TaqMan® Rodent GAPDH Control Reagents

VIC™ Probe

Protocol

Applied Biosystems

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Introduction

Purpose of the Kit

The TaqMan® Rodent GAPDH Control Reagents are designed to detect rat, mouse, and Chinese hamster glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. Either DNA or RNA can be used as a target template.

The TaqMan Rodent GAPDH Control Reagents can be used with TaqMan® PCR Core Reagents or TaqMan® Universal PCR Master Mix to perform polymerase chain reaction (PCR). Both TaqMan® EZ RT-PCR Core Reagents and the TaqMan® Gold RT-PCR Kit (one-step and two-step) can be used to perform reverse transcription–polymerase chain reaction (RT-PCR).

Materials and Equipment

Module Components

The TaqMan Rodent GAPDH Control Reagents (P/N 4308313) contain the probe and primers sufficient to perform one thousand 50-µL reactions. The module provides enough Control RNA (Rodent) for 100 control reactions. The module includes the following components:

Component	Description
Rodent GAPDH Probe (VIC)	One tube containing 500 µL of 20 µM probe in TE buffer.
Rodent GAPDH Forward Primer	One tube containing 500 µL of 10 µM primer in TE buffer.
Rodent GAPDH Reverse Primer	One tube containing 500 µL of 10 µM primer in TE buffer.
Control RNA (Rodent)	One tube containing 100 µL of 50 ng/µL total mouse RNA in 100 mM Tris-HCl, pH 7.0, 100 mM NaCl, and 1 mM EDTA.

IMPORTANT The TaqMan VIC dye must be configured as a Pure Dye on the ABI PRISM® 7700 Sequence Detection System for it to appear on the Reporter pull-down menu. See *User Bulletin #4: Generating New Spectra Components* (P/N 4306234) pages 6-7 to configure TaqMan VIC as a Pure Dye.

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Core Kits Supplied by the User

One of the TaqMan core reagent kits listed in the following table is required in addition to the reagents supplied in the TaqMan Rodent GAPDH Control Reagents.

Application	TaqMan Core Reagents[a]	Source
PCR	TaqMan PCR Core Reagents	Applied Biosystems (P/N N808-0228)
	TaqMan Universal PCR Master Mix	Applied Biosystems (P/N 4304437)
RT-PCR	TaqMan Gold RT-PCR Kit	Applied Biosystems (P/N N808-0232)
	TaqMan EZ RT-PCR Core Reagents	Applied Biosystems (P/N N808-0236)
RT	TaqMan® Reverse Transcription Reagents	Applied Biosystems (P/N N808-0234)

a. See your local Applied Biosystems representative for a current listing of available reagents.

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Materials Supplied by User

The items listed in the following tables are required in addition to the reagents supplied in the TaqMan Rodent GAPDH Control Reagents.

Equipment Item	Source
ABI PRISM® 7700 Sequence Detection System	See your local Applied Biosystems representative for the instrument best suited to meet your needs.
ABI PRISM® 7200 Sequence Detection System/GeneAmp® PCR System 9600	
TaqMan® LS-50B PCR Detection System/GeneAmp PCR System 9600	

Consumable or Reagent Item	Source
Sequence Detection Primers Minimum 4000 pmol, unlabeled Minimum 40,000 pmol, unlabeled Minimum 130,000 pmol, unlabeled	Applied Biosystems (P/N 4304970) (P/N 4304971) (P/N 4304972)
Custom TaqMan Probes 5000 pmol 15,000–25,000 pmol 50,000–100,000 pmol	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)
MicroAmp Optical Caps	Applied Biosystems (P/N N801-0935)
MicroAmp Optical Tubes	Applied Biosystems (P/N N801-0933)
96-Well Microplate (Portvair)[a]	Applied Biosystems (P/N L225-1692)
Microcentrifuge	Major laboratory suppliers (MLS)
Centrifuge with adapter for 96-well plate	MLS
Polypropylene tubes	MLS

Consumable or Reagent Item	Source
Pipettors, positive-displacement or air-displacement	MLS
Pipette tips, with filter plugs	MLS
Vortexer	MLS
Disposable gloves	MLS
Tris EDTA (TE) Buffer	MLS
RNase free water	MLS

a. The ABI PRISM 7700 and ABI PRISM 7200 Sequence Detectors use the MicroAmp Optical 96-Well Reaction Plate and MicroAmp Optical Caps. The TaqMan LS-50B PCR Detection System uses the 96-Well Microplate (Portvair). Do not use MicroAmp Optical Tubes with the ABI PRISM 7200 Sequence Detector.

Amplicon Size

The amplicon generated from the GAPDH gene for both rodent RNA and DNA is 177 bp in length.

Performance Specifications

The following performance specifications apply to the TaqMan Rodent GAPDH Control Reagents.

Description	Performance Specification
Detection limit of total RNA	Two picograms of Control RNA (Rodent) per 50-µL reaction
Dynamic range of Rodent GAPDH on ABI PRISM 7700 Sequence Detection System	Five log magnitude on the ABI PRISM 7700 Sequence Detection System

Storage and Stability

Upon receipt, store the TaqMan Rodent GAPDH Control Reagents at –15 to –25 °C in a constant-temperature freezer. If stored under the recommended conditions, the product will maintain performance for one year from time of receipt.

Preventing Contamination

Introduction

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).

AmpErase UNG

AmpErase® UNG (uracil-N-glycosylase, UNG), is a pure nuclease-free, 26-kDa recombinant enzyme encoded by the *Eschericia coli* uracil-N-glycosylase gene. This gene has been inserted into an *E. coli* host to direct expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UNG acts on single- and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA.

UNG in Two-Step Reactions

When two-step RT-PCR is performed with the TaqMan Gold RT-PCR Kit, AmpErase UNG treatment can prevent the reamplification of carry-over PCR products. When dUTP replaces dTTP during PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of the carry-over GAPDH amplicon per 50-µL reaction.

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UNG in One-Step Reactions

Amperase UNG can be used to remove amplicon contamination in one-step RT-PCR when using the TaqMan EZ RT-PCR Core Reagents. The rT*th* DNA Polymerase contained in the kit is thermally stable and is used at temperatures at which Amperase UNG is inactive. Because one-step EZ RT-PCR utilizes dUTP, amplicons generated during this reaction contain uridine residues.

AmpErase UNG cannot be used when one-step RT-PCR is performed using the TaqMan Gold RT-PCR Kit. UNG is active at the temperatures for reverse transcription. The active UNG enzyme would remove uracil bases that are incorporated into the newly synthesized complementary DNA (cDNA) strand (AmpErase UNG Product Insert, 1993).

If contamination is suspected from previous PCR runs, performing PCR with and without AmpErase UNG will help to identify the source of contamination. To do this, set up parallel No Template Control PCR reactions with and without UNG. A positive signal in the reaction without UNG indicates contamination of reaction components.

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General Guidelines for PCR

Certain laboratory practices are necessary in order to avoid false-positive amplifications (Kwok and Higuchi, 1989). This is because the PCR process is capable of amplifying single DNA molecules (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

- Wear a clean lab coat (one never worn while handling amplified PCR products or doing sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever contamination is possible.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification and detection
- 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.
- Clean lab benches and equipment regularly 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 and 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.

Preparing Reaction Mix Components

Overview

This procedure is optimized for the TaqMan Rodent GAPDH Control Reagents. The number of reactions depends upon the plate set up by the user. Between 2 pg and 200 ng of total RNA may be used for a one-step RT-PCR reaction.The No Template Control reaction is the complete RT-PCR formulation without the target RNA.

Preparation of Reagents

The kit is stored at –15 to –25 °C. Prior to use, thaw all reagents except the enzymes and the RNase Inhibitor. When the reagents are thawed, keep them on ice. Keep the enzymes in a freezer until immediately prior to use. Mix kit components, except enzymes and RNase Inhibitor, by vortexing and using a microcentrifuge to briefly spin down the tube contents. Protect the fluorescent dye labeled probe from excessive exposure to light. When finished with the kit, return it to the –15 to –25 °C freezer.

Reaction Mix Preparation

Preparing a Reaction Mix of RT-PCR or PCR components is recommended in order to increase the accuracy of the results. The use of a Reaction Mix reduces the number of reagent transfers and minimizes volume loss due to pipetting.

Step	Action
1	Prepare Reaction Mix by combining all the nonenzymatic components listed in the appropriate table.
2	Mix the components by pipetting up and down.
3	Vortex briefly.
4	Add the enzymatic components (for example: AmpliTaq Gold™ DNA Polymerase, MultiScribe™ Reverse Transcriptase, RNase Inhibitor) listed for the appropriate reaction mix.
5	Mix the components by inverting the microcentrifuge tube.

PCR Using the TaqMan PCR Core Reagents

Introduction

PCR can be performed using the TaqMan PCR Core Reagents (P/N N808-0228) by following the instructions described in the TaqMan PCR Core Reagents protocol (P/N 402823).

PCR Reaction Mix

The ingredients of a 50 µL reaction, PCR Reaction Mix are listed in the table below. To make the PCR Reaction Mix, follow the instructions described in “Reaction Mix Preparation” on page 9.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
10X TaqMan Buffer A	5	1X
25 mM Magnesium chloride	11	5.5 mM
10 mM deoxyATP	1	200 µM
10 mM deoxyCTP	1	200 µM
10 mM deoxyGTP	1	200 µM
20 mM deoxyUTP	1	400 µM
10 µM Rodent GAPDH Forward Primer	0.5	100 nM
10 µM Rodent GAPDH Reverse Primer	0.5	100 nM
20 µM Rodent GAPDH Probe (VIC)	0.5	200 nM
AmpErase UNG	0.5	0.01 Units/µL
AmpliTaq Gold DNA Polymerase	0.25	0.025 Units/µL

a. The volume of RNase-free water will be (27.75 µL – DNA sample volume).

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Thermal Cycling Parameters for PCR

Use the following thermal cycling parameters for PCR:

Step	UNG Digestion	AmpliTaq Gold 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

One-Step RT-PCR Using The TaqMan EZ RT-PCR Reagents

Introduction

RT-PCR can be performed with the TaqMan EZ RT-PCR Core Reagents (P/N N808-0236) by following the instructions described in the TaqMan EZ RT-PCR Core Reagents protocol (P/N 402877).

Description of One-Step RT-PCR

One-step RT-PCR is RT as well as PCR in a single buffer system using the r*Tth* DNA polymerase enzyme for both steps. 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. The carryover prevention enzyme, AmpErase UNG (uracil-N-glycosylase, UNG), can be used with one-step RT-PCR using the TaqMan EZ RT-PCR Core Reagents.

EZ Reaction Mix

The ingredients used for a 50-µL RT-PCR reaction mix using the TaqMan EZ RT-PCR Core Reagents is listed in the table below.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
5X TaqMan EZ Buffer	10	1X
25 mM Manganese acetate	6	3 mM
10 mM deoxyATP	1.5	300 µM
10 mM deoxyCTP	1.5	300 µM
10 mM deoxyGTP	1.5	300 µM
20 mM deoxyUTP	1.5	600 µM
10 µM Rodent GAPDH Forward Primer	0.5	100 nM
10 µM Rodent GAPDH Reverse Primer	0.5	100 nM
20 µM Rodent GAPDH Probe (VIC)	0.5	200 nM
AmpErase UNG	0.5	0.01 U/µL
r*Tth* DNA Polymerase	2	0.1 U/µL

a. The volume of RNase-free water will be (24 µL – RNA sample volume).

Note To run a control sample, use 1 µL of the the Control RNA (Rodent) provided with the TaqMan Rodent GAPDH Control Reagents.

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Reaction Preparation

For a description of how to prepare the reaction, refer to the TaqMan EZ RT-PCR Core Reagents protocol (P/N 402877).

Thermal Cycling for One-Step EZ RT-PCR

The following thermal cycling parameters are optimized for the Rodent GAPDH system. See thermal cycler manuals for details on operation.

Step	UNG Activation	RT	UNG Deactivation	PCR	
	HOLD	HOLD	HOLD	CYCLE (40 cycles)	
				Denature	Anneal/ Extend
Time	2 min	30 min	5 min	15 sec	1 min
Temp	50 °C	60 °C	95 °C	94 °C	60 °C

Run Types

The TaqMan EZ RT-PCR Kit is designed for two run types: Real Time Detection and Plate Read Detection. Real Time Detection monitors fluorescence during each PCR cycle. Plate Read (endpoint) Detection collects one fluorescence scan per tube after PCR is completed.The features of these detection systems are listed below.

Run Type	**Fluorescence Detection**	**Analysis Results**
Real Time	At each PCR cycle	Quantitation of initial template amount
Plate Read Only	Only endpoint	Detection of final amplified products

One-Step RT-PCR Using The TaqMan Gold RT-PCR Kit

Introduction

RT-PCR can be performed with the TaqMan Gold RT-PCR Kit (P/N N808-0232) by following the instructions in the TaqMan Gold RT-PCR Kit protocol (P/N 402876).

The TaqMan Gold RT-PCR Kit can be used to perform one-step or two-step RT-PCR.

Description of One-Step RT-PCR

One-step RT-PCR is 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 cannot be used with one-step RT-PCR when using the TaqMan Gold RT-PCR Kit.

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One-Step Reaction Mix

The ingredients for a 50 µL RT-PCR reaction mix using the TaqMan Gold RT-PCR Kit are listed in the table below.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
10X TaqMan Buffer A	5	1X
25 mM Magnesium chloride	11	5.5 mM
10 mM deoxyATP	1.5	300 µM
10 mM deoxyCTP	1.5	300 µM
10 mM deoxyGTP	1.5	300 µM
20 mM deoxyUTP	1.5	600 µM
10 µM Rodent GAPDH Forward Primer	0.5	100 nM
10 µM Rodent GAPDH Reverse Primer	0.5	100 nM
20 µM Rodent GAPDH Probe (VIC)	0.5	200 nM
RNase Inhibitor	1	0.4 U/µL
MultiScribe Reverse Transcriptase	0.25	0.25 U/µL
AmpliTaq Gold DNA Polymerase	0.25	0.025 U/µL

a. The volume of RNase-free water will be (25 µL – RNA sample volume).

Note To run a control sample, use 1 µL of the the Control RNA (Rodent) provided with the TaqMan Rodent GAPDH Control Reagents.

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Reaction Preparation

For a description of how to prepare the reaction, refer to the TaqMan Gold RT-PCR Core Reagents protocol (P/N 402876).

Thermal Cycling for One-Step Gold RT-PCR

The following thermal cycling parameters are optimized for the one-step RT-PCR for the Rodent GAPDH system. See thermal cycler manuals for details on operation.

Step	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

Two-Step RT-PCR Using The TaqMan Gold RT-PCR Kit

Two-Step RT-PCR Reaction

Two-step RT-PCR is performed in two separate reactions, reverse transcription (RT) of the RNA to the cDNA, followed by PCR amplification. This is useful when detecting multiple transcripts from a single cDNA reaction, or when storing a portion of the cDNA for later use. The advantage of the two-step RT-PCR amplification is that the UNG enzyme can be used to prevent carryover contamination.

RT-Reaction Mix Preparation

The ingredients of a 10-µL RT-reaction mix are listed below. A 10-µL reaction volume is sufficient to reverse transcribe up to 200 ng of total RNA. When more than 200 ng of total RNA is used for RT, the reaction volume must be increased. When changing the reaction volume, make sure that the final concentration is consistent with that described in the table below.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
10X TaqMan RT Buffer	1	1X
25 mM Magnesium chloride	2.2	5.5 mM
10 mM dNTP mixture	2.0	500 µM each
Random hexamers or Oligo $d(T)_{16}$[b]	0.5	2.5 µM
RNase Inhibitor	0.2	0.4 U/µL
MultiScribe Reverse Transcriptase	0.25	1.25 U/µL

a. The volume of RNase-free water will be (3.85 µL – RNA sample volume).

b. If using a sequence-specific reverse primer for the first strand cDNA synthesis, the final concentration should be 200 nM

Note To run a control sample, use 1 µL of the the Control RNA (Rodent) provided with the TaqMan Rodent GAPDH Control Reagents.

RT-Reaction Preparation

For a description of how to prepare the RT-reaction, refer to the TaqMan Gold RT-PCR Core Reagents protocol (P/N 402876). The RT reagents are available in a seperate module called TaqMan Reverse Transcription Reagents (P/N N808-0234).

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RT-Reaction Thermal Cycling Parameters

The cycling parameters for a 10-µL RT-reaction mix using the TaqMan Gold RT-PCR Kit are listed below.

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

a. If using random hexamers or oligo $d(T)_{16}$ primers for first-strand cDNA synthesis, a primer incubation step is necessary at 25 °C for 10 minutes. If using a sequence specific reverse primer, the incubation step is not necessary.

PCR Reaction Mix

The ingredients of a 50-µL PCR reaction are listed below.

Component	Volume/Tube (µL)	Final Concentration
RNase-free water	see below[a]	–
10X TaqMan Buffer A	5	1X
25 mM Magnesium chloride	11	5.5 mM
10 mM deoxyATP	1	200 µM
10 mM deoxyCTP	1	200 µM
10 mM deoxyGTP	1	200 µM
20 mM deoxyUTP	1	400 µM
10 µM Rodent GAPDH Forward Primer	0.5	100 nM
10 µM Rodent GAPDH Reverse Primer	0.5	100 nM
20 µM Rodent GAPDH Probe (VIC)	0.5	200 nM
AmpErase UNG	0.5	0.01 U/µL
AmpliTaq Gold DNA Polymerase	0.25	0.025 U/µL

a. The volume of RNase-free water will be (27.75 µL – cDNA sample volume).

Note To run a control sample, use 5 µL of the completed RT-reaction from the previous step.

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PCR Reaction Preparation

For a description of how to prepare the PCR reaction, refer to the TaqMan Gold RT-PCR Core Reagents protocol (P/N 402876).

PCR-Reaction Thermal Cycling Parameters

The cycling parameters for the PCR step (step two) of a 50-µL, two-step RT-PCR reaction using the TaqMan Gold RT-PCR Kit are listed below.

Step	UNG Incubation	AmpliTaq Gold 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

Interpretation of Results

Normalization

The Passive Reference is a dye included in the 10X TaqMan Buffer A and in the 5X TaqMan EZ Buffer and 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.

Multi-componenting

Multicomponenting is the term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the three pure dye components generate the composite spectrum. This spectrum represents one fluorescent reading from one well. For the TaqMan Rodent GAPDH Control system, the three dyes used for Multicomponent Analysis are:

Reporter	VIC
Quencher	TAMRA
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

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 (figure below), the threshold cycle occurs when the Sequence Detection Application begins to detect the increase in signal associated with an exponential growth of PCR product.

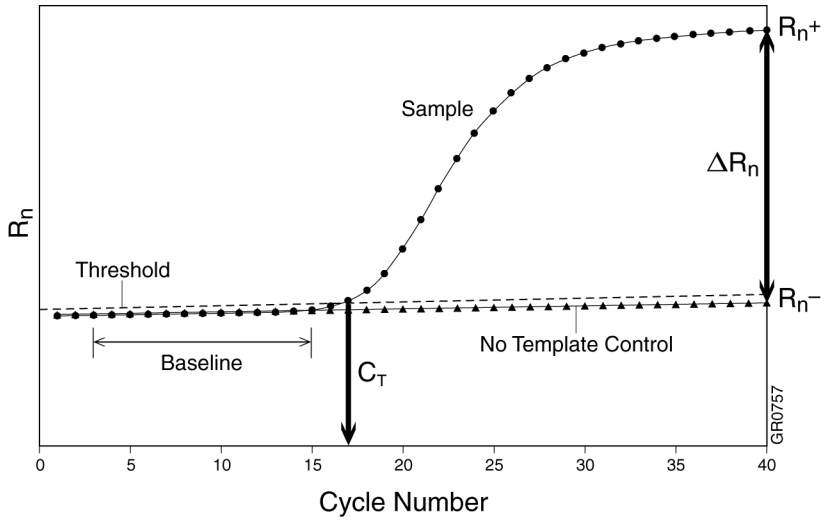

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Plate Read Analysis

Plate Read analysis relies on endpoint data to calculate ΔR_n. To ensure statistically high confidence levels using either the GAPDH probe or a custom probe, run the protocol with at least three No Template Controls per microplate. A positive result is defined as ΔR_n values greater than the threshold ΔR_n. The threshold ΔR_n is calculated by multiplying the standard deviation of three R_n^- values by an appropriate multiplier. The value for the appropriate multiplier is selected from a table of *t*-distribution values and depends upon the desired confidence level (Beyer, 1984). For 99% confidence levels, the multiplier is 6.965.

When more than three No Template Controls are run, the multiplier for the standard deviation decreases. Refer to a table of *t*-distribution values for the appropriate multiplier (Beyer, 1984).

To evaluate reproducibility, calculate the coefficient of variation on replicate samples. Inconsistent results (coefficients of variation exceeding 10%) may be caused by pipetting errors and incomplete mixing.

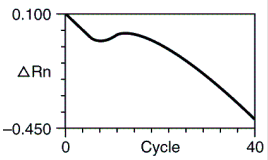
Determination of Threshold ΔR_n Value

To determine threshold ΔR_n value:

- On one microplate, measure both reporter and passive reference fluorescence, and determine the normalized reporter for each No Template Control tube (R_n^-).
- Determine the mean and standard deviation of R_n^-.
- When running three No Template Controls, multiply the R_n^- standard deviation by 6.965 to determine the threshold ΔR_n for the system.

For example, if the mean R_n^- for a system is 0.5 with a standard deviation of 0.02, the threshold ΔR_n is 6.965 × 0.02 or 0.14. Any ΔR_n >0.14 is a positive result and indicates that the sample contains target.

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 (cycles 0–5)	Fluorescent emissions have not stabilized to new buffer conditions of reaction mix. This does not affect PCR, or the final results.	Reset lower value of baseline range. Add probe to the buffer component and allow it to equilibrate at room temperature prior to Reagent Mix formulation.
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.
Small ΔR_n	PCR efficiency is poor	Reoptimize reaction conditions.
	Low copy number of target	Increase starting copy number.

Observation	Possible Cause	Recommended Action
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	Reduce sample amount.
	Template or amplicon contamination	Review “General Guidelines for PCR” on page 8.
Standard deviation of C_T value >0.16	Inaccurate pipetting	Prepare a Reagent Mix. Refer to “Reaction Mix Preparation” on page 9. Use positive-displacement pipettors.

Technical Support

To Reach Us on the Web

Applied Biosystems web site address is:

http://www.appliedbiosystems.com/techsupport

We strongly encourage you to visit our web site for answers to frequently asked questions, and to learn more about our products. You can also order technical documents and/or an index of available documents and have them faxed or e-mailed to you through our site (see the “Documents on Demand” section below).

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In the United States and Canada, technical support is available at the following times.

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Chemiluminescence	9:00 a.m. to 5:00 p.m. Eastern Time
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See the “Regional Offices Sales and Service” section below for how to contact local service representatives outside of the United States and Canada.

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Appendix A. References

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