Fast SYBR® Green Master Mix

Protocol

Applied Biosystems

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Contents

Preface

This preface contains:

Safety

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.

Definitions

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 (MSDSs) 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 in 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

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
   a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.

b. Select the language of your choice.

c. Click **Search**.

3. To view, download, or print the document of interest:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the document
      - **Save Target As** – To download a PDF version of the document to a destination that you choose
      - **Print Target** – To print the document
4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:
   a. Select **Fax** or **Email** below the document title.
   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
   c. Enter the required information.
   d. Click **View/Deliver Selected Documents Now**.

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical Waste Hazards

CAUTION **HAZARDOUS WASTE.** Refer to Material Safety Data Sheets and local regulations for handling and disposal.

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

WARNING **CHEMICAL STORAGE HAZARD.** Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

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

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, 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 equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories*
  http://bmbl.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**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

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.

Introduction 1

Overview

This chapter describes the Fast SYBR® Green Master Mix and provides important information on PCR practices.

Purpose of the Kit

About the Kit

Fast SYBR® Green Master Mix, supplied in a 2× concentration, is a convenient premix to perform real-time PCR using SYBR® Green I dye. The master mix contains:

- SYBR® Green I Dye
- AmpliTaq® Fast DNA Polymerase, UP (Ultra Pure)
- Uracil-DNA Glycosylase (UDG)
- ROX™ dye Passive Reference
- dNTPs
- Optimized buffer components

Primers, template, and water are the only components that you provide.

UDG

Fast SYBR® Green Master Mix contains uracil-DNA glycosylase (UDG). UDG is also known as uracil-N-glycosylase (UNG).

UDG treatment can prevent the reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded amplicons (Longo *et al*., 1990). UDG prevents reamplification of carryover PCR products in an assay if all previous PCR for that assay was performed using a dUTP-containing master mix. See “Prevent Contamination and Nonspecific Amplification” on page 1-10 for more information about UDG.

ROX Passive Reference

Fast SYBR® Green Master Mix contains ROX™ dye Passive Reference. The ROX™ dye Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations due to changes in concentration or volume.

Real-Time Instruments

Fast SYBR® Green Master Mix can be used to run experiments on the following Applied Biosystems Real-Time PCR Systems:

- Applied Biosystems StepOne™ Real-Time PCR System
- Applied Biosystems StepOnePlus™ Real-Time PCR System
- Applied Biosystems 7500 Fast Real-Time PCR System
- Applied Biosystems 7900HT Fast Real-Time PCR System

About This Protocol

This protocol provides:

- Background information about Fast gene quantification assays
- A list of equipment and materials for using the Fast SYBR® Green Master Mix
- Procedures for using the Fast SYBR® Green Master Mix

For details about specific procedures described in this protocol, see "Applied Biosystems Documents" on page 1-9.

Chemistry Overview

How the SYBR Green I Dye Chemistry Works

The SYBR Green I dye chemistry uses the SYBR Green I dye to detect PCR products by binding to double-stranded DNA formed during PCR. The process works as follows:

1. When Fast SYBR® Green Master Mix is added to a sample, SYBR Green I dye immediately binds to all double-stranded DNA.
2. During the PCR, AmpliTaq® Fast DNA Polymerase, UP amplifies the target sequence, which creates the PCR product, or "amplicon."
3. The SYBR Green I dye then binds to each new copy of double-stranded DNA.
4. As the PCR progresses, more amplicon is created.

   Because the SYBR Green I dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportional to the amount of double-stranded PCR product produced.

   The figure below illustrates this process.

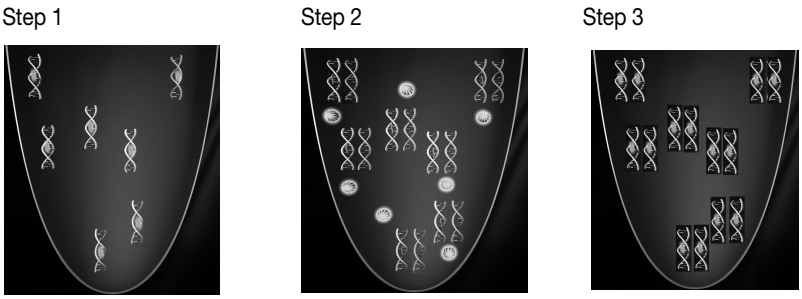


Figure 1-1 Representation of how the SYBR Green I dye acts on double-stranded DNA during one extension phase of PCR

Using the Master Mix in Two-Step RT-PCR

When performing a two-step RT-PCR reaction, total or mRNA must first be transcribed into cDNA:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the High-Capacity cDNA Reverse Transcription Kit.
2. In the PCR step, PCR products are synthesized from cDNA samples using the Fast SYBR® Green Master Mix.

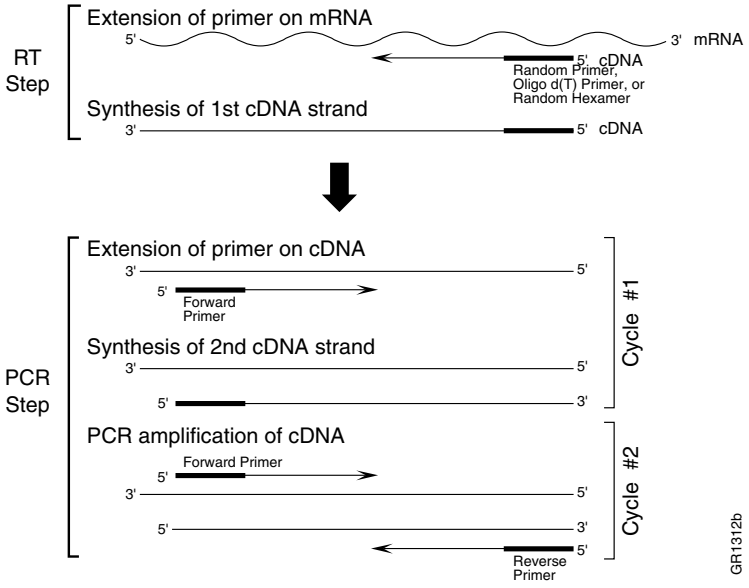


Figure 1-2 Two-step RT-PCR

Contents and Storage

Contents

The Fast SYBR® Green Master Mix is supplied in a 2× concentration.

Item	Part Number	Contents
Mini-Pack	4385610	One 1-mL tube (100 × 20-µL reactions)
1-Pack	4385612	One 5-mL tube (500 × 20-µL reactions)
2-Pack	4385616	2 × 5-mL tubes (1000 × 20-µL reactions)
5-Pack	4385617	5 × 5-mL tubes (2500 × 20-µL reactions)
10-Pack	4385618	10 × 5-mL tubes (5000 × 20-µL reactions)
Bulk Pack	4385614	One 50-mL tube (5000 × 20-µL reactions)

Storage

Store the Fast SYBR Green Master Mix at −20 °C.

Note: After the first thaw, Applied Biosystems recommends storing the Fast SYBR® Green Master Mix at 4 °C.

Materials Required but Not Supplied

Plates

Choose the plate appropriate for your real-time instrument.

Instrument	Plates‡	Part Number
StepOne™ system	MicroAmp™ Fast Optical 48-Well Reaction Plate, 20 plates	4375816
StepOnePlus™ 7500 Fast 7900 HT Fast§ systems	MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (0.1-mL)	
	• 20 plates	4346906
	• 200 plates	4366932
7900 HT 384-well	MicroAmp™ Optical 384-Well Reaction Plate with Barcode	
	• 50 plates	4309849
	• 500 plates	4326270
	• 1000 plates	4343814
	MicroAmp™ Optical 384-Well Reaction Plate, 1000 plates	4343370

‡ Seal all plates, except StepOne™ system plates, with MicroAmp Optical Adhesive Film (PN 4360954). Seal StepOne system plates with MicroAmp 48-Well Optical Adhesive Film (PN 4375323).

§ Requires a MicroAmp Snap-On Optical Film Compression Pad (PN 4333292).

Other Kits

Item	Source
Applied Biosystems High Capacity cDNA Reverse Transcription Kit:	
• 200 reactions	4368814
• 200 reactions with RNase Inhibitor	4374966
• 1000 reactions	4368813
• 1000 reactions with RNase Inhibitor	4374967

Other Consumables

Item	Source
Centrifuge with adapter for 96-well plates *or* Centrifuge with adapter for 384-well plates	Major laboratory supplier (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

Applied Biosystems Documents

You can download the following documents from:
http://docs.appliedbiosystems.com/search.taf

Document	Part Number
All Systems	
High-Capacity cDNA Reverse Transcription Kit Protocol	4375575
Primer Express® Software Version 3.0 Getting Started Guide	4362460
Real-Time PCR Systems Chemistry Guide	4348358
StepOne and StepOnePlus Systems	
Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T ($\Delta\Delta C_T$) Experiments	4376785
Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Standard Curve Experiments	4376784
Applied Biosystems StepOne™ Real-Time PCR System Installation, Maintenance, and Networking Guide	4376782
7500 Fast System	
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Getting Started Guide	4347828
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825
7900HT System	
Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide	4351684
Applied Biosystems 7900HT Fast Real-Time PCR System User Bulletin: Performing Fast Gene Quantification	4352533

Prevent Contamination and Nonspecific Amplification

Overview

PCR assays require special laboratory practices to avoid false positive amplifications. The high throughput and repetition of these assays can lead to amplification of a single DNA molecule.

Using UDG to Minimize Reamplification Carryover Products

Fast SYBR Green Master Mix contains uracil-DNA glycosylase (UDG). UDG is also known as uracil-N-glycosylase (UNG). UDG treatment is useful in preventing the reamplification of carryover PCR products.

UDG is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-DNA 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).

UDG 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).

Use NAC and NTC Controls

Because 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.

Design Primers to Avoid Primer-Dimers

Use primers that contain dA nucleotides near the 3′ ends so that any primer-dimer generated is efficiently degraded by UDG 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 can serve as templates for a subsequent PCR amplification.

Production of primer-dimers 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 UDG (Delort *et al.*, 1985) and, therefore, the primers are not degraded. Biotin-dUMP derivatives are not substrates for UDG.

For more information about designing primers, see "Guidelines for Designing Primers" on page A-2.

Do not use UDG in subsequent amplifications of dU-containing PCR template, such as in nested PCR protocols. The UNG degrades the dU-containing PCR product, preventing further amplification.

PCR Good Laboratory 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 a 10% bleach solution.

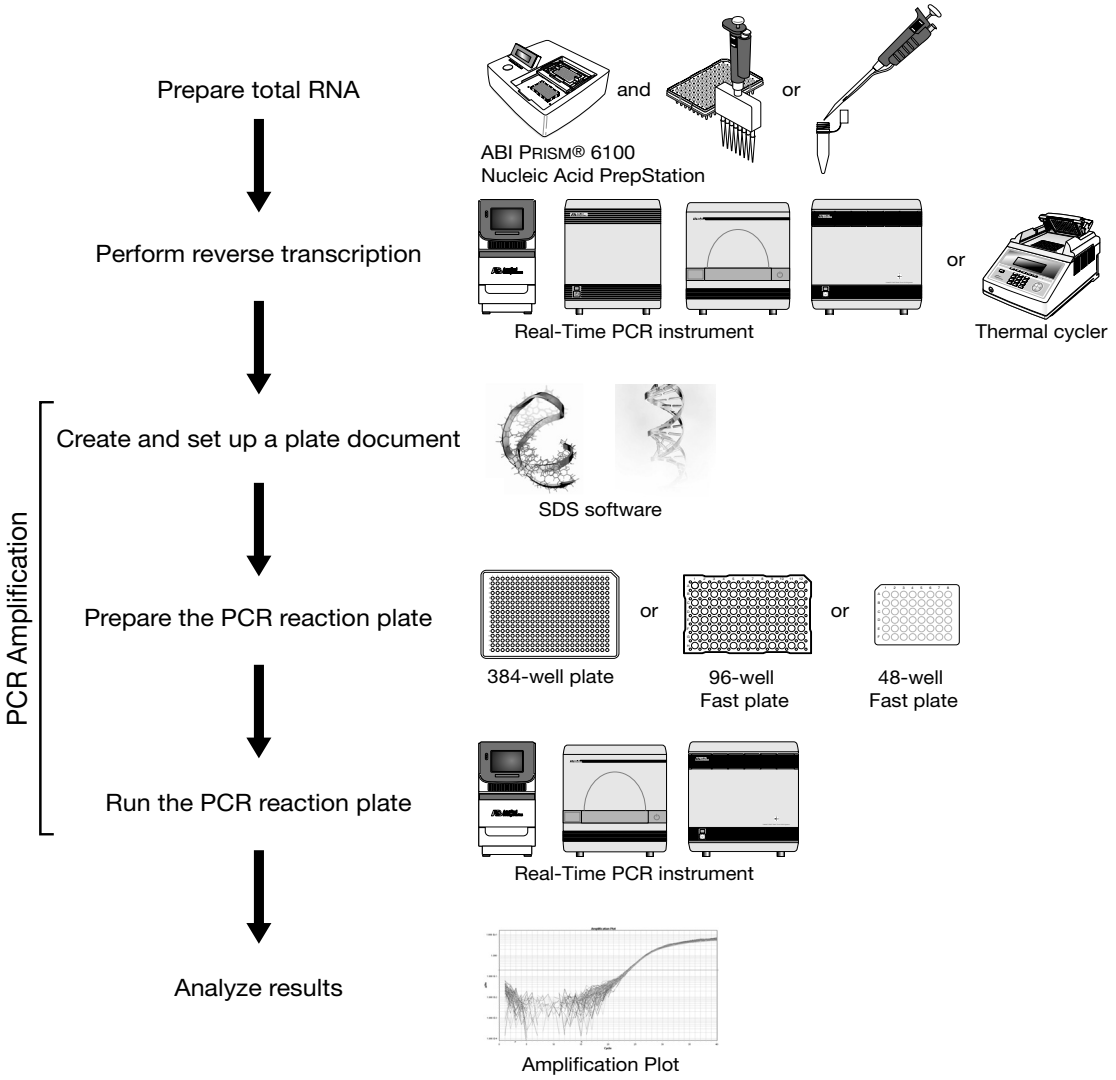
Prepare and Run the Samples 2

Overview

This chapter describes how to prepare your template and samples for PCR amplification.

Procedural Overview

This diagram is an overview of the procedures for performing gene expression experiments.



Prepare the Template

After isolating the template, examine its quality and quantity and store it properly.

Examine RNA Template Quality

Before using the Fast SYBR® Green Master Mix, you need to synthesize single-stranded cDNA from total RNA or mRNA samples. For optimal performance, Applied Biosystems recommends using RNA that is:

- Between 0.002 and 0.2 µg/µL
- Less than 0.005% of genomic DNA by weight
- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer
- Free of RNase activity

  IMPORTANT! If you suspect that the RNA contains RNase activity, add RNase inhibitor to the reverse transcription reaction at a final concentration of 1.0 U/µL. Adding RNase inhibitor to the reverse transcription reaction is not necessary if the RNA is purified using the ABI PRISM® 6100 Nucleic Acid PrepStation and Applied Biosystems nucleic acid purification reagents.

- Nondenatured

  IMPORTANT! It is not necessary to denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some gene targets.

Examine DNA Template Quality

Use both of the following methods to examine DNA quality:

- **Agarose gel electrophoresis** – Purified DNA should run as a single band on an agarose gel. Agarose gels reveal contaminating DNAs and RNAs, but not proteins.
- **Spectrophotometry** – The A_{260}/A_{280} ratio should be 1.8 to 2.0. Smaller ratios usually indicate contamination by protein or organic chemicals. Spectrophotometry can reveal protein contamination, but not DNA or RNA contamination.

Quantitate the Template

Template quantitation is critical for successful PCR reactions. The most common way to determine DNA quantity is to measure the absorbance (optical density or O.D.) of a sample at 260 nm in a spectrophotometer.

One O.D. unit is the amount of a substance dissolved in 1.0 mL that gives an absorbance reading of 1.00 in a spectrophotometer with a 1-cm path length. The wavelength is assumed to be 260 nm unless stated otherwise. A_{260} values can be converted into µg/µL using Beer´s Law:

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

The following formulas are derived from Beer's Law (Ausubel *et al.*, 1998):

- Concentration of single-stranded DNA = $A_{260} \times 33$ µg/µL.
- Concentration of double-stranded DNA = $A_{260} \times 50$ µg/µL.
- Concentration of single-stranded RNA = $A_{260} \times 40$ µg/µL

Note: Absorbance measurements of highly concentrated (O.D.>1.0) or very dilute (O.D.<0.05) DNA or RNA samples can be inaccurate. Dilute or concentrate the DNA/RNA to obtain a reading within the acceptable range.

Store the Template

Store the templates as follows:

- Store purified RNA templates at −20 °C or −70 °C in RNase-free water.
- Store purified DNA templates at −20 °C or −70 °C in Tris-HCl, pH 8.0.

Set Up the Plate Document

Select a Plate for PCR

Refer to page 1-7 for part numbers of the plates.

If you use...	Select...
Applied Biosystems StepOne™ Real-Time PCR System	MicroAmp™ Fast Optical 48-Well Reaction Plates
Applied Biosystems StepOnePlus™ Real-Time PCR System	MicroAmp™ Fast Optical 96-Well Reaction Plates
Applied Biosystems 7500 Fast Real-Time PCR System	MicroAmp™ Fast Optical 96-Well Reaction Plates
Applied Biosystems 7900HT Fast Real-Time PCR System (*with Fast block*)	MicroAmp™ Fast Optical 96-Well Reaction Plates
Applied Biosystems 7900HT Fast Real-Time PCR System (*with 384-well block*)	MicroAmp™ Optical 384-Well Reaction Plate

Configure the Plate Document

For information about configuring plate documents when performing real-time quantification, refer to the appropriate user guides listed in "Applied Biosystems Documents" on page 1-9.

Prepare the PCR Reaction Plate

Reminder About Your Primers

Before starting, check that the melting point for your primers is 58 to 60 °C. Refer to page A-2 for more information about identifying target sequences and designing primers.

Reagent Handling and Preparation

Follow these guidelines to ensure optimal PCR performance.
Prior to use:

- Mix the Fast SYBR® Green Master Mix thoroughly by swirling the bottle.
- Place frozen cDNA samples and primers on ice to thaw. After the samples are thawed, vortex them, then centrifuge the tubes briefly.

Prepare the PCR Reactions

CAUTION CHEMICAL HAZARD. Fast SYBR® Green Master Mix (2×) may cause eye, skin and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Applied Biosystems recommends performing four replicates of each reaction. For the recommended reaction volume of 20 µL, prepare the PCR reactions according to the following procedure.

1. Using an appropriate size tube, prepare the reactions for each sample:

Component	Volume for One 20-µL Reaction (µL)
Fast SYBR® Green Master Mix (2×)	10.0
Forward and Reverse Primers‡	Variable
cDNA template + RNase-free water§	Variable
Total Volume	20.0

‡ For optimal performance, use a minimum of 200 nM of each primer.

§ For optimal performance, use up to 20 ng of cDNA per 20-µL reaction, plus RNase-free water.

2. Cap the tube, mix by gentle inversion, then centrifuge the tube briefly to spin down the contents and eliminate any air bubbles.

3. Transfer the appropriate volume of each reaction to each well of an optical plate, as specified in the table below:

Plate Format	Reaction Volume (µL)
MicroAmp™ Fast Optical 48-Well Reaction Plate	20
MicroAmp™ Fast Optical 96-Well Reaction Plate	20
MicroAmp™ Optical 384-Well Reaction Plate	20

4.	Seal the plate with an optical adhesive cover, then centrifuge the plate briefly to spin down the contents and eliminate any air bubbles. **IMPORTANT!** Run the reaction plate within 2 hours after completing the reaction setup. If you cannot do so, store the plate at 4 °C.

Run the PCR Reaction Plate

Run the plate on an Applied Biosystems real-time quantitative PCR instrument. See the appropriate instrument user guide for help with programming the thermal-cycling conditions or with running the plate.

To run the plate:

1. Place the reaction plate in the instrument.

2. Set the thermal cycling conditions using the default PCR thermal-cycling conditions specified in the following table:

Select Fast Mode				
Instrument	**Step**	**Temperature (°C)**	**Duration**	**Cycles**
• Step One • StepOne Plus • 7500 Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	3 sec	40
	Anneal/Extend	60	30 sec	
7900HT Fast	AmpliTaq® Fast DNA Polymerase, UP Activation	95	20 sec	HOLD
	Denature	95	1 sec	40
	Anneal/Extend	60	20 sec	

3. Set the reaction volume according to the following table:

Plate Format	Reaction Volume (µL)
MicroAmp™ Fast Optical 48-Well Reaction Plate	20
MicroAmp™ Fast Optical 96-Well Reaction Plate	20
MicroAmp™ Optical 384-Well Reaction Plate	20

4. Start the run.

Analyze the Data 3

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

Analyze Your Results

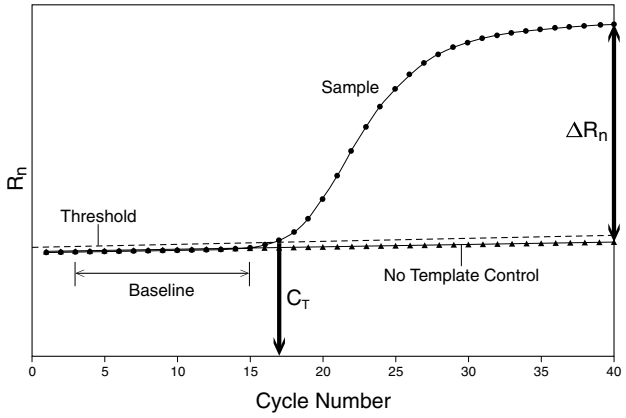
The general process for analyzing the data from gene expression assays requires that you:

- View the amplification plots.
- Adjust the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
- Use the standard curve method or the relative quantification ($\Delta\Delta C_T$) method to analyze the results.

Baseline and Threshold Values

Use the Sequence Detection System (SDS) software to *automatically calculate* or *manually set* the baseline and threshold for the amplification curves.

- *Baseline* refers to the initial cycles of PCR in which there is little change in fluorescence signal.
- The intersection of the threshold with the amplification plot defines the C_T in real-time PCR assays. The threshold is set above the background and within the exponential growth phase of the amplification curve.

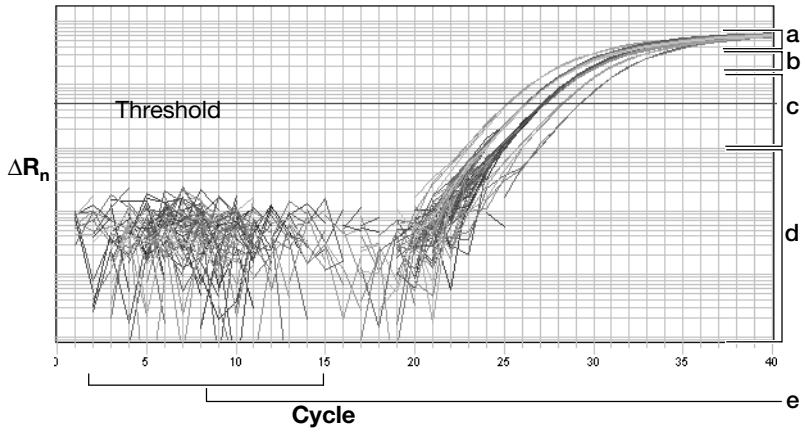


View the Amplification Plots

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 below, has a:

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



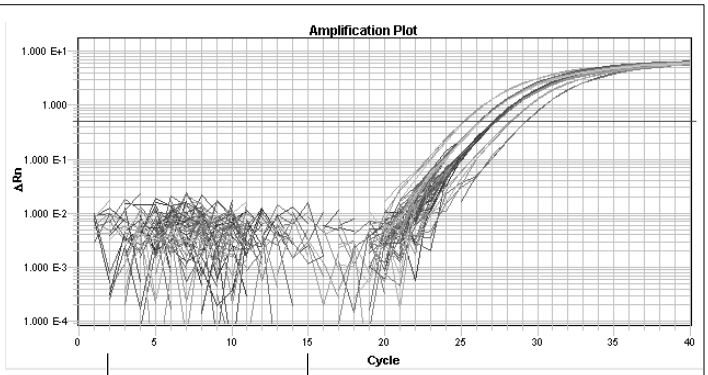
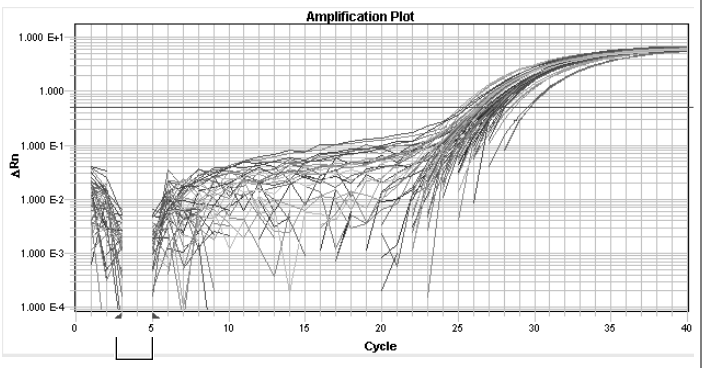
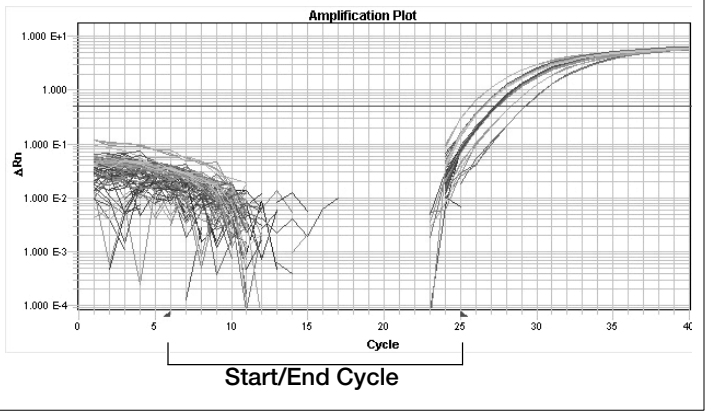
Manually Adjust the Baseline and Threshold

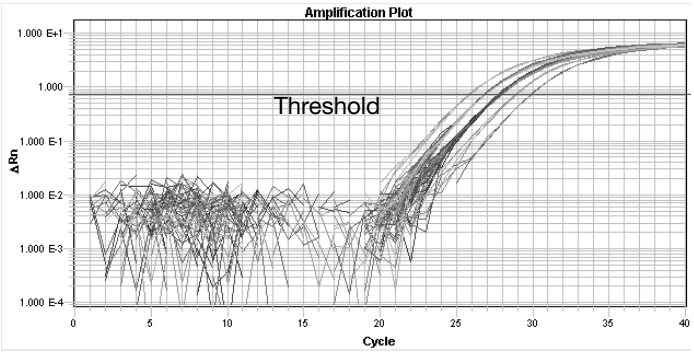
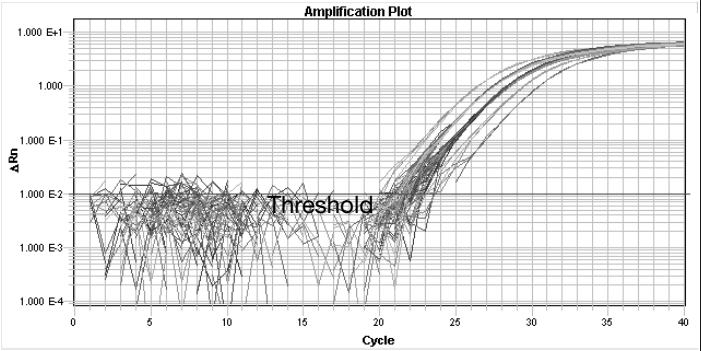
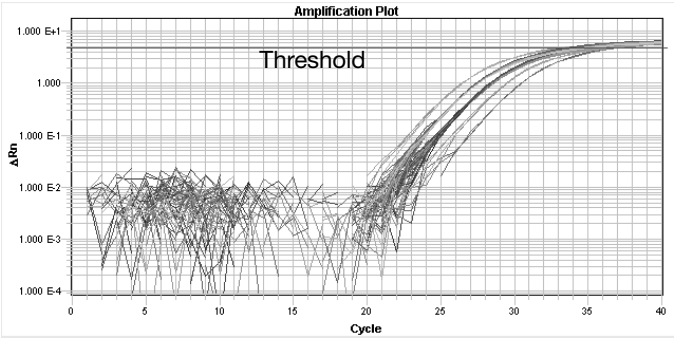
Experimental error (such as contamination or inaccurate pipetting) can produce data that deviate significantly from data for typical amplification curves. Such atypical data cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

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 example amplification plots below to determine whether or not the baseline and threshold settings were correctly set.

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

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.	

Analyzing the Results

Using the Fast SYBR® Green Master Mix, you can perform two types of quantitation: relative and absolute.

- Relative quantitation compares a target against an internal standard. You can perform relative quantitation using either the standard curve method or the comparative C_T method.
- Absolute quantitation compares the C_T of an unknown sample against a standard curve with known copy numbers.

Relative Quantitation Method

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

All quantitations are also normalized to an endogenous control (such as GAPDH) to account for variability in the initial concentration and quality of the total RNA, and in the conversion efficiency of the reverse transcription reaction.

Resources for Data Analysis

Refer to the following documents for more information about analyzing your data. You can download the documents from: **http://docs.appliedbiosystems.com/search.taf**

- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Relative Quantification Getting Started Guide* (PN 4347824)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide* (PN 4347825)
- *Real-Time PCR Systems Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems* (PN 4348358)

Detect Nonspecific Amplification

Because SYBR Green I dye detects any double-stranded DNA, check for nonspecific product formation by using dissociation-curve or gel analysis.

Dissociation Curves

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.

When to Generate Dissociation Curves

IMPORTANT! Because of the presence of UDG, you must generate a dissociation curve immediately after the real-time PCR run on any Applied Biosystems Real-Time PCR System.

An Example

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

Primer-dimers are most prevalent in NTC wells and sample wells containing a low concentration of template.

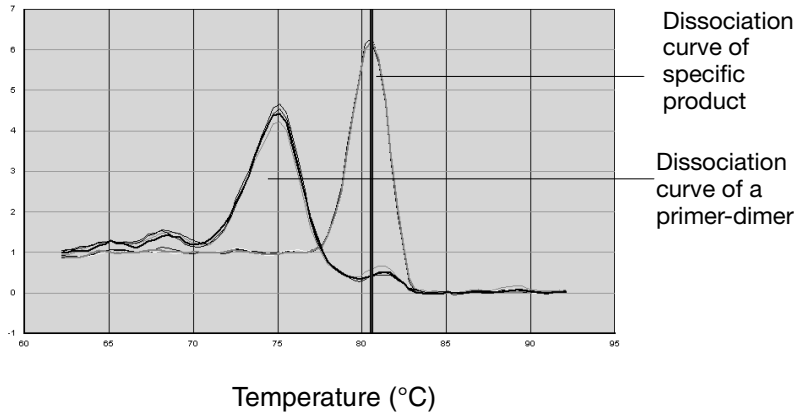


Figure 3-1 Example of two dissociation curves

Use Agarose Gels to Check PCR Product Purity

IMPORTANT! Because of the presence of UDG, *immediately* verify the absence of nonspecific amplification using agarose gel electrophoresis.

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 (that is, 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. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Run the gel: • For PCR fragments <100 bp, use 80 to 100 V for 45 to 60 min. • For PCR fragments 100 to 250 bp, use 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.

Troubleshoot

Observation	Possible Cause	Action
High C_T values/poor precision or failed PCR reactions	Insufficient cDNA template is present	Use up to 20 ng of cDNA template per 20-µL reaction.
	Quality of cDNA template is poor	1. Quantify the amount of cDNA template. 2. Test the cDNA template for the presence of PCR inhibitors.
	Sample degradation	Prepare fresh cDNA, then repeat the experiment.
	Incorrect pipetting.	Prepare the reactions as described on page 2-6.
	Reduced number of PCR cycles in the thermal cycler protocol	Increase the number of PCR cycles to the default setting of 40 (see page 2-8).
	Primer-dimer formation and residual polymerase activity	1. Prepare the reaction mixes and the reaction plate on ice. 2. To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, store the reaction plate at 4 °C.
Low ΔR_n or R_n values	Extension time is too short	Use the default thermal profile settings (see page 2-8).
	Primer-dimer formation and residual polymerase activity	1. Prepare the reaction mixes and the reaction plate on ice. 2. To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, store the reaction plate at 4 °C.

Observation	Possible Cause	Action
Run takes more than 40 minutes	Thermal cycler mode is set to Standard or 9600 Emulation	Make sure that the thermal cycler mode is set to Fast (see page 2-8).
R_n vs. Cycle plot is not displayed	ROX™ dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.
Extremely high ΔR_n or R_n values	ROX dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.
	Evaporation	Make sure that the reaction plate is sealed completely, especially around the edges.
Lower ΔR_n values obtained in early cycles	C_T value is less than 15	Adjust the upper baseline range to a value less than 15.
High variability across the reaction plate	ROX dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.
	Evaporation	Make sure that the reaction plate is sealed completely, especially around the edges.
High variability across replicates	Reaction mix was not mixed well	Mix the reaction mix gently by inversion, then centrifuge briefly before aliquoting to the reaction plate.

Design the Assay A

Overview

This appendix describes how to design primers to amplify custom target sequences for quantitation. You may not have to routinely do this part of the procedure.

Identify Target Sequences and Design Primers

Identify Target Sequence and Amplicon Size

A target template is a DNA sequence, including cDNA, genomic DNA, or plasmid nucleotide sequence that you want to amplify.

Using Primer Express Software, you design primers to amplify *amplicons* (segments of DNA) within the target sequence. Shorter amplicons work best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Guidelines for Designing Primers

Using Primer Express® Software

Design primers using Primer Express Software as described in the *Primer Express® Version 3.0 Getting Started Guide* (PN 4362460) and *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.
- **Important: 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 “Select an Amplicon Site for cDNA” on page A-3.
RNA	Design the primers as described above.

Select an Amplicon Site for cDNA

Selecting a good amplicon site ensures amplification of the target cDNA 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 must be specific to the target gene; the primer pair does not amplify pseudogenes or other related genes.
- Design primers according to Primer Express Software guidelines.
- Test the amplicons, then select those that have the highest signal-to-noise ratio (that is, low C_T with cDNA and no amplification with no template control or genomic DNA).
- If no good sequence is found, you may need 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 amplifies the mRNA sequence without amplifying the genomic sequence. In this case, you may need to run RT minus controls.

Optimize Primer Concentrations for PCR

Overview

By independently varying the forward and reverse primer concentrations, you can identify the primer concentrations that provide optimal assay performance. The primer concentrations you select should provide a low C_T and a high ΔR_n when run against the target template, but should not produce nonspecific product formation with NTCs.

Quantitate the Primers

1.	Measure the absorbance (at 260 nm of a 1:100 dilution) of each primer oligonucleotide in TE buffer.
2.	Calculate the sum of extinction coefficient contributions for each primer: extinction coefficient contribution = Σ(extinction coefficient × number of bases in oligonucleotide sequence) See "An Example Calculation of Primer Concentration" on page A-5 for an example calculation.
3.	Calculate the oligonucleotide concentration in µM for each primer: absorbance @ 260 nm = sum of extinction coefficient contribution × cuvette pathlength × concentration/100 Rearrange to solve for concentration: concentration = 100[absorbance @ 260 nm / (sum of extinction coefficient contribution × cuvette pathlength)]

An Example Calculation of Primer Concentration

In this example, the concentration of a primer (in TE buffer, diluted 1:100), with the sequence

CGTACTCGTTCGTGCTGC

is calculated using the following values:

Chromophore	Extinction Coefficient	Number of Specific Chromophores in Example Sequence	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**

measured absorbance @260 nm = 0.13

sum of extinction coefficient contributions for probe = **167,950 $M^{-1}cm^{-1}$**

cuvette pathlength = 0.3 cm

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

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

C = 258 μM

Determine the Optimal Primer Concentration

CHEMICAL HAZARD. Fast SYBR Green Master Mix is a combustible liquid and vapor (keep away from heat and flame). It may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing and gloves.

To optimize primer concentrations for PCR:

1. Prepare a 96-well reaction plate as shown in Table A-1 on page A-9.
   - Use 10 to 100 ng of genomic DNA or 1 to 10 ng of cDNA template.
   - The final concentration of Fast SYBR Green Master Mix is 1×.

   Note: The plate configuration accounts for four replicates of each of the following nine variations of primer concentration applied to both template and NTC wells:

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. Calibrate your instrument for SYBR Green Dye, if necessary. Refer to the instrument user manual for calibration instructions.

   Note: Applied Biosystems recommends that you calibrate your instrument every 6 months.

3. Load the plate into an Applied Biosystems real-time PCR system.

4. Program the thermal-cycling conditions according to the information in step 2 on page 2-8.

5. Run the plate.

To optimize primer concentrations for PCR: *(continued)*

6.	Compile the results for ΔR_n and C_T, then select the minimum forward and reverse primer concentrations that yield the maximum ΔR_n values and low C_T values.

Confirm the Absence of Nonspecific Amplification

Dissociation curves help you select the optimal primer concentrations for your SYBR quantification assays.

1.	Review the linear view of the amplification plot in your NTC wells. **Note:** In Figure A-1 on page A-8, part a, the strong amplification of the NTC wells indicates that significant nonspecific amplification is occurring.
2.	Generate a dissociation curve on your Applied Biosystems Real-Time PCR System. **Note:** In the example dissociation curve data shown in Figure A-1 on page A-8, part b, the melting temperature of the product generated in the absence of template is lower than the melting temperature of the specific product generated with template. This variation is typical of primer-dimer formation, and it indicates that lower primer concentration may provide optimal results.

Example of Nonspecific Amplification

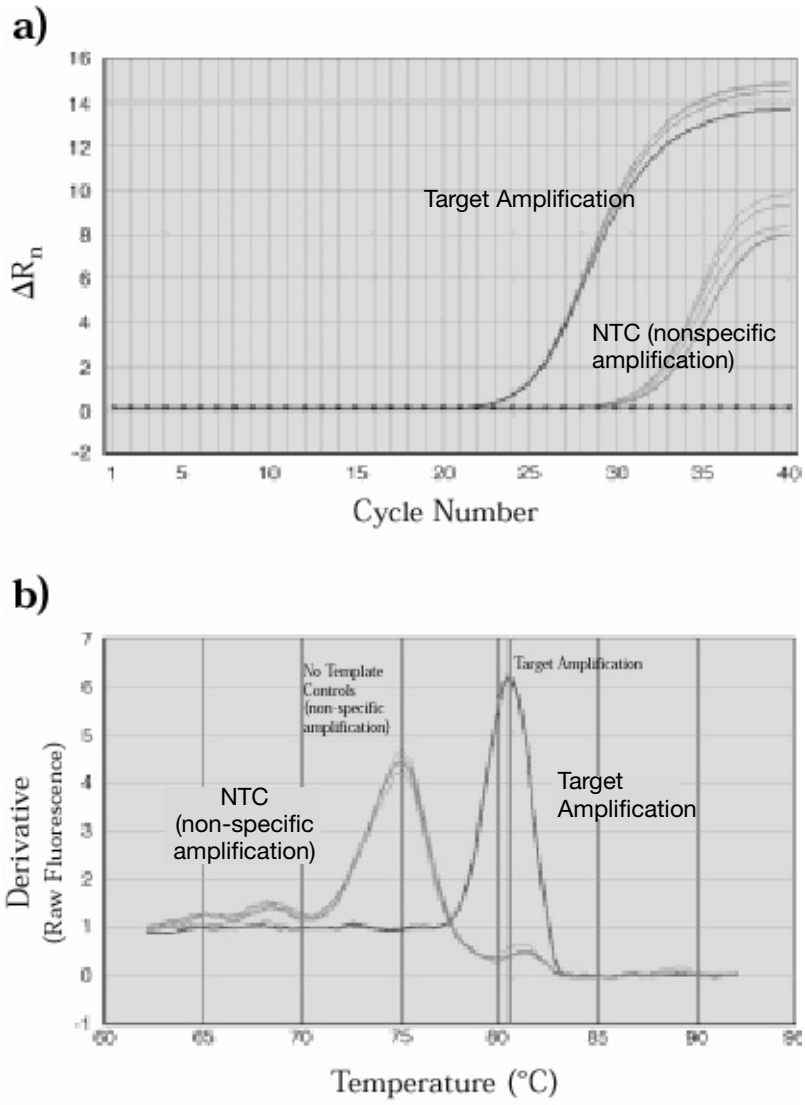


Figure A-1 Amplification data using SYBR Green I dye chemistry
(a) Amplification plot (linear view) demonstrating suspected nonspecific amplification in NTC wells.
(b) Dissociation curve analysis confirming that product in NTC wells has a melting temperature different from the specific product.

Table A-1 Plate Configuration for Primer Optimization for PCR

Wells	Fast SYBR Green Master Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
A1–A4	10	0.2	0.2	2.0	7.6	20
A5–A8	10	0.2	1.2	2.0	6.6	20
A9–A12	10	0.2	3.6	2.0	4.2	20
B1–B4	10	1.2	0.2	2.0	6.6	20
B5–B8	10	1.2	1.2	2.0	5.6	20
B9–B12	10	1.2	3.6	2.0	3.2	20
C1–C4	10	3.6	0.2	2.0	4.2	20
C5–C8	10	3.6	1.2	2.0	3.2	20
C9–C12	10	3.6	3.6	2.0	0.8	20
D1–D4	10	0.2	0.2	0	9.6	20
D5–D8	10	0.2	1.2	0	8.6	20
D9–D12	10	0.2	3.6	0	6.2	20
E1–E4	10	1.2	0.2	0	8.6	20
E5–E8	10	1.2	1.2	0	7.6	20
E9–E12	10	1.2	3.6	0	5.2	20
F1–F4	10	3.6	0.2	0	6.2	20
F5–F8	10	3.6	1.2	0	5.2	20
F9–F12	10	3.6	3.6	0	2.8	20

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