

SYBR[®] Green PCR and RT-PCR Reagents

Protocol

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Introduction

1

Overview

About This Chapter This chapter describes the SYBR® Green PCR Core Reagents 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	1-5
Safety	1-7

Purpose of the Kit

About the Kit The SYBR Green PCR Core Reagents (P/N 4304886) are designed to detect genomic, plasmid, and cDNA with SYBR Green dye. Direct detection of polymerase chain reaction (PCR) product is monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double-stranded (ds) DNA.

In RNA quantitation assays the SYBR Green PCR Core Reagents are used in the second step of a two-step reverse transcription-polymerase chain reaction (RT-PCR) protocol. The template is the cDNA generated from a reverse transcription reaction.

The SYBR Green PCR Core Reagents are designed for use with the ABI PRISM® 7700 Sequence Detection System or the GeneAmp® 5700 Sequence Detection System. The excitation-emission profile for the SYBR Green dye is similar to that of the FAM dye. This allows the use of the ROX Passive Reference molecule.

For the best quantitation results, use the following:

- ◆ Primer Express™ software (P/N 402089) for primer design
- ◆ Applied Biosystems® reagents
- ◆ Applied Biosystems universal thermal cycling conditions

Follow the instructions in “Amplifying Custom Target Sequences for Quantitation” on page 3-2.

Interpreting Results **Normalization**

The Passive Reference is a dye included in the 10X SYBR Green PCR Buffer that does not participate in the PCR amplification. 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 fluorescent fluctuations caused by changes in concentration or volume.

Multicomponenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescence spectra. The overlapping spectra from the pure dye components generate the composite spectrum. This spectrum represents one fluorescence intensity reading from one well. The ABI PRISM 7700 Sequence Detection System should

include in its Pure Dye spectra the SYBR Green and ROX spectra for multicomponenting data generated with the SYBR Green PCR Core Reagents.

To add SYBR Green to the Pure Dye spectra, use the Sequence Detection Systems Spectral Calibration Kit (P/N 4305822). Follow the detailed instructions in *ABI PRISM 7700 Sequence Detection System User Bulletin #4: Generating New Spectra Components* (P/N 4306234).

R_n and ΔR_n Values

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

♦ R_n^+ = R_n value of a reaction containing all components including the template

♦ R_n^- = R_n value of an unreacted sample

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

♦ $\Delta R_n = (R_n^+) - (R_n^-)$

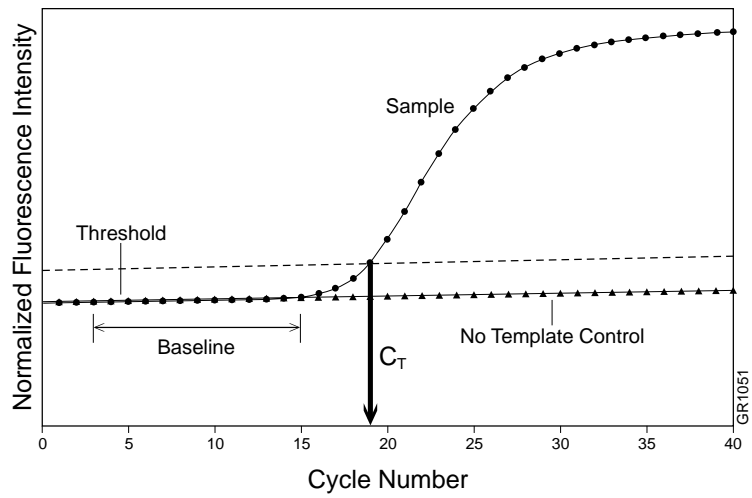
where:

$$R_n^+ = \frac{\text{Emission Intensity of SYBR Green}}{\text{Emission Intensity of Passive Reference}} \quad (\text{PCR with template})$$

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

ΔR_n indicates the magnitude of the signal generated by the given set of PCR conditions.

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 shown below, the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.



Materials and Equipment

Kit Contents The SYBR Green PCR Core Reagents (P/N 4304886) contain enough PCR reagents for up to 200 50- μ L reactions.

Reagent	Volume	Description
10X SYBR Green PCR Buffer	1.5 mL	One tube containing optimized 10X SYBR Green PCR Buffer, which includes Passive Reference 1
AmpErase [®] UNG	100 μ L	One vial containing 1 U/ μ L uracil-N-glycosylase
AmpliTaq Gold [®] DNA Polymerase	50 μ L	One vial containing 5 U/ μ L AmpliTaq Gold DNA Polymerase
dNTP Mix	1.0 mL	One tube containing 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 5.0 mM dUTP
25 mM MgCl ₂	3.0 mL	Two tubes containing 25 mM MgCl ₂ , 1.5 mL each

Storage and Stability Store the SYBR Green PCR Core Reagents at -15 to -25 °C. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

Materials Required but Not Supplied The following items are required in addition to the reagents supplied in the SYBR Green PCR Core Reagents.

Required items:

Item	Source
ABI PRISM 7700 Sequence Detection System or GeneAmp 5700 Sequence Detection System	See your local Applied Biosystems representative for the instrument or software best suited to meet your needs.
Primer Express [™] software (single-use license)	Applied Biosystems (P/N 402089)
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)

Required items: *(continued)*

Item	Source
MicroAmp® Optical Tubes	Applied Biosystems (P/N N801-0933)
MicroAmp® Optical Caps	Applied Biosystems (P/N N801-0935)
<p>Note The MicroAmp Optical 96-Well Reaction Plate may be sealed with:</p> <ul style="list-style-type: none"> ◆ MicroAmp Optical Caps or ◆ ABI PRISM™ Optical Adhesive Cover <p>The Optical Adhesive Cover must be used with a compression pad and applicator, which are included in the starter pack.</p>	
ABI PRISM Optical Adhesive Cover Starter Pack ◆ 20 Optical Adhesive Covers ◆ 1 Applicator ◆ 1 Compression Pad	Applied Biosystems (P/N 4313663)
MicroAmp® Reaction Tubes	Applied Biosystems (P/N N801-0540)
Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (P/N 4305822)
TaqMan® Reverse Transcription Reagents	Applied Biosystems (P/N N808-0234)
Unlabeled Sequence Detection primers: ◆ 40 nM ◆ 0.2 µM ◆ 1.0 µM	Applied Biosystems: ◆ P/N 450005 ◆ P/N 450004 ◆ P/N 450021
Centrifuge with adapter for 96-well plate	Major laboratory supplier (MLS)
Microcentrifuge	MLS
NuSieve 4% (3:1) agarose gels, for DNA <1 kb	FMC BioProducts (P/N 54928)
Polypropylene tubes	MLS
Pipettors, positive-displacement or air-displacement	MLS
Pipette tips, with filter plugs	MLS

Required items: *(continued)*

Item	Source
Vortexer	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Disposable gloves	MLS

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 and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.*, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.

- ◆ Do not leave chemical containers open. Use only with adequate ventilation.
- ◆ 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.

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.

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

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- ◆ 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.

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 MSDSs...	Then...							
Over the Internet	<p>a. Go to our Web site at www.appliedbiosystems.com/techsupp</p> <p>b. Click MSDSs</p> <table border="1"> <thead> <tr> <th>If you have...</th> <th>Then...</th> </tr> </thead> <tbody> <tr> <td>The MSDS document number or the Document on Demand index number</td> <td>Enter one of these numbers in the appropriate field on this page.</td> </tr> <tr> <td>The product part number</td> <td rowspan="2">Select Click Here, then enter the part number or keyword(s) in the field on this page.</td> </tr> <tr> <td>Keyword(s)</td> </tr> </tbody> </table> <p>c. You can open and download a PDF (using Adobe® Acrobat® Reader™) of the document by selecting it, or you can choose to have the document sent to you by fax or email.</p>	If you have...	Then...	The MSDS document number or the Document on Demand index number	Enter one of these numbers in the appropriate field on this page.	The product part number	Select Click Here , then enter the part number or keyword(s) in the field on this page.	Keyword(s)
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Keyword(s)								
By automated telephone service	Use "To Obtain Documents on Demand" under "Technical Support."							
By telephone in the United States	Dial 1-800-327-3002 , then press 1 .							
By telephone from Canada	<table border="1"> <thead> <tr> <th>To order in...</th> <th>Dial 1-800-668-6913 and...</th> </tr> </thead> <tbody> <tr> <td>English</td> <td>Press 1, then 2, then 1 again</td> </tr> <tr> <td>French</td> <td>Press 2, then 2, then 1</td> </tr> </tbody> </table>	To order in...	Dial 1-800-668-6913 and...	English	Press 1 , then 2 , then 1 again	French	Press 2 , then 2 , then 1	
To order in...	Dial 1-800-668-6913 and...							
English	Press 1 , then 2 , then 1 again							
French	Press 2 , then 2 , then 1							
By telephone from any other country	See the specific region under "To Contact Technical Support by Telephone or Fax" under "Technical Support."							

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

Preventing Contamination

2

Overview

About This Chapter This chapter covers ways to prevent contamination and detect its occurrence.

In This Chapter The following topics are discussed in this chapter:

Topic	See Page
Preventing Non-Specific Amplification	2-2
Amplicon-Independent Amplification (Including Primer-Dimers)	2-5

Preventing Non-Specific Amplification

Overview The DNA amplification capability of the PCR process and the non-specific detection format of SYBR Green chemistry make special laboratory practices necessary. Small levels of DNA carryover from samples with high DNA concentrations, from the DNA Template Controls, or from previous PCR amplifications can result in product even in the absence of added template DNA. Because the indicator dye will bind to any double-stranded DNA, unintended products will result in a positive signal.

For more information on the polymerase chain reaction, see Kwok and Higuchi, 1989. For more information on the prevention of unintended products, see 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 where 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 Recently, Applied Biosystems introduced a new PCR enzyme, 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.

AmpliTaQ Gold DNA Polymerase can be introduced into existing amplification systems with only minimal alterations to the reaction protocol, resulting in higher specificity, sensitivity, and product yield.

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

UNG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo *et. al.*, 1990).

AmpErase UNG treatment can prevent the reamplification of carryover PCR products. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50- μ L reaction.

General PCR Please follow these recommended procedures:

Practices

- ◆ Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- ◆ Change gloves whenever you suspect that they are contaminated.
- ◆ Maintain separate areas and dedicated equipment and supplies for:
 - Sample preparation
 - 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 pipet or aerosol-resistant pipet tips
- ◆ Clean lab benches and equipment periodically with 10% bleach solution.

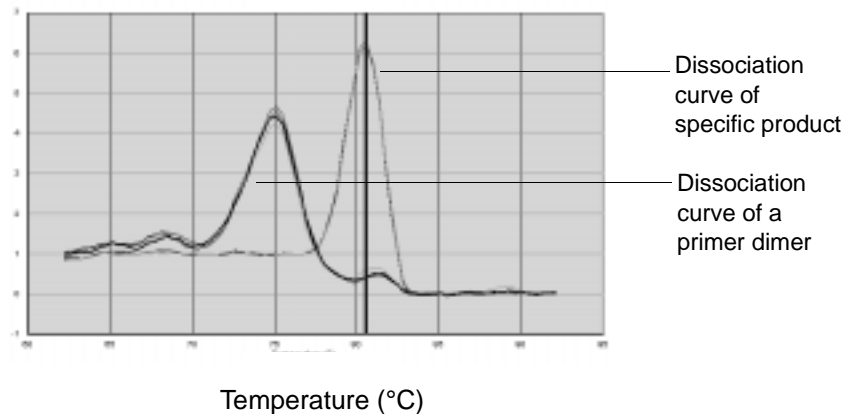
Fluorescent Contaminants Since fluorescent contaminants can interfere with this assay and give false-positive results, it may be necessary to include a No Amplification Control tube that contains sample, but no enzyme. If the absolute fluorescence of the No Amplification Control is greater than that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

Amplicon-Independent Amplification (Including Primer-Dimers)

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 The GeneAmp 5700 Sequence Detection System enables dissociation curves to be run to detect nonspecific amplification. 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-dimers 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 Sequence Detection System 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.

Refer to the *GeneAmp 5700 Sequence Detection System User's Manual* (P/N 4304472) for further details on generating a dissociation curve.

Using Agarose Gels to Check PCR Product Purity

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

To perform electrophoresis:

Step	Action
1	Load 12–15 μ L of sample per well on an ethidium bromide-stained 4% NuSieve 3:1 agarose gel. ⚠ WARNING Ethidium bromide causes eye, skin, and respiratory tract irritation and is a known mutagen (<i>i.e.</i> , it can change genetic material in a living cell and has the potential to cause cancer). Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2	Run the gel: <ul style="list-style-type: none">◆ For PCR fragments <100 bp, run the gel at 80–100 V for 45–60 minutes.◆ For PCR fragments 100–250 bp, run the gel at 100–115 V for 1–1.5 hours.
3	Run samples 1/3–1/2 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.

PCR

3

Overview

**About This
Chapter**

This chapter describes how to develop quantitative PCR assays.

In This Chapter

The following topic is discussed in this chapter:

Topic	See Page
Amplifying Custom Target Sequences for Quantitation	3-2

Amplifying Custom Target Sequences for Quantitation

Overview We recommend the following steps for the development of quantitative PCR assays.

To develop quantitative PCR assays:

Step	Action	See Page
1	Install Primer Express Software	—
2	Identify Target Sequence and Amplicon Size	3-2
3	Design Primers	3-2
4	Select an Amplicon Site for Gene Expression Assays	3-3
5	Order Reagents	3-3
6	Quantitate Primers	3-4
7	Perform RT Reactions for All Amplicons Except 18S	4-2
8	Perform RT for the 18S Amplicon	4-5

Identify Target Sequence and Amplicon Size A target template is a double-stranded DNA, cDNA, or plasmid containing the nucleotide sequence of interest.

Design primers to amplify short segments of DNA within the target sequence. These short segments are called amplicons. Shorter amplicons work the most efficiently: consistent results are obtained for amplicon size ranges from 50–150 bp.

Design Primers Design primers using Primer Express software as described in the *Primer Express Applications-Based Primer Design Software User's Manual* (P/N 4303014).

Follow these guidelines:

- ◆ Primers can be designed as close as possible to each other provided that they do not overlap.
- ◆ Keep the GC content in the 20–80% 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 58–60 °C.
- ◆ The five nucleotides at the 3' end should have no more than two G and/or C bases.

Select an Amplicon Site for Gene Expression Assays

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.

Order Reagents

See "Materials Required but Not Supplied" on page 1-5 for a list of required reagents and equipment.

Quantitate 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 below.

Chromophore	Extinction Coefficient	Number	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
Total	—	—	167,950

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

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

$$C = 258 \mu\text{M}$$

Reverse Transcription

4

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
Perform RT Reactions for All Amplicons Except 18S	4-2
Reverse Transcription for the 18S Amplicon	4-5

Perform RT Reactions for All Amplicons Except 18S

Overview Synthesis of cDNA from total RNA 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 reverse transcription using MultiScribe Reverse Transcriptase.

Guidelines Follow these guidelines to achieve optimal RT performance:

- ◆ A 100- μ L reaction efficiently converts a maximum of 2 μ g of total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more than 2 μ g 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.

RT Procedure The procedure for generating cDNA using the TaqMan Reverse Transcription Reagents (P/N N808-0234) 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.

Performing Reverse Transcription

To perform RT:

Step	Action
1	Prepare the RT Reaction Mix by combining all the nonenzymatic components listed in "RT Reaction Mix" above.
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	Using MicroAmp Optical Caps, cap the tubes and briefly centrifuge to remove air bubbles and collect the liquid at the bottom of the tube. Transfer the plates to the thermal cycler block.
7	Perform RT as described in "Thermal Cycling Parameters for RT Reactions" on page 4-4.
8	Remove the 96-Well Reaction Plate after thermal cycling is complete. IMPORTANT After thermal cycling, store all cDNA samples at -15 to -25 °C.

RT Reaction Mix

Component	Volume/Tube (μL)	Final Concentration
RNase-free water	See below ^a	—
10X TaqMan RT Buffer	10.0	1X
25 mM MgCl ₂	22.0	5.5 mM
deoxyNTPs Mixture	20.0	500 μM per dNTP
Random Hexamers ^b	5.0	2.5 μM
RNase Inhibitor	2.0	0.4 U/μL
MultiScribe™ Reverse Transcriptase (50 U/μL)	2.5	1.25 U/μL
Total	61.5 ^c	—

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

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

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

Thermal Cycling Parameters for RT Reactions

Step	Incubation ^a	Reverse Transcription	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temperature	25 °C	48 °C	95 °C
Time	10 min	30 min	5 min
Volume	100 μL		

a. If using random hexamers or oligo d(T)₁₆ primers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 minutes) is necessary to maximize primer–RNA template binding.

After thermal cycling, store all cDNA samples at –15 to –25 °C.

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

Recommended Template Use only total RNA samples to generate cDNA for the 18S amplicon. The following table lists the known template incompatibilities:

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

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

Template Quantity If possible, use spectrophotometric analysis to determine the concentrations of purified total RNA samples before 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- μ L RT reaction)
Total RNA	60 ng–2 μ g

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- μ L RT reaction will efficiently convert a maximum of 2 μ g total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more than 2 μ g total RNA.
- ◆ Use only random hexamers to reverse transcribe the total RNA samples for gene expression assays.

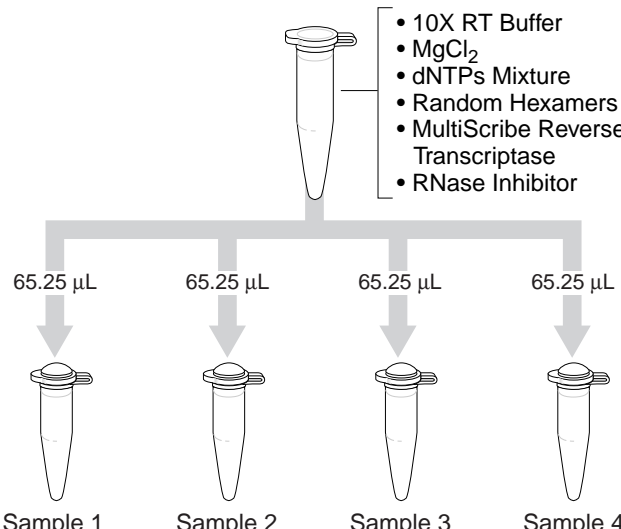
Preparing the Reactions The following procedure describes the preparation of four different test samples for reverse transcription. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents (P/N N808-0234).

⚠ 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:

Step	Action																																						
1	In a 1.5-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed. If preparing four samples, follow the recommended volumes shown below.																																						
	<table border="1"> <thead> <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> </thead> <tbody> <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>10.0</td> <td>40.0</td> <td>1X</td> </tr> <tr> <td>25 mM MgCl_2</td> <td>22.0</td> <td>88.0</td> <td>5.5 mM</td> </tr> <tr> <td>deoxyNTPs Mixture</td> <td>20.0</td> <td>80.0</td> <td>500 μM per dNTP</td> </tr> <tr> <td>Random Hexamers</td> <td>5.0</td> <td>20.0</td> <td>2.5 μM</td> </tr> <tr> <td>RNase Inhibitor</td> <td>2.0</td> <td>8.0</td> <td>0.4 U/μL</td> </tr> <tr> <td>MultiScribe Reverse Transcriptase (50 U/μL)</td> <td>6.25</td> <td>25.0</td> <td>3.125 U/μL</td> </tr> <tr> <td>Total^b</td> <td>65.25</td> <td>261.0</td> <td>—</td> </tr> </tbody> </table>	Component	Volume (μL)		Final Conc.	Per Sample	Reaction Mix (x4)	RNase-free water	See below ^a	See below ^a	—	10X RT Buffer	10.0	40.0	1X	25 mM MgCl_2	22.0	88.0	5.5 mM	deoxyNTPs Mixture	20.0	80.0	500 μM per dNTP	Random Hexamers	5.0	20.0	2.5 μM	RNase Inhibitor	2.0	8.0	0.4 U/ μL	MultiScribe Reverse Transcriptase (50 U/ μL)	6.25	25.0	3.125 U/ μL	Total ^b	65.25	261.0	—
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MultiScribe Reverse Transcriptase (50 U/ μL)	6.25	25.0	3.125 U/ μL																																				
Total ^b	65.25	261.0	—																																				
a. The volume of RNase-free water (μL) will be 34.75–RNA sample volume in a 100- μL reaction.																																							
b. If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.																																							
2	Label four 1.5-mL microcentrifuge tubes for the four test samples.																																						
3	Transfer 60 ng–2 μg (up to 34.75 μL) of each total RNA sample to the corresponding microcentrifuge tube.																																						
4	If necessary, dilute each total RNA sample to a volume of 34.75 μL with RNase-free, deionized water.																																						
5	Cap the tubes and gently tap each to mix the diluted samples.																																						
6	Briefly centrifuge the tubes to eliminate air bubbles in the mixture.																																						
7	Label four 0.2-mL MicroAmp Reaction Tubes for the four total RNA test samples.																																						

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

Step	Action
8	<p>Pipet 65.25 μL of the reaction mix (from step 1) to each MicroAmp Reaction Tube (from step 7).</p>  <ul style="list-style-type: none"> • 10X RT Buffer • MgCl₂ • dNTPs Mixture • Random Hexamers • MultiScribe Reverse Transcriptase • RNase Inhibitor <p>65.25 μL 65.25 μL 65.25 μL 65.25 μL</p> <p>Sample 1 Sample 2 Sample 3 Sample 4</p>
9	Transfer 34.75 μ L 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 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 reverse transcription thermal cycling:

Step	Action																				
1	Load the reactions into a thermal cycler.																				
2	Program your thermal cycler with the following conditions: <table border="1"><thead><tr><th>Step</th><th>Hexamer Incubation^a</th><th>Reverse Transcription</th><th>Reverse Transcriptase Inactivation</th></tr></thead><tbody><tr><td></td><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">100 µL</td></tr></tbody></table>	Step	Hexamer Incubation ^a	Reverse Transcription	Reverse Transcriptase Inactivation		HOLD	HOLD	HOLD	Temp.	25 °C	37 °C	95 °C	Time	10 min	60 min	5 min	Volume	100 µL		
Step	Hexamer Incubation ^a	Reverse Transcription	Reverse Transcriptase Inactivation																		
	HOLD	HOLD	HOLD																		
Temp.	25 °C	37 °C	95 °C																		
Time	10 min	60 min	5 min																		
Volume	100 µL																				
3	Begin reverse transcription. IMPORTANT After thermal cycling, store all cDNA samples at -15 to -25 °C.																				

Optimizing Primer Concentrations

5

Overview

About This Chapter This chapter describes how to optimize primer concentrations for one-step and two-step reverse transcription–polymerase chain reaction (RT-PCR).

In This Chapter The following topics are discussed in this chapter:

Topic	See Page
Optimize Primer Concentrations for Two-Step RT-PCR	5-2
Optimize Primer Concentrations for One-Step RT-PCR	5-6

Optimize Primer Concentrations for Two-Step RT-PCR

Overview The purpose of this procedure is to determine the minimum primer concentrations giving the maximum ΔR_n and minimum non-specific amplification. The reaction volumes are 50 μ L. Use 10–100 ng of genomic DNA or 1–10 ng of cDNA template.

The ABI PRISM 7700 Sequence Detection System or the GeneAmp 5700 Sequence Detection System can provide additional data for optimization using the minimum threshold cycle (C_T). See “Real Time Detection” on page 1-4 for more information regarding C_T .

Determine the Optimal Primer Concentrations

To determine the optimal primer concentrations:

Step	Action																				
1	Prepare a PCR master mix to run four replicates of each of the nine conditions as shown. The master mix is described in “PCR Master Mix for Primer Optimization” on page 5-3.																				
		<table border="1"> <thead> <tr> <th rowspan="2">Reverse Primer (nM)</th> <th colspan="3">Forward Primer (nM)</th> </tr> <tr> <th>50</th> <th>300</th> <th>900</th> </tr> </thead> <tbody> <tr> <th>50</th> <td>50/50</td> <td>300/50</td> <td>900/50</td> </tr> <tr> <th>300</th> <td>50/300</td> <td>300/300</td> <td>900/300</td> </tr> <tr> <th>900</th> <td>50/900</td> <td>300/900</td> <td>900/900</td> </tr> </tbody> </table>	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
		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																		
2	Load the plate for both a template and a No Template Control (NTC) matrix as shown in “Plate Configuration for Primer Optimization” on page 5-4.																				
3	Place the plate in the ABI PRISM 7700 Sequence Detection System or the GeneAmp 5700 Sequence Detection System.																				
4	Begin thermal cycling. Use the thermal cycling conditions in “Thermal Cycling Parameters for Two-Step Primer Optimization” on page 5-5. Note If using the ABI PRISM 7700 Sequence Detection System, confirm that a SYBR Green Pure Dye spectrum is accessed by the SDS software before starting the run.																				

To determine the optimal primer concentrations: *(continued)*

Step	Action
5	<p>At the end of the run:</p> <ul style="list-style-type: none"> ◆ 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 non-specific 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 combustible 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.

Component	Volume (μL) for One 50-μL Reaction	Volume (μL) for 100 50-μL Reactions
10X SYBR Green PCR Buffer	5.0	500
25 mM MgCl ₂	6.0	600
dNTP Blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5.0 mM dUTP)	4.0	400
AmpliTaq Gold (5 U/μL)	0.25	25
AmpErase UNG (1 U/μL)	0.50	50
Total Volume	15.75	1575

**Plate
Configuration for
Primer
Optimization**

Plate configuration:

Wells	PCR Master Mix (μL)	5 μM Forward Primer (μL)	5 μM Reverse Primer (μL)	Template	Deionized Water (μL)	Total Volume (μL)
A1–A4	15.75	0.5	0.5	5.0	28.25	50
A5–A8	15.75	0.5	3.0	5.0	25.75	50
A9–A12	15.75	0.5	9.0	5.0	19.75	50
B1–B4	15.75	3.0	0.5	5.0	25.75	50
B5–B8	15.75	3.0	3.0	5.0	23.25	50
B9–B12	15.75	3.0	9.0	5.0	17.25	50
C1–C4	15.75	9.0	0.5	5.0	19.75	50
C5–C8	15.75	9.0	3.0	5.0	17.25	50
C9–C12	15.75	9.0	9.0	5.0	11.25	50
D1–D4	15.75	0.5	0.5	0	33.25	50
D5–D8	15.75	0.5	3.0	0	30.75	50
D9–D12	15.75	0.5	9.0	0	24.75	50
E1–E4	15.75	3.0	0.5	0	30.75	50
E5–E8	15.75	3.0	3.0	0	28.25	50
E9–E12	15.75	3.0	9.0	0	22.25	50
F1–F4	15.75	9.0	0.5	0	24.75	50
F5–F8	15.75	9.0	3.0	0	22.25	50
F9–F12	15.75	9.0	9.0	0	16.25	50

**Thermal Cycling
Parameters for
Two-Step Primer
Optimization**

Step	AmpErase UNG Incubation ^a	AmpliTaq Gold Activation ^b	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	50.0 °C	95.0 °C	95.0 °C	60.0 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

a. Required for optimal AmpErase UNG activity.

b. Required for optimal AmpliTaq Gold DNA Polymerase activation.

**Confirm the
Absence of
Non-Specific
Amplification**

To confirm the absence of non-specific amplification:

Step	Action
1	Analyze the PCR products by agarose gel electrophoresis (see “Using Agarose Gels to Check PCR Product Purity” on page 2-6).
2	Generate a dissociation curve on the GeneAmp 5700 Sequence Detection System (see “Using Dissociation Curves” on page 2-5).

Optimize Primer Concentrations for One-Step RT-PCR

Overview One-step RT-PCR reactions with MultiScribe Reverse Transcriptase are optimized following a similar protocol. In this case, the “PCR Master Mix for Primer Optimization” on page 5-3 is replaced with the corresponding One-Step RT-PCR Master Mix. See the table below.

Note Procedures for one-step RT-PCR are the same as for two-step RT-PCR. See “Optimize Primer Concentrations for Two-Step RT-PCR” on page 5-2.

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 combustible 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.

Component	Volume (μL) for One 50-μL Reaction	Volume (μL) for 100 50-μL Reactions
10X SYBR Green PCR Buffer	5.0	500
25 mM MgCl ₂	6.0	600
dNTP Blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 5.0 mM dUTP)	6.0	600
AmpliTaq Gold (5 U/μL)	0.25	25
MultiScribe Reverse Transcriptase (50 U/μL)	0.25	25
RNase Inhibitor	1.0	100
Total Volume	18.50	1850

**Thermal Cycling
Conditions for
One-Step Primer
Optimization**

Step	Reverse Transcription	AmpliTaq Gold Activation ^a	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	48.0 °C	95.0 °C	95.0 °C	60.0 °C
Time	30 min	10 min	15 sec	1 min
Volume	50 µL			

a. Required for optimal AmpliTaq Gold DNA Polymerase activation.

**Confirm the
Absence of
Non-Specific
Amplification**

To confirm the absence of non-specific amplification:

Step	Action
1	Analyze the PCR products by agarose gel electrophoresis (see "Using Agarose Gels to Check PCR Product Purity" on page 2-6).
2	Generate a dissociation curve on the GeneAmp 5700 Sequence Detection System (see "Using Dissociation Curves" on page 2-5).

Data Analysis

6

Overview

About This Chapter This 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	6-2

Absolute and Relative Quantitation

Overview Two types of quantitation are possible with the SYBR Green reagents:

- ◆ 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 of Plasmids or Genomic DNA

Absolute quantitation compares the C_T of an unknown sample against a standard curve with known copy numbers. Absolute quantitation on the ABI PRISM 7700 Sequence Detection System or the GeneAmp 5700 Sequence Detection System can be carried out with the plate configuration shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC A1	NTC A2	NTC A3	NTC A4	STND A5 1.0e+03	STND A6 1.0e+03	STND A7 1.0e+03	STND A8 1.0e+03	STND A9 2.0e+03	STND A10 2.0e+03	STND A11 2.0e+03	STND A12 2.0e+03
B	STND B1 3.0e+03	STND B2 3.0e+03	STND B3 3.0e+03	STND B4 3.0e+03	STND B5 1.0e+04	STND B6 1.0e+04	STND B7 1.0e+04	STND B8 1.0e+04	STND B9 2.0e+04	STND B10 2.0e+04	STND B11 2.0e+04	STND B12 2.0e+04
C	UNKN C1	UNKN C2	UNKN C3	UNKN C4	UNKN C5	UNKN C6	UNKN C7	UNKN C8	UNKN C9	UNKN C10	UNKN C11	UNKN C12
D	UNKN D1	UNKN D2	UNKN D3	UNKN D4	UNKN D5	UNKN D6	UNKN D7	UNKN D8	UNKN D9	UNKN D10	UNKN D11	UNKN D12
E	UNKN E1	UNKN E2	UNKN E3	UNKN E4	UNKN E5	UNKN E6	UNKN E7	UNKN E8	UNKN E9	UNKN E10	UNKN E11	UNKN E12
F	UNKN F1	UNKN F2	UNKN F3	UNKN F4	UNKN F5	UNKN F6	UNKN F7	UNKN F8	UNKN F9	UNKN F10	UNKN F11	UNKN F12
G	UNKN G1	UNKN G2	UNKN G3	UNKN G4	UNKN G5	UNKN G6	UNKN G7	UNKN G8	UNKN G9	UNKN G10	UNKN G11	UNKN G12
H	UNKN H1	UNKN H2	UNKN H3	UNKN H4	UNKN H5	UNKN H6	UNKN H7	UNKN H8	UNKN H9	UNKN H10	UNKN H11	UNKN H12

Each well in this plate receives 45 μ L of a master mix with forward primer, and reverse primer concentrations and 5 μ L of RNase-free water (NTC wells), template standard (STND wells) or unknown template (UNKN wells).

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.

The plate below shows a typical configuration for relative quantitation with SYBR Green reagents:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibrator	Calibrator	Calibrator	Calibrator	Sample 1	Sample 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2	Sample 2
B	ENDO1 Sample 3	ENDO1 Sample 3	ENDO1 Sample 3	ENDO1 Sample 3	ENDO1 Sample 4	ENDO1 Sample 4	ENDO1 Sample 4	ENDO1 Sample 4	ENDO1 Sample 5	ENDO1 Sample 5	ENDO1 Sample 5	ENDO1 Sample 5
C	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1
D	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1
E	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2
F	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2
G	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3
H	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3

In this configuration, endogenous controls are run for each sample in rows A and B and targets T1–T3 are run on rows C–H. Each row shows wells corresponding to a calibrator sample and up to five experimental samples. All wells are run in quadruplicate. The analysis of relative quantitation for a target template in samples 1–5 requires the following:

- ◆ The mean C_T value of the replicate wells run for each sample
- ◆ The difference (ΔC_T) between the mean C_T values of the samples in the target wells (T1–T3) and those of the endogenous controls in the ENDGNS wells
- ◆ The difference ($\Delta\Delta C_T$) between the ΔC_T values of the samples for each target and the mean C_T value of the Calibrator for that target

The relative quantitation value is expressed as $2^{-\Delta\Delta C_T}$.

A semilog plot of these values provides a graphical representation of the relative quantitation values obtained in this plate.

References

A

Faloona, F., Weiss, S., Ferre, F., and Mullis, K. 1990. Direct detection of HIV sequences in blood high-gain polymerase chain reaction [abstract]. In: *6th International Conference on AIDS*, University of California, San Francisco: San Francisco (CA). Abstract 1019.

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Longo, M.C., Berninger, M.S., and Hartley, J.L. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93:125–128.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.

Technical Support

B

Technical Support

Contacting Technical Support

You can contact Applied Biosystems for technical support by telephone or fax, by e-mail, or through the Internet. You can order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents 24 hours a day. In addition, you can download documents in PDF format from the Applied Biosystems Web site (please see the section "To Obtain Documents on Demand" following the telephone information below).

To Contact Technical Support by E-Mail

Contact technical support by e-mail for help in the following product areas:

Product Area	E-mail address
Genetic Analysis (DNA Sequencing)	galab@appliedbiosystems.com
Sequence Detection Systems and PCR	pcrlab@appliedbiosystems.com
Protein Sequencing, Peptide and DNA Synthesis	corelab@appliedbiosystems.com
◆ Biochromatography ◆ PerSeptive DNA, PNA and Peptide Synthesis systems ◆ FMAT™ 8100 HTS System ◆ CytoFluor® 4000 Fluorescence Plate Reader ◆ Voyager™ Mass Spectrometers ◆ Mariner™ Mass Spectrometers	tssupport@appliedbiosystems.com
LC/MS (Applied Biosystems/MDS Sciex)	support@sciex.com
Chemiluminescence (Tropix)	tropix@appliedbiosystems.com

Hours for Telephone Technical Support

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

Product	Hours
Chemiluminescence	8:30 a.m. to 5:30 p.m. Eastern Time
Framingham support	8:00 a.m. to 6:00 p.m. Eastern Time
All Other Products	5:30 a.m. to 5:00 p.m. Pacific Time

To Contact Technical Support by Telephone or Fax

In North America

To contact Applied Biosystems Technical Support, use the telephone or fax numbers given below. (To open a service call for other support needs, or in case of an emergency, dial **1-800-831-6844** and press **1**.)

Product or Product Area	Telephone Dial...	Fax Dial...
ABI PRISM® 3700 DNA Analyzer	1-800-831-6844 , then press 8	1-650-638-5981
DNA Synthesis	1-800-831-6844 , then press 2 , then 1	1-650-638-5981
Fluorescent DNA Sequencing	1-800-831-6844 , then press 2 , then 2	1-650-638-5981
Fluorescent Fragment Analysis (includes GeneScan® applications)	1-800-831-6844 , then press 2 , then 3	1-650-638-5981
Integrated Thermal Cyclers (ABI PRISM® 877 and Catalyst 800 instruments)	1-800-831-6844 , then press 2 , then 4	1-650-638-5981
ABI PRISM® 3100 Genetic Analyzer	1-800-831-6844 , then press 2 , then 6	1-650-638-5981
Peptide Synthesis (433 and 43X Systems)	1-800-831-6844 , then press 3 , then 1	1-650-638-5981
Protein Sequencing (Pro-cise® Protein Sequencing Systems)	1-800-831-6844 , then press 3 , then 2	1-650-638-5981

Product or Product Area	Telephone Dial...	Fax Dial...
PCR and Sequence Detection	1-800-762-4001 , then press 1 for PCR, 2 for the 7700, 7900 or 5700, 6 for the 6700 or dial 1-800-831-6844 , then press 5	1-240-453-4613
<ul style="list-style-type: none"> ◆ Voyager™ MALDI-TOF Biospectrometry ◆ Mariner™ ESI-TOF Mass Spectrometry Workstations 	1-800-899-5858 , then press 1, then 3	1-508-383-7855
Biochromatography (BioCAD® Workstations and POROS® Perfusion Chromatography Products)	1-800-899-5858 , then press 1, then 4	1-508-383-7855
Expedite™ Nucleic acid Synthesis Systems	1-800-899-5858 , then press 1, then 5	1-508-383-7855
Peptide Synthesis (Pioneer™ and 9050 Plus Peptide Synthesizers)	1-800-899-5858 , then press 1, then 5	1-508-383-7855
PNA Custom and Synthesis	1-800-899-5858 , then press 1, then 5	1-508-383-7855
<ul style="list-style-type: none"> ◆ FMAT™ 8100 HTS System ◆ Cytofluor® 4000 Fluorescence Plate Reader 	1-800-899-5858 , then press 1, then 6	1-508-383-7855
Chemiluminescence (Tropix)	1-800-542-2369 (U.S. only), or 1-781-271-0045	1-781-275-8581
LC/MS (Applied Biosystems/MDS Sciex)	1-800-952-4716	1-508-393-7899

Outside North America

Region	Telephone Dial...	Fax Dial...
Africa and the Middle East		

Region	Telephone Dial...	Fax Dial...
Africa (English Speaking) and West Asia (Fairlands, South Africa)	27 11 478 0411	27 11 478 0349
Africa (French Speaking; Courtaboeuf Cedex, France)	33 1 69 59 85 11	33 1 69 59 85 00
South Africa (Johannesburg)	27 11 478 0411	27 11 478 0349
Middle Eastern Countries and North Africa (Monza, Italia)	39 (0)39 8389 481	39 (0)39 8389 493

Eastern Asia, China, Oceania		
Australia (Scoresby, Victoria)	61 3 9730 8600	61 3 9730 8799
China (Beijing)	86 10 64106608 or 86 800 8100497	86 10 64106617
Hong Kong	852 2756 6928	852 2756 6968
India (New Delhi)	91 11 653 3743/3744	91 11 653 3138
Korea (Seoul)	82 2 593 6470/6471	82 2 593 6472
Malaysia (Petaling Jaya)	60 3 79588268	603 79549043
Singapore	65 896 2168	65 896 2147
Taiwan (Taipei Hsien)	886 2 2358 2838	886 2 2358 2839
Thailand (Bangkok)	66 2 719 6405	66 2 319 9788
Europe		
Austria (Wien)	43 (0)1 867 35 75 0	43 (0)1 867 35 75 11
Belgium	32 (0)2 532 4484	32 (0)2 582 1886
Czech Republic and Slovakia (Praha)	420 2 35365189	420 2 35364314
Denmark (Naerum)	45 45 58 60 00	45 45 58 60 01
Finland (Espoo)	358 (0)9 251 24 250	358 (0)9 251 24 243
France (Paris)	33 (0)1 69 59 85 85	33 (0)1 69 59 85 00
Germany (Weiterstadt)	49 (0) 6150 101 0	49 (0) 6150 101 101
Hungary (Budapest)	36 (0)1 270 8398	36 (0)1 270 8288
Italy (Milano)	39 (0)39 83891	39 (0)39 838 9492

Region	Telephone Dial...	Fax Dial...
Norway (Oslo)	47 23 12 06 05	47 23 12 05 75
Poland, Lithuania, Latvia, and Estonia (Warszawa)	48 (22) 866 40 10	48 (22) 866 40 20
Portugal (Lisboa)	351 (0)22 605 33 14	351 (0)22 605 33 15
Russia (Moskva)	7 502 935 8888	7 502 564 8787
South East Europe (Zagreb, Croatia)	385 1 34 91 927/838	385 1 34 91 840
Spain (Tres Cantos)	34 (0)91 806 1210	34 (0)91 806 1206
Sweden (Stockholm)	46 (0)8 619 4400	46 (0)8 619 4401
Switzerland (Rotkreuz)	41 (0)41 799 7777	41 (0)41 790 0676
The Netherlands (Nieuwerkerk a/d IJssel)	31 (0)180 392400	31 (0)180 392409 or 31 (0)180 392499
United Kingdom (Warrington, Cheshire)	44 (0)1925 825650	44 (0)1925 282502
All other countries not listed (Warrington, UK)	44 (0)1925 282481	44 (0)1925 282509
Japan		
Japan (Hacchobori, Chuo-Ku, Tokyo)	8120 477392 (Toll free within Japan) or 81 3 5566 6230	8120 477120 (Toll free within Japan) or 81 3 5566 6507
Latin America		
Caribbean countries, Mexico, and Central America	52 55 35 3610	52 55 66 2308
Brazil	0 800 704 9004 or 55 11 5070 9654	55 11 5070 9694/95
Argentina	800 666 0096	55 11 5070 9694/95
Chile	1230 020 9102	55 11 5070 9694/95
Uruguay	0004 055 654	55 11 5070 9694/95

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We strongly encourage you to visit our Web site for answers to frequently asked questions and for more information about our products. You can also order technical documents or an index of available documents and have them faxed or e-mailed to you through our site. The Applied Biosystems Web site address is

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2	Under the Troubleshooting heading, click Support Request Forms , then select the relevant support region for the product area of interest.
3	In the Personal Assistance form, enter the requested information and your question, then click Ask Us RIGHT NOW .
4	In the Customer Information form, enter the requested information and your question, then click Ask Us RIGHT NOW . Within 24 to 48 hours, you will receive an e-mail reply to your question from an Applied Biosystems technical expert.

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