TaqMan® Universal PCR Master Mix

Protocol

Applied Biosystems

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Introduction

1

Overview

About This Chapter

This chapter describes the TaqMan® Universal PCR Master Mix and provides important information about safety.

In This Chapter

The following topics are covered in this chapter:

Topic	See Page
Purpose of the Kit	1-2
Materials and Equipment	1-6
Safety	1-9
Preventing Contamination	1-12

Purpose of the Kit

About the Kit

To amplify your DNA target of choice, primers and probe are designed according to guidelines stated in this protocol, and universal thermal cycling parameters are followed. The purpose of this kit is to detect known sequences of genomic, plasmid, or complementary DNA (cDNA).

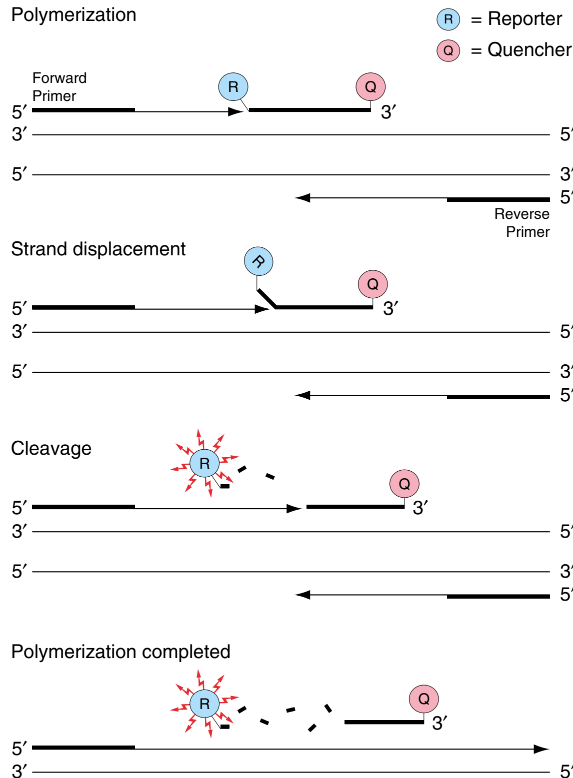
In RNA quantitation assays the TaqMan Universal PCR Master Mix is used in the second step of a two-step reverse transcription–polymerase chain reaction (RT-PCR) protocol. The template is cDNA generated from a reverse transcription reaction.

The TaqMan Universal PCR Master Mix may be used for real-time or plate read (endpoint) detection of DNA or cDNA. Analysis is performed using the ABI PRISM® 7700 Sequence Detection System, the ABI PRISM® 7900HT Sequence Detection System, ABI PRISM® 7000 Sequence Detection System, or the GeneAmp® 5700 Sequence Detection System.

Basics of the 5´ Nuclease Assay

The PCR reaction exploits the 5´ nuclease activity of AmpliTaq Gold® DNA Polymerase 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, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The figure below shows the forklike-structure-dependent, polymerization-associated 5´ to 3´ nuclease activity of AmpliTaq Gold enzyme during PCR.



Note The forklike-structure-dependent, polymerization-associated, 5´ to 3´ nuclease activity of AmpliTaq Gold DNA Polymerase during PCR.

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 between the forward and reverse primer sites.

The 5´ to 3´ nucleolytic activity of the AmpliTaq Gold 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 does not interfere with the exponential accumulation of product.

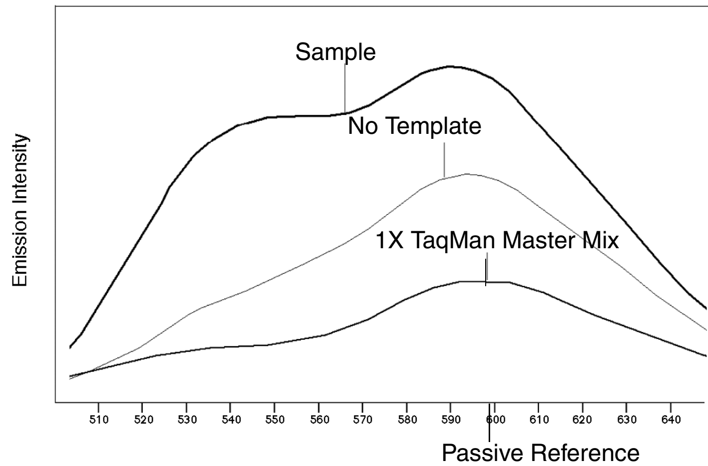


Figure 1-1 An overlay of three emission scans, post-PCR.

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

TaqMan Probe

The probe consists of an oligonucleotide with a 5´-reporter dye and a 3´-quencher dye. A fluorescent reporter dye, such as FAM™ dye, is covalently linked to the 5´ end of the oligonucleotide. TET™ dye , and VIC™ dye are also used as reporter dyes. Each of the reporters is quenched by TAMRA™ dye at the 3´ end or non-fluorescent quencher.

AmpliTaq Gold DNA Polymerase

The AmpliTaq Gold enzyme is a thermal stable DNA polymerase. The enzyme has a 5´ to 3´ nuclease activity, but lacks a 3´ to 5´ exonuclease activity (Innis *et al.*, 1988; Holland *et al.*,1991). When using AmpliTaq Gold enzyme, you can introduce Hot Start PCR and Time Release PCR into existing amplification systems with little or no modification of cycling parameters or reaction conditions. These techniques improve amplification of most templates by lowering background and increasing amplification of specific products.

TaqMan Universal PCR Master Mix

TaqMan Universal PCR Master Mix consists of one module, containing one tube of the TaqMan Universal PCR Master Mix.

TaqMan Universal Master Mix Reagents provide a PCR mix that may be used with any appropriately designed primer and probe to detect any DNA or cDNA sequence.

AmpErase® uracil-N-glycosylase (UNG) (P/N N808-0096) is a component of the TaqMan Universal PCR Master Mix (P/N 4304437, 4326708). AmpErase UNG treatment can prevent the reamplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded DNA (Longo *et al.*, 1990).

Materials and Equipment

Kit Components

The TaqMan Universal PCR Master Mix (P/N 4304437 and 4326708) is supplied in a 2X concentration and contains sufficient reagent to perform 200 reactions (50 µL each). The mix is optimized for TaqMan reactions and contains AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer components. The TaqMan Universal PCR Master Mix (P/N 4324018 and 4326614) contains all the above ingredients except AmpErase UNG.

Materials Required but Not Supplied

The following items are required when using TaqMan Universal PCR Master Mix, but are not supplied. See the table for source information.

User-Supplied Materials

Item	Source
7900HT Sequence Detection System 7000 Sequence Detection System	See your local Applied Biosystems representative for the instrument or software best suited to meet your needs.
Primer Express™ software (single-use license)	
Sequence Detection primers ♦ Min 4000 pmol purified for sequence detection ♦ Min 40,000 pmol purified for sequence detection ♦ Min 130,000 pmol purified for sequence detection	Applied Biosystems ♦ P/N 4304970 ♦ P/N 4304971 ♦ P/N 4304972
TaqMan® MGB Probe ♦ 5000 to 6000 pmoles ♦ 15,000 to 25,000 pmoles ♦ 50,000 to 100,000 pmoles	Applied Biosystems ♦ P/N 4316034 ♦ P/N 4316033 ♦ P/N 4316032
TaqMan® TAMRA Probe ♦ 5000 to 6000 pmoles ♦ 15,000 to 25,000 pmoles ♦ 50,000 to 100,000 pmoles	Applied Biosystems ♦ P/N 450025 ♦ P/N 450024 ♦ P/N 450003

User-Supplied Materials *(continued)*

Item	Source
MicroAmp® Optical 96-Well Reaction Plate and Optical Caps	Applied Biosystems (P/N 403012)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems (P/N N801-0560)
MicroAmp® 96-well Tray/Retainer Set (10 sets)	Applied Biosystems (P/N 403081)
ABI PRISM™ 384-Well Clear Optical Reaction Plate with Barcode	Applied Biosystems (P/N 4309849)
Note The MicroAmp Optical 96-Well Reaction Plate may be sealed with: ♦ MicroAmp Optical Caps or ♦ ABI PRISM™ Optical Adhesive Cover	
MicroAmp® Optical Caps	Applied Biosystems (P/N 4323032)
ABI PRISM Optical Adhesive Cover Starter Pack containing 20 optical adhesive covers, one applicator, and one compression pad. **Note** The MicroAmp Optical 96-well Reaction Plate may be sealed with MicroAmp Optical caps or ABI PRISM Optical Adhesive Cover	Applied Biosystems (P/N 4313663)
MicroAmp® Optical Tubes	Applied Biosystems (P/N N801-0933)
Sequence Detection Systems Spectral Calibration Kit (for 7700 instrument only)	Applied Biosystems (P/N 4305822)
Sequence Detection Systems 384-Well Spectral Calibration Kit	Applied Biosystems (P/N 4323977)
ABI PRISM® 7900 Sequence Detection Systems 96-Well Spectral Calibration Kit	Applied Biosystems (P/N 4328639)
ABI PRISM® 7000 Sequence Detection Systems Spectral Calibration Kit	Applied Biosystems (P/N 4328895)

User-Supplied Materials *(continued)*

Item	Source
Centrifuge with adapter for 96-well plate	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS
NuSieve 4% (3:1) agarose gels, for DNA <1 kb	FMC BioProducts (P/N 54928)
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement or air-displacement	MLS
Polypropylene tubes	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

Storage and Stability

Upon receipt, store the TaqMan Universal PCR Master Mix at 2 to 8 °C.

If TaqMan Universal PCR Master Mix is mistakenly stored at –20 °C, please transfer it to 2 to 8 °C. Before using, make sure the Master Mix is thoroughly thawed and mixed.

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

⚠ CAUTION Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

⚠ WARNING Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

⚠ DANGER Indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

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

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Hazard Warning

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

- Read and understand the material safety data sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current material safety data sheet (MSDS) before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ WARNING **CHEMICAL HAZARD**. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below:

To order documents by automated telephone service:

Step	Action
1	From the U.S. or Canada, dial **1.800.487.6809**.
2	Follow the voice instructions to order documents (for delivery by fax). **Note** There is a limit of five documents per fax request.

To order documents by telephone:

In the U.S.	Dial **1.800.345.5224**, and press **1**.
In Canada	Dial **1.800.668.6913**, and press **1** for English or **2** for French.

To view, download, or order documents through the Applied Biosystems Web site:

Step	Action
1	Go to **http://www.appliedbiosystems.com**
2	Click **SERVICES & SUPPORT** at the top of the page, click **Documents on Demand**, then click **MSDS.**
3	Click **MSDS Index**, search through the list for the chemical of interest to you, then click on the MSDS document number for that chemical to open a PDF version of the MSDS.

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

Preventing Contamination

Overview

Due to the high throughput and repetitive nature of the 5´ nuclease assay, special laboratory practices are necessary in order to avoid false positive amplifications (Kwok and Higuchi, 1989). This is because of the capability for single DNA molecule amplification during the PCR process (Saiki *et al.*, 1985; Mullis *et al.*, 1987).

False Positives

Special laboratory practices are necessary in order to avoid false positive amplifications (Higuchi, *et al.*, 1989). This is because of the capability for single DNA molecule amplification provided by the PCR process (Saiki *et al.*, 1985; Mullis *et al.*, 1987; Saiki *et al.*, 1988). Because of the enormous amplification possible with PCR, amplicon carryover can result in sample contamination. Other sources of contamination could be from samples with high DNA levels or from positive control templates.

When dUTP replaces dTTP as a dNTP substrate in PCR and the method described below is used, AmpErase UNG treatment can prevent the reamplification of carryover PCR products in subsequent experiments Sninsky and Gelfand, pers. comm.) This method uses enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to degrade specifically PCR products from previous PCR amplifications or to degrade mis-primed, non-specific products produced prior to specific amplifications, but not degrade native nucleic acid templates.

The method used to make PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR mix and treating subsequent PCR mixes with the enzyme uracil N-glycosylase (UNG, EC 3.2.2-) prior to amplification (Longo *et al.*, 1990).

The AmpErase UNG provided in this product is a pure, nuclease-free, 26-kDa enzyme encoded by the *Escherichia coli* uracil N-glycosylase gene which has been inserted into an *E. coli* host to direct the expression of the native form of the enzyme (Higuchi *et al.*, 1989).

Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried over PCR products, we encourage users to continue using the specific devices and suggestions described in this protocol booklet and in Kwok (1990) and Higuchi(1989) to minimize cross-contamination from non-dU-containing PCR products or other samples.

AmpErase UNG Inactivation

Ten-minute incubation at 95 °C is necessary to cleave the dU-containing PCR product generated in the low temperature (18 to 50 °C) incubation, to substantially reduce AmpErase UNG activity, and to denature the native DNA in the experimental sample. Because UNG is not completely deactivated during the 95 °C incubation, it is important to keep the reaction temperatures greater than 55 °C, to prevent amplicon degradation.

Prevention of PCR Product Carryover

Use primers that contain dA nucleotides near the 3´ ends so that any primer-dimer generated is efficiently degraded by AmpErase UNG at least as well as any dU-containing PCR products. The further a dA nucleotide is from the 3´ end, the more likely that partially degraded primer-dimer molecules may serve as templates for a subsequent PCR amplification.

Production of primer dimer could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3' terminal dU-nucleotides should be considered. Single-stranded DNA with terminal dU nucleotides are not substrates for AmpErase UNG (Delort *et al.*, 1985) and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for AmpErase UNG.

The concentration of AmpErase UNG and the time of the incubation step necessary to prevent amplification of contaminating dU-containing PCR product depends on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination expected. In most cases, using AmpErase UNG at 1 U/l00 µL reaction and incubation at 50 °C for two minutes is sufficient.

Do not attempt to use AmpErase UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR product, preventing further amplification.

General PCR Practices

Please follow these recommended procedures:

- ♦ Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- ♦ Change gloves whenever you suspect that they are contaminated.

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

Fluorescent Contaminants

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

Amplifying Custom Target Sequences for Quantitation

2

Overview

About This Chapter

This chapter describes how to design a custom 5′ nuclease assay for quantitating DNA or cDNA templates.

In This Chapter

The following topics are covered in this chapter:

Topic	See Page
Identifying Target Sequence and Amplicon Size	2-2
Designing TaqMan Probes and Primers	2-3
Quantitating Probes and Primers	2-4
Optimizing Primer and Probe Concentrations	2-5
Performing Routine Analysis	2-9

Identifying Target Sequence and Amplicon Size

Target Template Defined

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

Amplicon Defined

Design primers to amplify short segments of DNA within the target sequence. These short segments are called amplicons.

Amplicon Size

The shortest amplicons work the best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Designing TaqMan Probes and Primers

Probes

Probes can be designed using Primer Express software as described in the *Primer Express User Bulletin* (P/N 4317594). Follow these guidelines when designing probes:

- ♦ Keep the G-C content in the 20 to 80% range.
- ♦ Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- ♦ Do not put a G on the 5´ end.
- ♦ Select the strand that gives the probe more Cs than Gs.
- ♦ Both probes should be on the same strand.
- ♦ For single-probe assays, the melting temperature (T_m) should be 68 to 70 °C when using Primer Express software.
- ♦ Use Primer Express software v 1.5a or v 2.0 when designing TaqMan MGB probes.

Primers

Primers can be designed using Primer Express software as described in the *Primer Express User Bulletin*. Follow these guidelines when designing primers:

- ♦ Choose the primers after 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. This is especially true 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

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

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

Chromophore	Extinction Coefficient	Number	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
FAM	20,958	1	20,958
TAMRA	31,980	1	31,980
TET	16,255	0	—
JOE	12,000	—	—
VIC	30,100	—	—
Total	—	—	220,888

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

$0.13 = 220{,}888\ M^{-1}cm^{-1} \times 0.3\ cm \times C/100$

$C = 196\ \mu M$

Optimizing Primer and Probe Concentrations

Determining Optimal Primer Concentration

The purpose of this procedure is to determine the minimum primer concentrations giving the maximum ΔR_n. The ABI PRISM Sequence Detection Systems can provide additional data for optimization using the minimum threshold cycle (C_T).

To determine the optimal primer concentration:

Step	Action
1	Prepare a PCR reaction mix to run at least four replicates of each of the nine conditions as shown in the table below. The reaction mix is described in “PCR Reaction Mix for Primer Optimization” on page 2-6.
2	Load the plate 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	UNKN 50/50	UNKN 50/50	UNKN 50/50	UNKN 50/50	UNKN 300/50	UNKN 300/50	UNKN 300/50	UNKN 300/50	UNKN 900/50	UNKN 900/50	UNKN 900/50	UNKN 900/50
B	UNKN 50/300	UNKN 50/300	UNKN 50/300	UNKN 50/300	UNKN 300/300	UNKN 300/300	UNKN 300/300	UNKN 300/300	UNKN 900/300	UNKN 900/300	UNKN 900/300	UNKN 900/300
C	UNKN 50/900	UNKN 50/900	UNKN 50/900	UNKN 50/900	UNKN 300/900	UNKN 300/900	UNKN 300/900	UNKN 300/900	UNKN 900/900	UNKN 900/900	UNKN 900/900	UNKN 900/900

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

Step	Action
3	Place the plate in the ABI PRISM Sequence Detection System and follow the thermal cycling conditions described in “Thermal Cycling Conditions for Primer Optimization” on page 2-7.
4	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.

PCR Reaction Mix for Primer Optimization

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

Reaction Component	Volume (µL) Per Sample	Final Concentration
TaqMan Universal PCR Master Mix (2X)	25.0	1X
Forward primer	5.0	50 to 900 nM
Reverse primer	5.0	50 to 900 nM
TaqMan probe (2.5 µM)	5.0	250 nM
DNA sample	5.0	10 to 100 ng
Water	5.0	—
Total	50.0[a]	—

a. If you are using the 7900HT 384-well block, this reaction setup may be scaled down appropriately to accomodate smaller reaction volumes.

Thermal Cycling Conditions for Primer Optimization

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	HOLD[a]	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

a. If using TaqMan Universal Master Mix without UNG AmpErase, this step is not necessary.

IMPORTANT The 2-min, 50 °C step is required for optimal UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold enzyme.

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.

For the vast majority of TaqMan assays that are designed and run following Applied Biosystems assay development guidelines, using a concentration of 900-nM primers and a 250-nM probe provides for a highly reproducible and sensitive assay when using cDNA or DNA as a substrate in a singleplex assay.

Procedure

To determine the optimal probe concentration:

Step	Action
1	For single-probe assays, determine the optimal probe concentration by running four replicates at each 50-nM interval from 50 to 250 nM. Prepare a PCR reaction mix as described in “Reaction Mix for Probe Optimization” on page 2-8. **Note** Use the forward- and reverse-primer concentrations determined above in the reaction mix.
2	Place the plate in the Sequence Detection System and follow the thermal cycling conditions described in “Thermal Cycling Conditions for Probe Optimization” on page 2-8.

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

Step	Action
3	Tabulate the results for C_T. Choose the minimum probe concentrations that yield the minimum C_T.

Reaction Mix for Probe Optimization

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

Reaction Component	Volume (µL) Per Sample	Final Concentration
TaqMan Universal PCR Master Mix (2X)	25.0	1X
Forward primer	5.0	Optimal
Reverse primer	5.0	Optimal
TaqMan probe	5.0	50 to 250 nM
DNA sample	5.0	10 to 100 ng
Water	5.0	—
Total	50.0	—

Thermal Cycling Conditions for Probe Optimization

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	HOLD[a]	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

a. If using TaqMan Universal Master Mix without UNG AmpErase, this step is not necessary.

IMPORTANT The 2-min, 50 °C step is required for optimal UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold enzyme.

Performing Routine Analysis

Overview

For routine assays that are optimized as described here, perform analysis using optimum probe and primer concentrations and specified thermal cycling conditions.

Use optimized conditions to amplify DNA, or cDNA obtained from the first step of two-step RT-PCR. For routine analysis the following ranges of RNA and DNA can be used:

- RNA 10 pg to 100 ng
- Genomic DNA 0.1 ng to 1 µg

Amplifying Custom Sequences for Allelic Discrimination

3

Overview

About This Chapter This chapter describes how to optimize probe and primer concentrations.

In This Chapter The following topic is covered in this chapter:

Topic	See Page
Identifying Target Sequence and Amplicon Size	3-2
Designing the TaqMan Probe and Primers	3-3
Quantitating Probes and Primers	3-4
Optimizing Probe and Primer Concentrations	3-5

Identifying Target Sequence and Amplicon Size

Target Template Defined

A target is a nucleotide sequence, two primers, and a probe.

For allelic discrimination, each allele associated with a target has a probe labeled with its own fluorescent reporter dye.

Primers are common and have complete homology for both alleles.

Amplicon Size

The shortest amplicons work the best. Consistent results are obtained for amplicon ranges from 50 to 150 bp.

Designing the TaqMan Probe and Primers

Probes

The location of the polymorphism dictates the placement of the probe. Because mismatches near the end of probes tend not to be as disruptive to hybridization, Applied Biosystems generally recommends designing probes so that the polymorphic site is near the center of the probe.

Guidelines for Designing TaqMan Probes

- Use the VIC™ and FAM™ reporter dyes to label the allelic discrimination probes.
- Avoid runs of an identical nucleotide. This is especially true for guanosine, where runs of four or more Gs should be avoided.
- The 5´ end of a probe cannot be a guanosine residue. A guanosine residue adjacent to the reporter dye will quench the reporter fluorescence somewhat, even after cleavage.
- Using Primer Express® software, the melting temperature (T_m) should be 65 to 67 °C.
- Position the polymorphic site approximately in the middle third of the sequence.

Primers

After selecting probes for the assay, choose primers based on the guidelines below. Consequently, amplicons are usually 75 to 150 bp. By limiting the parameters for amplicon design (such as amplicon size), it is possible to run all reactions with a single reaction buffer (TaqMan® Universal PCR Master Mix (P/N 4304437)) and a single thermal cycling protocol.

Guidelines for Designing Primers

- Keep the G-C content in 20 to 80% range.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- Using Primer Express software, the T_m should be 58 to 60 °C.
- The five nucleotides at the 3´ end should have no more than two G and/or C bases.
- Place the forward and reverse primers as close as possible to the probe without overlapping the probe.

Quantitating Probes and Primers

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

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

Chromophore	Extinction Coefficient	Number	Extinction Coefficient Contribution
A	15,200	1	15,200
C	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
FAM	20,958	1	20,958
TAMRA	31,980	1	31,980
TET	16,255	0	—
VIC	30,100	—	—
Total	—	—	220,888

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

0.13 = $220{,}888\ M^{-1}cm^{-1} \times 0.3\ cm \times C/100$

C = 196 µM

Optimizing Probe and Primer Concentrations

Probe Concentrations

The purpose of this procedure is to determine the probe concentrations that give the most reliable autocalls.

For the vast majority of allelic discrimination assays designed and run following Applied Biosystems assay development guidelines, using a concentration of 900-nM primers and 200-nM probes provides for a highly reproducible and sensitive assay.

Determining Optimal Probe Concentrations

The following points should be noted when determining optimal probe concentrations.

- ♦ The initial fluorescence signals from the two probes are matched approximately.
- ♦ Fluorescence is measured directly. No thermal cycling is required.

Default Assay Conditions

The Applied Biosystems assay development guidelines enable allelic discrimination assays to be designed and optimized rapidly and efficiently. Since thousands of allelic discrimination assays have been developed this way using the ABI PRISM 7700 Sequence Detector and probes labeled with FAM and VIC, the following conclusion can be drawn:

For the vast majority of allelic discrimination assays designed and run following Applied Biosystems assay development guidelines, using a concentration of 900-nM primers and 200-nM probes provides for a highly reproducible and sensitive assay.

Determining Optimal Primer Concentrations

The purpose of this procedure is to determine the minimum primer concentrations that give the maximum R_n. The ABI PRISM Sequence Detection Systems can provide additional data for optimization using the minimum threshold cycle (C_T).

- ♦ Use the TaqMan Universal PCR Master Mix.

♦ Use the thermal cycler conditions in the table below.

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	92 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

IMPORTANT The 2-min, 50 °C step is required for optimal UNG activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold enzyme.

♦ Use one of the allelic discrimination probes with its target at a concentration of 100 nM.

♦ Run at least four replicates of each of the nine conditions defined by the 3 × 3 matrix below, as well as four No Template Control (NTC) and four No Amplification Control (NAC) replicates. The NTC and NAC replicates should be run at 900 nM forward and reverse primer concentrations.

	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

Plate Configuration

Wells	Universal PCR Master Mix 2X (µL)	10-µM FAM Probe (µL)	FAM Template Target (µL)	20-µM Forward Primer (µL)	20-µM Reverse Primer (µL)	Deionized Water	Total Volume Per Well (µL)
A1–A4	25	0.5	5.0	0.125	0.125	19.25	50
A5–A8	25	0.5	5.0	0.125	0.75	18.625	50
A9–A12	25	0.5	5.0	0.125	2.25	17.125	50

Plate Configuration *(continued)*

Wells	Universal PCR Master Mix 2X (µL)	10-µM FAM Probe (µL)	FAM Template Target (µL)	20-µM Forward Primer (µL)	20-µM Reverse Primer (µL)	Deionized Water	Total Volume Per Well (µL)
B1–B4	25	0.5	5.0	0.75	0.125	18.625	50
B5–B8	25	0.5	5.0	0.75	0.75	18.0	50
B9–B12	25	0.5	5.0	0.75	2.25	16.5	50
C1–C4	25	0.5	5.0	2.25	0.125	17.125	50
C5–C8	25	0.5	5.0	2.25	0.75	16.5	50
C9–C12	25	0.5	5.0	2.25	2.25	15.0	50
D1–D4 (NTC)	25	0.5	0	2.25	2.25	20.0	50
D5–D8 (NAC)[a]	25	0.5	5.0	2.25	2.25	14	50[a]

a. Add 1 µL of 0.5% sodium dodecyl sulfate (SDS) to each of the four NAC wells to inhibit any enzyme activity in those wells.

Reverse Transcription

4

Overview

About This Chapter

This chapter provides procedures for performing reverse transcription (RT). Guidelines and procedures are provided to ensure optimal RT performance.

In This Chapter

The following topics are covered in this chapter.

Topic	See Page
Reverse Transcription for All Amplicons Except 18S	4-2
Reverse Transcription for the 18S Amplicon	4-6

Reverse Transcription for All Amplicons Except 18S

Overview

Synthesis of cDNA from total RNA samples is the first step in the two-step RT-PCR gene expression quantification experiment. In this step, random hexamers, oligo $d(T)_{16}$, or sequence-specific reverse primers from the TaqMan Reverse Transcription Reagents (P/N N808-0234) prime total RNA samples for reverse transcription using MultiScribe™ Reverse Transcriptase.

Note N808-0234 is good for 200 reactions at 10 µL per reaction

Guidelines

Follow the guidelines below to ensure optimal RT performance.

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

Choice of Primers

The choice of primers for RT is best made after experimentally evaluating all three priming systems. For short RNA sequences containing no hairpin loops, any of the trhee priming systems work equally well. For longer RNA transcripts or sequences containing hairpin loops, consider the following guidelines.

Primers	Selection Guidelines
Random hexamers	♦ Try first for use with long reverse transcripts or reverse transcripts containing hairpin loops ♦ Use to transcribe all RNA (rRNA, mRNA, and tRNA)
Sequence-specific reverse primer	♦ Use to reverse transcribe RNA-containing complementary sequences only
Oligo $d(T)_{16}$	♦ Use to reverse transcribe only eukaryotic mRNAs and retroviruses with poly-A tails ♦ Avoid long mRNA transcripts or amplicons greater than two kilobases upstream

Performing RT Reactions

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

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

To perform RT reactions:

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

RT Reaction Mix

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

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

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

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

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

Thermal Cycling

To conduct RT thermal cycling:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Load the reactions into a thermal cycler.</td></tr>
<tr><td>2</td><td>Program the thermal cycler or Sequence Detection System with the following conditions:
<table>
<tr><th rowspan="2">Step</th><th>Incubation[a]</th><th>RT</th><th>Reverse Transcriptase Inactivation</th></tr>
<tr><td>HOLD</td><td>HOLD</td><td>HOLD</td></tr>
<tr><td>Temp</td><td>25 °C</td><td>48 °C</td><td>95 °C</td></tr>
<tr><td>Time</td><td>10 min</td><td>30 min</td><td>5 min</td></tr>
<tr><td>Volume</td><td colspan="3">10 µL</td></tr>
</table>
a. If using random hexamers or oligo $d(T)_{16}$ primers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 min) is necessary to maximize primer-RNA template binding.</td></tr>
<tr><td>3</td><td>Begin RT.

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

Reverse Transcription for the 18S Amplicon

Overview

Synthesis of cDNA from total RNA samples is the first step in the two-step RT-PCR gene expression quantification experiment. In this step, random hexamers from the TaqMan Reverse Transcription Reagents prime total RNA samples for reverse transcription using MultiScribe Reverse Transcriptase.

Recommended Template

The following table lists the known template incompatibilities.

Template	Explanation
Poly A^+	The 18S rRNA endogenous control assay cannot accurately evaluate cDNA generated from poly A^+ RNA samples because most of the rRNA has been removed from them.
Non-human	Except for 18S rRNA, all assays are human-specific.

Template Quality

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

Template Quantity

If possible, use spectrophotometric analysis to determine the concentrations of purified total RNA samples before reverse transcription. The table below lists the recommended range of initial template quantities for the RT step.

Initial Template	Quantity of total RNA (per 100-µL RT reaction)
Total RNA	60 ng to 2 µg

Guidelines

Follow the guidelines below to ensure optimal RT performance.

- A 100-µL RT reaction efficiently converts a maximum of 2 µg total RNA to cDNA. Perform multiple RT reactions in multiple wells if using more than 2 µg total RNA.
- Use only random hexamers to reverse transcribe the total RNA samples for gene expression assays.

Preparing the Reactions

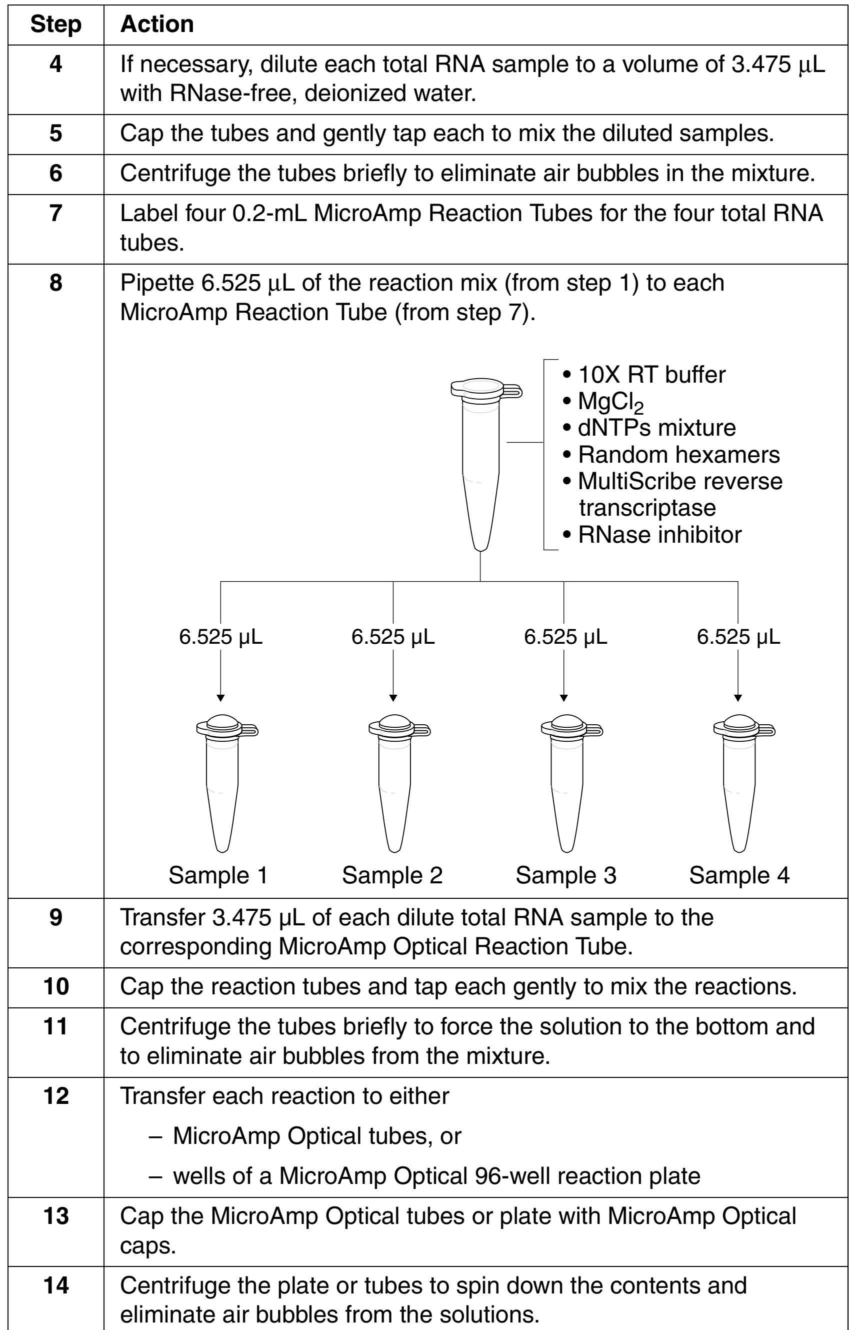
The following procedure describes the preparation of four different test samples for RT. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents.

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

To prepare the RT reactions:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 0.2-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed. If preparing four reactions, follow the recommended volumes shown below.
<table>
<tr><th rowspan="2">Component</th><th colspan="2">Volume (µL)</th><th rowspan="2">Final Conc.</th></tr>
<tr><th>Per Sample</th><th>Reaction Mix (x4)</th></tr>
<tr><td>RNase-free water</td><td>see below[a]</td><td>see below[a]</td><td>—</td></tr>
<tr><td>10X RT Buffer</td><td>1.0</td><td>4.0</td><td>1X</td></tr>
<tr><td>25 mM $MgCl_2$</td><td>2.2</td><td>8.8</td><td>5.5 mM</td></tr>
<tr><td>deoxyNTPs Mixture</td><td>2.0</td><td>8.0</td><td>500 µM per dNTP</td></tr>
<tr><td>Random Hexamers</td><td>0.5</td><td>2.0</td><td>2.5 µM</td></tr>
<tr><td>RNase Inhibitor</td><td>0.2</td><td>0.8</td><td>0.4 U/µL</td></tr>
<tr><td>MultiScribe Reverse Transcriptase (50 U/µL)</td><td>0.625</td><td>2.5</td><td>3.125 U/µL</td></tr>
<tr><td>Total[b]</td><td>6.525</td><td>26.1</td><td>—</td></tr>
</table>
a. The volume of RNase-free water (µL) is 3.475–RNA sample volume in a 10-µL reaction.
b. If changing the reaction volume, make sure the final proportions are</td></tr>
<tr><td>2</td><td>Label four 0.2-mL microcentrifuge tubes for the four reactions.</td></tr>
<tr><td>3</td><td>Transfer 60 ng to 2 µg (up to 3.475 µL) of each total RNA sample to the corresponding microcentrifuge tube.</td></tr>
</table>

To prepare the RT reactions: *(continued)*



Step	Action
4	If necessary, dilute each total RNA sample to a volume of 3.475 µL with RNase-free, deionized water.
5	Cap the tubes and gently tap each to mix the diluted samples.
6	Centrifuge the tubes briefly to eliminate air bubbles in the mixture.
7	Label four 0.2-mL MicroAmp Reaction Tubes for the four total RNA tubes.
8	Pipette 6.525 µL of the reaction mix (from step 1) to each MicroAmp Reaction Tube (from step 7).
9	Transfer 3.475 µL of each dilute total RNA sample to the corresponding MicroAmp Optical Reaction Tube.
10	Cap the reaction tubes and tap each gently to mix the reactions.
11	Centrifuge the tubes briefly to force the solution to the bottom and to eliminate air bubbles from the mixture.
12	Transfer each reaction to either – MicroAmp Optical tubes, or – wells of a MicroAmp Optical 96-well reaction plate
13	Cap the MicroAmp Optical tubes or plate with MicroAmp Optical caps.
14	Centrifuge the plate or tubes to spin down the contents and eliminate air bubbles from the solutions.

Thermal Cycling

To conduct RT thermal cycling:

Step	Action
1	Load the reactions into a thermal cycler.
2	Program your thermal cycler with the following conditions:
3	Begin RT. **IMPORTANT** After thermal cycling, store all cDNA samples at –15 to –25 °C.

Step	Hexamer Incubation[a]	RT	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temp	25 °C	37 °C[b]	95 °C
Time	10 min	60 min[c]	5 min
Volume	10 µL		

a. When using random hexamers for first-strand cDNA synthesis, a primer incubation step (25 °C for 10 min) is necessary to maximize primer-RNA template binding.

b. This temperature is specific for reverse transcribing 18S only.

c. This time is specific for reverse transcribing 18S only.

Data Analysis

5

Overview

About This Chapter

This chapter describes how to analyze the data generated in your experiments.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Interpreting the Results	5-2
Real-Time Detection	5-4

Interpreting the Results

Normalization

The Passive Reference 1 is a dye included in the 10X TaqMan Buffer A and does not participate in the 5´ nuclease PCR. The Passive Reference provides an inte5rnal 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.

Multicomponenting

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	FAM™, TET™, JOE™, VIC™
Quenchers	TAMRA™, NON-FLUORESCENT QUENCHER
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 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:

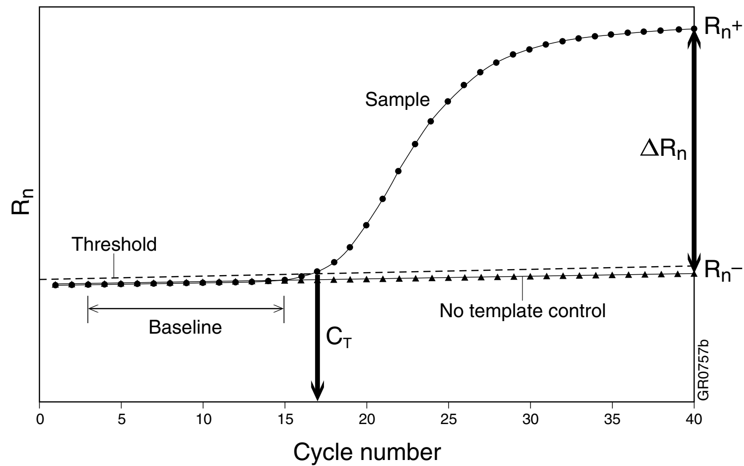
$R_n^+ =$	$\frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$	PCR with template
$R_n^- =$	$\frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$	PCR without template or early cycles of a real-time reaction

Real-Time Detection

Threshold Cycle

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 Sequence Detection System begins to detect the increase in signal associated with an exponential growth of PCR product.



Troubleshooting

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.
$\Delta R_n \leq$ 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)	Fluorescent emissions have not stabilized to buffer conditions of reaction mix. This does not affect PCR, or the final results.	Reset lower value of baseline range. Preformulate the probe, primer, and Universal PCR 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.

Troubleshooting *(continued)*

Observation	Possible Cause	Recommended Action
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.
Small ΔR_n	PCR efficiency is poor	Recheck the optimization.
	Low copy number of target	Increase starting copy number.
C_T value is higher than expected	Less template added than expected	Increase sample amount.
	Sample is degraded	Evaluate sample integrity.
C_T value is lower than expected	More sample added than expected Template or amplicon contamination	Reduce sample amount. Review "Preventing Contamination" on page 1-12.
Standard deviation of C_T value >0.16	Inaccurate pipetting	Prepare a Reaction Mix for each sample type. Mix well. Use positive-displacement pipettors.

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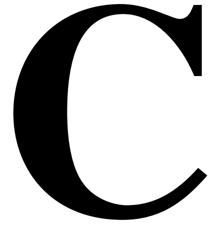

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- ♦ Download PDF documents
- ♦ Obtain information about customer training
- ♦ Download software updates and patches

In addition, the Applied Biosystems Web site provides a list of telephone and fax numbers that can be used to contact Technical Support.

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