

Power SYBR[®] Green PCR Master Mix and RT-PCR

Protocol

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Preface

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Safety


Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT**, **CAUTION**, **WARNING**, **DANGER**—implies a particular level of observation or action, as defined below:


IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

 **CAUTION** – Indicates a potentially hazardous situation that, 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 that, if not avoided, could result in death or serious injury.

 **DANGER** – Indicates an imminently hazardous situation that, 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.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About MSDSs” on page vi.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, 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 (for example, 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.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **<https://docs.appliedbiosystems.com/msdssearch.html>**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
 - **Open** – To view the document

- **Print Target** – To print the document
 - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
 5. After you enter the required information, click **View/Deliver Selected Documents Now**.

Chemical Waste Hazard



WARNING CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- 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.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, 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 (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- 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.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety



WARNING

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; <http://bmb1.od.nih.gov>)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html).

Additional information about biohazard guidelines is available at: <http://www.cdc.gov>

How to Obtain Support

For the latest services and support information for all locations, go to <http://www.appliedbiosystems.com>, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Overview This chapter describes the Power SYBR[®] Green PCR Master Mix and provides important information on PCR practices.

This chapter covers:

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Preventing Contamination and Nonspecific Amplification	1-8
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Purpose of the Kit

About the Kit The Power SYBR® Green PCR Master Mix is a convenient premix of the components (except primers, template, and water) necessary to perform real-time polymerase chain reaction (PCR) using SYBR® Green I dye with enhanced sensitivity and specificity. The SYBR Green dye binds to double-stranded (ds) DNA, thus providing a fluorescent signal that reflects the amount of dsDNA product generated during PCR.

You can perform One-Step or Two-Step RT-PCR using the Power SYBR® Green RT-PCR Reagents Kit (see [“Materials and Equipment” on page 1-4](#)).

In RNA quantitation assays, you use the Power SYBR Green PCR Master Mix in the second step of a Two-Step reverse-transcription polymerase chain reaction (RT-PCR) protocol. In a One-Step RT-PCR protocol, you add MultiScribe™ Reverse Transcriptase and RNase Inhibitor to the Power SYBR Green PCR Master Mix.

You can use the Power SYBR Green PCR Master Mix with the Applied Biosystems 7900HT Real-Time PCR System, the Applied Biosystems 7500 Real-Time PCR System, the Applied Biosystems 7300 Real-Time PCR System, the ABI PRISM® 7700 Sequence Detection System (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

Note: For optimal results, Applied Biosystems recommends using the 9600 Emulation mode. However, using the Standard (default) run mode with the Power SYBR Green PCR Master Mix provides comparable results. Refer to the troubleshooting section of the appropriate instrument user’s manual if you encounter poor performance.

Advantages of the Kit

The Power SYBR Green PCR Master Mix delivers highly sensitive nucleic acid quantitation, detecting as few as 1-10 copies of a target gene over a broad range of template concentrations. The master mix design also produces reliable DNA amplification results, with minimal lot to lot variation in assay performance (see [“Performance Characteristics of the Kit”](#) on page 1-5 for more information).

The proprietary master mix formulation contains a blend of dTTP/dUTP, which maintains optimal PCR results and compatibility with AmpErase[®] UNG treatment. In addition, the master mix includes a highly purified version of AmpliTaq Gold[®] DNA Polymerase, AmpliTaq Gold[®] DNA Polymerase, LD (Low DNA). The enzyme purification process minimizes non-specific, false positive DNA products due to potential bacterial DNA contamination during PCR. The enzyme is provided in an inactive state to automate the Hot Start PCR technique and allow flexibility in the reaction setup, including pre-mixing of PCR reagents at room temperature (see [“Preventing Contamination and Nonspecific Amplification”](#) on page 1-8 for more information).

About This Protocol

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

Materials and Equipment

Description of Master Mix

The Power SYBR[®] Green PCR Master Mix is supplied in a 2X concentration. The mix is optimized for SYBR[®] Green reagent reactions, and it contains:

- SYBR[®] Green I Dye
- AmpliTaq Gold[®] DNA Polymerase, LD
- dNTPs
- Passive reference
- Optimized buffer components

For Power SYBR[®] Green reagent-based real-time PCR and One-Step or Two-Step RT-PCR, the following components are available:

Item	Part Number	Contents
Power SYBR [®] Green PCR Master Mix: <ul style="list-style-type: none"> • Mini-Pack • 1-Pack • Bulk Pack • 2-Pack • 5-Pack • 10-Pack 	<ul style="list-style-type: none"> • 4368577 • 4367659 • 4367660 • 4368706 • 4368702 • 4368708 	<ul style="list-style-type: none"> • One 1 mL tube (40 × 50 µL reactions) • One 5 mL tube (200 × 50 µL reactions) • One 50 mL tube (2000 × 50 µL reactions) • 2 × 5 mL tubes (400 × 50 µL reactions) • 5 × 5 mL tubes (1000 × 50 µL reactions) • 10 × 5 mL tubes (2000 × 50 µL reactions)
Power SYBR [®] Green RT-PCR Reagents Kit	4368711	<ul style="list-style-type: none"> • Power SYBR[®] Green PCR Master Mix (200 × 50 µL reactions) • TaqMan[®] Reverse Transcription Reagents[‡] (200 × 10 µL reactions)
Related Documentation: <ul style="list-style-type: none"> • Protocol • Quick Reference Card 	<ul style="list-style-type: none"> • 4367218 • 4367219 	<ul style="list-style-type: none"> • — • —

[‡] The TaqMan[®] Reverse Transcription Reagents contains the components required to perform RT reactions; it does not contain TaqMan[®] probes.

Performance Characteristics of the Kit

The performance criteria listed in the following table are verified against the β -actin gene sequence in 10-50 μ L total reaction volume for each manufactured lot of Power SYBR Green PCR Master Mix.

Performance Specification	Metric
High sensitivity (requires low sample input)	≤ 10 copies detected per well
Wide dynamic range (provides accurate DNA quantitation)	≥ 5 orders of magnitude dynamic range
Consistent lot to lot reproducibility (produces reliable results)	± 1.0 fluorescence threshold cycle (C_T) lot variation

Storage and Stability

Upon receipt, store the Power SYBR Green PCR Master Mix at 2 °C to 8 °C for short term storage (less than 6 months), or at -15 °C to -25 °C for long term storage (more than 6 months). Store the TaqMan Reverse Transcription Reagents at -15 °C to -25 °C.

Note: If stored under the recommended conditions, Applied Biosystems guarantees product performance through the expiration date (control date) printed on the label. However the kit components are most stable long term at -15 °C to -25 °C.

Materials Required but Not Supplied

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

User-Supplied Materials

Item	Source
AmpErase [®] uracil-N-glycosylase (UNG)	Applied Biosystems (PN N808-0096)
<ul style="list-style-type: none"> • Applied Biosystems 7900HT Real-Time PCR System • Applied Biosystems 7500 Real-Time PCR System • Applied Biosystems 7300 Real-Time PCR System • ABI PRISM[®] 7700 Sequence Detection System (SDS) • ABI PRISM[®] 7000 SDS • GeneAmp[®] 5700 SDS 	Contact your Applied Biosystems representative for the instrument best suited to meet your needs
Applied Biosystems 7900HT Real-Time PCR System 96-well Spectral Calibration Kit	Applied Biosystems (PN 4328639)
ABI PRISM [®] 7000 Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (PN 4328895)
Sequence Detection Systems Spectral Calibration Kit (for 7700 instrument only)	Applied Biosystems (PN 4305822)
Sequence Detection Systems 384-Well Spectral Calibration Kit	Applied Biosystems (PN 4323977)
Primer Express [®] Software	Contact your Applied Biosystems representative
<i>ABI PRISM[®] 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression</i>	Applied Biosystems (PN 4303859)
ABI PRISM [®] 384-Well Clear Optical Reaction Plate with Barcode	Applied Biosystems (PN 4309849)

User-Supplied Materials (continued)

Item	Source
MicroAmp® Optical 96-well Reaction Plate Note: The MicroAmp Optical 96-well Reaction Plate may be sealed with: <ul style="list-style-type: none"> • MicroAmp® Optical Caps or • ABI PRISM® Optical Adhesive Cover 	Applied Biosystems (PN N801-0560)
MicroAmp® Optical 96-well Reaction Plate and Optical Caps	Applied Biosystems (PN 403012)
ABI PRISM® Optical Adhesive Cover Starter Pack containing 20 optical adhesive covers, one applicator, and one compression pad	Applied Biosystems (PN 4313663)
MicroAmp® Optical Caps	Applied Biosystems (PN 4323032)
ABI PRISM® Cap Installing Tool	Applied Biosystems (PN 4330015)
MicroAmp® 96-well Tray/Retainer Set (10 sets)	Applied Biosystems (PN 403081)
MicroAmp® Optical Tubes	Applied Biosystems (PN N801-0933)
<ul style="list-style-type: none"> • Centrifuge with adapter for 96-well plates • Centrifuge with adapter for 384-well plates 	<ul style="list-style-type: none"> • Major laboratory supplier (MLS) • MLS
Disposable gloves	MLS
Microcentrifuge	MLS
NuSieve 4% (3:1) agarose gels, for DNA <1 kb	FMC BioProducts (PN 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

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 Dye detection, any double stranded DNA will be detected. Therefore, Applied Biosystems recommends that you check for nonspecific product formation by dissociation curve or gel analysis.

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

Hot Start PCR The Hot Start technique (Faloona *et al.*, 1990) improves PCR specificity and sensitivity by controlling mispriming events. Hot Start PCR is a simple modification of the original PCR process in which the amplification reaction is started at an elevated temperature.

This technique can be performed manually, by adding an essential component of the reaction to the reaction mixture only after that mixture is heated to an elevated temperature. However, this approach is often cumbersome and time consuming, especially when using large numbers of samples.

AmpliTaq Gold DNA Polymerase, LD

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.

The AmpliTaq Gold[®] DNA Polymerase, LD (Low DNA) enzyme is identical to AmpliTaq Gold DNA Polymerase, but the enzyme is further purified through a proprietary process to reduce bacterial DNA introduced from the host. The purification process ensures that non-specific, false positive DNA products due to DNA contamination are minimized during PCR.

False Positives

Special laboratory practices are necessary in order to avoid false positive amplifications (Kwok and Higuchi, 1989), due to the capability for single DNA molecule amplification provided by the PCR process (Saiki *et al.*, 1985; Mullis and Faloona, 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 samples with high DNA levels or positive control templates.

When dUTP replaces dTTP as a dNTP substrate in PCR and you use the method described below, AmpErase UNG treatment can prevent the reamplification of carryover PCR products in subsequent experiments. This method uses enzymatic and chemical reactions analogous to cellular restriction-modification and excision-repair systems to specifically degrade PCR products from previous PCR amplifications or mis-primed, non-specific products produced prior to specific amplifications. This method does 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).

AmpErase[®] uracil-N-glycosylase (UNG) 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 (Lawyer *et al.*, 1989).

Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried-over PCR products, Applied Biosystems encourages users to continue using the specific devices and suggestions described in this protocol and in Kwok (1990) and Kwok 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. The Power SYBR Green PCR Master Mix contains a dUTP/dTTP ratio optimized for use with UNG. If PCR carryover contamination is suspected, UNG should be used to troubleshoot the problem. UNG can be purchased individually from Applied Biosystems (PN N808-0096).

Fluorescent Contaminants

Since fluorescent contaminants can interfere with SYBR Green I Dye assays and give false-positive results, it may be necessary to include a No Amplification Control (NAC) tube that contains sample, but no enzyme. If the absolute fluorescence of the NAC is greater than that of the No Template Control (NTC) 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 farther a dA nucleotide is from the 3' end, the more likely 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, consider using primers with 3' terminal dU-nucleotides. 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 depend on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination expected. In most cases, using AmpErase UNG at 1 U/100 μ L 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 and agarose gel electrophoresis 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, due to a dye or probe interacting with double-stranded DNA, is plotted against temperature.

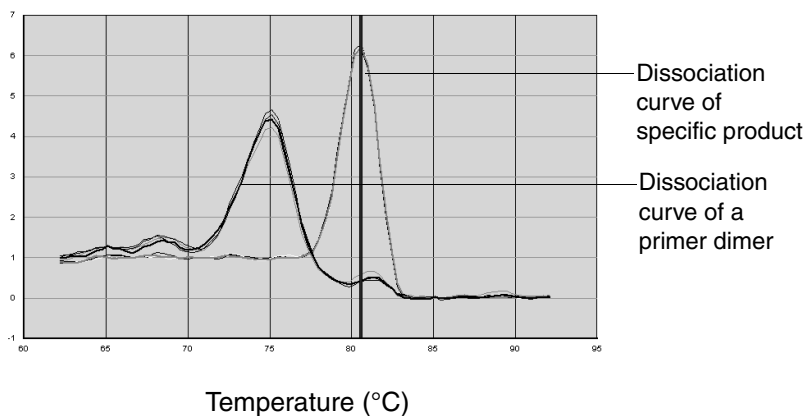
Using Dissociation Curves

General Information

The following systems enable you to run dissociation curves to detect nonspecific amplification through computer software:

- Applied Biosystems 7900HT Real-Time PCR System
- Applied Biosystems 7500 Real-Time PCR System
- Applied Biosystems 7300 Real-Time PCR System
- ABI PRISM[®] 7700 SDS
- ABI PRISM[®] 7000 SDS
- GeneAmp[®] 5700 SDS

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 NTC wells and sample wells containing low concentrations of template.

When to Generate Dissociation Curves

You can set up the Applied Biosystems 7900HT Real-Time PCR System, the Applied Biosystems 7500 Real-Time PCR System, the Applied Biosystems 7300 Real-Time PCR System, the ABI PRISM[®] 7700 SDS, the ABI PRISM[®] 7000 SDS, and the GeneAmp[®] 5700 SDS 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 instrument 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

Confirm the absence of nonspecific amplification 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>i.e.</i> , 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.

To check PCR product purity with agarose gels: *(continued)*

Step	Action
2.	Run the gel: <ul style="list-style-type: none">• 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.

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

This chapter covers:

Designing Custom Target Sequences for Quantitation2-2

Amplifying Custom Target Sequences for Quantitation2-4

Designing Custom Target Sequences for Quantitation

Overview 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, a plasmid containing the nucleotide sequence of interest, genomic DNA, cDNA, or RNA.

Design primers to amplify short segments of a target (DNA, cDNA, or RNA) within the target sequence. These short segments are called amplicons. Shorter amplicons work most efficiently, 50- to 150-bp sequences yielding the most consistent results.

Designing Primers

Design primers using Primer Express Software as described in the *Primer Express[®] Version 3.0 Getting Started Guide* (PN 4362460).

Note: For more information on design guidelines, refer to the *Primer Express[®] Software Version 3.0 Online Help*.

General Guidelines

- Do not overlap primer and probe sequences. The optimal primer length is 20 bases.
- Keep the GC content in the 30 to 80% range.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- Keep the T_m between 58 to 60 °C.
- Make sure the last five nucleotides at the 3' end contain no more than two G and/or C bases.

If the template is...	Then...
DNA	Design the primers as described above.
plasmid DNA	
genomic DNA	
cDNA	Design the primers as described above. Also see “Selecting an Amplicon Site for cDNA” on page 2-3 below.
RNA	Design the primers as described above.

Selecting an Amplicon Site for cDNA

Overview

Selecting a good amplicon site ensures amplification of the target mRNA without co-amplifying the genomic sequence, pseudogenes, and related genes.

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; the primer pair does not amplify pseudogenes or other related genes.
- Design primers following Primer Express Software 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 to 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: <ul style="list-style-type: none"> • PCR • One-Step RT-PCR • Two-Step RT-PCR 	4-3 4-9 4-13

Ordering Reagents See “[Materials Required but Not Supplied](#)” on page 1-6 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

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

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

Reverse Transcription

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

This chapter covers:

Reverse Transcription for All Amplicons Except 18S3-2

Reverse Transcription for the 18S Amplicon3-5

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 prime total RNA samples for RT using Multiscribe[™] Reverse Transcriptase.

Note: The TaqMan Reverse Transcription Reagents contains the components required to perform RT reactions; it does not contain TaqMan[®] probes.

Guidelines Follow the guidelines below to ensure optimal RT performance.

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

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	<ul style="list-style-type: none"> • 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	<ul style="list-style-type: none"> • Use to reverse transcribe RNA-containing complementary sequences only
Oligo d(T) ₁₆	<ul style="list-style-type: none"> • 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**

Component	Volume/Tube (μL)	Final Concentration
RNase-free water	Variable [‡]	—
10X 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 [§] (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 [#]	—

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

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

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

The RT reaction volume can vary from 10 μL to 100 μL. Increasing the RT reaction volume will reduce the total number of reactions.

Thermal Cycling Parameters for RT Reactions

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

[‡] 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



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 using the TaqMan Reverse Transcription Reagents:

Step	Action
1.	Prepare the RT Reaction Mix by combining all the nonenzymatic components.
2.	Vortex briefly.
3.	Add the enzymatic components (for example, 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.
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- μ L RT reaction)
Human Total RNA	60 ng to 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 RT. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents.

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

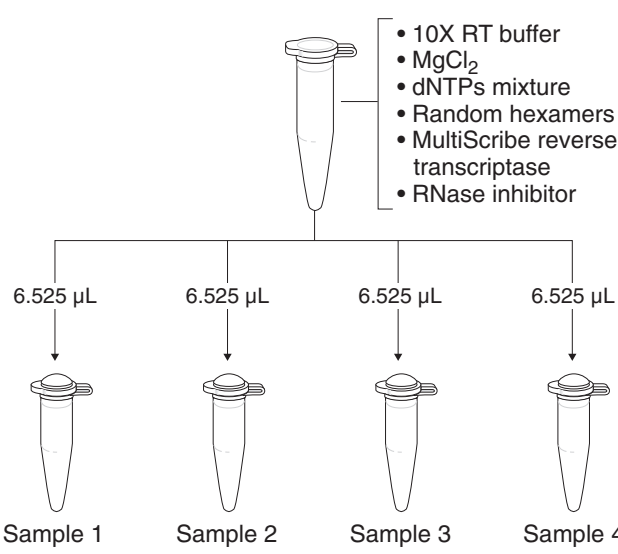


CAUTION CHEMICAL HAZARD. TaqMan Reverse Transcription Reagents may cause eye and skin irritation. They may cause discomfort if swallowed or inhaled. Always use adequate ventilation such as that provided by a fume hood. Please read the MSDS, and follow the handling instructions. Wear appropriate protective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

To prepare the RT reactions for 18S amplicon:

Step	Action																																						
1.	<p>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.</p> <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>Variable[‡]</td> <td>Variable[‡]</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[§]</td> <td>6.525</td> <td>26.1</td> <td>—</td> </tr> </tbody> </table> <p>[‡] The volume of RNase-free water (μL) will be 3.475–RNA sample volume in a 10-μL reaction.</p> <p>[§] If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.</p>	Component	Volume (μL)		Final Conc.	Per Sample	Reaction Mix (x4)	RNase-free water	Variable [‡]	Variable [‡]	—	10X RT Buffer	1.0	4.0	1X	25 mM MgCl_2	2.2	8.8	5.5 mM	deoxyNTPs Mixture (2.5 mM)	2.0	8.0	500 μM per dNTP	Random Hexamers (50 μM)	0.5	2.0	2.5 μM	RNase Inhibitor (20 U/ μL)	0.2	0.8	0.4 U/ μL	MultiScribe Reverse Transcriptase (50 U/ μL)	0.625	2.5	3.125 U/ μL	Total[§]	6.525	26.1	—
Component	Volume (μL)		Final Conc.																																				
	Per Sample	Reaction Mix (x4)																																					
RNase-free water	Variable [‡]	Variable [‡]	—																																				
10X RT Buffer	1.0	4.0	1X																																				
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deoxyNTPs Mixture (2.5 mM)	2.0	8.0	500 μM per dNTP																																				
Random Hexamers (50 μM)	0.5	2.0	2.5 μM																																				
RNase Inhibitor (20 U/ μL)	0.2	0.8	0.4 U/ μL																																				
MultiScribe Reverse Transcriptase (50 U/ μL)	0.625	2.5	3.125 U/ μL																																				
Total[§]	6.525	26.1	—																																				
2.	Label four 0.2-mL microcentrifuge tubes for the four test samples.																																						
3.	Transfer 60 ng to 2 μg (up to 3.475 μL) of each total RNA sample to the corresponding microcentrifuge tube.																																						

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

Step	Action
4.	If necessary, dilute each total RNA sample to a volume of 3.475 μL with RNase-free, deionized water.
5.	Cap the tubes and gently tap each to mix the diluted samples.
6.	Centrifuge the tubes briefly to eliminate air bubbles in the mixture.
7.	Label four 0.2-mL MicroAmp [®] Optical Tubes for the four total RNA test samples.
8.	<p>Pipet 6.525 μL of the reaction mix (from step 1) to each MicroAmp Optical Tube (from step 7).</p>  <p>The diagram illustrates the process of adding 6.525 μL of reaction mix to four separate MicroAmp Optical Tubes labeled Sample 1, Sample 2, Sample 3, and Sample 4. A central pipette tip is shown dispensing the mix into each tube. A legend lists the components of the reaction mix: 10X RT buffer, MgCl_2, dNTPs mixture, Random hexamers, MultiScribe reverse transcriptase, and RNase inhibitor.</p>
9.	Transfer 3.475 μL of each dilute total RNA sample to the corresponding MicroAmp Optical Tube (see step 4).
10.	Cap the reaction tubes and tap each gently to mix the reactions.

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

Step	Action
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 <ul style="list-style-type: none"> • MicroAmp Optical Tubes <i>or</i> • 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:

Step	Action																				
1.	Load the reactions into a thermal cycler.																				
2.	Program your thermal cycler with the following conditions: <table border="1" data-bbox="553 1119 1239 1466"> <thead> <tr> <th>Step</th> <th>Hexamer Incubation[‡]</th> <th>RT</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">10 µL</td> </tr> </tbody> </table> <p>‡ When using random hexamers for first-strand cDNA synthesis, include a primer incubation step (25 °C for 10 min) to maximize primer-RNA template binding.</p>	Step	Hexamer Incubation [‡]	RT	Reverse Transcriptase Inactivation		HOLD	HOLD	HOLD	Temp	25 °C	37 °C	95 °C	Time	10 min	60 min	5 min	Volume	10 µL		
Step	Hexamer Incubation [‡]	RT	Reverse Transcriptase Inactivation																		
	HOLD	HOLD	HOLD																		
Temp	25 °C	37 °C	95 °C																		
Time	10 min	60 min	5 min																		
Volume	10 µL																				

To conduct RT thermal cycling: *(continued)*

Step	Action
3.	Begin RT. IMPORTANT! After thermal cycling, store all cDNA samples at -15 to -25 °C.

Optimizing Primer Concentrations

4

Overview This chapter describes how to optimize primer concentrations for PCR, One-Step RT-PCR, and Two-Step RT-PCR.

This chapter covers:

Optimizing Primer Concentrations for PCR4-2

Optimizing Primer Concentrations for One-Step RT-PCR4-7

Optimizing Primer Concentrations for Two-Step RT-PCR4-12

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 μ L. Use 10 to 100 ng of genomic DNA or 1 to 10 ng of cDNA template.

Use PCR Master Mix 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 4-4.

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 4-5.
2.	<p>Place the plate in the appropriate instrument.</p> <p>Use the thermal cycling conditions in “Thermal Cycling Parameters for Primer Optimization” on page 4-6.</p> <p>Note: For optimal results, Applied Biosystems recommends using the 9600 Emulation mode. However, using the Standard (default) run mode with the Power SYBR[®] Green PCR Master Mix provides comparable results. Refer to the troubleshooting section of the appropriate instrument user’s manual if you encounter poor performance.</p> <p>Note: SYBR[®] Green dye must be calibrated on the instrument. Please refer to the appropriate instrument user’s manual to calibrate the instrument with SYBR Green reagents.</p>
3.	<p>At the end of the run:</p> <ul style="list-style-type: none"> • Tabulate the results for the yield. This analysis identifies the optimum concentrations of primers for PCR yield. • Tabulate the results for the C_T value. This analysis identifies the optimum primer concentrations for C_T and for the absence of nonspecific amplification.

PCR Master Mix for Primer Optimization



WARNING CHEMICAL HAZARD. Power SYBR Green PCR Master Mix is a combustible liquid and vapor (keep away from heat and flame). It may cause eye, skin, and respiratory tract irritation. 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
Power SYBR Green PCR Master Mix (2X)	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
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® Polymerase 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, LD.

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 Applied Biosystems 7900HT Real-Time PCR System, the Applied Biosystems 7500 Real-Time PCR System, the Applied Biosystems 7300 Real-Time PCR System, the ABI PRISM® 7700 SDS, the ABI PRISM® 7000 SDS, or the GeneAmp® 5700 SDS.

Optimizing Primer Concentrations for One-Step RT-PCR

Overview Use One-Step RT-PCR Master Mix 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 4-9.

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. The AmpliTaq Gold[®] DNA Polymerase, LD 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 Power 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 4-10.
2.	<p>Place the plate in the instrument.</p> <p>Use the thermal cycling conditions in “Thermal Cycling Parameters for Primer Optimization” on page 4-11.</p> <p>Note: For optimal results, Applied Biosystems recommends using the 9600 Emulation mode. However, using the Standard (default) run mode with the Power SYBR Green PCR Master Mix provides comparable results. Refer to the troubleshooting section of the appropriate instrument user’s manual if you encounter poor performance.</p> <p>Note: SYBR[®] Green dye must be calibrated on the instrument. Please refer to the appropriate instrument user’s manual on how to calibrate the instrument for SYBR Green reagents.</p>
3.	<p>At the end of the run:</p> <ul style="list-style-type: none"> • Tabulate the results for the yield. This analysis identifies the optimum concentrations of primers for PCR yield. • Tabulate the results for the C_T value. This analysis identifies 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. Power SYBR Green PCR Master Mix is a combustible liquid and vapor (keep away from heat and flame). It may cause eye, skin, and respiratory tract irritation. 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.
Power SYBR Green PCR Master Mix (2X)	25	2500	1X
Reverse Transcription Reagents: <ul style="list-style-type: none"> • MultiScribe™ Reverse Transcriptase (50 U/μL) • RNase Inhibitor (20 U/μL) 	<ul style="list-style-type: none"> • 0.25 • 1.0 	<ul style="list-style-type: none"> • 25 • 100 	<ul style="list-style-type: none"> • 0.25 U/μL • 0.4 U/μL
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) [‡]	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

[‡] 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® Polymerase Activation	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)			

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

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 Applied Biosystems 7900HT Real-Time PCR System, the Applied Biosystems 7500 Real-Time PCR System, the Applied Biosystems 7300 Real-Time PCR System, the ABI PRISM® 7700 SDS, the ABI PRISM® 7000 SDS, or the GeneAmp® 5700 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 μ L. Use 10 to 100 ng of genomic DNA or 1 to 10 ng of cDNA template.

Use Two-Step RT-PCR Master Mix to run four replicates of each of the nine conditions shown in the table below. The master mix is described in [“Two-Step RT-PCR Master Mix for Primer Optimization”](#) on page 4-14.

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.	<p>Load the plate for both a template and a No Template Control (NTC) matrix.</p> <p>Refer to “Plate Configuration for Primer Optimization for Two-Step RT-PCR” on page 4-15.</p>
2.	<p>Place the plate in the appropriate instrument.</p> <p>Use the thermal cycling conditions in “Thermal Cycling Parameters for Primer Optimization” on page 4-16.</p> <p>Note: For optimal results, Applied Biosystems recommends using the 9600 Emulation mode. However, using the Standard (default) run mode with the Power SYBR[®] Green PCR Master Mix provides comparable results. Refer to the troubleshooting section of the appropriate instrument user’s manual if you encounter poor performance.</p> <p>Note: SYBR[®] Green dye 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 reagents.</p>
3.	<p>At the end of the run:</p> <ul style="list-style-type: none"> • Tabulate the results for the yield. This analysis identifies the optimum concentrations of primers for PCR yield. • Tabulate the results for the C_T value. This analysis identifies 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. Power SYBR Green PCR Master Mix is a combustible liquid and vapor (keep away from heat and flame). It may cause eye, skin, and respiratory tract irritation. 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 (μ L) for One 50- μ L Reaction	Volume (μ L) for 100 50- μ L Reactions	Final Concentration
Power SYBR Green PCR Master Mix (2X)	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 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
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® Polymerase 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, LD.

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 Applied Biosystems 7900HT Real-Time PCR System, the Applied Biosystems 7500 Real-Time PCR System, the Applied Biosystems 7300 Real-Time PCR System, the ABI PRISM® 7700 SDS, the ABI PRISM® 7000 SDS, or the GeneAmp® 5700 SDS.

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

This chapter covers:

Absolute and Relative Quantitation of Target DNA5-2

Interpreting the Results5-4

Absolute and Relative Quantitation of Target DNA

Overview Two types of quantitation are possible when using the Power 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.

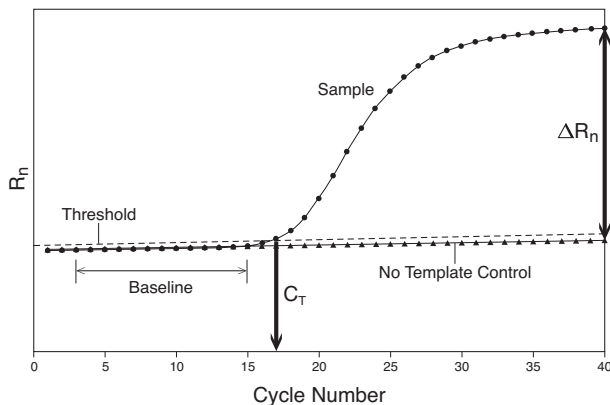
All quantitations are also normalized to an endogenous control such as 18S rRNA to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. All amplicons in these determinations should follow the amplicon 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* (PN 4303859) for additional information about relative quantitation.

Passive Reference ROX The Passive Reference (ROX[™] dye) is a dye molecule, included in the Power SYBR Green PCR Master Mix, that does not participate in the PCR amplification. On the Applied Biosystems 7900HT Real-Time PCR System, the Applied Biosystems 7500 Real-Time PCR System, the Applied Biosystems 7300 Real-Time PCR System, the ABI PRISM[®] 7700 SDS, the ABI PRISM[®] 7000 SDS, and the GeneAmp[®] 5700 SDS, the Passive Reference provides an internal reference to which the SYBR[®] Green dye/dsDNA complex signal can be normalized during data analysis. Normalization is necessary to correct for well-to-well fluorescent fluctuations.

Terms Used in Quantitation Analysis

Term	Definition
Baseline	The initial cycles of PCR in which there is little change in fluorescence signal.
Threshold	A level of ΔR_n —automatically determined (or manually set) by the SDS software—used for C_T determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the C_T .
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.
Reporter dye	The dye used to detect the PCR product. The Power SYBR Green PCR Master Mix uses SYBR® Green I Dye to provide a fluorescent signal that reflects the amount of PCR product.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by the specified set of PCR conditions ($\Delta R_n = R_n - \text{baseline}$).

The figure below shows a representative amplification plot that includes some of the terms defined on [page 5-3](#).



Interpreting the Results

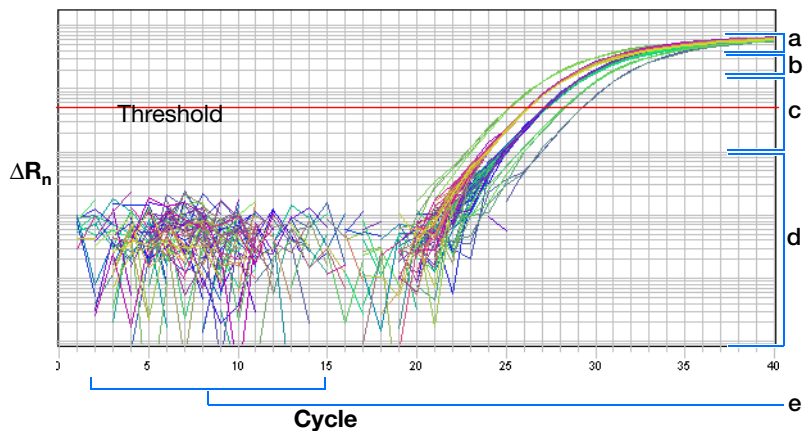
Adjusting the Baseline and Threshold

Automatic Baseline and Threshold Determination

The SDS software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the “typical” amplification curve.

A typical amplification curve, as shown on [page 5-5](#), has a:

- Plateau phase (a)
- Linear phase (b)
- Exponential (geometric phase) (c)
- Background (d)
- Baseline (e)



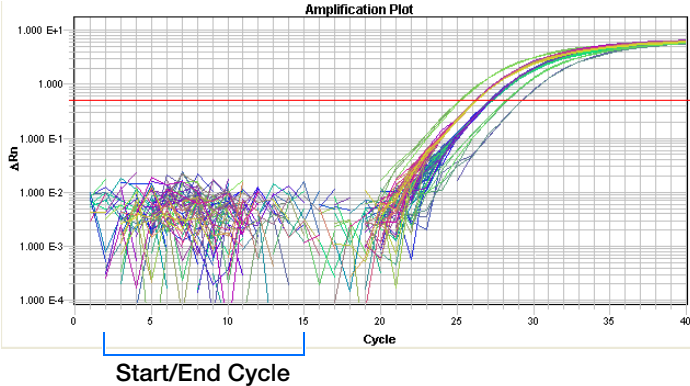
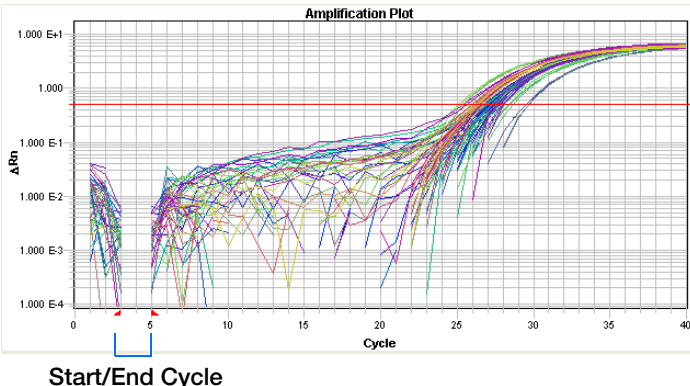
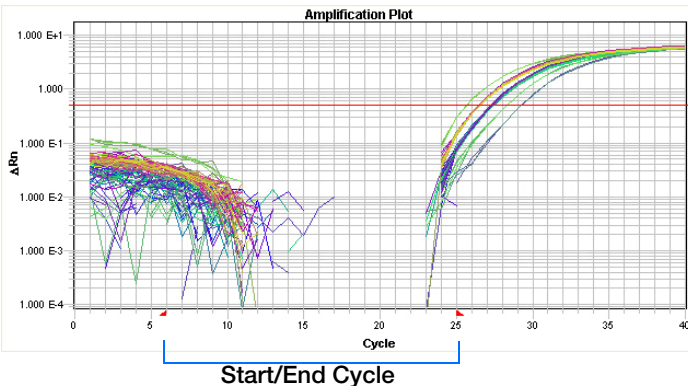
Manually Setting Baseline and Threshold Settings

Experimental error (such as contamination, pipetting errors, and so on) can produce data that deviate significantly from data for typical amplification curves. Such atypical data cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

Therefore, Applied Biosystems recommends reviewing all baseline and threshold values after analysis of the study data. If necessary, adjust the values manually as described in the appropriate instrument user manual.

IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots.

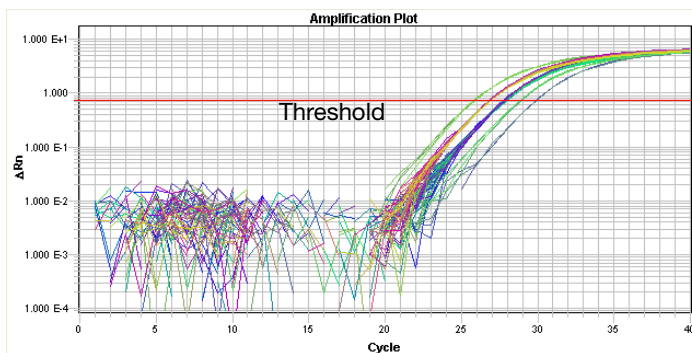
See the sample amplification plots below to determine whether the baseline and threshold settings were correctly set.

<p>Baseline Set Correctly</p> <p>The amplification curve begins after the maximum baseline. No adjustment necessary.</p>	
<p>Baseline Set Too Low</p> <p>The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.</p>	
<p>Baseline Set Too High</p> <p>The amplification curve begins before the maximum baseline. Decrease the End Cycle value.</p>	

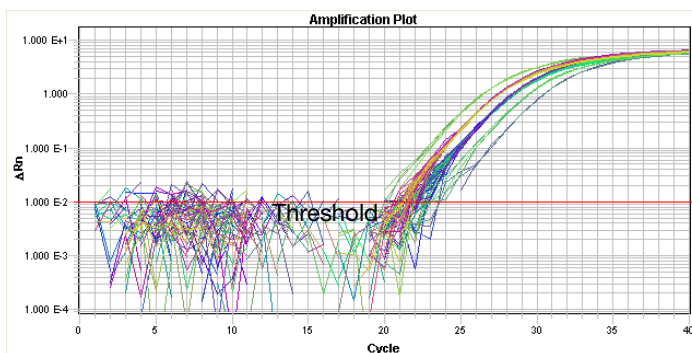
Threshold Set Correctly

The threshold is set in the exponential phase of the amplification curve.

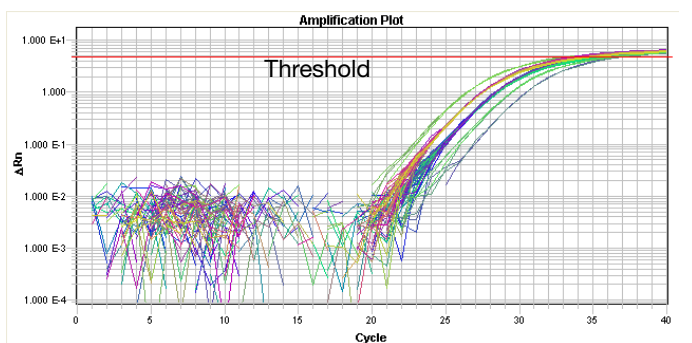
Threshold settings above or below the optimum increase the standard deviation of the replicate groups.

**Threshold Set Too Low**

The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Set the threshold up into the exponential phase of the curve.

**Threshold Set Too High**

The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Set the threshold down into the exponential phase of the curve.



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