TaqMan® Gene Expression Master Mix

Protocol

Applied Biosystems

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Contents

Preface

This preface contains:

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.

– 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.

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

– 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

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

Purpose

TaqMan® Gene Expression Master Mix is a convenient mix of components (except primers, probes, template, and water) necessary to perform real-time quantitative polymerase chain reaction (PCR). Use TaqMan Gene Expression Master Mix with the DNA target of your choice, including cDNA, genomic DNA, or plasmid DNA. You can use TaqMan Gene Expression Master Mix with any TaqMan assay or any quantitative PCR application, such as:

- Pathogen detection
- Copy number analysis (gene dosage analysis)
- Microarray validation
- Differential gene expression analysis
- Viral load quantitation
- Methylation analysis
- MicroRNA quantitation

You can use TaqMan Gene Expression Master Mix with any of these instruments:

- Applied Biosystems StepOne™ Real-Time PCR System
- Applied Biosystems 7300/7500 Real-Time PCR Systems
- Applied Biosystems 7500 Fast/7900HT Fast Real-Time PCR Systems (in standard or 9600 emulation mode)
- The ABI PRISM® 7000/7700/7900HT Sequence Detection Systems

About this Protocol

This protocol provides detailed information for performing PCR using TaqMan Gene Expression Master Mix with TaqMan® Gene Expression Assays, Custom TaqMan® Gene Expression Assays, or Custom TaqMan® Probes and Sequence Detection Primers. This protocol also includes:

- Background information about gene expression assays
- A list of equipment and materials for using the TaqMan Gene Expression Master Mix

- An overview of procedures for performing gene expression experiments
- Information on analyzing PCR results
- Troubleshooting information

For detailed information about specific procedures outlined in this protocol, consult the appropriate instrument user guide. A procedural overview is also provided in the *TaqMan Gene Expression Master Mix Quick Reference Card* (PN 4371134).

Chemistry Overview

Two-Step RT-PCR

Gene quantitation assays using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays are performed in a two-step RT-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from RNA.
2. In the PCR step, PCR products are quantitatively synthesized from cDNA samples using the TaqMan Gene Expression Master Mix.

The figure below illustrates two-step PCR.

Note: Figure 1 does not show hybridization of the TaqMan® MGB probe. See Figure 2 on page 6 for details on how the TaqMan MGB probe is used in the PCR step.

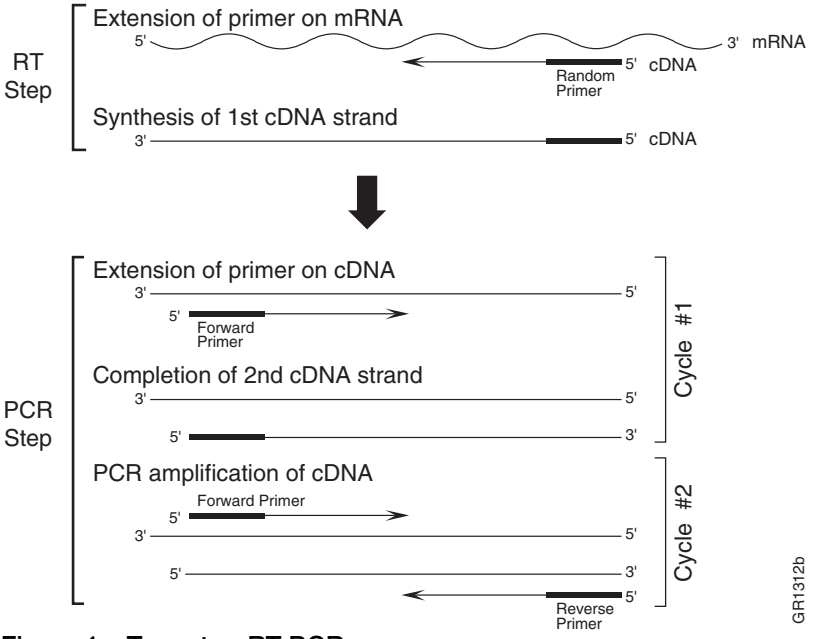


Figure 1 Two-step RT-PCR

About TaqMan Gene Expression Master Mix

TaqMan Gene Expression Master Mix reagents provide a PCR mix that may be used with any appropriately designed primer and probe to detect any DNA target (including cDNA, genomic, or plasmid DNA). The mix is optimized for TaqMan assays, and it contains AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), Uracil-DNA Glycosylase (UDG), deoxyribonucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP), ROX™ Passive Reference, and buffer components optimized for sensitivity, precision, specificity, and duplexing.

AmpliTaq Gold DNA Polymerase, (UP) Ultra Pure

AmpliTaq Gold® DNA Polymerase, UP, a chemically modified form of AmpliTaq® DNA Polymerase, is a key ingredient in an automated, convenient, and efficient Hot Start PCR. The thermal 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, UP (Ultra Pure) 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 organism. The purification process ensures that non-specific, false-positive DNA products due to bacterial DNA contamination are minimized during PCR.

About Uracil-DNA Glycosylase

Uracil-DNA Glycosylase [UDG, also know as uracil-N-glycosylase (UNG)] 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 “Preventing Contamination” on page 16 for more information about UDG.

About ROX Passive Reference

The ROX™ Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

About TaqMan MGB Probes

The TaqMan® MGB Probes consists of a target-specific oligonucleotide with:

- A reporter dye (for example, 6FAM™ dye) linked to the 5′ end of the probe
- A minor groove binder (MGB), which increases the melting temperature (T_m) without increasing probe length (Afonina *et al.,* 1997; Kutyavin *et al.,* 1997), thereby allowing the design of probes that are shorter and more specific than traditional dual-labeled probes.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe, which offers the advantage of a lower background signal, resulting in better precision quantitation.

Basics of the 5′ Nuclease Assay

The PCR reaction exploits the 5′ nuclease activity of AmpliTaq® Gold DNA Polymerase, UP (Ultra Pure) to cleave a TaqMan® probe during PCR. The TaqMan probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe.

During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Figure 2 on page 6 shows the 5′ to 3′ nuclease activity of AmpliTaq Gold, UP enzyme during PCR. The nuclease activity is fork-like and structure dependent.

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983). During PCR, if the target of interest is present, the probe specifically anneals to the target.

The 5′ to 3′ nucleolytic activity of the AmpliTaq Gold, UP enzyme cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle, and it does not interfere with the exponential accumulation of product.

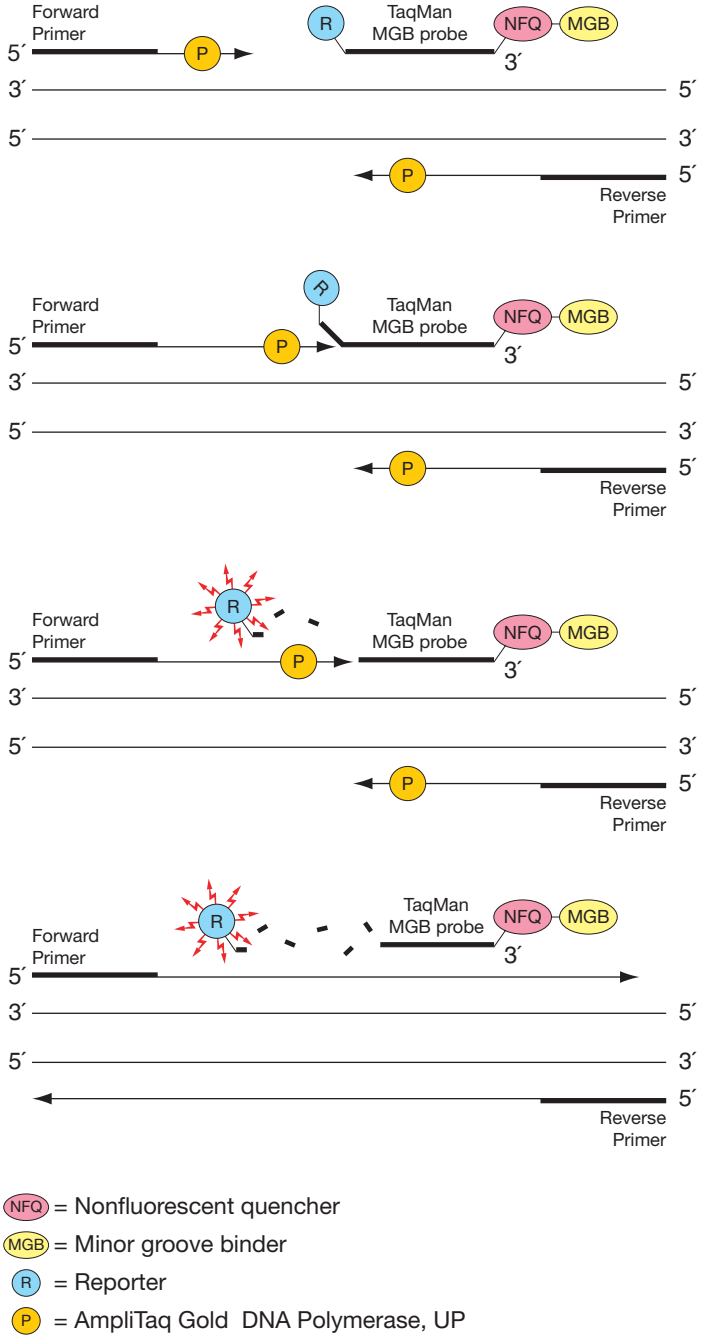
The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and if it is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Polymerization

Strand displacement

Cleavage

Completion of polymerization



NFQ = Nonfluorescent quencher

MGB = Minor groove binder

R = Reporter

P = AmpliTaq Gold DNA Polymerase, UP

Figure 2 The 5′ nuclease assay

Multi-componenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the pure dye components generate the composite spectrum. This spectrum represents one fluorescent reading from one well. Dyes available for multicomponent analysis are:

Types of Dyes	Dyes
Reporters	6FAM™, CY3™, CY5™, JOE™, NED™, TET™, VIC®
Quenchers	TAMRA™, MGB/NFQ
Passive Reference	ROX™

R_n and ΔR_n Values

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the ROX Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube.

R_n^+ is the R_n value of a reaction containing all components including the template.

R_n^- is the R_n value of an unreacted sample. This value may be obtained from the early cycles of a real-time run, those cycles prior to a detectable increase in fluorescence. This value may also be obtained from a reaction not containing template.

ΔR_n is the difference between the R_n^+ value and the R_n^- value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

$$\Delta R_n = (R_n^+) - (R_n^-)$$

where:

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

$$R_n^- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$ PCR without template or early cycles of a real-time reaction

Threshold Cycle (C_T)

The threshold cycle or C_T value is the cycle at which a statistically significant increase in ΔR_n is first detected. Threshold is defined as the average standard deviation of R_n for the early cycles, multiplied by an adjustable factor.

On the graph of R_n versus cycle number shown below, the threshold cycle occurs when the signal associated with an exponential growth of PCR product increases.

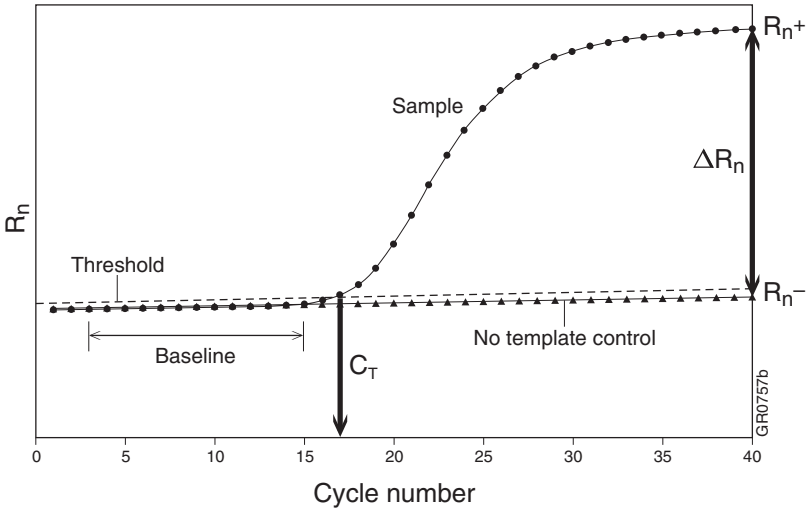


Figure 3 R_n as a function of cycle number

See "Analyzing Results" on page 31 for information on how these terms are used in PCR data analysis.

Materials and Equipment

Contents

The TaqMan Gene Expression Master Mix contains:

- AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)
- Uracil-DNA glycosylase
- dNTPs with dUTP
- ROX™ Passive Reference
- Optimized buffer components

TaqMan Gene Expression Master Mix is supplied in a 2× concentration and is available from Applied Biosystems in the following volumes:

Item	Contents	Part Number
Mini-Pack	One 1 mL tube (40 × 50 µL reactions)	4370048
1-Pack	One 5 mL bottle (200 × 50 µL reactions)	4369016
2-Pack	2 × 5 mL bottles (400 × 50 µL reactions)	4369514
5-Pack	5 × 5 mL bottles (1000 × 50 µL reactions)	4369510
10-Pack	10 × 5 mL bottles (2000 × 50 µL reactions)	4369542
Bulk Pack	One 50 mL bottle (2000 × 50 µL reactions)	4370074

Storage and Stability

Upon receipt, store the TaqMan Gene Expression Master Mix at 2 to 8 °C. TaqMan Gene Expression Master Mix is stable through the date on the package and bottle label when stored at 2 to 8 °C.

If TaqMan Gene Expression Master Mix is stored at –20 °C, transfer it to 2 to 8 °C. Applied Biosystems does not recommend storing TaqMan Gene Expression Master Mix at temperatures other than 2 to 8 °C or using TaqMan Gene Expression Master Mix after the date printed on the package and bottle label.

Before using, make sure the Master Mix is thoroughly thawed and mixed.

Instruments Not Included

The following instruments are recommended when using TaqMan Gene Expression Master Mix.

Instruments	Source
Applied Biosystems StepOne™ Real-Time PCR System	Contact your Applied Biosystems sales representative.
Applied Biosystems 7300 Real-Time PCR System	
Applied Biosystems 7500 Real-Time PCR System	
Applied Biosystems 7500 Fast Real-Time PCR System	
Applied Biosystems 7900HT Fast Real-Time PCR System	
ABI PRISM® 7000 Sequence Detection System	

Reagents and Plastics Not Supplied

The following items are not supplied with the TaqMan Gene Expression Master Mix.

User-Supplied Materials from Applied Biosystems

Materials	Part Number
TaqMan® Reverse Transcription Reagents	N8080234
High-Capacity cDNA Reverse Transcription Kits:	
• 200 reactions	4368814
• 1000 reactions	4368813
• with RNase Inhibitor, 200 reactions	4374966
• with RNase Inhibitor, 1000 reactions	4374967
RNase inhibitor	N8080119
TaqMan® Gene Expression Assays, inventoried	4331182
TaqMan® Gene Expression Assays, Made-to-order	4351372

User-Supplied Materials from Applied Biosystems *(continued)*

Materials	Part Number
Custom TaqMan® Gene Expression Assays • Small-Scale (20×, 144 × 50µL reactions) • Medium-Scale (20×, 300 × 50µL reactions) • Large-Scale (60×, 1160 × 50µL reactions)	 4331348 4332078 4332079
Sequence Detection Primers • 10,000 pmol • 80,000 pmol • 130,000 pmol	 4304970 4304971 4304972
TaqMan® MGB Probe • 6,000 pmol • 20,000 pmol • 50,000 pmol	 4316034 4316033 4316032
TaqMan® TAMRA Probe • 6,000 pmol • 20,000 pmol • 50,000 pmol	 450025 450024 450003
TaqMan® PreAmp Master Mix Kit, 40 reactions	4384267
StepOne™ consumables: • MicroAmp™ Fast Optical 48-Well Reaction Plates • MicroAmp™ Optical 48-Well Adhesive Film • MicroAmp™ Fast 48-Well Trays • MicroAmp™ 48-Well Base Adaptor	 4375816 4375816 4375282 4375284
Fast consumables: • MicroAmp™ Fast 8-Tube Strips • MicroAmp™ Optical 8-Cap Strips • MicroAmp™ Fast Reaction Tubes with Caps	 4358293 4323032 4358297
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, (quantity 500)	4326659

User-Supplied Materials from Applied Biosystems *(continued)*

Materials	Part Number
MicroAmp™ Optical 96-Well Reaction Plate with Barcode, (quantity 20)	4306737
MicroAmp™ Optical 384-Well Reaction Plate with Barcode, (quantity 50)	4309849
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1-mL (quantity 20)	4346906
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (quantity 200)	4366932
MicroAmp™ Optical 8-Tube Strip, 0.2-mL (quantity 1000 tubes in strips of 8)	4316567
MicroAmp™ Optical 8-Cap Strip (quantity 300 strips)	4323032
MicroAmp™ Optical Adhesive Film (quantity 100)	4311971
MicroAmp™ Optical Adhesive Film Kit	4313663
MicroAmp™ Optical Adhesive Film (quantity 25)	4360954
MicroAmp™ Splash Free 96-Well Base	4312063
MicroAmp™ Optical Film Compression Pad (quantity 5) **Note:** This is *not* compatible with the Applied Biosystems 7300, 7500, and 7500 Fast Real-Time PCR Systems.	4312639
MicroAmp™ Snap-On Optical Film Compression Pad	4333292
MicroAmp® Multi Removal Tool	4313950
6700 Reagent Tubes, 10-mL (quantity 40)	4305932

User-Supplied Materials from Other Sources

Materials	Source
Accessories for tubes of assay mixes • Decapper for single caps • Decapper for eight caps • TPE cap cluster for simultaneously capping 96 individual polypropylene tubes, 50 capmats/bag	Micronic BV‡ PO Box 604 8200 AP Lelystad Netherlands Telephone: 0031.320.277.090 Fax: 0031.320.277.088 United States Telephone: 724.941.6411 Fax: 724.941.8662 www.micronic.com
RNase-free, sterile-filtered water	major laboratory supplier (MLS)
Centrifuge with plate adapter	MLS
Disposable gloves	MLS
Microcentrifuge	MLS
Pipet tips, aerosol resistant	MLS
Pipettors: • Positive-displacement • Air-displacement • Multichannel	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

‡ Other vendors supply similar products

Optional User-Supplied Reagents

For a description of these reagents, go to:

www.ambion.com/techlib/index

Materials	Source
RNAqueous® Kit, 50 purifications	AM 1912
RNAqueous® -4PCR Kit, 30 purifications	AM1914
Turbo DNA-*free*™, 50 reactions	AM1907
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE, 40 purifications	AM 1975
RiboPure™ RNA Isolation Kit, 50 purifications	AM1924
RiboPure™ Bacterial Kit	AM1925
RiboPure™ Yeast Kit	AM1926
RiboPure™ Blood Kit, 40 purifications	AM1928
mirVana™ miRNA Isolation Kit, 40 purifications	AM1560
MagMax™ Viral RNA Isolation Kit, 50 purifications	AM1939
MagMax™ AI/ND Viral RNA Isolation Kit, 50 purifications	AM1929
RNase *Zap*® RNase Decontamination Solution, 250 mL	AM9780
RNA*later*® Solution, 100 mL	AM7020
RT-PCR Grade Water, 10, 1.75-mL bottles	AM9935
TRI Reagent®, 100-mL	AM9738
RNAlater® ICE Solution, 25-mL	AM7030

Applied Biosystems Documents

You can download these and other documents from the Applied Biosystems Documents on Demand Web site at **http://docs.appliedbiosystems.com/search.taf**

Document	Part Number
TaqMan® Gene Expression Master Mix Protocol Quick Reference Card	4371134
Real-Time PCR Systems Chemistry Guide	4348358
High-Capacity cDNA Reverse Transcription Kit Protocol	4375575
TaqMan® Gold RT-PCR Kit Protocol	402876
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825
Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Presence/Absence Experiments	4376787
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 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide	4347828
Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide	4351684
ABI PRISM® 7000 Sequence Detection System User Guide	4330228
Applied Biosystems TaqMan® Low Density Array User Bulletin	4371129

Preventing Contamination

Overview

PCR assays require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these assays can lead to amplification of a single DNA molecule (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

UDG

The UDG provided in the TaqMan Gene Expression Master Mix is a pure, nuclease-free, 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-DNA glycosylase gene which has been inserted into an *E. coli* host to direct the expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UDG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, and creates an alkali-sensitive apyrimidic site in the DNA. Apyrimidic sites block replication by DNA polymerases. The enzyme has no activity on RNA or dT-containing DNA.

UDG incubation at 50 °C is necessary to cleave any dU-containing PCR carryover products. Ten-minute incubation at 95 °C is necessary to substantially reduce UDG activity, and to denature the native DNA in the experimental sample. Because UDG is not completely deactivated during the 95 °C incubation, it is important to keep the annealing temperatures greater than 55 °C and to refrigerate PCR products at 2 to 8 °C in order to prevent amplicon degradation.

General PCR Practices

Please follow these recommended procedures:

- Wear a clean lab coat and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification

  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or 3% hydrogen peroxide.

Procedural Overview

The following diagram provides a simplified overview of the procedures for performing gene expression experiments. This protocol includes detailed procedures for using TaqMan Gene Expression Master Mix in PCR amplification, and general information about reverse transcription and data analysis procedures. Refer to the appropriate instrument user manuals for additional information on performing gene expression experiments.

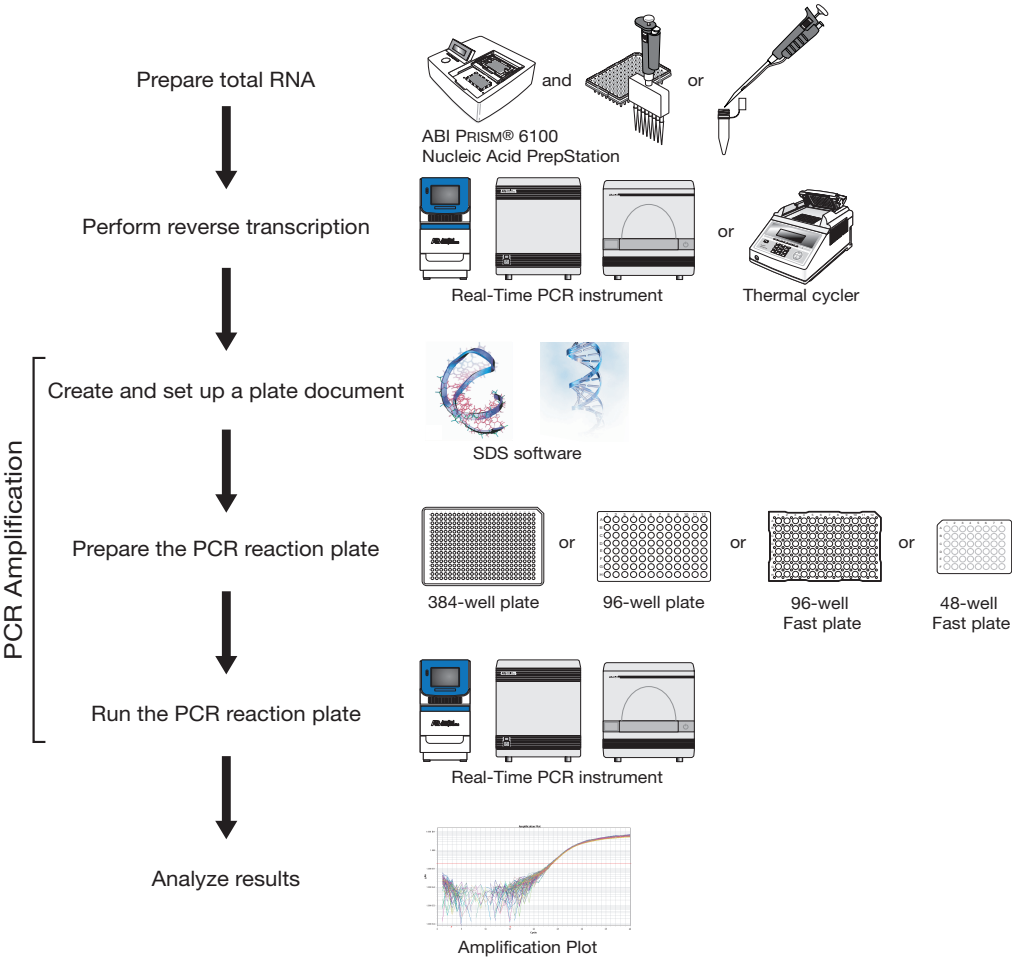


Figure 4 Overview of a gene expression experiment

Performing Reverse Transcription

Overview

Synthesis of single-stranded cDNA from RNA is the first step in the RT-PCR process, and involves the following procedures:

1. Preparing the reverse transcription (RT) master mix
2. Preparing the RT reaction plate
3. Performing reverse transcription

Applied Biosystems recommends using an Applied Biosystems reverse transcription kit to obtain cDNA from RNA samples. For additional RT guidelines and instructions, refer to the appropriate protocol:

If using...	Refer to...‡
Applied Biosystems High Capacity cDNA Reverse Transcription Kit: 200 reactions (PN 4368814) 1000 reactions (PN 4368813)	*High-Capacity cDNA Reverse Transcription Kit Protocol* (PN 4375575)
Applied Biosystems TaqMan® Reverse Transcription Reagents (PN N8080234)	*TaqMan® Gold RT-PCR Kit Protocol* (PN 402876)

‡ You can download the protocols from the Applied Biosystems Documents on Demand Web site at **http://docs.appliedbiosystems.com/search.taf**

IMPORTANT! Applied Biosystems has optimized TaqMan Gene Expression Assays and the TaqMan Gene Expression Master Mix for use with samples reverse transcribed from total RNA using an Applied Biosystems reverse transcription kit. Refer to "User-Supplied Materials from Applied Biosystems" on page 10 for a list of kits.

RNA Template Guidelines

For optimal performance, Applied Biosystems recommends using RNA with the following characteristics:

- Between 0.002 and 0.2 µg/µL in concentration of RNA
- 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. It is not necessary to add RNase inhibitor to the reverse transcription reaction if the RNA was 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.

Reagent and Sample Preparation Guidelines

Follow the guidelines below to ensure optimal performance.

- Use nuclease-free pipet tips and reagents to minimize degradation of the RNA.
- Observe standard laboratory practices when handling RNA.

Performing Real-Time PCR Amplification

Overview

Target amplification, using cDNA as the template, is the second step in the RT-PCR process. In this step, the DNA polymerase (from the TaqMan Gene Expression Master Mix) amplifies target cDNA synthesized from the RNA sample, using sequence-specific primers and a TaqMan probe (for example, a probe from the TaqMan Gene Expression Assay mix).

IMPORTANT! You must perform the PCR step on a Real-Time PCR System. Traditional thermal cyclers cannot be used because they cannot detect and record the fluorescent signals generated by the cleavage of TaqMan probes.

Performing the PCR step for simplex or duplex assays in 384-, 96-, or 48-well formats requires the following procedures:

- Configuring the plate document
- Preparing the PCR reaction plate
- Running the PCR reaction plate

Note: If you choose to use Custom TaqMan Probes and Sequence Detection Primers, rather than a TaqMan Gene Expression Assay or a Custom TaqMan Gene Expression Assay, see "Appendix A Using TaqMan Gene Expression Master Mix with Custom TaqMan Probes and Primers" on page 36 for more information.

IMPORTANT! TaqMan Gene Expression Master Mix is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan Gene Expression Master Mix on the StepOne™, 7500 Fast, or 7900HT Fast instruments, use Standard mode thermal cycling conditions. If you use assays other than the TaqMan Gene Expression assays, or use thermal cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal cycling conditions as needed. Refer to *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for more information on selecting thermal cycling conditions.

Configuring the Plate Document

Select a plate for the reaction:

Table 1 Instruments and reaction plates appropriate for PCR amplification

If using the...	Select...
Applied Biosystems StepOne™ Real-Time PCR System	• MicroAmp™ Fast Optical 48-Well Reaction Plates • MicroAmp™ Fast Optical 96-Well Reaction Plates
• Applied Biosystems 7300 Real-Time PCR System • Applied Biosystems 7500 Real-Time PCR System • ABI PRISM® 7000 Sequence Detection System	MicroAmp™ Optical 96-Well Reaction Plate
Applied Biosystems 7500 Fast Real-Time PCR System (*in standard mode*)	MicroAmp™ Fast Optical 96-Well Reaction Plates
Applied Biosystems 7900HT Fast Real-Time PCR System (*in standard mode*)	• MicroAmp™ Optical 384-Well Reaction Plate • MicroAmp™ Optical 96-Well Reaction Plate (with Standard 96-Well Block) • TaqMan® Low Density Array
Applied Biosystems 7900HT Fast Real-Time PCR System (*with Fast block*)	MicroAmp™ Fast Optical 96-Well Reaction Plates

For information about configuring plate documents when performing real-time quantification, refer to the appropriate user guide:

- *Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Presence/Absence Experiments*
- *Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T ($\Delta\Delta C_T$) Experiments*
- *Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Standard Curve Experiments*

- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (PN 4347825)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide* (PN 4347824)
- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- *ABI PRISM® 7000 Sequence Detection System User Guide* (PN 4330228)

Preparing the PCR Reaction Plate

PCR Reagent Handling and Preparation

Following these guidelines ensures optimal PCR performance:

- Keep all TaqMan reagents protected from light, in the freezer, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Mix the TaqMan Gene Expression Master Mix thoroughly by swirling the bottle.
  - Thaw frozen TaqMan reagents by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.
  - Resuspend the TaqMan reagents (for example, the TaqMan Gene Expression Assay mix) by vortexing and then centrifuge the tube briefly.
  - Thaw frozen cDNA samples by placing them on ice. When thawed, resuspend the samples by vortexing and then centrifuge the tubes briefly.
- Prepare the PCR reaction mix before transferring it to the reaction plate for thermal cycling and fluorescence analysis.

PCR Mix Components

CAUTION CHEMICAL HAZARD. TaqMan Gene Expression Master Mix may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING CHEMICAL HAZARD. Gene Expression Assay (<2% formamide). Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! For optimal performance of TaqMan Gene Expression Assays, use 10 to 100 ng of cDNA per 20- or 50-µL reaction.

Table 2 PCR reaction mix components

Component	Volume (µL) / Reaction			
	50-µL Reactions (Standard 96-Well Plate Setup)		20-µL Reactions (384-Well Plate, 96- or 48-Well Fast Plate Setup‡)	
	Simplex PCR Setup	Duplex PCR Setup	Simplex PCR Setup	Duplex PCR Setup
TaqMan Gene Expression Master Mix (2X)	25.0	25.0	10.0	10.0
TaqMan Gene Expression Assay (20X)§	2.5	2.5	1.0	1.0
TaqMan® Endogenous Control (20X)	—	2.5	—	1.0
cDNA template + H_2O#	22.5	20.0	9.0	8.0
Total Volume	50.0	50.0	20.0	20.0

‡ When using TaqMan Gene Expression Master Mix, use Standard mode thermal cycling conditions.
§ See **www.allgenes.com** for TaqMan Gene Expression Assay information.
# Use 10 to 100 ng of cDNA plus RNase-free water.

Applied Biosystems recommends performing four replicates of each reaction. For more information about selecting the number of replicates to run, see "Appendix B Example: Statistically Significant Less Than Two-Fold Differences in Gene Expression" on page 45.

Select the reaction size depending on the type of reaction plate used. As shown in Table 3, for every four reactions, include volume for a fifth reaction to provide excess volume for the loss that occurs during reagent transfers.

Table 3 Example: Total reaction volume for four replicates plus excess

Component	Volume (µL) / Four Reactions Plus Excess‡			
	50-µL Reactions (Standard 96-Well Plate Setup)		20-µL Reactions (384-Well Plate, 96- or 48-Well Fast Plate Setup§)	
	Simplex PCR Setup	Duplex PCR Setup	Simplex PCR Setup	Duplex PCR Setup
TaqMan Gene Expression Master Mix (2×)	125.0	125.0	50.0	50.0
TaqMan Gene Expression Assay (20×)	12.5	12.5	5.0	5.0
TaqMan Endogenous Control (20×)	—	12.5	—	5.0
cDNA template + H_2O#	112.5	100.0	45.0	40.0
Total Volume	250.0	250.0	100.0	100.0

‡ For every four reactions, include volume for a fifth reaction to provide excess volume for the loss that occurs during reagent transfers.

§ When using TaqMan Gene Expression Master Mix, use Standard mode thermal cycling conditions.

Use 10 to 100 ng of cDNA per reaction, plus RNase-free water.

PCR Plate Set-Up

To prepare the reaction plate:

1.	Prepare the reaction mix for each sample using the components listed in Table 2 on page 24. • Calculate the volume of each component of the PCR reaction mix by multiplying the volume of each component by the number of replicates for each sample. • Include excess volume for the loss that occurs during reagent transfers. • Use 10 to 100 ng of cDNA per replicate.  **CAUTION CHEMICAL HAZARD. TaqMan Gene Expression Master Mix** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.  **WARNING CHEMICAL HAZARD. Gene Expression Assay (<2% formamide).** Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
2.	Cap the tube(s).
3.	Vortex the tube(s) briefly to mix the solutions.
4.	Centrifuge the tube(s) briefly to spin down the contents and eliminate any air bubbles from the solutions.

To prepare the reaction plate: ***(continued)***

5. Transfer the appropriate volume of each reaction mixture to each well of an optical plate, as specified in the following table.

Plate Format	Reaction Volume
MicroAmp™ Optical 96-Well Reaction Plate	50 µL
MicroAmp™ Fast Optical 96-Well Reaction Plate	20 µL
MicroAmp™ Optical 384-Well Reaction Plate	20 µL
MicroAmp™ Fast Optical 48-Well Reaction Plate	20 µL

6. Cover the plate with a MicroAmp™ Optical Adhesive Film. For standard 96-well plates, you may use MicroAmp™ Optical Caps.

   IMPORTANT! Use a MicroAmp™ Optical Film Compression Pad when using MicroAmp™ Optical Adhesive Film with a MicroAmp™ Optical 96-Well Reaction Plate on the ABI PRISM 7000 Sequence Detection System or the 7900HT Fast Real-Time PCR System.

7. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

Running 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:

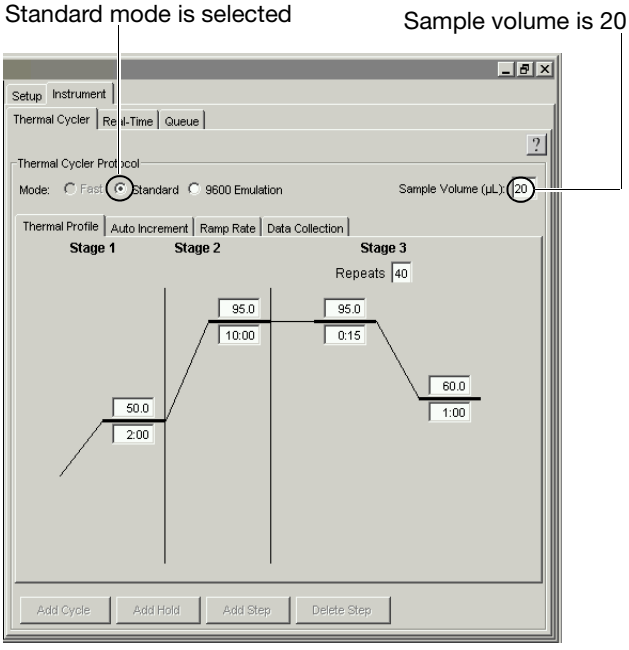
1. Place the reaction plate in the instrument.
2. Set the thermal cycling conditions using the default PCR thermal cycling conditions specified in the following table:

Step	UDG Incubation	AmpliTaq Gold, UP Enzyme Activation	PCR	
	HOLD	HOLD	CYCLE (40 Cycles)	
			Denature	Anneal/ Extend
Time	2 min	10 min	15 sec	1 min
Temp	50 °C	95 °C	95 °C	60 °C

IMPORTANT! The 2-minute, 50 °C step is required for optimal UDG enzyme activity. The 10-minute, 95 °C step is required to activate the AmpliTaq Gold, UP enzyme.

To run the plate: ***(continued)***

3. Select Standard mode, as shown below:



IMPORTANT! TaqMan Gene Expression Master Mix is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan Gene Expression Master Mix on the StepOne™, 7500 Fast, or 7900HT Fast instruments use Standard mode thermal cycling conditions. If you use assays other than the TaqMan Gene Expression assays, or use thermal cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal cycling conditions as needed. Refer to *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for more information on selecting thermal cycling conditions.

To run the plate: ***(continued)***

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

Plate Format	Reaction Volume
MicroAmp™ Optical 96-Well Reaction Plate	50 µL
MicroAmp™ Fast Optical 96-Well Reaction Plate	20 µL
MicroAmp™ Optical 384-Well Reaction Plate	20 µL
MicroAmp™ Fast Optical 48-Well Reaction Plate	20 µL

5. Start the run.

Analyzing Results

General Process Overview

The general process for analyzing the data from gene expression assays involves:

1. Viewing the amplification plots for the entire plate
2. Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves
3. Using the relative standard curve method or the comparative C_T method to analyze your data

Baseline and Threshold Values

When using Applied Biosystems Real-Time PCR instruments, you can use the Sequence Detection System (SDS) software to either automatically calculate or manually set the baseline and threshold for the amplification curves.

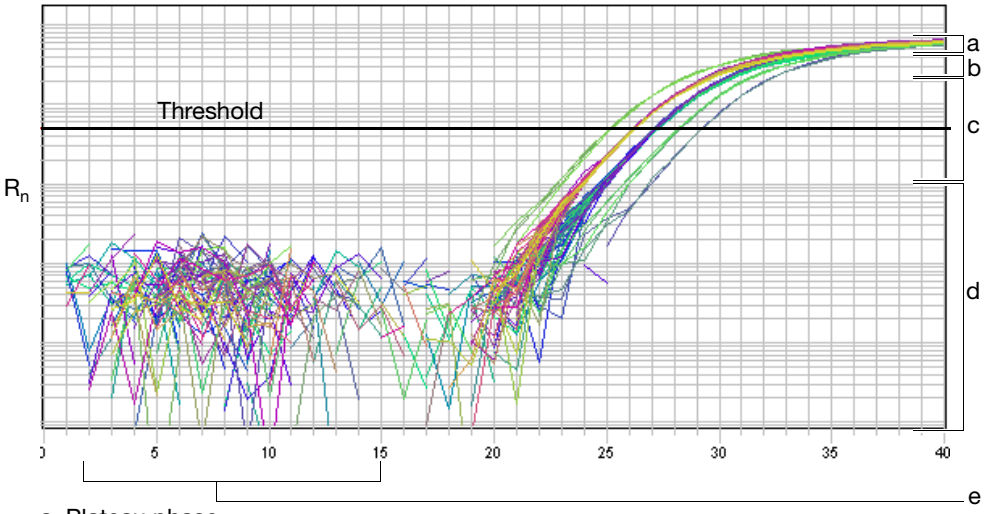
- *Baseline* refers to the initial cycles of PCR in which there is little change in fluorescence signal.
- The intersection of the threshold with the amplification plot defines the C_T in real-time PCR assays. The threshold is set above the background and within the exponential growth phase of the amplification curve. See "Threshold Cycle (C_T)" on page 8 for definition of this term.

Setting the Baseline and Threshold Automatically

The SDS software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the "typical" amplification curve shown in Figure 5 on page 32.

Experimental error (such as contamination or pipetting errors) can produce atypical data that can cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

IMPORTANT! After analysis, verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots, and adjust the values manually if necessary.



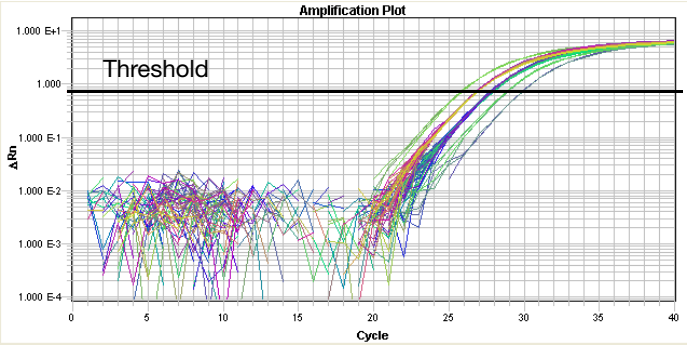
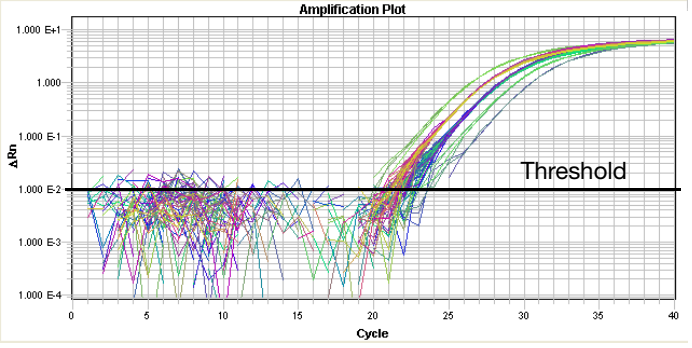
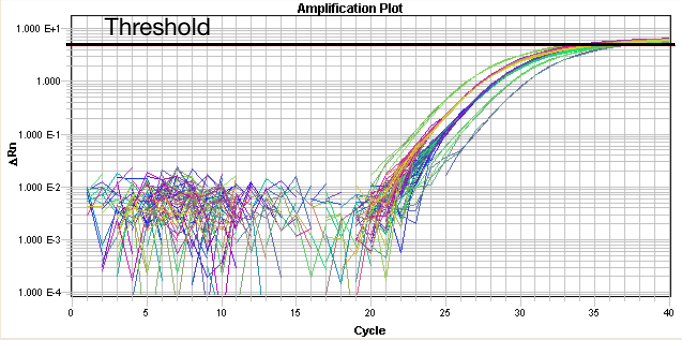
a. Plateau phase
b. Linear phase
c. Exponential (geometric) phase
d. Background
e. Baseline

Figure 5 Typical amplification curve

Setting the Baseline and Threshold Manually

If you use the SDS software to set the baseline and threshold values manually for any detector in the study, perform an adjustment procedure for each detector. The guidance referenced in "Resources for Data Analysis" on page 34 provides information on setting and adjusting your threshold and baseline manually.

Table 4 Correct and incorrect threshold settings

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 Data

You can perform two types of quantitation; relative and absolute using the Gene Expression Master Mix:

- Relative quantitation compares a target against an internal standard. You may 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.

Quantitation of cDNA Relative to a Calibrator Sample

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 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 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. All amplicons in these determinations should follow the amplicon design criteria defined previously around the Primer Express® Software. Refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for additional information about relative quantitation.

Resources for Data Analysis

Data analysis varies depending on the instrument. Refer to the following documents for information on setting threshold and baseline values:

- Analyze the Experiment chapter in *Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Presence/Absence Experiments*
- Analyze the Experiment chapter in *Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T ($\Delta\Delta C_T$) Experiments*
- Analyze the Experiment chapter in *Applied Biosystems StepOne™ Real-Time PCR System Getting Started Guide for Standard Curve Experiments*

- Analyzing AQ Data chapter in *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide* (PN 4347825)
- Analyzing Data in an RQ Study chapter in *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Relative Quantification Getting Started Guide* (PN 4347824)
- Analyzing Real-Time Data chapter in *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- Basic Operation chapter in *ABI PRISM® 7000 Sequence Detection System User Guide* (PN 4330228)

Additional data analysis information is provided in:

- Gene Expression and Other Quantitative Assays chapter in *Real-Time PCR Systems Chemistry Guide* (PN 4348358).
- The appropriate instrument user guide.
- The Applied Biosystems support web site for a variety of tutorials on performing TaqMan® Gene Expression Assays. See "How to Obtain Support" on page x.

Appendix A Using TaqMan Gene Expression Master Mix with Custom TaqMan Probes and Primers

This appendix describes how to design custom probes and primers for a real-time quantitative PCR assay. The process involves the following procedures:

- Determining your target template and amplicon
- Designing Custom TaqMan® Probes and Sequence Detection Primers
- Quantitating Custom TaqMan Probes and Sequence Detection Primers
- Determining optimal Custom TaqMan Probe and Sequence Detection Primer concentrations
- Performing real-time quantitative PCR

Determining Target Template and Amplicon

A target template is a DNA sequence, including a cDNA, genomic DNA, or plasmid nucleotide sequence.

Design primers to amplify *amplicons* (short segments of DNA) within the target sequence. The shortest amplicons work the best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Designing Custom TaqMan Probes and Primers

Designing Custom TaqMan Probes

Probes can be designed using Primer Express software as described in the *Primer Express Software Version 3.0 Getting Started Guide* (PN 4362460). Follow these guidelines when designing probes:

- Keep the G-C content in the 20 to 80% range.
- Avoid runs of an identical nucleotide, especially for guanine, where runs of four or more Gs should be avoided.
- Do not put a G on the 5′ end.
- Select the strand that gives the probe more Cs than Gs.
- The probe melting temperature (T_m) should be 68 to 70 °C when using Primer Express software.
- Use Primer Express software v 1.5a or later when designing TaqMan MGB probes.

- For duplex assays, both probes should be on the same strand.

Designing Sequence Detection Primers for TaqMan Assays

Primers can be designed using Primer Express software as described in the *Primer Express Software Version 3.0 Getting Started Guide*. Follow these guidelines when designing primers:

- Choose the primers after selecting the probe.
- Design the primers as close as possible to the probe without overlapping the probe.
- Keep the G-C content in the 20 to 80% range.
- Avoid runs of an identical nucleotide, especially for guanine, where runs of four or more Gs should be avoided.
- The T_m of each primer should be 58 to 60 °C.
- The five nucleotides at the 3′ end of each primer should have no more than two G and/or C bases.

Quantitating Probes and Primers

Use a spectrophotometric method to determine the concentrations of the probes and primers received.

To quantitate probes and primers:

1.	Measure the absorbance (at 260 nm of a 1:100 dilution) of each probe and primer oligonucleotide in TE buffer.
2.	Calculate the sum of extinction coefficient contributions for each probe and primer: extinction coefficient contribution = Σ(extinction coefficient × number of bases in oligonucleotide sequence) See the example in Table 5 on page 39 for the extinction coefficients of each chromophore.

To quantitate probes and primers: *(continued)*

3. Calculate the oligonucleotide concentration in µM for each probe and primer:

$$\text{absorbance @ 260 nm} = \text{sum of extinction coefficient contribution} \times \text{cuvette pathlength} \times \text{concentration}/100$$

Rearranging to solve for concentration:

$$\text{concentration} = 100[\text{absorbance @ 260 nm} / (\text{sum of extinction coefficient contribution} \times \text{cuvette pathlength})]$$

Example calculation of probe concentration

In the following example, the concentration of a probe (in TE buffer, diluted 1:100), with the sequence (6FAM™)CGTACTCGTTCGTGCTGC(MGB/NFQ) is calculated using the following values:

measured absorbance @260 nm	=	0.13
sum of extinction coefficient contributions for probe	=	268,208 $M^{-1}cm^{-1}$ (See Table 5)
cuvette pathlength	=	0.3 cm

Table 5 Extinction coefficients with example

Chromophore	Extinction Coefficient at A_{260}	Example	
		Number in Example Probe Sequence	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7,050	6	42,300
G	12,010	5	60,050
T	8,400	6	50,400
6FAM	20,958	1	20,958
TAMRA	—	—	—
TET	16,255	—	—
JOE	12,000	—	—
VIC	30,100	—	—
NED	31,050	—	—
CY3	4,900	—	—
CY5	10,000	—	—
MGB/NFQ	79,300	1	79,300
Total	—	—	**268,208**

Using the formula for concentration (from step 3 on page 38), concentration = 196 µM (A_{260}).

concentration = 100[0.13/(268,208 $M^{-1}cm^{-1} \times 0.3$ cm)]

concentration = 162 µM

Determining Optimal Primer Concentration

The purpose of this procedure is to determine the minimum primer concentrations giving the maximum ΔR_n. The Applied Biosystems Real-Time PCR Systems can provide additional data for optimization using the minimum threshold cycle (C_T). Refer to your instrument user manual for more information.

To determine the optimal primer concentration:

1. Prepare a PCR reaction mix for primer optimization:

   CHEMICAL HAZARD. TaqMan Gene Expression Master Mix may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

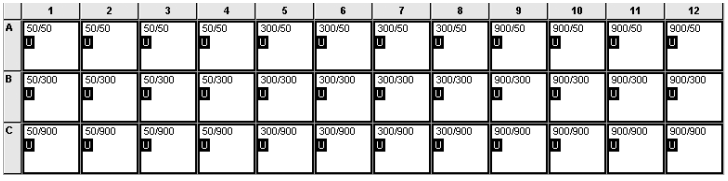
Reaction Component	Volume Per 50-µL Sample	Volume Per 20-µL Sample	Final Concentration
TaqMan Gene Expression Master Mix (2×)	25.0	10.0	1×
Forward primer	5.0	2.0	50 to 900 nM
Reverse primer	5.0	2.0	50 to 900 nM
TaqMan probe (2.5-µM)	5.0	2.0	250 nM
DNA sample	5.0	2.0	10 to 100 ng
Water	5.0	2.0	—
Total	50.0	20.0	—

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

2. Run at least four replicates of each of the nine conditions as shown:

	Forward Primer (nM)		
Reverse 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

3. Load the plate with four replicates of each condition as shown:



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

4. Place the plate in the Applied Biosystems Real-Time PCR System and follow the thermal cycling conditions:

Step	UDG Incubation	AmpliTaq Gold, UP Enzyme Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temp	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Vol	20 or 50 µL‡			

‡ Select appropriate volume for reaction plate.

IMPORTANT! The 2-minute, 50 °C step is required for optimal UDG enzyme activity. The 10-minute, 95 °C step is required to activate the AmpliTaq Gold, UP enzyme.

5. At the end of runs, tabulate the results for ΔR_n. Choose the minimum forward- and reverse-primer concentrations that yield the maximum ΔR_n.

Determining Optimal Probe Concentration

The purpose of this procedure is to determine the minimum probe concentrations that give the minimum C_T for each probe target.

The majority of TaqMan assays are designed and run following Applied Biosystems assay development guidelines, a concentration of 900-nM primers and a 250-nM probe provides for a highly reproducible and sensitive assay.

To determine the optimal probe concentration:

1. Prepare a PCR reaction mix:

   CAUTION CHEMICAL HAZARD. TaqMan Gene Expression Master Mix may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Reaction Component	Volume Per 50-µL Sample	Volume Per 20-µL Sample	Final Concentration
TaqMan Gene Expression Master Mix (2×)	25.0	10.0	1×
Forward primer	5.0	2.0	Optimal
Reverse primer	5.0	2.0	Optimal
TaqMan probe	5.0	2.0	50 to 250 nM
DNA sample	5.0	2.0	10 to 100 ng
Water	5.0	2.0	—
Total	50.0	20.0	—

2. For single-probe assays, determine the optimal probe concentration by running four replicates at each 50-nM interval from 50 to 250 nM.

   Note: Use the forward- and reverse-primer concentrations (determined using the procedure on page 40) in the reaction mix.

To determine the optimal probe concentration: ***(continued)***

3. Place the plate in the Real-Time PCR System and follow the thermal cycling conditions:

Step	UDG Incubation	AmpliTaq Gold, UP Enzyme Activation	PCR	
	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/ Extend
Temp	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Vol	20 or 50 µL‡			

‡ Select appropriate volume for reaction plate.

IMPORTANT! The 2-minute, 50 °C step is required for optimal UDG enzyme activity. The 10-minute, 95 °C step is required to activate the AmpliTaq Gold, UP enzyme.

4. Tabulate the results for C_T. Choose the minimum probe concentrations that yield the minimum C_T.

Recommended sample input for real-time quantitative PCR

For routine assays that are optimized as described here, perform real-time quantitative PCR using:

- 0.1 ng to 1 µg of DNA
- The determined optimum probe and primer concentrations
- The appropriate volume of TaqMan Gene Expression Master Mix as described in "PCR Mix Components" on page 24
- The thermal cycling conditions specified in the appropriate instrument user guide

Note: For the RT step of this RT-PCR reaction, use 10 pg to 100 ng of RNA.

Appendix B Example: Statistically Significant Less Than Two-Fold Differences in Gene Expression

Overview

The goal of performing a gene expression assay is to identify differential gene expression between your samples. You can statistically analyze your real-time quantitative PCR results to determine whether a measured difference in gene expression of less than two-fold is statistically significant. This appendix provides an example to show how the statistical significance of your results relates to experimental variability.

Definitions

The following terms are used in this appendix:

Term	Definition
Statistically significant (to a 99.7% confidence level)	A result with a low probability (0.3%) of being due to chance
Full replicate	Repeated wells of the same sample with the same assay, where the contents of each well go through all experimental steps (sample preparation, reverse transcription, and PCR) separately
PCR or Technical replicate	Repeated wells of the same sample with the same assay, where the contents of each well go through all the same pre-PCR steps together
Fold difference	The measured ratio of the quantity of template in Sample A over the quantity of template in Sample B, (where quantity A > quantity B, so that the ratio is > 1)
Minimum fold difference	The smallest fold difference that is statistically significant

Minimum Fold Difference

The minimum fold difference for each assay depends on your experimental design and variability. The minimum fold difference decreases (improves) with:

- an increase in the number of full replicates run of each sample
- a decrease in the standard deviation between full replicates

Table 6 on page 47 demonstrates how the minimum fold difference depends on the threshold cycle (C_T) standard deviation and the number of full replicates run. The Table 6 values are calculated from a 99.7% 2-tail confidence range using a z-test on the difference between the C_T means of the two samples, where

$$\text{standard error in the } C_T \text{ difference} = \frac{\text{expected } C_T \text{ standard deviation} \times \sqrt{2}}{\sqrt{\text{number of full replicates per sample}}}$$

and using the following assumptions:

- The standard deviation between full replicates is consistent (based on at least 30 previous measurements) and takes into account the variability from sample preparation, reverse transcription, and PCR
- The PCR efficiency is greater than 90%
- A 99.7% level of confidence is required
- Each sample has the same number of full replicates

Table 6 is provided as an example. However, if your experiment meets the assumptions in the paragraph above, you may use Table 6 to determine the statistical significance of your results. If the variability from PCR is your dominant source of variability, then you may use PCR replicates rather than full replicates to determine "Number of Replicates" in Table 6 on page 47. Take into consideration any additional variability resulting from low final template quantity (high C_T values). If your standard deviation is variable due to sample preparation or other issues, use a t-test instead to determine statistical differences between samples.

Table 6 Minimum Fold Difference

Minimum Fold Difference‡								
Expected Standard Deviation§	Number of Full Replicates							
	1	2	3	**4#**	5	6	7	8
0.066	1.21	1.14	1.12	**1.10**	1.09	1.08	1.07	1.07
0.125	1.44	1.29	1.23	**1.20**	1.18	1.16	1.15	1.14
0.180	1.69	1.45	1.35	**1.30**	1.26	1.24	1.22	1.20
0.231	1.96	1.61	1.47	**1.40**	1.35	1.32	1.29	1.27
0.279	2.25	1.77	1.60	**1.50**	1.44	1.39	1.36	1.33
0.323	2.56	1.94	1.72	**1.60**	1.52	1.47	1.43	1.39
0.365	2.89	2.12	1.85	**1.70**	1.61	1.54	1.49	1.46
0.404	3.24	2.30	1.97	**1.80**	1.69	1.62	1.56	1.52
0.441	3.61	2.48	2.10	**1.90**	1.78	1.69	1.62	1.57
0.477	4.00	2.67	2.23	**2.00**	1.86	1.76	1.69	1.63

‡ Minimum Fold Difference calculated using a 99.7% level of confidence. Note that lower minimum fold difference values would result from using lower confidence levels (99 or 95%).

§ This is the expected standard deviation between full replicates for this assay at the measured C_T values, based on previously measured variability, not the standard deviation within full replicates from one sample.

Applied Biosystems recommends four replicates.

Example Using Minimum Fold Difference Table

In this example, an assay is run to screen a sample for differences relative to a control. The assay meets the conditions used to develop Table 6:

- The Pre-PCR (sample preparation and reverse transcription) variability has been measured, and PCR is known to be the dominant source of variability.
- Based on 30 runs of this assay, the typical standard deviation between PCR replicates at these C_T values has been calculated.

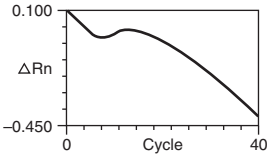
Four PCR replicates are run of both the sample and control. An average of 12,500 copies of sample and 10,000 copies of control are measured, so the measured fold difference is 12,500/10,000 = 1.25.

Although the C_T standard deviation for this particular run is very small, the typical C_T standard deviation for this assay is known to be 0.12 C_Ts. The closest expected standard deviation value from Table 6 on page 47 is 0.125.

The Minimum Fold Difference from Table 6, (using number of replicates = 4 and expected standard deviation = 0.125), is 1.20.

Because the measured fold difference 1.25 is greater than the minimum fold difference 1.20, the difference in gene expression between the sample and control is statistically significant (to a confidence level of 99.7%).

Appendix C Troubleshooting

Observation	Possible Cause	Recommended Action
$\Delta R_n \leq$ No Template Control ΔR_n, and no amplification plot	Inappropriate reaction conditions	Troubleshoot RT-PCR optimization.
	Incorrect dye components chosen	Check dye component prior to data analysis.
	Reaction component omitted	Check that all the correct reagents were added.
	Incorrect primer or probe sequence	Resynthesize with appropriate sequence.
	Degraded template or no template added	Repeat with fresh template.
	Reaction inhibitor present	Repeat with purified template.
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.
$\Delta R_n \leq$ No Template Control ΔR_n, and both reactions show an amplification plot	Amplicon contamination of reagents Template contamination of reagents	Check technique and equipment to confine contamination. Use fresh reagents.
Shifting R_n value during the early cycles of PCR (cycle 0–5)	Fluorescence emissions have not stabilized to buffer conditions of reaction mix. **Note:** This condition does not affect PCR or the final results.	Reset lower value of baseline range. Pre-mix the probe, primer, and TaqMan® Gene Expression Master Mix to allow the reaction mix to equilibrate.
Abnormal amplification plot: 	C_T value <15, amplification signal detected in early cycles	Reset upper value of baseline range. Dilute the sample to increase the C_T value.
Multicomponent signal for ROX is not linear	Pure dye component's spectra are incorrect	Rerun pure dye spectra.
	Incorrect dye components choosen	Choose correct dyes for data analysis.

Observation	Possible Cause	Recommended Action
Small ΔR_n	PCR efficiency is poor	Recheck the optimization.
	Low copy number of target	Increase starting copy number.
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.
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.
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.
High C_T values/poor precision or failed PCR reactions	Insufficient cDNA template is present	Use 10 to 100 ng of cDNA template per 20- or 50-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.
	Reduced number of PCR cycles in the thermal cycler protocol	Increase the number of PCR cycles to the default setting of 40 (see **page 28**).
C_T value is lower than expected	More sample added than expected Template or amplicon contamination	Reduce sample amount. Review "Preventing Contamination" on page 16.

Observation	Possible Cause	Recommended Action
Standard deviation of C_T value >0.16	Inaccurate pipetting	Prepare a Reaction Mix for each sample type. Mix well. Use positive-displacement pipettors.
	Threshold set incorrectly	See Table 4, “Correct and incorrect threshold settings,” on page 33.
Standard deviation of C_T value for duplex reaction >0.225	Inaccurate pipetting	Prepare a Reaction Mix for each sample type. Mix well.
	Reaction Mix not mixing well	Use positive-displacement pipettors.
	Threshold set incorrectly	See Table 4, “Correct and incorrect threshold settings,” on page 33.

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