5.0	9.25	50
C5–C8	26.25	9.0	3.0	5.0	6.75	50
C9–C12	26.25	9.0	9.0	5.0	0.75	50
D1–D4	26.25	0.5	0.5	0	22.75	50
D5–D8	26.25	0.5	3.0	0	20.25	50
D9–D12	26.25	0.5	9.0	0	14.25	50
E1–E4	26.25	3.0	0.5	0	20.25	50
E5–E8	26.25	3.0	3.0	0	17.75	50
E9–E12	26.25	3.0	9.0	0	11.75	50
F1–F4	26.25	9.0	0.5	0	14.25	50
F5–F8	26.25	9.0	3.0	0	11.75	50
F9–F12	26.25	9.0	9.0	0	5.75	50

a. Volume of 26.25 µL includes 25 µL of PCR Master Mix plus 1.25 µL of RT Reagents.

Thermal Cycling Parameters for Primer Optimization

Step	RT	AmpliTaq Gold Activation[a]	PCR	
			Cycle (40 cycles)	
			Denature	Anneal/Extend
	HOLD	HOLD	CYCLE	
Temperature/ TIme	48 °C 30 min	95 °C 10 min	95 °C 15 sec	60 °C 1 min
Volume	50 (µL)			

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

Confirm the Absence of Nonspecific Amplification

To confirm the absence of nonspecific amplification:

Step	Action
1	Analyze the PCR products by agarose gel electrophoresis.
2	Generate a dissociation curve on the GeneAmp 5700 SDS, ABI PRISM 7700 SDS, ABI PRISM 7900HT SDS or ABI PRISM 7000 SDS.

Optimizing Primer Concentrations for Two-Step RT-PCR

Overview

The purpose of the procedure below is to determine the minimum primer concentrations giving the lowest threshold cycle (C_T) and maximum ΔR_n while minimizing nonspecific amplification. The reaction volumes are 50 µL. Use 10 to 100 ng of genomic DNA or 1 to 10 ng of cDNA template.

Two-Step RT-PCR Master Mix is used in the procedure on page 4-9 to run four replicates of each of the nine conditions shown in the table below. The master mix is described in "One-Step RT-PCR Master Mix for Primer Optimization" on page 4-6.

Reverse Primer (nM)	Forward Primer (nM)		
	50	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Optimizing Primer Concentrations for Two-Step RT-PCR

To optimize primer concentrations for two-step RT-PCR:

Step	Action
1	Load the plate for both a template and a No Template Control (NTC) matrix. Refer to "Plate Configuration for Primer Optimization for Two-Step RT-PCR on page 4-10.
2	Place the plate in the Sequence Detection System. Use the thermal cycling conditions in "Thermal Cycling Parameters for Primer Optimization" on page 4-11. **Note** SYBR Green must be calibrated on the instrument. Please refer to the appropriate instrument User's Manual for instructions on how to calibrate the instrument for SYBR Green.
3	At the end of the run: ♦ Tabulate the results for the yield. This analysis will identify the optimum concentrations of primers for PCR yield. ♦ Tabulate the results for the C_T value. This analysis will identify the optimum primer concentrations for C_T and for the absence of nonspecific amplification.

Two-Step RT-PCR Master Mix for Primer Optimization

⚠ WARNING CHEMICAL HAZARD. SYBR Green may cause eye, skin, and respiratory tract irritation. It is readily absorbed through the skin and is a combustable liquid and vapor (keep away from heat and flame). This product contains material which may cause liver and blood damage. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves..

Two-Step RT-PCR Master Mix for Primer Optimization

Component	Volume (µL) for One 50-µL Reaction	Volume (µL) for 100 50-µL Reactions	Final Concentration
2X SYBR Green PCR Master Mix	25	2500	1X
Forward Primer	Variable	Variable	50 to 900 nM
Reverse Primer	Variable	Variable	50 to 900 nM
Template	Variable	Variable	1ng to 100 ng
Water	Variable	Variable	—
Total	50	5000	—

Plate Configuration for Primer Optimization for Two-Step RT-PCR

Wells	PCR Master Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
A1–A4	25	0.5	0.5	5.0	19.0	50
A5–A8	25	0.5	3.0	5.0	16.5	50
A9–A12	25	0.5	9.0	5.0	10.5	50
B1–B4	25	3.0	0.5	5.0	16.5	50
B5–B8	25	3.0	3.0	5.0	14.0	50
B9–B12	25	3.0	9.0	5.0	8.0	50
C1–C4	25	9.0	0.5	5.0	10.5	50
C5–C8	25	9.0	3.0	5.0	8.0	50
C9–C12	25	9.0	9.0	5.0	2.0	50
D1–D4	25	0.5	0.5	0	24.0	50
D5–D8	25	0.5	3.0	0	21.5	50
D9–D12	25	0.5	9.0	0	15.5	50
E1–E4	25	3.0	0.5	0	21.5	50

Plate Configuration for Primer Optimization for Two-Step RT-PCR *(continued)*

Wells	PCR Master Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
E5–E8	25	3.0	3.0	0	19.0	50
E9–E12	25	3.0	9.0	0	13.0	50
F1–F4	25	9.0	0.5	0	15.5	50
F5–F8	25	9.0	3.0	0	13.0	50
F9–F12	25	9.0	9.0	0	7.0	50

Thermal Cycling Parameters for Primer Optimization

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

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

Confirm the Absence of Nonspecific Amplification

To confirm the absence of nonspecific amplification:

Step	Action
1	Analyze the PCR products by agarose gel electrophoresis.
2	Generate a dissociation curve on the GeneAmp 5700 SDS, ABI PRISM 7700 SDS, ABI PRISM 7900HT SDS, or ABI PRISM 7000 SDS.

5 Data Analysis

Overview

About This Chapter

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

In This Chapter

The following topic is discussed in this chapter:

Topic	See Page
Absolute and Relative Quantitation of Target DNA	5-2
Interpreting the Results	5-3

Absolute and Relative Quantitation of Target DNA

Overview

Two types of quantitation are possible when using the SYBR Green® PCR Master Mix:

- Relative quantitation of a target against an internal standard is particularly useful for gene expression measurements.
- Absolute quantitation is possible if the isolation procedure and sample contents do not impact the PCR results. The quantitation of genomic DNA may lend itself for absolute quantitation against a standard curve.

Absolute Quantitation

Absolute quantitation compares the C_T of an unknown sample against a standard curve with known copy numbers.

Quantitation of cDNA Relative to a Calibrator Sample

Gene expression can be measured by the quantitation of cDNA converted from a messenger RNA corresponding to this gene relative to a calibrator sample serving as a physiological reference. In a typical experiment, gene expression levels are studied as a function of either a treatment of cells in culture, of patients, or of tissue type. The calibrator sample in each case is the cDNA from either the untreated cells or patients, or a specific tissue type.

All quantitations are also normalized to an endogenous control such as 18S rRNA to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. All amplicons in these determinations should follow the amplicon design criteria defined previously around the Primer Express software. Refer to *ABI PRISM 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression* (P/N 4303859) for additional information about relative quantitation.

Interpreting the Results

Passive Reference ROX

The Passive Reference (ROX) is a dye molecule included in the SYBR Green PCR Master Mix that does not participate in the PCR amplification. On the ABI PRISM® 7700 SDS, the ABI PRISM® 7900HT SDS, the ABI PRISM® 7000 SDS, and the GeneAmp® SDS, the Passive Reference provides an internal reference to which the SYBR Green/dsDNA complex signal can be normalized during data analysis. Normalization is necessary to correct for well-to-well fluorescent fluctuations.

References

A

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Technical Support

B

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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