TaqMan® One-Step RT-PCR Master Mix Reagents Kit

Protocol

Applied Biosystems

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Printed in the USA, 07/2010
Part Number 4310299 Rev.E

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1 Introduction

Overview

About This Chapter

This chapter describes the TaqMan® One-Step RT-PCR Master Mix Reagents Kit and provides important information about safety.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Purpose of the Kit	1-2
Materials and Equipment	1-7
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Purpose of the Kit

RNA Detection

The TaqMan® One-Step RT-PCR Master Mix Reagents Kit is designed for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of a specific target RNA from either total RNA or mRNA. The reagents in this kit can be used for one-step RT-PCR with the ABI PRISM® 7700 Sequence Detection System (SDS), the ABI PRISM® 7900HT SDS, the ABI PRISM® 7000 SDS, or the GeneAmp® 5700 SDS.

One-step RT-PCR performs RT as well as PCR in a single buffer system. The reaction proceeds without the addition of reagents between the RT and PCR steps. This offers the convenience of a single-tube preparation for RT and PCR amplification. However, the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), cannot be used with the TaqMan One-Step RT-PCR Master Mix Reagents Kit. UNG is active at the same temperature as that required for the RT reaction. Therefore UNG would act to remove any uracil incorporated into the cDNA strand synthesized during the RT step.

One-Step RT-PCR Method

Method	Primer for cDNA synthesis	Features
One-step	Sequence-specific reverse primer	♦ Requires single Reaction Mix ♦ UNG cannot be used

The TaqMan One-Step RT-PCR Reagents Kit can be used for real- time or plate read (endpoint) detection of RNA. Analysis is performed using the ABI PRISM 7700 SDS, ABI PRISM 7900HT SDS, ABI PRISM 7000 SDS or GeneAmp 5700 SDS (real-time PCR only).

For the best quantitation results, use the following:

- Primer Express™ software for primer design
- Applied Biosystems reagents
- Applied Biosystems universal thermal cycling conditions

Normalization

The Passive Reference (ROX) is a dye included in the RT-PCR Master Mix which does not participate in the PCR amplification. The Passive Reference provides an internal reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for well-to-well fluorescent fluctuations.

One-Step RT-PCR

As shown in the figure below, one-step RT-PCR uses a single buffer that enables RT and PCR amplification to occur without interruption.

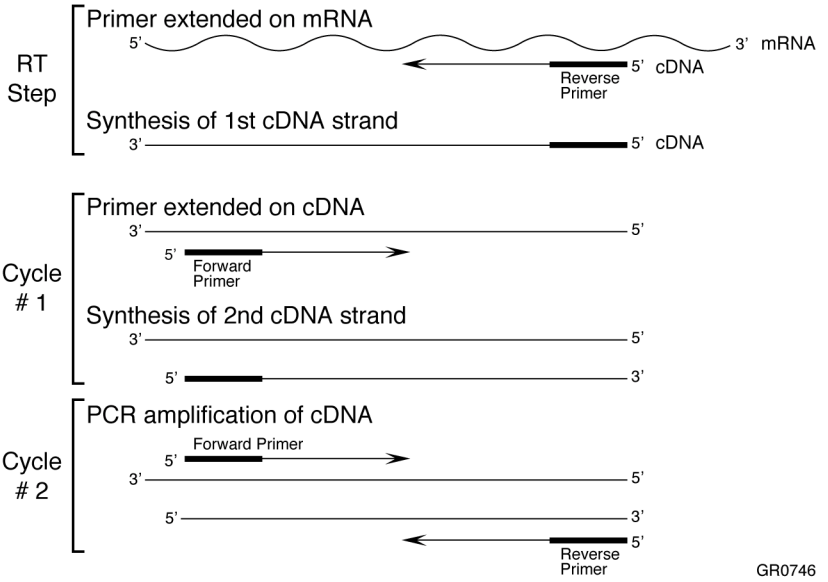
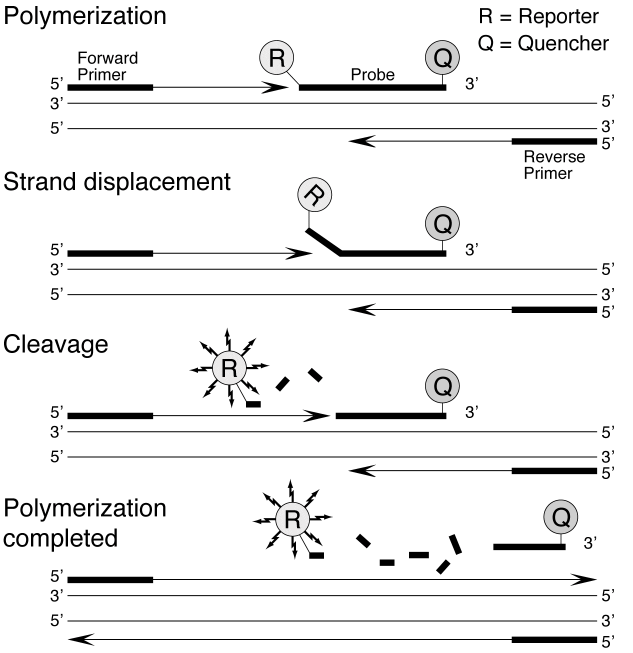


Figure 1-1 Schematic representation of RT-PCR using the TaqMan One-Step RT-PCR Reagents Kit. Hybridization of the TaqMan® probe is not shown.

Basics of the 5´ Nuclease Assay

The RT-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, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye, shown below.



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 DNA Polymerase 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.

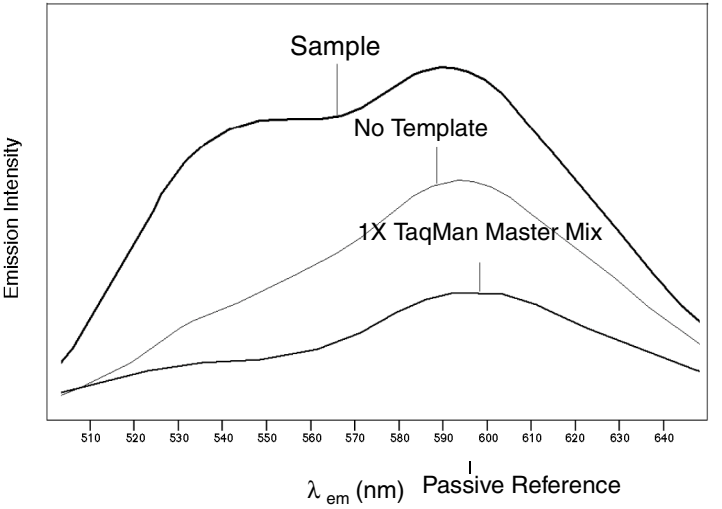


Figure 1-2 An overlay of three emission scans, post-PCR.

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.

MultiScribe Reverse Transcriptase

MultiScribe™ Reverse Transcriptase is a recombinant Moloney Murine Leukemia Virus (MuLV) Reverse Transcriptase (P/N N808-0018). MultiScribe Reverse Transcriptase is similar to MuLV Reverse Transcriptase, but differs in its recommended usage.

AmpliTaq Gold DNA Polymerase

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). With AmpliTaq Gold enzyme, Hot Start PCR and Time Release PCR can be introduced 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 One-Step RT-PCR

The TaqMan One-Step RT-PCR Reagents Kit provides a RT-PCR mix which may be used with any appropriately designed primer and probe set to detect total RNA or mRNA.

UNG cannot be used with one-step RT-PCR using the TaqMan One-Step RT-PCR Master Mix Reagents Kit. UNG is active at the same temperature as that required for the reverse transcription reaction. Therefore UNG would act to remove any uracil incorporated into the cDNA strand synthesized during the RT step (AmpErase UNG Product Insert, 1993).

Materials and Equipment

Kit Components

The TaqMan One-Step RT-PCR Master Mix Reagents Kit contains sufficient reagent to perform 200 50-µL reactions. The mix is optimized for TaqMan One-Step RT-PCR and contains AmpliTaq Gold enzyme, dNTPs with dUTP, Passive Reference, and optimized buffer components. An additional vial, MultiScribe and RNase Inhibitor Mix, is present in the kit.

For TaqMan One-Step RT-PCR Kit, the following components are available:

Kit	P/N	Contents
TaqMan One-Step RT-PCR Master Mix Reagents Kit	4309169	♦ 2X Master Mix without UNG ♦ 40X MultiScribe and RNase Inhibitor Mix
Protocol	4310299	–

Materials Required but Not Supplied

The following items are required when using TaqMan One-Step RT-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

User-Supplied Materials *(continued)*

Item	Source
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
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)
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	
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® 96-well Tray/Retainer Set (10 sets)	Applied Biosystems (P/N 403081)
MicroAmp® Optical Caps	Applied Biosystems (P/N 4323032)
MicroAmp® Optical Tubes	Applied Biosystems (P/N N801-0933)

User-Supplied Materials *(continued)*

Item	Source
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)
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 One-Step RT-PCR Master Mix Reagents Kit at 2 to 8 °C. Store the product away from light. This product is light sensitive. If stored under the recommended conditions, the product will maintain performance through the control date printed on the label.

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 provided by the PCR process (Saiki *et al.*, 1985; Mullis *et al.*, 1987). Potential contamination can be introduced by small levels of carryover from previous runs.

Hot Start PCR

To improve PCR specificity and sensitivity by controlling mispriming events, the Hot Start technique was introduced (Faloona *et al.*, 1990). Hot Start PCR is a simple modification of the original PCR process in which the amplification reaction is started at an elevated temperature. This was initially performed manually, by adding an essential component of the reaction to the reaction mixture only after that mixture had been heated to an elevated temperature. However, this approach was often cumbersome and time consuming, especially when using large numbers of samples.

UNG in One-Step Reactions

UNG cannot be used when one-step RT-PCR is performed with reagents from the TaqMan One-Step RT-PCR Master Mix Reagents Kit. Because UNG is active at temperatures required to complete reverse transcription, the active UNG enzyme would remove uracil bases incorporated into the newly synthesized cDNA strand.

Fluorescent Contaminants

Since fluorescent contaminants may interfere with TaqMan One-Step RT-PCR assays 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.

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 positive displacement pipette or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution.

Designing Custom Target Sequences

2

Overview

About This Chapter

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

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Designing Custom Target Sequences for Quantitation	2-2
Designing Probes and Primers	2-4

Designing Custom Target Sequences for Quantitation

Overview

We recommend the following steps to design custom target sequences for quantitation.

Step	Action	See Page
1	Install Primer Express Software	–
2	Selecting an Amplicon Site for Gene Expression Assays	2-2
3	Identifying Target Sequence and Amplicon Size	2-3
4	Design of Probes	2-4
5	Design of Primers	2-4

For best results, use Primer Express primer design software, Applied Biosystems reagents, and the universal thermal cycling parameters.

Selecting an Amplicon Site for Gene Expression Assays

Overview

Selecting a good amplicon site ensures amplification of the target mRNA without co-amplifying the genomic sequence, pseudogenes, and related genes. SYBR Green chemistry can be useful for screening Amplicon sites when using TaqMan chemistry for Gene Expression. To guarantee the absence of pseudogene amplification, the use of SYBR Green is recommended.

Guidelines

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair has to be specific to the target gene and does not amplify pseudogenes or other related genes.
- Primers must be designed following Primer Express guidelines.
- Test the amplicons and select ones that have the highest signal-to-noise ratio (*i.e.*, low C_T with cDNA and no amplification with no template control or genomic DNA).
- If no good sequence is found, it may be necessary to examine the sequence and redesign the amplicon or simply screen for more sites.

If the gene you are studying does not have introns, then it is not possible to design an amplicon that will amplify the mRNA sequence without amplifying the gene sequence. In this case, it may be necessary to run RT minus controls.

Identifying Target Sequence and Amplicon Size

A target template is a total RNA or mRNA nucleotide sequence.

Design primers to amplify short segments of a target (total RNA or mRNA) within the target sequence. These short segments are called amplicons. The shorter amplicons work most efficiently: the most consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Designing Probes and Primers

Design of Probes

Probes can be designed using Primer Express software as described in the “TaqMan Probe and Primer” section of *Primer Express User Bulletin* (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 Gs on the 5´ end.
- Select the strand that gives the probe more Cs than Gs.
- For single-probe assays, T_m should be 68 to 70 °C when using Primer Express software.

Design of Primers

Primers can be designed using Primer Express software as described in the “TaqMan Probe and Primer” section of 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.
- When 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.

Amplifying Custom Target Sequences

3

Overview

About This Chapter

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

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Amplifying Custom Target Sequences for Quantitation	3-2
Optimizing Primer Concentration	3-3
Optimizing Probe Concentration	3-6

Amplifying Custom Target Sequences for Quantitation

Overview

We recommend the following steps to amplify the custom target sequences and complete development of quantitative PCR assays.

Step	Action	See Page
1	Quantitating Probes and Primers	3-2
2	Optimizing Primer Concentration	3-3
3	Optimizing Probe Concentration	3-6

Quantitating Probes and Primers

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 in μM using the method shown 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

$$\text{Absorbance (260 nm)} = \text{sum of extinction coefficient contributions} \times \text{cuvette pathlength} \times \text{concentration}/100$$

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

$$C = 196\ \mu M$$

Optimizing Primer Concentration

Determining Minimum Primer Concentration

The purpose of this procedure is to determine the minimum primer concentrations giving the lowest threshold cycle (C_T) and maximum ΔR_n. The reaction volumes are 50 µL. Use 10 pg to 100 ng of RNA template.

RT-PCR Master Mix is used to run four replicates of each of the nine conditions shown in the table below.

Reverse Primer (nM)	Forward Primer (nM)		
	50	300	900
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Reducing Non-Specific Amplification

For one-step RT-PCR, this protocol requires an initial incubation of the reaction mixture for 30 minutes at 48 °C. This RT step co-incubates the PCR primers and probe at a temperature below their annealing temperatures. AmpliTaq Gold enzyme will slowly activate at 48 °C and may lead to non-specific amplification. To minimize any potential non-specific amplification in TaqMan One-Step RT-PCR reactions, primer and probe concentration optimizations are strongly recommended.

Tables for Primer Optimization Procedure

This subsection provides three tables for use with the primer optimization procedure:

- RT-PCR Reaction Mix for Primer Optimization
- Plate Configuration for Primer Optimization for One-Step RT-PCR
- Thermal Cycling Parameters for RT Reaction, Primer Optimization, and Probe Optimization

RT-PCR Reaction Mix for Primer Optimization

RT-PCR Reaction Mix for Primer Optimization

Reaction Component	Volume per Reaction (µL)	Volume per 100 Reactions (µL)	Final Value
2X Master Mix without UNG	25	2500	1X
40X MultiScribe and RNase Inhibitor Mix	1.25	125	0.25 U/µL 0.4 U/µL
Forward primer	Variable	Variable	50 to 900 nM
Reverse primer	Variable	Variable	50 to 900 nM
TaqMan probe (25 µM)	Variable	Variable	250 nM
RNA sample, 50 ng	Variable	Variable	10 pg to 10 ng
Water	Variable	Variable	—
Total	50	5000	—

Plate and Master Mix Configuration for Primer Optimization for One-Step RT-PCR

Wells	2X Master Mix without UNG (µL)	40X MultiScribe and RNase Inhibitor Mix (µL)	5 µM Forward Primer (µL)	5 µM Reverse Primer (µL)	25 µM TaqMan Probe (µL)	Template (µL)	Deionized Water (µL)	Total Volume (µL)
A1–A4	25	1.25	0.5	0.5	0.5	5.0	17.25	50
A5–A8	25	1.25	3.0	0.5	0.5	5.0	14.75	50
A9–A12	25	1.25	9.0	0.5	0.5	5.0	8.75	50
B1–B4	25	1.25	0.5	3.0	0.5	5.0	14.75	50
B5–B8	25	1.25	3.0	3.0	0.5	5.0	12.25	50
B9–B12	25	1.25	9.0	3.0	0.5	5.0	6.25	50
C1–C4	25	1.25	0.5	9.0	0.5	5.0	8.75	50
C5–C8	25	1.25	3.0	9.0	0.5	5.0	6.25	50
C9–C12	25	1.25	9.0	9.0	0.5	5.0	0.25	50
D1–D4	25	1.25	9.0	9.0	0.5	0	5.25	50

Thermal Cycling Parameters

	RT	AmpliTaq Gold Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Time	30 min	10 min	15 sec	1 min
Temperature	48 °C	95 °C	95 °C	60 °C

Procedure for Optimizing Primer Concentrations

To optimize primer concentrations:

Step	Action
1	Use the plate configuration for primer optimization.
2	Place the plate in the appropriate ABI PRISM Sequence Detection System (SDS) or GeneAmp 5700 SDS and follow the thermal cycling conditions described in the table above.
3	At the end of the run: ♦ Tabulate the results for the yield by looking at the ΔRn. This analysis will identify the optimum concentrations of primers for PCR yield. ♦ Tabulate the results for C_T value. This analysis will identify the optimum primer concentrations for C_T and detect any potential non-specific amplification in the No Template controls.

Optimizing Probe Concentration

Determining Probe Minimum Concentration

The purpose of this procedure is to determine the minimum probe concentrations that give the minimum C_T for each probe target and reduce non-specific amplification.

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 the table below. **Note** Use the forward- and reverse-primer concentrations determined by the primer optimization experiment on page 3-3.
2	Place the plate in the Sequence Detection System and follow the thermal cycling conditions.
3	Tabulate the results for C_T. Choose the probe concentration that yields the minimum C_T and highest ΔR_n.

RT-PCR Reaction Mix for Probe Optimization

Reaction Component	Volume per Reaction (µL)	Volume per 100 Reactions (µL)	Final Value
2X Master Mix without UNG	25	2500	1X
40X MultiScribe and RNase Inhibitor Mix	1.25	125	0.25 U/µL 0.4 U/µL
Forward primer	5	500	Optimal
Reverse primer	5	500	Optimal
TaqMan probe	Variable	Variable	50 to 250 nM
RNA sample	5	500	10 to 100 ng
Water	Variable	Variable	
Total	50	5000	–

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

For routine analysis the following ranges of RNA can be used:

- RNA 0.1 ng to 1 µg
- mRNA 10 pg to 100 ng

4 Data Analysis

Overview

About This Chapter

This chapter discusses data analysis.

In This Chapter

The following topics are covered in this chapter.

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

Interpreting the Results

Normalization

The Passive Reference, ROX, is a dye included in the TaqMan Universal PCR Master Mix that does not participate in the 5´ nuclease assay. The 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.

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:

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

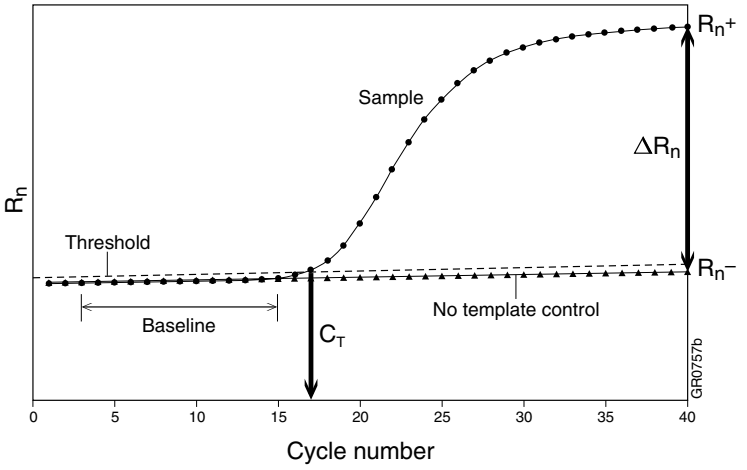
where:

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

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.



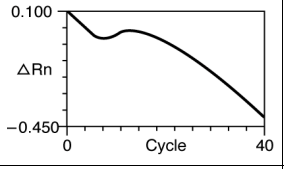
Troubleshooting

A

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. Run on a gel to verify if PCR worked.
	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 to 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 TaqMan One-Step RT-PCR Master Mix to allow the reaction mixture to equilibrate.

Troubleshooting *(continued)*

Observation	Possible Cause	Recommended Action
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. See on-line tutorial http://www.appliedbiosystems.com /support/tutorials/baseline
Multicomponent signal for ROX is not linear	Pure dye component's spectra are incorrect	Rerun pure dye spectra.
	Incorrect dye components chosen	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.
Standard deviation of C_T value >0.16	Inaccurate pipetting	Prepare a Reagent Mix. Use positive-displacement pipettors.

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C

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Printed in the USA, 07/2010
Part Number 4310299 Rev. E