TaqMan® Human Endogenous Control Plate

Protocol

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Introduction 1

Overview

About This Product

The TaqMan® Human Endogenous Control Plate is a research tool designed to simplify the selection of endogenous controls for gene expression studies. The plate evaluates the expression of eleven select housekeeping genes in total RNA samples using a two-step, reverse transcription–polymerase chain reaction (RT-PCR). The plate also features a unique internal positive control (IPC) designed to detect the presence of PCR inhibitors in test samples.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Control Plate Assay System	1-2
Preventing Contamination	1-9
Materials and Equipment	1-10
Safety	1-13

Control Plate Assay System

Purpose of the Kit

Applied Biosystems® developed the TaqMan Human Endogenous Control Plate to simplify endogenous control selection by eliminating several major developmental obstacles. The following table explains difficulties researchers face when investigating potential controls and how the plate alleviates these problems.

Obstacle	Solution
Assay development and optimization is expensive and time-consuming.	The TaqMan Human Endogenous Control Plate features 11 preoptimized, ready-to-use control gene assays.
Several studies indicate that expression of traditional housekeeping genes, such as GAPDH and beta-actin, varies among tissues and developmental stages (Bonini and Hofmann, 1991; Spanakis, 1993).	The TaqMan Human Endogenous Control Plate simultaneously evaluates eleven candidate controls that cover a broad range of biological functions and vary in expression levels.
Recent studies indicate that pseudogenes and related genes make RT-PCR results unreliable unless the PCR primers are cDNA-specific (Raff *et al.*, 1997; Multimer *et al.*, 1998).	TaqMan endogenous control assays are cDNA-specific, and their performance was tested using cDNA prepared from human total RNA samples.

Instruments

This protocol describes how to evaluate candidate control gene expression in total RNA samples using the plate and the following sequence detection systems:

- ABI PRISM® 7700 Sequence Detection System
- GeneAmp® 5700 Sequence Detection System

About TaqMan Endogenous Control Assays

With the exception of 18S rRNA, all assays present on the TaqMan Human Endogenous Control Plate are cDNA-specific. Each assay has been experimentally proven not to detect up to 10,000 copies of contaminating DNA.

The 18S rRNA assay is not cDNA-specific. However, because of the extremely high expression level of rRNA, amplification from contaminant DNA has a negligible effect on gene expression values obtained from the plate. In spite of these design characteristics, Applied Biosystems recommends using only purified total RNA samples.

About the Internal Positive Control

Applied Biosystems designed the TaqMan Internal Positive Control (IPC) to help interpret negative results caused by PCR inhibitors. In the absence of inhibitors, IPC is co-amplified with the target DNA and gives a consistent signal. If inhibitors are present, the signal generated by the IPC assay diminishes or becomes nonexistent. The IPC sequence is artificial to prevent nonspecific amplification.

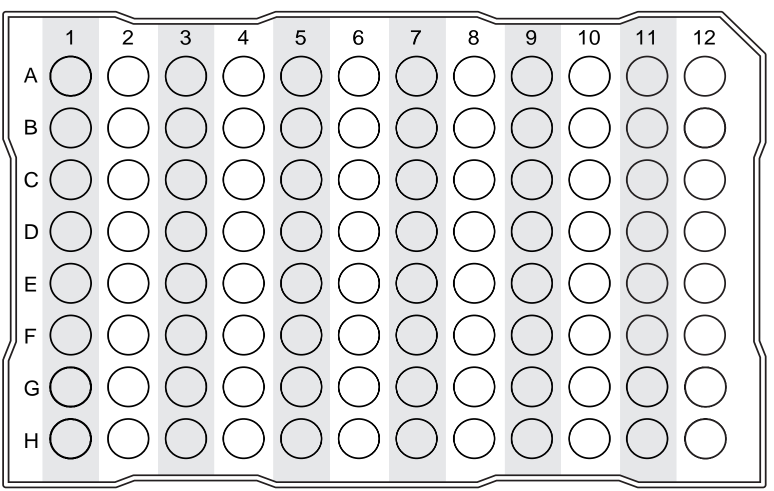
Product Guidelines

Read the following information before proceeding:

- ♦ The endogenous control plate cannot be used to conduct multiplex experiments. It is designed only as a tool to aid in the selection of endogenous controls.
- ♦ The endogenous control plate should not be used to assay poly A^+ RNA samples. The 18S rRNA assay cannot evaluate poly A^+ RNA samples because most of the ribosomal RNA has been removed. Applied Biosystems designed the plate to evaluate only total RNA.
- ♦ Reverse transcription of total RNA to cDNA must be done using random hexamers.
- ♦ ABI PRISM 7700 Sequence Detection Systems must be calibrated for the VIC™ dye before running the TaqMan Human Endogenous Control Plate. See "Configuring the ABI Prism 7700 Software for the VIC Dye" on page 3-2 for more information.
- ♦ The endogenous control plate is optimal for use with the following:
  - – ABI PRISM 7700 Sequence Detection System and GeneAmp 5700 Sequence Detection System
  - – TaqMan® Universal PCR Master Mix (P/N 4304437)
  - – TaqMan® Reverse Transcription Reagents (P/N N808-0234)

About the TaqMan Human Endogenous Control Plate

The TaqMan Human Endogenous Control Plate is a MicroAmp® Optical 96-Well Reaction Plate divided into 12 columns, one for every control assay. Each column consists of eight identical wells containing TaqMan primers and probes for the detection of one target gene. The figure below illustrates the assay configurations on the plate.

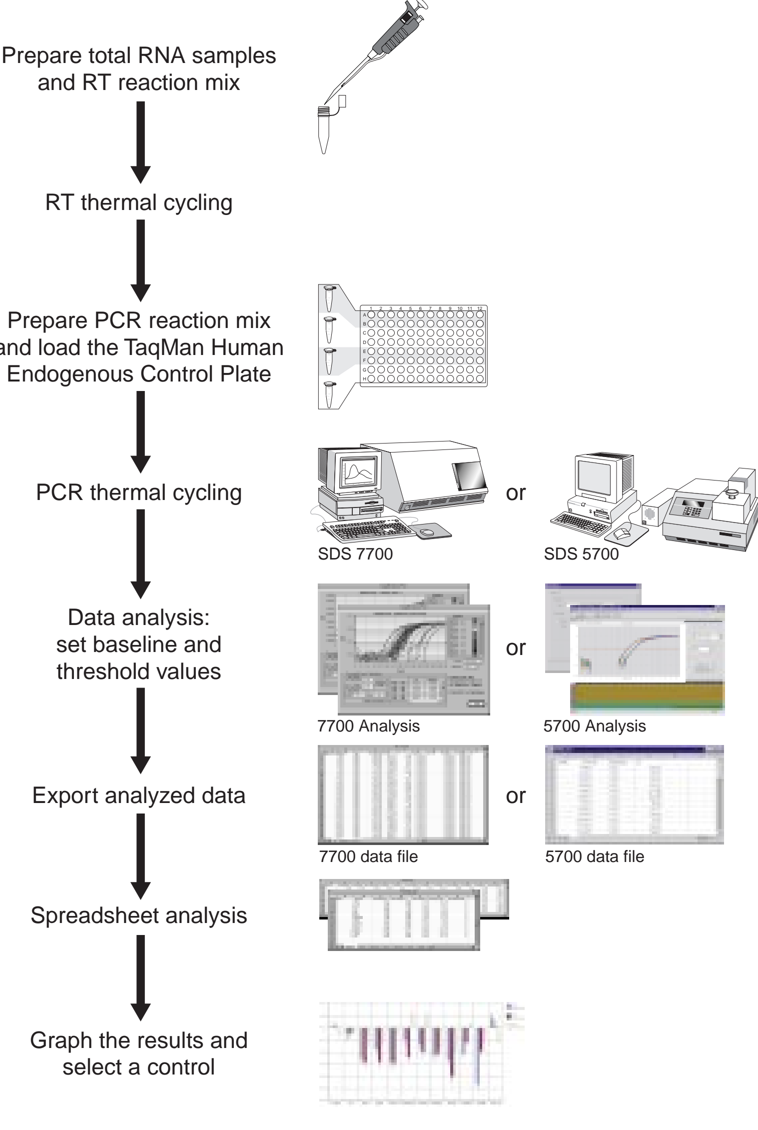


Column	Control Assay	Abbreviation
1	Internal Positive Control	IPC
2	18S rRNA	18S
3	Acidic ribosomal protein	huPO
4	Beta-actin	huβA
5	Cyclophilin	huCYC
6	Glyceraldehyde-3-phosphate dehydrogenase	huGAPDH
7	Phosphoglycerokinase	huPGK
8	β_2-Microglobulin	huβ2m
9	β-Glucronidase	huGUS
10	Hypoxanthine ribosyl transferase	huHPRT
11	Transcription factor IID, TATA binding protein	huTBP
12	Transferrin receptor	huTfR

Note See Appendix B, “About These Assays,” for a list of the TaqMan assays and their functions.

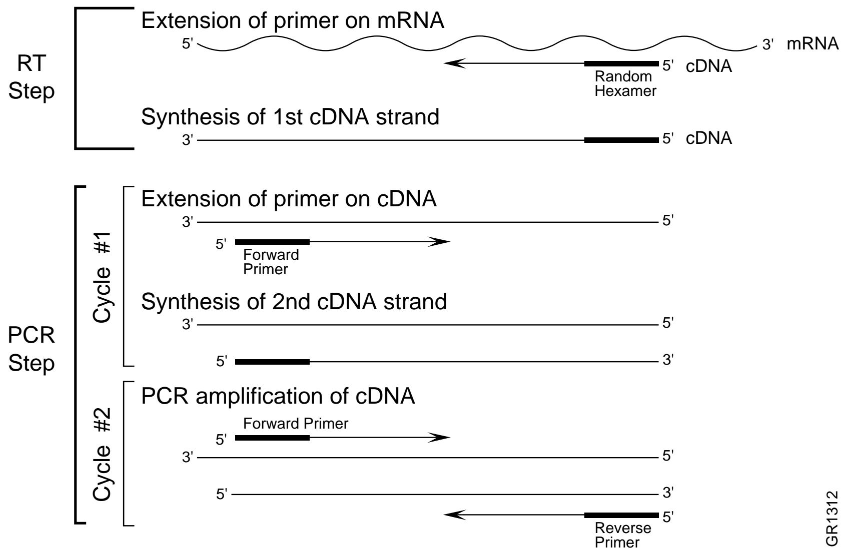
Procedure Flowchart

The following diagram is a simplified overview of this protocol:



How TaqMan Endogenous Control Assays Work

The TaqMan Human Endogenous Control Plate kit evaluates RNA expression in a two-step reverse transcription–polymerase chain reaction (RT-PCR). The figure below illustrates the assay steps.

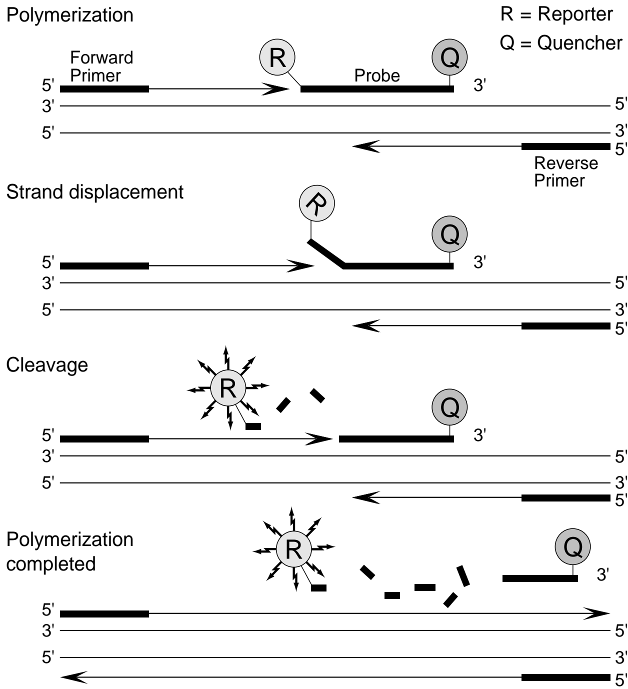


In the RT step, cDNA is reverse transcribed from total RNA samples using random hexamers from the TaqMan Reverse Transcription Reagents. In the PCR step, products are synthesized from cDNA samples using the TaqMan Universal PCR Master Mix.

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 incorporates a VIC 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 VIC 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 as shown in the figure below.



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´→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.

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, any nonspecific amplification is not detected.

About AmpliTaq Gold DNA Polymerase

AmpliTaq Gold is a thermal stable DNA polymerase. The enzyme has a 5´→3´ nuclease activity, but lacks a 3´→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 is 2X in 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.

Preventing Contamination

Introduction

Because of 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 and Faloona, 1987).

About AmpErase UNG

AmpErase uracil-N-glycosylase (UNG) is a pure, nuclease-free, 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. This gene was 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 (Longo *et. al.*, 1990).

General PCR Practices

Please follow these recommended procedures:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, 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 pipettes or aerosol-resistant pipette tips.
- Regularly clean benches and equipment with 10% bleach solution.

Materials and Equipment

Kit Components

TaqMan Human Endogenous Control Plates are available in the following configurations:

P/N	Component	P/N
4309920	TaqMan Human Endogenous Control Plates (2)	—
	TaqMan Universal PCR Master Mix	4304437
	TaqMan Human Control Total RNA	4307281
	TaqMan Human Endogenous Control Plate Protocol	4308134
4309921	TaqMan Human Endogenous Control Plates (2)	—
	TaqMan Universal PCR Master Mix	4304437

Materials Storage Guidelines

The table below lists the storage conditions for the TaqMan Human Endogenous Control Plate Kit and reagents used in this protocol.

Kit Component/Reagent	Storage Conditions
TaqMan Human Endogenous Control Plate	2 to 8 °C, dark
TaqMan Universal PCR Master Mix	2 to 8 °C, dark
TaqMan Human Control Total RNA	–15 to –25 °C

IMPORTANT Do not remove the TaqMan Human Endogenous Control Plate from its packaging until ready to use. Excessive exposure to light damages the fluorescent probes.

Materials and Equipment Not Included

Some equipment and materials are required in addition to the reagents supplied with the TaqMan Human Endogenous Control Plate. Many of the items listed are available from major laboratory suppliers (MLS). Equivalent sources are acceptable where noted.

Sequence Detection Systems	Source
ABI PRISM 7700 Sequence Detection System	Contact your local Applied Biosystems sales office for the instrument best suited to meet your needs.
GeneAmp 5700 Sequence Detection System	

User-supplied materials:

Materials	Source
MicroAmp Optical 96-Well Reaction Plate/Optical Caps	Applied Biosystems (P/N 403012)
Note The MicroAmp Optical 96-Well Reaction Plate may be sealed with: ♦ MicroAmp Optical Caps or ♦ ABI PRISM™ Optical Adhesive Cover The Optical Adhesive Cover must be used with a compression pad and applicator, which are included in the starter pack.	
ABI PRISM Optical Adhesive Cover Starter Pack ♦ 20 optical adhesive covers ♦ 1 applicator ♦ 1 compression pad	Applied Biosystems (P/N 4313663)
Sequence Detection Systems Spectral Calibration Kit[a]	Applied Biosystems (P/N 4305822)
TaqMan Reverse Transcription Reagents	Applied Biosystems (P/N N808-0234)
Centrifuge with 96-well plate adapter	MLS
Disposable gloves	MLS
Microcentrifuge	MLS
Microcentrifuge tubes, sterile, 1.5 mL	MLS
MicroAmp Reaction Tubes with Caps	Applied Biosystems (P/N N802-0540)
Microsoft® Excel (or equivalent software)	Software vendors

User-supplied materials: *(continued)*

Materials	Source
Pipette tips, aerosol resistant	MLS
Pipettors: ♦ Positive-displacement ♦ Air-displacement	MLS
Polypropylene tubes	MLS
Water, RNase-free, distilled, deionized	MLS

a. Only for 7700 instruments not calibrated with the VIC dye. See “Configuring the ABI Prism 7700 Software for the VIC Dye” on page 3-2 for more information.

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 and inhalation of chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Do not leave chemical containers open. Use only with adequate ventilation.
- 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.

Ordering MSDSs

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

<table>
<tr><th>To order MSDSs...</th><th>Then...</th></tr>
<tr><td>Over the Internet</td><td>a. Go to our Web site at www.appliedbiosystems.com/techsupp
b. Click MSDSs
<table>
<tr><th>If you have...</th><th>Then...</th></tr>
<tr><td>The MSDS document number or the Document on Demand index number</td><td>Enter one of these numbers in the appropriate field on this page.</td></tr>
<tr><td>The product part number</td><td rowspan="2">Select Click Here, then enter the part number or keyword(s) in the field on this page.</td></tr>
<tr><td>Keyword(s)</td></tr>
</table>
c. You can open and download a PDF (using Adobe® Acrobat® Reader™) of the document by selecting it, or you can choose to have the document sent to you by fax or email.</td></tr>
<tr><td>By automated telephone service from any country</td><td>Use “To Obtain Documents on Demand” on page D-7.</td></tr>
<tr><td>By telephone in the United States</td><td>Dial 1-800-327-3002, then press 1.</td></tr>
<tr><td>By telephone from Canada</td><td>
<table>
<tr><th>To order in...</th><th>Then dial 1-800-668-6913 and...</th></tr>
<tr><td>English</td><td>Press 1, then 2, then 1 again</td></tr>
<tr><td>French</td><td>Press 2, then 2, then 1</td></tr>
</table></td></tr>
<tr><td>By telephone from any other country</td><td>See the specific region in “To Contact Technical Support by Telephone or Fax” on page D-3.</td></tr>
</table>

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

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.

Reverse Transcription 2

Overview

About This Chapter

This chapter covers reverse transcription (RT), a process in which cDNA is synthesized from total RNA samples. Reverse transcription is the first step in the two-step RT-PCR gene expression quantification experiment, as described in "How TaqMan Endogenous Control Assays Work" on page 1-6. In this step, random hexamers from the TaqMan® Reverse Transcription Reagents prime total RNA samples for reverse transcription using MultiScribe™ Reverse Transcriptase.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Sample Preparation	2-2
Reverse Transcription for All Amplicons Except 18S	2-4
Reverse Transcription for the 18S Amplicon	2-8

Sample Preparation

Recommended Template

Based on the template conflicts explained below, Applied Biosystems recommends evaluating only human total RNA samples using the TaqMan Human Endogenous Control Plate.

The following table lists the known template incompatibilities:

Template	Explanation
Poly A^+	The 18S rRNA assay cannot accurately evaluate poly A^+ RNA samples, because most of the rRNA has been removed.
Non-human	Except for the 18S rRNA and the internal positive control (IPC), all assays on the endogenous control plate are human-specific.

RNA Template Preparation and Quality

Because the quality of results is directly related to the purity of the RNA template, Applied Biosystems recommends using only well-purified samples with the TaqMan Human Endogenous Control Plate. Because ribonuclease and genomic DNA contamination are common problems in gene expression studies, purify your samples accordingly to ensure the best results.

IMPORTANT Each TaqMan endogenous control assay has been experimentally proven not to detect up to 10,000 copies of contaminating DNA. In spite of this design characteristic, Applied Biosystems recommends using purified total RNA samples to obtain the best results.

Recommended Quantity

If possible, use spectrophotometric analysis to determine the concentrations of your purified total RNA samples. The table below contains the recommended range of template quantity.

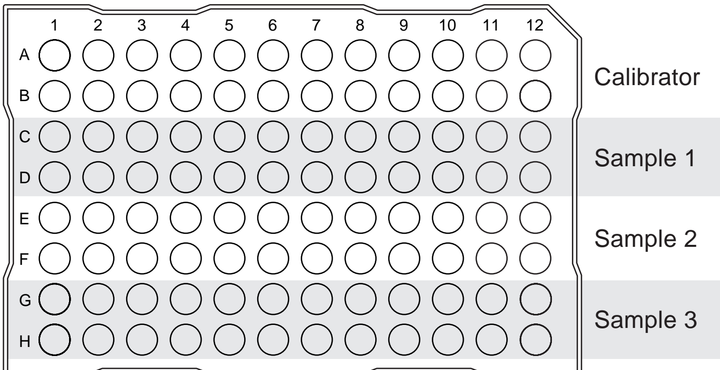
Initial Template	Quantity Per Well[a]
Human Total RNA	10–100 ng

a. Initial RNA converted to cDNA

IMPORTANT Enough sample-specific cDNA must be generated for each sample to fill 24 wells on the TaqMan Human Endogenous Control Plate.

About the Calibrator Sample

Applied Biosystems recommends evaluating duplicate rows of three test samples and a calibrator sample on the TaqMan Human Endogenous Control Plate. The figure below illustrates the recommended plate configuration.



The calibrator sample serves the following purposes:

- Provides a baseline for comparison with the other samples on the plate.
- Serves as a basis for comparing sample data from multiple, independently run plates.

Note The calibrator sample can be used to compare sample data from independently run plates only if the same calibrator sample is present on all plates.

Reverse Transcription for All Amplicons Except 18S

Reverse Transcription Guidelines

The following guidelines ensure optimal RT performance:

- Depending on gene expression levels in your samples, Applied Biosystems recommends using 10–100 ng of total RNA (converted to cDNA) per well. Enough sample-specific cDNA must be generated for each sample to fill 24 wells on the TaqMan Human Endogenous Control Plate.
- Perform multiple RT reactions in multiple wells if using more than 2 µg total RNA. A maximum of 2 µg total RNA per 100 µL RT reaction efficiently converts to cDNA.
- Prior to use, thaw all reagents except the enzyme and the RNase Inhibitor. When the reagents are thawed, keep them on ice.
- Keep the MultiScribe Reverse Transcriptase RNase Inhibitor in a freezer until immediately prior to use.

Instruments for Reverse Transcription

Because the data acquired during the RT reaction is not needed for analysis, any of the thermal cyclers listed below can be used:

- ABI PRISM 7700 Sequence Detection System
- GeneAmp 5700 Sequence Detection System
- GeneAmp® PCR System 9700 Thermal Cycler
- GeneAmp® PCR System 9600 Thermal Cycler

Performing the RT Reaction

⚠ CAUTION **CHEMICAL HAZARD**. **TaqMan Reverse Transcription Reagents** may cause eye and skin irritation. 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 the RT reaction:

Step	Action
1	In a 1.5-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed.

Component	Volume (µL)		Final Conc.
	Per Sample	Reaction Mix (x4)	
RNase-free water	See below[a]	See below[a]	—
10X RT Buffer	10.0	40.0	1X
25 mM $MgCl_2$	22.0	88.0	5.5 mM
deoxyNTPs Mixture	20.0	80.0	500 µM (per dNTP)
Random Hexamers	5.0	20.0	2.5 µM
RNase Inhibitor	2.0	8.0	0.4 U/µL
MultiScribe Reverse Transcriptase (50 U/µL)	2.5	10.0	1.25 U/µL
Total[b, c]	61.5	246.0	—

a. The volume of RNase-free water (µL) is 38.5 µL–RNA sample volume in a 100-µL reaction.

b. If changing the reaction volume, make sure that the final proportions are consistent with the recommendd values above.

c. Perform multiple RT reactions in multiple wells if using more than 2 µg total RNA.

Note The calibrator is a sample used as a basis for comparison with the other samples on the plate (see “About the Calibrator Sample” on page 2-3 for more information).

Step	Action
2	Label four 1.5-mL microcentrifuge tubes for three test samples and a calibrator sample.
3	Transfer 60 ng to 2 µg (up to 38.5 µL) of each total RNA sample to the corresponding microcentrifuge tube.

To perform the RT reaction: *(continued)*

Step	Action
4	If necessary, dilute each total RNA sample to a volume of 38.5 µL with RNase-free, deionized water.
5	Cap the tubes and gently tap each to mix the diluted samples.
6	Briefly centrifuge the tubes to eliminate air bubbles in the mixture.
7	Label four 0.2-mL MicroAmp Reaction tubes for the three total RNA samples and a calibrator sample.
8	Pipette 61.5 µL of reaction mix (from step 1) into each MicroAmp Reaction Tube (from step 7). • 10X RT buffer • $MgCl_2$ • dNTPs mixture • Random hexamers • MultiScribe reverse transcriptase • RNase inhibitor 61.5 µL 61.5 µL 61.5 µL 61.5 µL Calibrator Sample 1 Sample 2 Sample 3
9	Transfer 38.5 µL of each dilute total RNA sample to the corresponding MicroAmp Reaction tube.
10	Cap the reaction tubes and gently mix the reactions.
11	Breifly centrifuge the tubes to force the solution to the bottom of the tube and eliminate air bubbles from the mixture.
12	Transfer each reaction to: ♦ MicroAmp Optical Tube(s), or ♦ Wells of a MicroAmp Optical 96-Well Reaction Plate.
13	Cap the MicroAmp Optical tubes or plate.
14	Centrifuge the plate or tubes to spin down the contents and eliminate air bubbles from the solutions.

To perform the RT reaction: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>15</td><td>Load the reactions into a thermal cycler.</td></tr>
<tr><td>16</td><td>Program your thermal cycler with the following conditions:

IMPORTANT If using a 9700 thermal cycler, select MAX Mode to perform 100-μL reactions.

<table>
<tr><th></th><th>Incubation</th><th>Reverse Transcription</th><th>Reverse Transcriptase Inactivation</th></tr>
<tr><td>**Step**</td><td>HOLD</td><td>HOLD</td><td>HOLD</td></tr>
<tr><td>Temperature</td><td>25.0 °C[a]</td><td>48.0 °C</td><td>95.0 °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">100 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.

Note The thermal cycling parameters are optimal for the Applied Biosystems thermal cyclers listed in “Instruments for Reverse Transcription” on page 2-4. Due to differences in ramp rates and thermal accuracy, you may need to adjust the settings if using another thermal cycler.

Note See your thermal cycler user’s manual for help on setting thermal cycling conditions.</td></tr>
<tr><td>17</td><td>Load the plate into your thermal cycler and begin thermal cycling.

IMPORTANT Remove the 96-well reaction plate immediately after thermal cycling is complete. The cDNA can be used immediately for PCR amplification or stored at –15 to –25 °C for later use.</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 (P/N N808-0234) prime total RNA samples for reverse transcription using MultiScribe™ Reverse Transcriptase.

Recommended Template

Use only human total RNA samples to generate cDNA for the TaqMan Human Endogenous Control Plate.

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 and the IPC, all assays on the TaqMan Human Endogenous Control Plate 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 with the TaqMan Human Endogenous Control Plate. 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 reverse transcription (RT) step.

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

Guidelines

Follow the guidelines below to ensure optimal RT performance:

- Poly A^+ RNA samples are not recommended for endogenous control experiments because most rRNA has been removed from them.
- 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 endogenous control gene expression assays.

Preparing the Reactions

The following procedure describes the preparation of three different test samples and a calibrator sample for reverse transcription. Scale the recommended volumes accordingly for the number of samples needed using the TaqMan Reverse Transcription Reagents (P/N N808-0234).

⚠ CAUTION **CHEMICAL HAZARD**. **TaqMan Reverse Transcription Reagents** may cause eye and skin irritation. 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 reverse transcription reactions:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>In a 1.5-mL microcentrifuge tube, prepare a reaction mix for all total RNA samples to be reverse transcribed.
<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>10.0</td><td>40.0</td><td>1X</td></tr>
<tr><td>25 mM $MgCl_2$</td><td>22.0</td><td>88.0</td><td>5.5 mM</td></tr>
<tr><td>deoxyNTPs Mixture</td><td>20.0</td><td>80.0</td><td>500 µM per dNTP</td></tr>
<tr><td>Random Hexamers</td><td>5.0</td><td>20.0</td><td>2.5 µM</td></tr>
<tr><td>RNase Inhibitor</td><td>2.0</td><td>8.0</td><td>0.4 U/µL</td></tr>
<tr><td>MultiScribe Reverse Transcriptase (50 U/µL)</td><td>6.25</td><td>25.0</td><td>3.125 U/µL</td></tr>
<tr><td>Total[b]</td><td>65.25</td><td>261.0</td><td>—</td></tr>
</table>
a. The volume of RNase-free water (µL) is 34.75–RNA sample volume in a 100-µL reaction.
b. If changing the reaction volume, make sure the final proportions are consistent with the recommended values above.</td></tr>
<tr><td>2</td><td>Label four 1.5-mL microcentrifuge tubes for the three test samples and a calibrator sample.</td></tr>
<tr><td>3</td><td>Transfer 60 ng–2 µg (up to 34.75 µL) of each total RNA sample to the corresponding microcentrifuge tube.</td></tr>
<tr><td>4</td><td>If necessary, dilute each total RNA sample to a volume of 34.75 µL with RNase-free, deionized water.</td></tr>
<tr><td>5</td><td>Cap the tubes and gently tap each to mix the diluted samples.</td></tr>
<tr><td>6</td><td>Briefly centrifuge the tubes to eliminate air bubbles in the mixture.</td></tr>
<tr><td>7</td><td>Label four 0.2-mL MicroAmp® Reaction Tubes for the three total RNA test samples and the calibrator sample.</td></tr>
</table>

To prepare the reverse transcription reactions: *(continued)*

Step	Action
8	Pipette 65.25 µL of the reaction mix (from step 1) to each MicroAmp Reaction Tube (from step 7). • 10X RT buffer • $MgCl_2$ • dNTPs mixture • Random hexamers • MultiScribe reverse Transcriptase • RNase inhibitor 65.25 µL 65.25 µL 65.25 µL 65.25 µL Calibrator Sample 1 Sample 2 Sample 3
9	Transfer 34.75 µL of each dilute total RNA sample to the corresponding MicroAmp Reaction Tube.
10	Cap the reaction tubes and gently tap each to mix the reactions.
11	Briefly centrifuge the tubes to force the solution to the bottom and to eliminate air bubbles from the mixture.
12	Transfer each reaction to MicroAmp Optical tubes or wells of a MicroAmp Optical 96-Well Reaction plate.
13	Cap the MicroAmp Optical tubes or plate.
14	Centrifuge the plate or tubes to spin down the contents and eliminate air bubbles from the solutiions.

Thermal Cycling

To conduct reverse transcription thermal cycling:

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

Thermal cycler conditions (Step 2):

Step	Hexamer Incubation[a]	Reverse Transcription	Reverse Transcriptase Inactivation
	HOLD	HOLD	HOLD
Temp.	25 °C	37 °C	95 °C
Time	10 min	60 min	5 min
Volume	100 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.

PCR 3

Overview

About This Chapter

This chapter covers PCR, or the amplification of cDNA. PCR is the second step in the two-step RT-PCR experiment, as described in “How TaqMan Endogenous Control Assays Work” on page 1-6. In this step, AmpliTaq® Gold DNA polymerase amplifies cDNA synthesized from the original total RNA sample.

Note See “Basics of the 5´ Nuclease Assay” on page 1-7 for more information on AmpliTaq Gold DNA polymerase and the 5´ nuclease assay.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Preparing the Sequence Detection System for PCR	3-2
Preparing and Running the PCR Reactions	3-5

Preparing the Sequence Detection System for PCR

Instruments

IMPORTANT Because the data acquired during the PCR is needed for analysis, you must use one of the following sequence detectors for PCR:

- ♦ ABI PRISM 7700 Sequence Detection System
- ♦ GeneAmp 5700 Sequence Detection System

Configuring the ABI PRISM 7700 Software for the VIC Dye

If your ABI PRISM 7700 Sequence Detection System is not calibrated for the VIC dye, it must be calibrated using the Sequence Detection Systems Spectral Calibration Kit (P/N 4305822). The kit provides the standards needed to configure the ABI PRISM 7700 Sequence Detector for use with products containing TaqMan® VIC or SYBR® Green dyes. If the instrument is not calibrated for the VIC dye, the instrument software will be unable to configure the VIC dye layer for the endogenous control gene expression assay.

Note For more information about the Sequence Detection Systems Spectral Calibration Kit or the calibration procedure, see the *ABI PRISM 7700 Sequence Detection Systems User Bulletin #4: Generating New Spectra Components* (P/N 4306234). User bulletin #4 can be obtained from Applied Biosystems. See "To Obtain Documents on Demand" on page D-7.

Programming the Sequence Detector for PCR

To run the TaqMan Human Endogenous Control Plate on a sequence detection system instrument, you must configure a plate document with the appropriate assay and sample information. The TaqMan Human Endogenous Control Plate compares gene expression levels based on the data collected during the PCR run. By configuring the plate document with the sample and assay locations, the SDS software can collect and organize the florescence data for analysis.

To configure the PCR plate document:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Open the Sequence Detection System (SDS) software.</td></tr>
<tr><td>2</td><td>Create a plate document with the following attributes:
<table>
<tr><th>7700 Plate Document</th><th>5700 Plate Document</th></tr>
<tr><td>♦ Single Reporter
♦ 7700 Sequence Detector
♦ Real Time</td><td>♦ 5700
♦ 5700 Quantitation</td></tr>
</table>
</td></tr>
</table>

To configure the PCR plate document: *(continued)*

Step	Action
3	Choose from one of the following:

If using an…	Then…
ABI PRISM 7700 Sequence Detection System	From the **Dye Layer** menu, select **VIC**. **Note** If VIC does not appear on the Dye Layer menu, the instrument is not calibrated for the VIC dye. See “Configuring the ABI Prism 7700 Software for the VIC Dye” on page 3-2 for more information.
GeneAmp 5700 Sequence Detection System	a. From the **Primer/Probe Setup** dialog box, create the following primer/probe entry: **Acronym**: TAQ1 **Description**: TaqMan VIC b. Apply the probe to all wells.

Step	Action
4	Configure the plate document as shown in the figure below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
B	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
C	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
D	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
E	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
F	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
G	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR
H	UNKN IPC	UNKN 18S	UNKN huPO	UNKN huBA	UNKN huCYC	UNKN huGAP	UNKN huPGK	UNKN huB2m	UNKN huGUS	UNKN huHPRT	UNKN huTBP	UNKN huTfR

Note See Appendix B, “About These Assays,” for a list of target names, abbreviations, and descriptions.

To configure the PCR plate document: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>5</td><td>Program the thermal cycler with the following conditions:

<table>
<tr><th rowspan="3">Step</th><th>UNG Activation[a]</th><th>AmpliTaq Gold Activation[b]</th><th colspan="2">PCR</th></tr>
<tr><td rowspan="2">HOLD</td><td rowspan="2">HOLD</td><td colspan="2">CYCLE (40 cycles)</td></tr>
<tr><td>Denature</td><td>Anneal/ Extend</td></tr>
<tr><td>Temp.</td><td>50.0 °C</td><td>95.0 °C</td><td>95.0 °C</td><td>60.0 °C</td></tr>
<tr><td>Time</td><td>2 min</td><td>10 min</td><td>15 sec</td><td>1 min</td></tr>
<tr><td>Volume</td><td colspan="4">50 µL</td></tr>
</table>

a. Required for optimal AmpErase UNG activation.

b. Required for optimal AmpliTaq Gold DNA Polymerase activation.

Note See your sequence detection systems user’s manual for help on setting thermal cycling conditions.</td></tr>
</table>

Preparing and Running the PCR Reactions

PCR Guidelines

The following guidelines ensure optimal PCR performance:

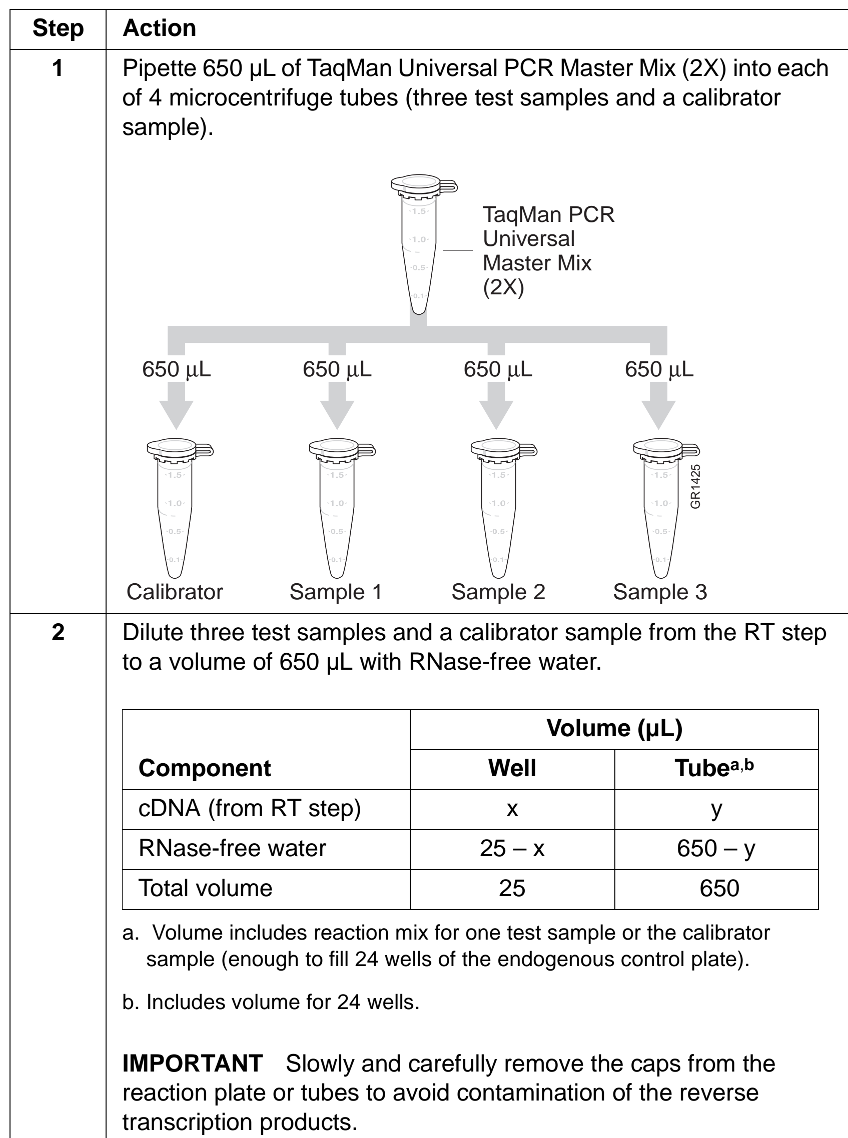
- Do not remove the TaqMan Human Endogenous Control Plate from its foil packaging until you are ready to load the PCR reaction mix. Excessive exposure to light can damage the florescent probes.
- Prior to use, thaw frozen cDNA samples by placing them on ice. When thawed, vortex and briefly centrifuge the contents of each tube to resuspend the samples.
- Prepare PCR reaction mixture for each sample in separate microcentrifuge tube before aliquoting it to the reaction plate for thermal cycling and fluorescence analysis.
- The volume of the PCR reaction mix per well must be 50 µL minus the volume of the cDNA sample from the RT step.
- Do not mix the PCR mixture and cDNA samples in the MicroAmp Optical 96-Well Reaction Plate.

Note The cDNA amplification reaction is optimized with TaqMan Universal PCR Master Mix.

Performing PCR

⚠ 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.

To perform the PCR:

Step	Action
1	Pipette 650 µL of TaqMan Universal PCR Master Mix (2X) into each of 4 microcentrifuge tubes (three test samples and a calibrator sample). 
2	Dilute three test samples and a calibrator sample from the RT step to a volume of 650 µL with RNase-free water.

Component	Volume (µL)	
	Well	Tube[a,b]
cDNA (from RT step)	x	y
RNase-free water	25 – x	650 – y
Total volume	25	650

a. Volume includes reaction mix for one test sample or the calibrator sample (enough to fill 24 wells of the endogenous control plate).

b. Includes volume for 24 wells.

IMPORTANT Slowly and carefully remove the caps from the reaction plate or tubes to avoid contamination of the reverse transcription products.

To perform the PCR: *(continued)*

Step	Action
3	Pipette 650 µL of each cDNA sample to a microcentrifuge tube containing TaqMan Universal PCR Master Mix. Calibrator + RNase-free H_2O → 650 µL → Calibrator: 650 µL Universal Master Mix Sample 1 + RNase-free H_2O → 650 µL → Sample 1: 650 µL Universal Master Mix Sample 2 + RNase-free H_2O → 650 µL → Sample 2: 650 µL Universal Master Mix Sample 3 + RNase-free H_2O → 650 µL → Sample 3: 650 µL Universal Master Mix
4	Cap the microcentrifuge tubes and mix the solutions by gentle inversion.
5	Centrifuge the tubes to spin down the contents and eliminate air bubbles from the solutions.

To perform the PCR: *(continued)*

Step	Action
6	Using a positive displacement pipette, transfer the three test samples and a calibrator sample 50 µL aliquots to the wells of the TaqMan Human Endogenous Control Plate. cDNA/Master Mix/ RNase-free H_2O mixture (1.3 mL) Calibrator Sample 1 Sample 2 Sample 3 Transfer 50 µL to each well 1 2 3 4 5 6 7 8 9 10 11 12 A B C D E F G H GR1280b
7	Seal the wells with MicroAmp Optical Caps or a MicroAmp Optical Adhesive Cover.
8	Centrifuge the 96-well plate to spin down the contents and eliminate any air bubbles from the solutions.
9	Load the TaqMan Human Endogenous Control Plate into your sequence detection system and begin thermal cycling. Refer to the thermal cycling conditions on page 3-4.

Data Analysis

4

Overview

About This Chapter

This chapter covers data analysis, which requires adjustment of the baseline and threshold values within the Sequence Detection Systems (SDS) software. After the adjustments, the data can be exported from the SDS software for spreadsheet analysis.

IMPORTANT If the threshold value is set before the baseline, the resulting C_T values may be invalid and produce errors when calculating gene expression.

In This Chapter

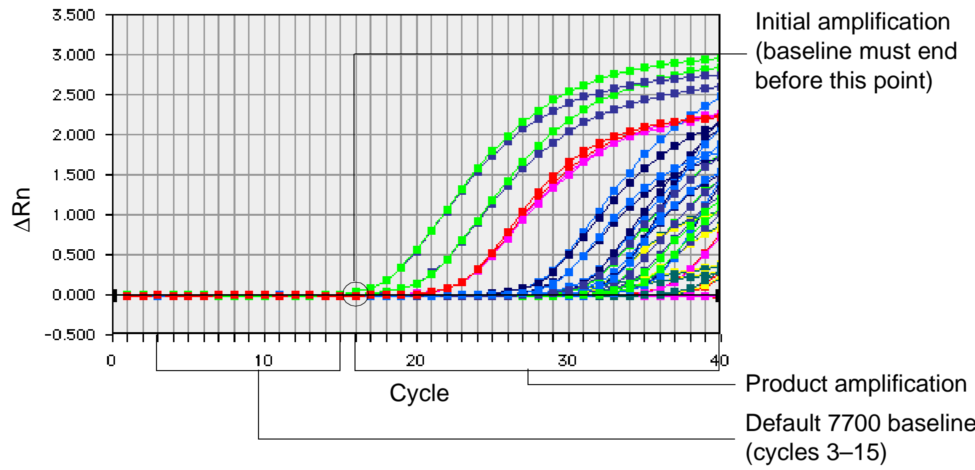
The following topics are discussed in this chapter:

Topic	See Page
Setting the Baseline	4-2
Setting the Threshold Value	4-6

Setting the Baseline

Baseline Basics

The baseline is a defined range of cycles before the Sequence Detection Systems (SDS) software detects the amplification of PCR product. The SDS software uses a default range of cycles 3–15 on 7700 instruments and cycles 6–15 on 5700 instruments to establish the baseline. The figure below illustrates the important characteristics of the baseline on a 7700 amplification plot.



Because of the abundance of rRNA, low C_T values can be obtained in TaqMan RT-PCR applications with the 18S assays. When the amplification of the 18S target reaches a detectable level at a very early cycle, it can limit the number of cycles over which the software calculates the baseline. In rare cases, this interferes with the detection of less abundant targets. See Appendix A, "Troubleshooting Early Amplification," for more information.

Guidelines for Setting the Baseline

Correct placement of the baseline is a crucial step in data analysis. Follow the guidelines below to ensure the baseline is set properly.

- Set the baseline so that the initial amplification curve begins at a cycle that is greater than the maximum value of the baseline.
- Do not adjust the default baseline if the amplification curve growth begins after cycle 15. For example, the default can be used for the plot above because initial amplification occurs at cycle 16.

Setting the Baseline for the ABI PRISM 7700 Instrument

Before setting the baseline, you must first

- ♦ Display the results on an amplification plot
- ♦ Change the Y-axis to linear scale

Displaying Results on an Amplification Plot

To display the results on an amplification plot:

Step	Action
1	Select **Analysis** > **Analyze**. The SDS software analyzes the raw data and displays an amplification plot.
2	If the SDS software does not display an Amplification Plot, then select **Analysis** > **Amplification Plot**. The SDS software displays an amplification plot (log ΔR_n vs. Cycle).

Changing the Y-Axis to Linear Scale

To change the Y-axis to linear scale:

Step	Action
1	Double-click the $\Delta \mathbf{R_n}$ label on the Y-axis of the amplification plot. Double-click here The **Scale** dialog box appears.
2	Click the **Linear Scale** radio button to graph the data on a linear scale. Display Linear Scale Logarithmic Scale Click here
3	Click **OK**. The amplification plot appears in a linear scale format.

Procedure for Setting the Baseline for the ABI PRISM 7700 Instrument

To set the baseline:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Identify the components of the linear scale amplification plot as shown on page 4-2.</td></tr>
<tr><td>2</td><td>Click the Stop text field in the Baseline box.

Click here</td></tr>
<tr><td>3</td><td>Following the guidelines from the previous page, choose from one of the following actions:
<table>
<tr><th>If the amplification plot looks like...</th><th>Then...</th></tr>
<tr><td></td><td>the amplification curve begins after the maximum baseline.

Do not adjust the baseline.</td></tr>
<tr><td></td><td>the maximum baseline is set too high.

Decrease the Stop baseline value.</td></tr>
<tr><td></td><td>the maximum baseline is set too low.

Increase the Stop baseline value.</td></tr>
</table>
</td></tr>
<tr><td>4</td><td>Click Update Calculations.

The SDS software updates the C_T and standard deviation values.</td></tr>
</table>

Setting the Baseline for the GeneAmp 5700 Instrument

To set the baseline for the GeneAmp 5700 instrument:

Step	Action
1	In the **Plate** window, select all wells for analysis.
2	Select **Analysis** > **Analyze.** An SDS warning message appears.
3	Click **OK** to continue.
4	In the **Plate** window, click the **Results** tab. **Note** The tabs just above the wells in the Plate window let you toggle between the Setup, Instrument, and Results views.
5	In the **Results** view, click the **Amp Plot** tab. The Amplification Plot window appears.
6	Identify the components of the linear scale amplification plot as shown in “Baseline Basics” on page 4-2.
7	Click the **Analysis Preferences** button or select **Edit** > **Preferences**. The Preferences dialog box appears.
8	In the **Baseline** box, highlight the current Start and Stop values and type in new values. **IMPORTANT** When selecting a baseline, refer to the guidelines listed in “Guidelines for Setting the Baseline” on page 4-2.
9	Click **OK**.
10	Select **Analysis** > **Analyze**. The software performs the analysis. The system beeps when the analysis is complete.

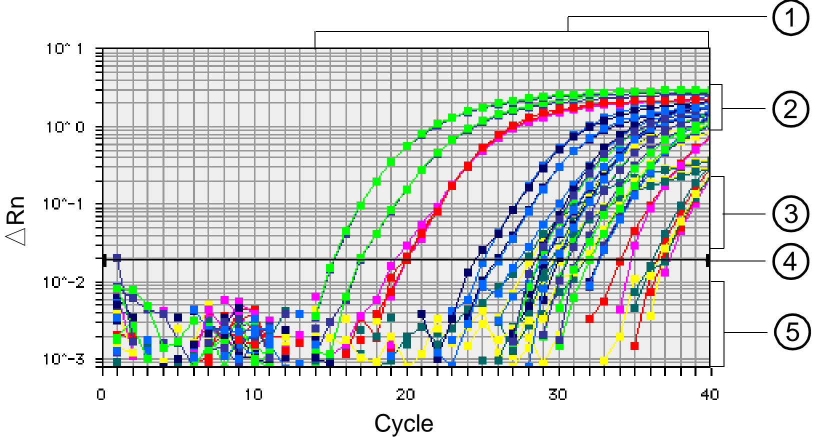
Note For help on setting the baseline, see the *GeneAmp 5700 Sequence Detection System User’s Manual* (P/N 4304472).

Setting the Threshold Value

Threshold Value Basics

For the 7700 instrument, the default threshold value is the average standard deviation of ΔR_n within the defined baseline region, multiplied by an adjustable factor. The SDS software calculates the threshold value as ten standard deviations from the baseline. For this reason, the baseline must be set before you adjust the threshold value. The threshold value must be set manually for the 5700 instrument.

The figure below illustrates the important characteristics of the threshold on a 7700 plot.



Characteristic	Description
1	Product amplification
2	Plateau phase
3	Exponential phase
4	Threshold value
5	Background (spectral noise)

Guidelines for Setting the Threshold

Note Correct placement of the threshold is a crucial step in data analysis. Follow the guidelines below to ensure the threshold is set properly.

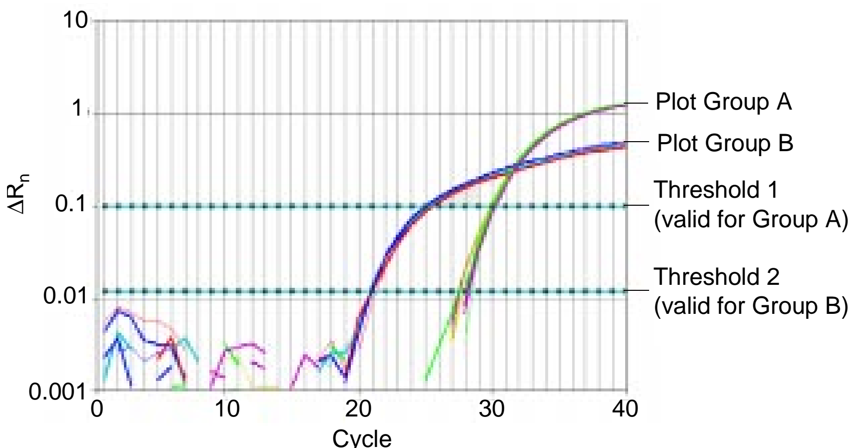
To obtain accurate results:

- Set the threshold value within the exponential phase of the logarithmic scale amplification plots. The exponential phase occurs within the range of data points that increase linearly when graphed.
- Set the threshold value so that it is within the exponential phase of all amplification plots. If a single threshold cannot be set to satisfy all plots, then it must be set multiple times.

Setting Multiple Thresholds

Because the expression levels and ΔR_n values of TaqMan endogenous control assays can vary significantly, it may be necessary to set the threshold more than once to obtain accurate results. If a single threshold value does not intersect the exponential phase of all amplification plots, the data must be analyzed (and subsequently exported) with multiple threshold values.

The figure below shows a 5700 amplification plot where the threshold must be set independently for each group of curves. As shown, Threshold 1 is within the exponential phase of the plots in Group A; however, it intersects with the plateau phase of the plots in Group B. The results from this setting would be accurate for the plots in Group A, but invalid for the plots in Group B. If reset for Group B (Threshold 2), the threshold intersects Group A at a point very early in the exponential phase where background noise causes non-reproducibility. The solution for this situation is to set the threshold separately for both groups.



To set multiple thresholds:

Step	Action
1	Following the appropriate procedure for your instrument, set a threshold value that is valid for the majority of plots on the logarithmic graph.
2	Export the data as explained in “Exporting and Viewing the Results File” on page 5-2. The software saves the data to a file.
3	From the logarithmic amplification plot, identify the plots for which the threshold set in step 1 was invalid.
4	Reset the threshold value for the second group of plots.
5	Export the data as explained in “Exporting and Viewing the Results File” on page 5-2. **IMPORTANT** Save the file with a name different than that used in step 2. The software overwrites files with identical names. There are now two files on the disk: ♦ The file created in step 2 containing valid data for the majority of plots from the experiment ♦ The file created in step 5 containing valid data for the remaining plots **Note** The data in the files is combined later during spreadsheet analysis.

To set multiple thresholds: *(continued)*

Step	Action
6	Follow the procedure for spreadsheet analysis as described in “Calculating the Relative Quantification Using a Spreadsheet” on page 5-5.

Setting the Threshold Value for the ABI PRISM 7700 Instrument

Changing the Y-Axis to Logarithmic Scale

To view the threshold value:

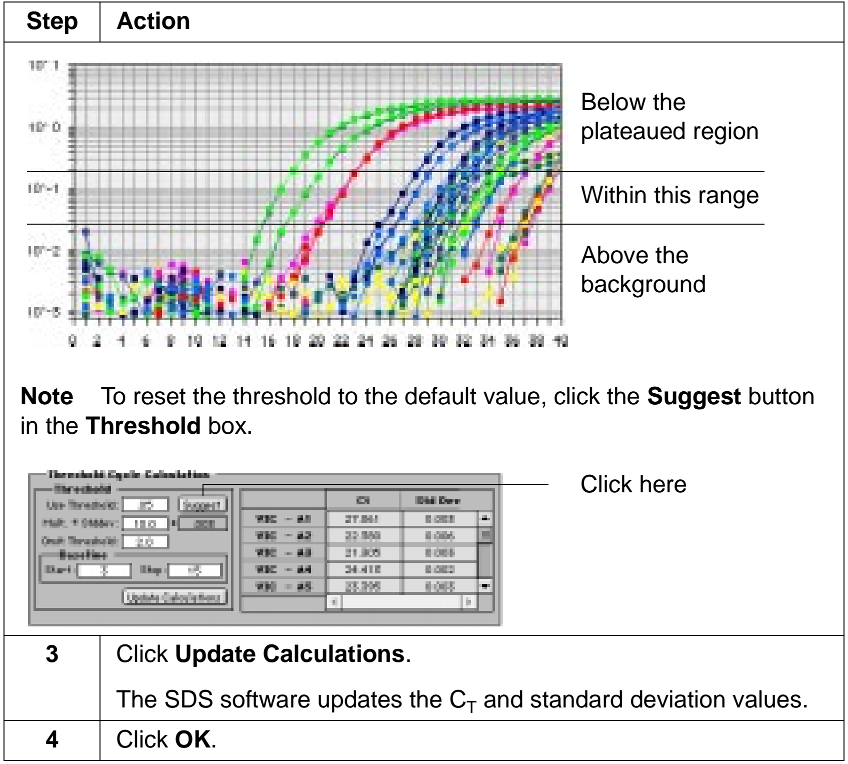
Step	Action
1	Double-click the ΔR_n label on the Y-axis of the graph. The Scale dialog box appears.
2	Click the **Logarithmic Scale** radio button from the **Display** box. Display Linear Scale Logarithmic Scale Click here
3	Click **OK**. The amplification plot appears in logarithmic format.

Procedure for Setting the Baseline for the ABI PRISM 7700 Instrument

To set the threshold value:

Step	Action
1	Identify the components of the amplification curve as shown in “Threshold Value Basics” on page 4-6.
2	Click and drag the threshold line so that it is: ♦ Above the background noise ♦ Below the plateaued region ♦ Within the exponential phase of the amplification curve

To set the threshold value: *(continued)*

Step	Action
	 **Note** To reset the threshold to the default value, click the **Suggest** button in the **Threshold** box.
3	Click **Update Calculations**. The SDS software updates the C_T and standard deviation values.
4	Click **OK**.

Setting the Threshold Value for the GeneAmp 5700 Instrument

To set the threshold for the GeneAmp 5700 instrument:

Step	Action
1	In the **Plate** window, click the **Results** tab. **Note** The tabs just above the wells in the Plate window let you toggle between the Setup, Instrument, and Results views.
2	In the **Results** view, click the **Amp Plot** tab. The Amplification Plot window appears.
3	Identify the components of the amplification curve as shown in “Threshold Value Basics” on page 4-6.
4	Determine a value for threshold that is: ♦ Above the background noise ♦ Below the plateaued region ♦ Within the exponential phase of the amplification curve **IMPORTANT** When selecting a threshold, refer to the guidelines listed in “Guidelines for Setting the Threshold” on page 4-7.
5	Click the **Analysis Preferences** button, or select **Edit** > **Preferences**. The Preferences dialog box appears.
6	In the **Threshold** box, enter the value you determined in step 4 above.
7	Click **OK**.
8	Select **Analysis** > **Analyze** . The software performs the analysis. The system beeps when the analysis is complete.

Note For help on setting the threshold value, see the *GeneAmp 5700 Sequence Detection System User’s Manual* (P/N 4304472).

Calculating Relative Quantification

5

Overview

About This Chapter

This chapter explains how to calculate relative quantification values from C_T values with the use of a spreadsheet application such as Microsoft® Excel. Applied Biosystems® recommends using a professional spreadsheet software package to analyze the results from the TaqMan Human Endogenous Control Plate. Although calculation of relative quantification values can be done manually, spreadsheet packages speed the process considerably.

In This Chapter

The following topics are discussed in this chapter:

Topic	See Page
Exporting and Viewing the Results File	5-2
Calculating the Relative Quantification Using a Spreadsheet	5-5
Interpreting Results	5-17

Exporting and Viewing the Results File

Creating a Results File

To analyze data from the TaqMan Human Endogenous Control Plate, export the results to a results file. The SDS software can export raw data from a sequence detection run in formats that are compatible with most spreadsheet applications. The type of file the software exports depends on the model instrument used to collect the data.

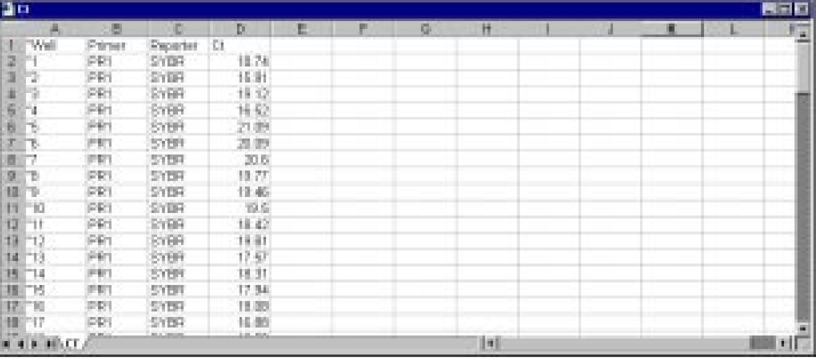
Instrument	Exported Format
ABI PRISM 7700 Instrument	Tab-delimited text file
GeneAmp 5700 Instrument	Comma-separated text file (.csv)

Exporting Results from a GeneAmp 5700 Sequence Detection System

To export the data from the endogenous control gene expression assay:

Step	Action
1	Select **Analysis** > **Export** > **Ct**. The Save As dialog box appears. **Note** You can also click the **Export** button in the **Report** window to open the **Save As** dialog box.
2	Click the **Save as text** box and type a name for the results file.
3	Click **Save**. The SDS software exports the data to a comma-separated text file.
4	Close the SDS software.

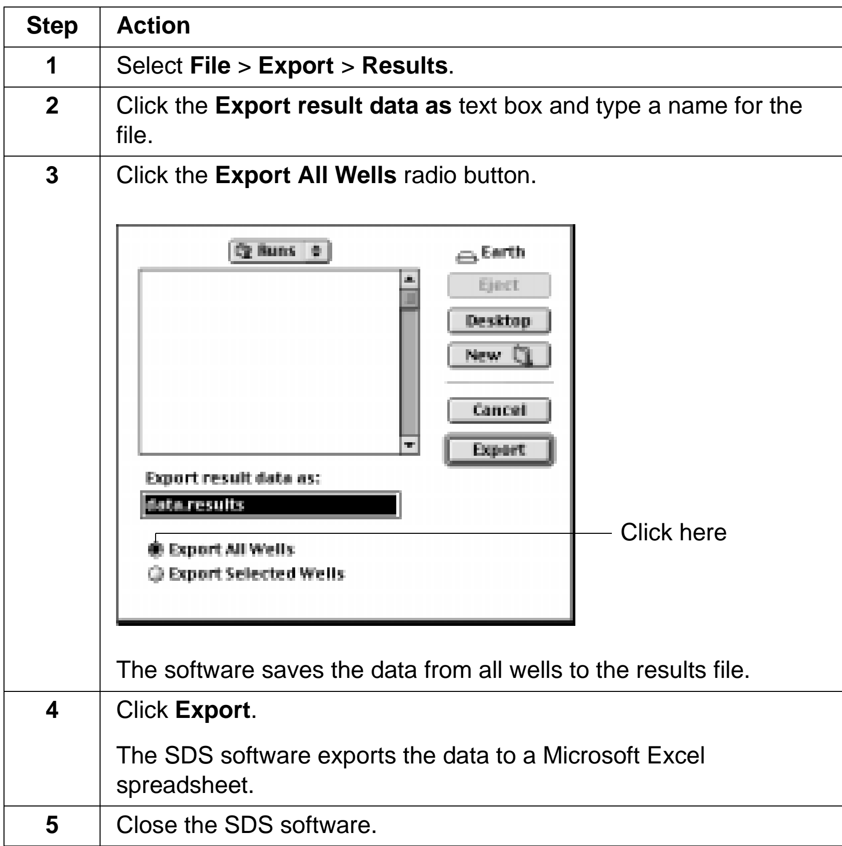
The figure below is an example of an exported 5700 results file as viewed with the Microsoft Excel spreadsheet.



Well	Primer	Reporter	Ct
1	PR1	SYBR	18.74
2	PR1	SYBR	15.91
3	PR1	SYBR	19.12
4	PR1	SYBR	16.52
5	PR1	SYBR	21.09
6	PR1	SYBR	20.09
7	PR1	SYBR	20.6
8	PR1	SYBR	19.77
9	PR1	SYBR	19.46
10	PR1	SYBR	19.5
11	PR1	SYBR	18.42
12	PR1	SYBR	19.81
13	PR1	SYBR	17.57
14	PR1	SYBR	18.31
15	PR1	SYBR	17.94
16	PR1	SYBR	18.08
17	PR1	SYBR	16.88

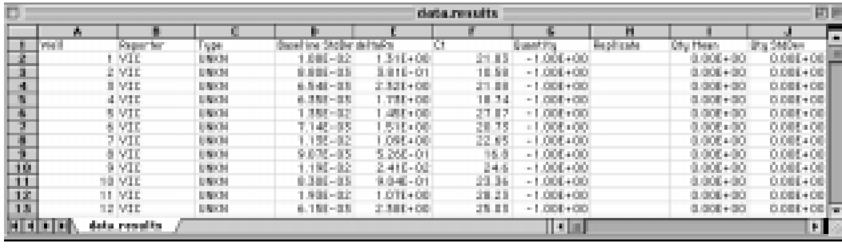
Exporting Results from a ABI PRISM 7700 Sequence Detection System

To export the data from the endogenous control gene expression assay:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Select File > Export > Results.</td></tr>
<tr><td>2</td><td>Click the Export result data as text box and type a name for the file.</td></tr>
<tr><td>3</td><td>Click the Export All Wells radio button.


The software saves the data from all wells to the results file.</td></tr>
<tr><td>4</td><td>Click Export.
The SDS software exports the data to a Microsoft Excel spreadsheet.</td></tr>
<tr><td>5</td><td>Close the SDS software.</td></tr>
</table>

The figure below is an example of an exported 7700 results file as viewed with the Microsoft Excel spreadsheet.



Viewing the Results File

The exported SDS file from the data analysis procedure can be viewed using almost any spreadsheet application.

To view the exported results file:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Open the spreadsheet software.</td></tr>
<tr><td>2</td><td>Select File > Open.</td></tr>
<tr><td>3</td><td>Select from one of the following:
<table>
<tr><th>If you created…</th><th>Then select the…</th></tr>
<tr><td>one results file</td><td>exported results file and click Open.</td></tr>
<tr><td>two results files as explained in the “Setting Multiple Thresholds” on page 4-7</td><td>exported file created in steps 1–2 and click Open.</td></tr>
<tr><td>two results files as explained in “How to Correct for Early Amplification” on page A-2</td><td>exported file created in steps 1–4 and click Open.</td></tr>
</table>
</td></tr>
</table>

Calculating the Relative Quantification Using a Spreadsheet

Overview

Applied Biosystems recommends using a spreadsheet to create comparative gene expression profiles from TaqMan Human Endogenous Control Plate data.

Constructing a C_T Table

To construct a C_T table:

Step	Action
1	Select **File** > **New**. A new spreadsheet appears.
2	From the **Window** menu, select the results file. The endogenous control plate results spreadsheet reappears.
3	Select cells **A2–A13**.
4	Select **Edit** > **Copy**.
5	From the **Window** menu, select the new spreadsheet. The new spreadsheet file reappears.
6	Click cell **A2**.
7	Select **Edit** > **Paste**. Excel pastes the data into the new spreadsheet.

To construct a C_T table: *(continued)*

Step	Action
8	Type the labels for the C_T table as specified in the following table.

Click on cell…	Type…
A1	Column
B1	Ct Calibrator
C1	Ct Calibrator
D1	Ct Sample 1
E1	Ct Sample 1
F1	Ct Sample 2
G1	Ct Sample 2
H1	Ct Sample 3
I1	Ct Sample 3

The ΔC_T table appears as shown below.

Importing Data to the C_T Table

Note This section also consolidates the data from additional files created in the sections:

- “Setting Multiple Thresholds” on page 4-7
- Appendix A, “Troubleshooting Early Amplification.”

To transfer data from the results file to the C_T table:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>From the Window menu, select the exported results file.

The endogenous control plate results spreadsheet reappears.</td></tr>
<tr><td>2</td><td>From the results file spreadsheet select:

<table>
<tr><th>If viewing a…</th><th>Select cells…</th></tr>
<tr><td>5700 results file</td><td>D2–D13</td></tr>
<tr><td>7700 results file</td><td>F2–F13</td></tr>
</table>

Note The columns of the selected cells contain the C_T values for the wells in row A of the TaqMan Human Endogenous Control Plate.</td></tr>
<tr><td>3</td><td>Select Edit > Copy.</td></tr>
<tr><td>4</td><td>From the Window menu, select the new spreadsheet.

The new spreadsheet file reappears.</td></tr>
<tr><td>5</td><td>Click on cell B2.</td></tr>
<tr><td>6</td><td>Select Edit > Paste.

Excel pastes the data into the new spreadsheet.</td></tr>
<tr><td>7</td><td>Using the cut-and-paste procedure from steps 1–6, copy the C_T values of the remaining wells into the new spreadsheet as shown below.

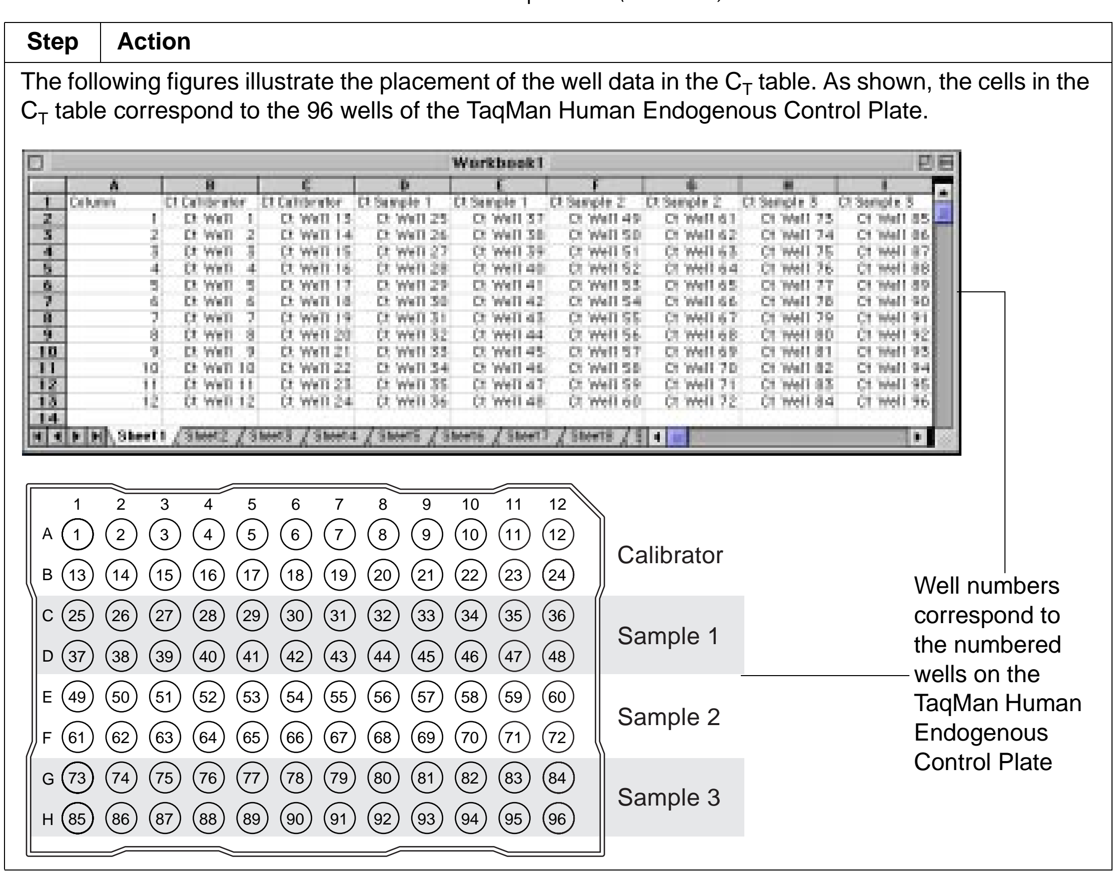
<table>
<tr><th colspan="2">Select and copy the following cells…</th><th rowspan="2">Paste to cells…</th></tr>
<tr><th>5700 results file</th><th>7700 results file</th></tr>
<tr><td>D14–D25</td><td>F14–F25</td><td>C2–C13</td></tr>
<tr><td>D26–D37</td><td>F26–F37</td><td>D2–D13</td></tr>
<tr><td>D38–D49</td><td>F38–F49</td><td>E2–E13</td></tr>
<tr><td>D50–D61</td><td>F50–F61</td><td>F2–F13</td></tr>
<tr><td>D62–D73</td><td>F62–F73</td><td>G2–G13</td></tr>
<tr><td>D74–D85</td><td>F74–F85</td><td>H2–H13</td></tr>
<tr><td>D86–D97</td><td>F86–F97</td><td>I2–I13</td></tr>
</table></td></tr>
</table>

To transfer data from the results file to the C_T table: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>8</td><td>Choose one of the following:
<table>
<tr><th>If the baseline and/or threshold values were set...</th><th>Then...</th></tr>
<tr><td>once for all targets</td><td>go to “Deleting Invalid CT Values” on page 5-10.</td></tr>
<tr><td>separately for the targets as done in:
♦ “Setting Multiple Thresholds” on page 4-7, or
♦ Appendix A, “Troubleshooting Early Amplification.”</td><td>using the figure below as a reference, replace the C_T values for the invalid wells as follows:
a. Open the second results file.
b. Copy and paste the C_T values of the valid wells from the second file to the C_T table, replacing the invalid values from the first results file.
c. “Deleting Invalid CT Values” on page 5-10.</td></tr>
</table></td></tr>
</table>

To transfer data from the results file to the C_T table: *(continued)*

Step	Action
	The following figures illustrate the placement of the well data in the C_T table. As shown, the cells in the C_T table correspond to the 96 wells of the TaqMan Human Endogenous Control Plate.



Workbook1

	A	B	C	D	E	F	G	H	I
1	Column	Ct Calibrator	Ct Calibrator	Ct Sample 1	Ct Sample 1	Ct Sample 2	Ct Sample 2	Ct Sample 3	Ct Sample 3
2	1	Ct Well 1	Ct Well 13	Ct Well 25	Ct Well 37	Ct Well 49	Ct Well 61	Ct Well 73	Ct Well 85
3	2	Ct Well 2	Ct Well 14	Ct Well 26	Ct Well 38	Ct Well 50	Ct Well 62	Ct Well 74	Ct Well 86
4	3	Ct Well 3	Ct Well 15	Ct Well 27	Ct Well 39	Ct Well 51	Ct Well 63	Ct Well 75	Ct Well 87
5	4	Ct Well 4	Ct Well 16	Ct Well 28	Ct Well 40	Ct Well 52	Ct Well 64	Ct Well 76	Ct Well 88
6	5	Ct Well 5	Ct Well 17	Ct Well 29	Ct Well 41	Ct Well 53	Ct Well 65	Ct Well 77	Ct Well 89
7	6	Ct Well 6	Ct Well 18	Ct Well 30	Ct Well 42	Ct Well 54	Ct Well 66	Ct Well 78	Ct Well 90
8	7	Ct Well 7	Ct Well 19	Ct Well 31	Ct Well 43	Ct Well 55	Ct Well 67	Ct Well 79	Ct Well 91
9	8	Ct Well 8	Ct Well 20	Ct Well 32	Ct Well 44	Ct Well 56	Ct Well 68	Ct Well 80	Ct Well 92
10	9	Ct Well 9	Ct Well 21	Ct Well 33	Ct Well 45	Ct Well 57	Ct Well 69	Ct Well 81	Ct Well 93
11	10	Ct Well 10	Ct Well 22	Ct Well 34	Ct Well 46	Ct Well 58	Ct Well 70	Ct Well 82	Ct Well 94
12	11	Ct Well 11	Ct Well 23	Ct Well 35	Ct Well 47	Ct Well 59	Ct Well 71	Ct Well 83	Ct Well 95
13	12	Ct Well 12	Ct Well 24	Ct Well 36	Ct Well 48	Ct Well 60	Ct Well 72	Ct Well 84	Ct Well 96
14									

Deleting Invalid C_T Values

Before averaging the C_T values of duplicate wells, clear the data for all wells containing values outside the given dynamic range of the assays. Invalid C_T values may be the result of experimental error (degraded samples, pipetting inaccuracy). If averaged using the C_T values from the duplicate wells, the invalid data can skew the results and indicate an incorrect level of gene expression.

Guidelines for Deleting Invalid C_T Values

Use the following guidelines to identify invalid data for deletion:

Guideline	Description
Use the 18S rRNA assay as an indicator of sample concentration and quality	Because cells generally express 18S rRNA at extremely high levels, the target is usually a good indicator of sample concentration. Typically, the 18S assay yields C_T values ≤ 22. If sample produces C_T values above 22 for the 18S assay, it may not contain enough cDNA for accurate analysis. Therefore, the sample must be cleared from the spreadsheet as shown below. The calibrator data must be deleted because the C_T values for the 18S assay (cells B2 and C2) are greater than 22 cycles.
Look for individual outlying C_Ts	Occasionally, a single well produces a C_T outside the average for its target group. Abnormalities of this kind are typically due to experimental error rather than differences in gene expression. To obtain accurate C_T values for the sample, the C_T must be cleared from the spreadsheet as shown below. This value is beyond the average C_T for this row and must be deleted.

How to Delete Invalid C_T Values

To delete an invalid C_T value from the spreadsheet:

Step	Action
1	Click the cell containing an invalid C_T to select it.
2	Select **Edit** > **Clear** > **All**.

Averaging Duplicate C_T Values

Before calculating ΔC_T values for the calibrator and samples, average the C_T values from duplicate wells. Because the samples and calibrator are arrayed twice across the endogenous control plate, the exported data for every sample contains two C_T values for each target control. To calculate ΔC_T values, you must average the values for these duplicate wells.

To add Average C_T columns to your C_T table:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Create columns for the average calibrator and sample C_T values by inserting new columns into the spreadsheet as follows:
<table>
<tr><th>Click cell…</th><th>Select…</th></tr>
<tr><td>D1</td><td>Select Insert > Columns.

Excel inserts a new column before column D.</td></tr>
<tr><td>G1</td><td>Select Insert > Columns.

Excel inserts a new column before column G.</td></tr>
<tr><td>J1</td><td>Select Insert > Columns.

Excel inserts a new column before column J.</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>Type the labels for the C_T table as specified in the following table:
<table>
<tr><th>Click on cell…</th><th>Type…</th></tr>
<tr><td>D1</td><td>Avg Ct Calibrator</td></tr>
<tr><td>G1</td><td>Avg Ct Sample 1</td></tr>
<tr><td>J1</td><td>Avg Ct Sample 2</td></tr>
<tr><td>M1</td><td>Avg Ct Sample 3</td></tr>
</table>
</td></tr>
</table>

To add Average C_T columns to your C_T table: *(continued)*

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>3</td><td>Average the C_T values of duplicate calibrator and sample wells by typing the following formulas into the specified cells:

<table>
<tr><th>Click cell…</th><th>Type…</th></tr>
<tr><td>D2</td><td>=AVERAGE(B2:C2)

Excel averages the C_T values of cells B2 and C2 and displays it in cell D2.</td></tr>
<tr><td>G2</td><td>=AVERAGE(E2:F2)

Excel averages the C_T values of cells E2 and F2 and displays it in cell G2.</td></tr>
<tr><td>J2</td><td>=AVERAGE(H2:I2)

Excel averages the C_T values of cells H2 and I2 and displays it in cell J2.</td></tr>
<tr><td>M2</td><td>=AVERAGE(K2:L2)

Excel averages the C_T values of cells K2 and L2 and displays it in cell M2.</td></tr>
</table>
</td></tr>
<tr><td>4</td><td>Copy the formulas entered into the spreadsheet in the previous step and paste them to the remaining cells of each column, as follows:

<table>
<tr><th>Select and copy cell…</th><th>Paste to cells…</th></tr>
<tr><td>D2</td><td>D3–D13</td></tr>
<tr><td>G2</td><td>G3–G13</td></tr>
<tr><td>J2</td><td>J3–J13</td></tr>
<tr><td>M2</td><td>M3–M13</td></tr>
</table>
</td></tr>
<tr><td colspan="2">Excel averages the C_T values for the two cells to the left of each copied cell and displays the averaged C_T.</td></tr>
</table>

Average C_T values of duplicate calibrator wells

Average C_T values of duplicate wells for Sample 1

Average C_T values of duplicate wells for Sample 2

Average C_T values of duplicate wells for Sample 3

About the ΔC_T Equation

Derivation of ΔC_T values from the average C_T values of the calibrator and samples is the final step in comparative gene expression analysis. The following equation describes the ΔC_T calculation.

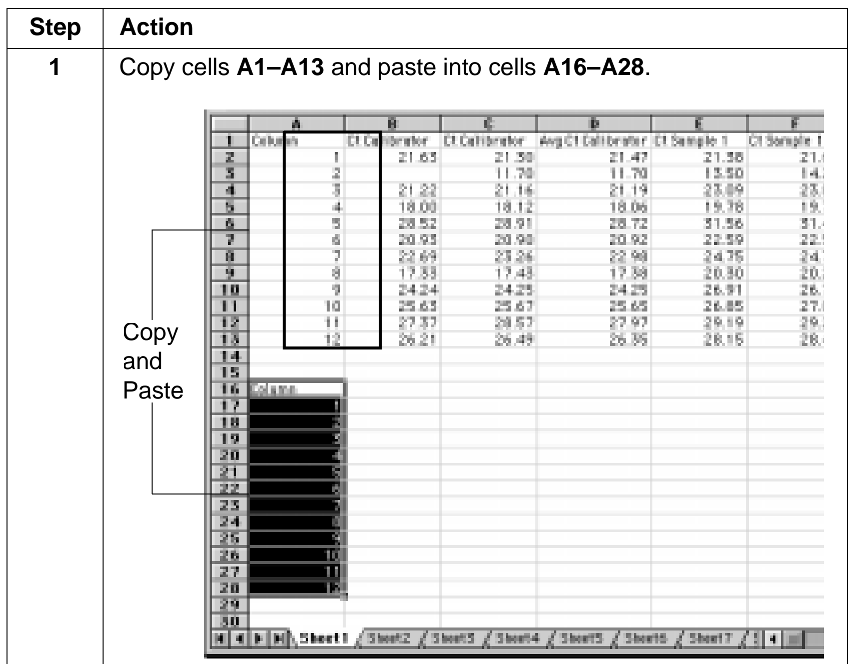
$$\Delta C_{T(Sample)} = \text{Average}C_{T(Calibrator)} - \text{Average}C_{T(Sample)}$$

The equation above uses the average C_T of the calibrator as a baseline for evaluating target gene expression in each sample.

- Samples with initial template concentrations higher than the calibrator have lower average C_T values and yield positive numbers.
- Samples with lower initial template concentrations have higher average C_T values and yield negative numbers.

Constructing a ΔC_T Table

To construct a ΔC_T table:

Step	Action
1	Copy cells **A1–A13** and paste into cells **A16–A28**. 

To construct a ΔC_T table: *(continued)*

Step	Action
2	Type the following labels into the specified cells in the table:

Click on cell…	Type…
B16	Target
B17	IPC
B18	18S
B19	huPO
B20	huBA
B21	huCYC
B22	huGAPDH
B23	huPGK
B24	huB2m
B25	huGUS
B26	huHPRT
B27	huTBP
B28	huTfR

Step	Action
3	Type the following ΔC_T labels into the specified cells in the table:

Click on cell…	Type…
C16	ΔCt Sample 1
D16	ΔCt Sample 2
E16	ΔCt Sample 3
F16	Average ΔCt
G16	ΔCt Calibrator

The ΔC_T table appears as shown below.

	A	B	C	D	E	F	G
16	Column	Target	ΔCt Sample 1	ΔCt Sample 2	ΔCt Sample 3	Average ΔCt	ΔCt Calibrator
17	1	IPC					
18	2	18S					
19	3	hu PO					
20	4	hu BA					
21	5	hu CYC					
22	6	hu GAPDH					
23	7	hu PGK					
24	8	hu B2m					
25	9	hu GUS					
26	10	hu HPRT					
27	11	hu TBP					
28	12	hu TfR					

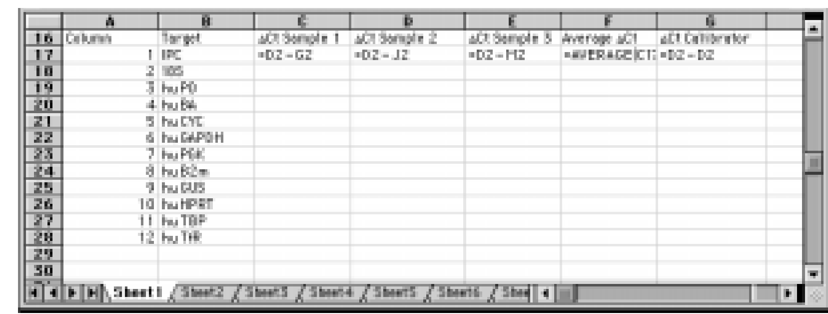
Calculating ΔC_T Values

To calculate ΔC_T values for the calibrator and samples:

Step	Action
1	Type the following formulas into the specified cells:

Click cell…	Type…
C17	=D2–G2 Excel subtracts the averaged C_T value for Sample 1 (cell G2) from the averaged C_T value for the calibrator (cell D2).
D17	=D2–J2 Excel subtracts the averaged C_T value for Sample 2 (cell J2) from the averaged C_T value for the calibrator (cell D2).
E17	=D2–M2 Excel subtracts the averaged C_T value for Sample 3 (cell M2) from the averaged C_T value for the calibrator (cell D2).
F17	=AVERAGE(C17:D17:E17) Excel averages ΔC_T values for the three samples yielding an overall mean for the IPC endogenous control.
G17	=D2–D2 Excel subtracts the averaged calibrator C_T (cell D2) from itself to verify the calibrator.

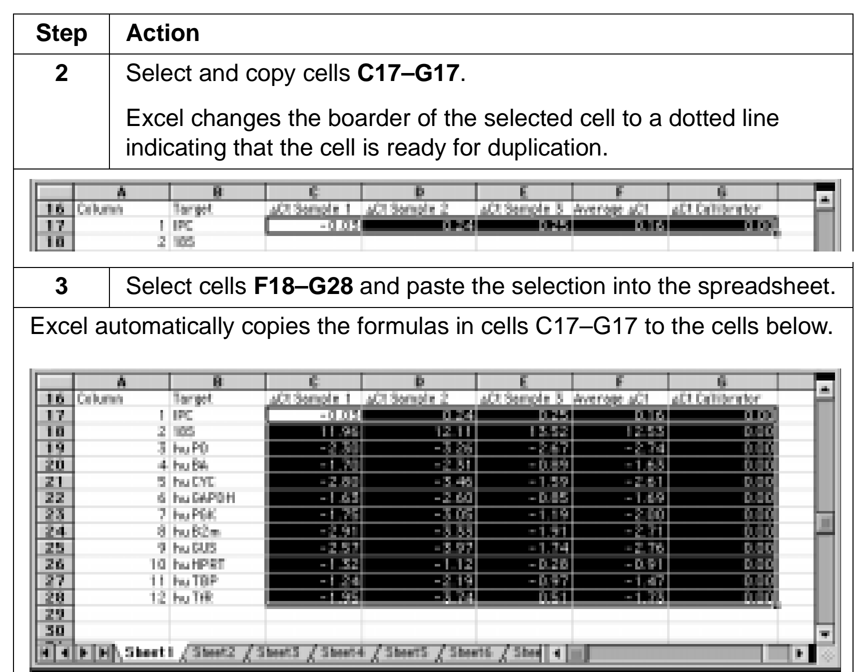
The ΔC_T table appears as shown below.



	A	B	C	D	E	F	G
16	Column	Target	ΔCt Sample 1	ΔCt Sample 2	ΔCt Sample 3	Average ΔCt	ΔCt Calibrator
17	1	IPC	=D2 – G2	=D2 – J2	=D2 – M2	=AVERAGE(C1	=D2 – D2
18	2	18S					
19	3	hu PO					
20	4	hu BA					
21	5	hu CYC					
22	6	hu GAPDH					
23	7	hu PGK					
24	8	hu B2m					
25	9	hu GUS					
26	10	hu HPRT					
27	11	hu TBP					
28	12	hu TfR					
29							
30							

Sheet1 / Sheet2 / Sheet3 / Sheet4 / Sheet5 / Sheet6 / Shee

To calculate ΔC_T values for the calibrator and samples: *(continued)*



Step	Action
2	Select and copy cells **C17–G17**. Excel changes the boarder of the selected cell to a dotted line indicating that the cell is ready for duplication.
3	Select cells **F18–G28** and paste the selection into the spreadsheet.

Excel automatically copies the formulas in cells C17–G17 to the cells below.

Interpreting Results

Overview

To interpret the results from the spreadsheet analysis, create a profile of control gene expression from the data in the ΔC_T table.

Interpreting results consists of the following steps:

Topic	See Page
Generating a Gene Expression Profile	5-17
Interpreting the Gene Expression Profile	5-18
The Relationship Between ΔC_T and Gene Expression	5-18
Choosing an Endogenous Control	5-19
Demonstrating Performance with TaqMan Human Control Total RNA	5-20

Generating a Gene Expression Profile

The following procedure describes how to generate a profile using the Excel Chart Wizard.

To graph your results using the Excel Chart Wizard:

Step	Action
1	Select cells **A16–E28**.

	A	B	C	D	E	F	G
16	Column	Target	ΔCt Sample 1	ΔCt Sample 2	ΔCt Sample 3	Average ΔCt	ΔCt Calibrator
17	1	IPC	-0.05	0.24	0.25	8.18	0
18	2	18S	11.96	12.11	13.52	7.51	0
19	3	hu PO	-2.38	-3.26	-2.67	7.53	0
20	4	hu BA	-1.70	-2.31	-0.85	5.68	0
21	5	hu CYC	-2.80	-3.46	-1.55	2.64	0
22	6	hu GAPDH	-1.63	-2.60	-0.85	2.79	0
23	7	hu PGK	-1.75	-3.05	-1.19	1.05	0
24	8	hu B2m	-2.91	-3.53	-1.91	1.48	0
25	9	hu GUS	-2.57	-3.97	-1.74	-2.18	0
26	10	hu HPRT	-1.32	-1.12	-0.26	-0.23	0
27	11	hu TBP	-1.24	-2.19	-0.97	-0.70	0
28	12	hu TfR	-1.95	-3.74	0.51	-0.16	0

Step	Action
2	Select **Insert** > **Chart** > **On This Sheet**. The Excel chart wizard requests data for the new graph.
3	Click the selected data. The chart wizard prompts you for information.
4	Follow the instructions as directed by the wizard.

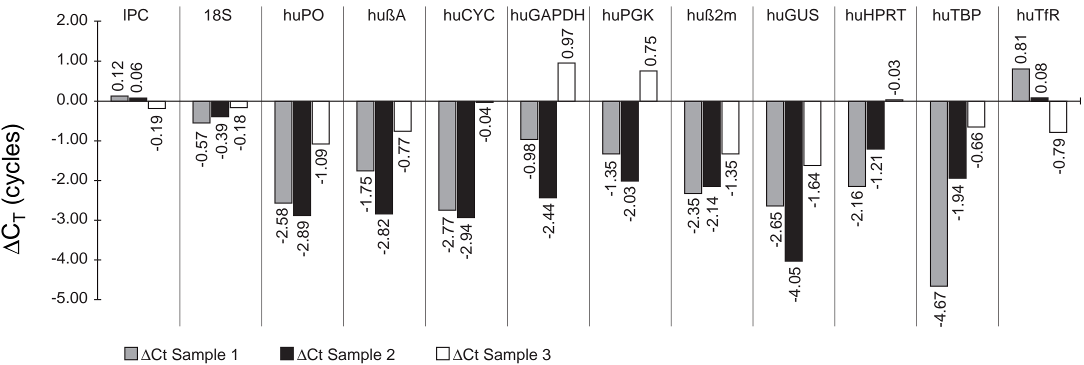
Interpreting the Gene Expression Profile

The results of the Endogenous Control Plate are expressed in ΔC_T, greater than or less than the calibrator ΔC_T. Thus, the calibrator serves as a baseline for the assays and is shown as zero on the graph.

- Samples with positive ΔC_T values have initial template concentrations higher than that of the calibrator sample.
- Samples with negative ΔC_T values have initial template concentrations lower than that of the calibrator sample.

Note See “About the ΔC_T Equation” on page 5-13 for more information.

The plot below illustrates a typical gene expression profile.



The Relationship Between ΔC_T and Gene Expression

One ΔC_T is equal to a twofold difference in initial template concentration. This relationship is shown with the following equation:

$$X_n = X_0(1 + E_X)^n$$

Where:

X_n	=	Copy number at cycle n	E_X	=	Amplicon efficiency
X_0	=	Copy number at cycle 0	n	=	Cycle number

Because amplicons designed and optimized according to Applied Biosystems guidelines have equivalent efficiencies approaching 100%, it can be stated that $E_X = 1$. Also, because we are interested in the difference in initial template for one cycle, it can be stated that n = 1.

Substituting values for the appropriate variables, the equation becomes:

$$X_1 = X_0(1+1)^1 = 2X_0$$

Choosing an Endogenous Control

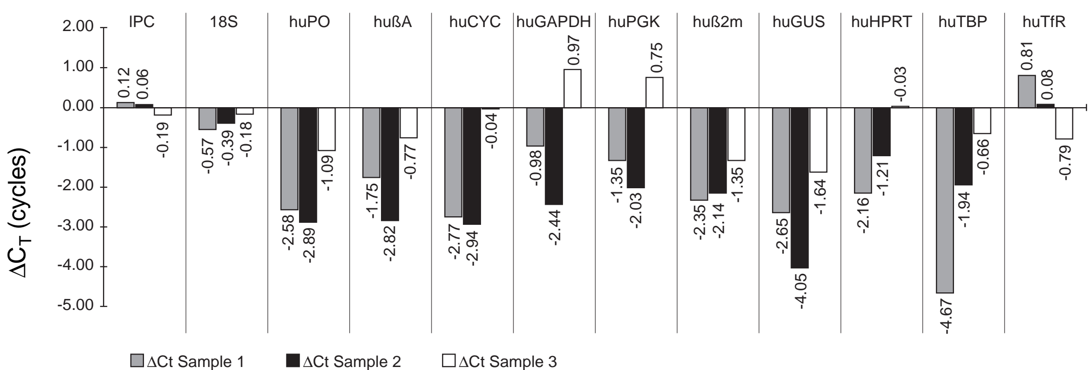
Choose the control with the least variation in ΔC_T levels. Ideally, the best control is expressed at a constant level in all samples regardless of cell cycle, cell type, or tissue. Because the ΔC_T indicates the level of gene expression relative to the calibrator, the ΔC_T values of a good control do not vary much from zero. It is important to remember that a difference of one cycle equates to a twofold difference in initial template. For example, a control with ΔC_T values that vary over a two cycle range would have nearly a fourfold difference in expression levels. Stable expression provides a reliable basis for comparison with other genes.

Good Endogenous Control Candidates

From the ΔC_T profile shown below, the 18S ribosomal RNA (18S) and transferrin receptor (huTfR) genes are good candidate controls because their expression remains relatively consistent across the test samples. Both assays produced ΔC_T values that deviate little from zero, indicating a fairly stable level of gene expression relative to the other candidate controls.

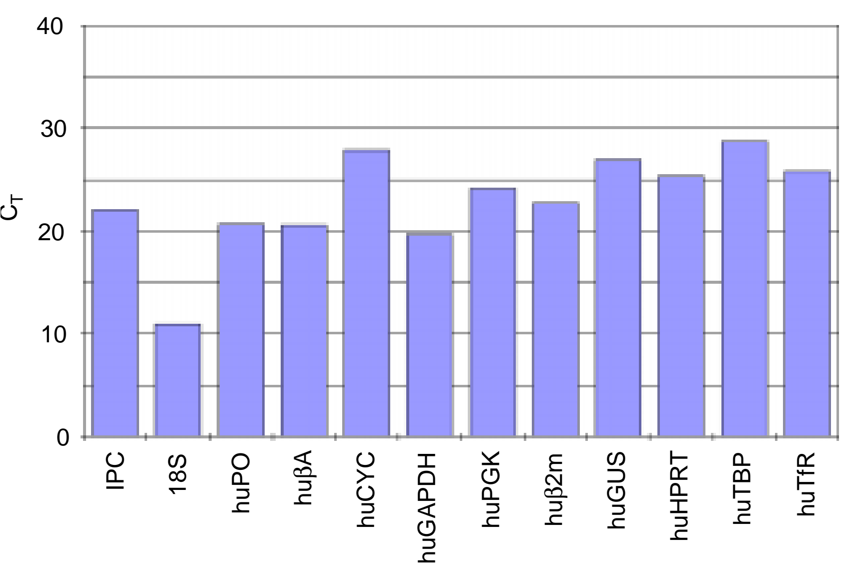
Poor Endogenous Control Candidates

In contrast to the 18S and huTfR controls, the TATA-binding protein (huTBP) and β-Glucronidase (huGUS) genes are the least desirable choices from the profile in the figure below. The expression of both controls vary widely, exhibiting ΔC_T values that fluctuate in excess of 4 cycles (this represents a 16-fold difference in gene expression).



Demonstrating Performance with TaqMan Human Control Total RNA

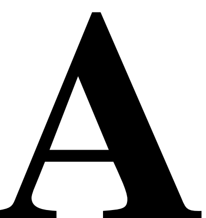
TaqMan Human Control Total RNA is available to demonstrate the performance of the TaqMan Human Endogenous Control Plate. The figure below shows a typical gene expression profile for the sample.



To generate the profile shown above:

Step	Action
1	Perform the reverse transcription step as described in “Reverse Transcription for All Amplicons Except 18S” on page 2-4 using the TaqMan Human Control Total RNA (10 ng per well).
2	Perform the PCR step as described in Chapter 3, “PCR,” configuring the plate with duplicate wells for the control sample.
3	Analyze and export the data. See Chapter 4, “Data Analysis.”
4	Construct a ΔC_T table and import data to it by following the procedures on pages 5-4 to 5-7.
5	Select the column of cells containing the C_T data for the Human Control Total RNA.
6	Select **Insert** > **Chart** > **On This Sheet**.
7	Click the selected data.
8	Follow the instructions as directed by the wizard.

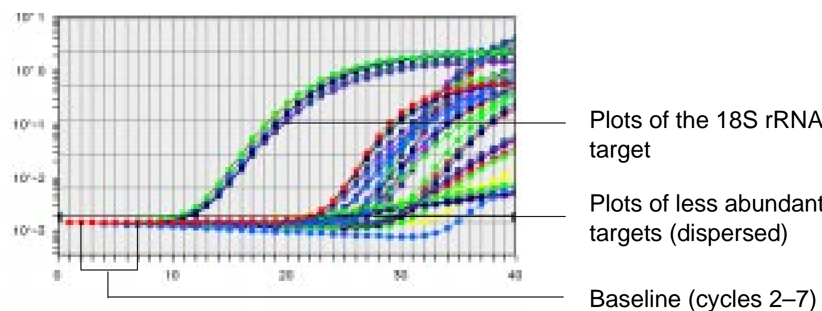
Troubleshooting Early Amplification



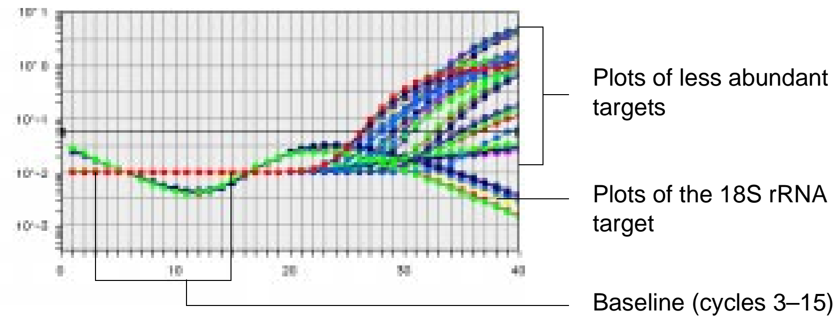
Effects of Early Amplification of the 18S Assay

In rare cases, the amplification of the 18S assay can interfere with the detection of less abundant targets. When amplification of the 18S target reaches a detectable level at a very early cycle, it limits the number of cycles over which the software can calculate the baseline. As the available baseline is compressed, the amplification plots of the less abundant targets may appear to disperse. This can lead to poor reproducibility and inaccurate quantification.

For example, in the figure below the baseline is set correctly for the 18S amplification (baseline is set for cycles 2–7), however the plots of the less abundant targets have become dispersed. As a result, C_T values from this plot are valid only for the 18S amplifications.



The baseline in the figure below is reset for the less abundant targets (baseline is set for cycles 3–15). Notice that the amplification plots of these targets are now well pronounced and allow the SDS software to determine accurate C_T values. In contrast, the plots of 18S targets now exhibit a sigmoidal curve and do not yield valid data points.



How to Correct for Early Amplification

When early amplification of the 18S rRNA target interferes with the detection of less abundant genes, set the baseline and threshold values independently for each group of plots. The following procedure explains how to configure each group of plots independently and export the data. The results from the results files are combined during spreadsheet analysis.

To set the baseline and threshold separately:

Step	Action
1	From the amplification plot, deselect the 18S wells that amplify during the very early cycles of the PCR.
2	Following the guidelines in “Guidelines for Setting the Baseline” on page 4-2, set the baseline for those plots that amplify during the later cycles of the PCR.
3	Following the guidelines in “Guidelines for Setting the Threshold” on page 4-7, set the threshold for the plots that amplify during the later cycles of the PCR.
4	Export the data as explained in “Exporting and Viewing the Results File” on page 5-2. The software saves the data. The results in the file are valid only for the wells that amplify during the later cycles.
5	From the amplification plot, deselect the wells that amplify during the later cycles of the PCR.
6	Following the guidelines in “Guidelines for Setting the Baseline” on page 4-2, reset the baseline for the 18S wells that amplify during the very early cycles of the PCR.
7	Following the guidelines in “Guidelines for Setting the Threshold” on page 4-7, set threshold for the 18S wells that amplify during the very early cycles of the PCR.

To set the baseline and threshold separately: *(continued)*

Step	Action
8	Export the data as explained in “Exporting and Viewing the Results File” on page 5-2. The software saves the data. The results in the file are valid only for the wells that amplify during the very early cycles. You now have two results files: ♦ A file containing valid data for plots appearing in the later cycles ♦ A file containing valid data for plots appearing in the early cycles
9	Follow the procedure for spreadsheet analysis as described in “Calculating the Relative Quantification Using a Spreadsheet” on page 5-5.

About These Assays B

Overview

The TaqMan Human Endogenous Control Plate evaluates the expression of eleven common “housekeeping” genes and an internal positive control in total RNA samples. Applied Biosystems designed TaqMan assay primers and probes to be cDNA specific to avoid problems associated with pseudogenes, related genes, and contaminant genomic DNA.

Quality Control

Applied Biosystems tests the preloaded primers and probes on the TaqMan Human Endogenous Control Plate as part of a manufacturing quality control process. In this process, the performance of each endogenous control target was gauged using cDNA prepared from human total RNA samples. Each assay demonstrated that it did not detect up to 10,000 copies of contaminating genomic DNA.

Description of Endogenous Controls

The following table lists the potential controls and their cellular functions:

Endogenous Control	Role
IPC	Applied Biosystems designed the TaqMan Exogenous Internal Positive Control (IPC) to help interpret negative results caused by PCR inhibitors. In the absence of inhibitors, IPC co-amplifies with target DNA and gives a specific signal. The IPC sequence is artificial so that PCR primers do not amplify anything in the test samples.
18S rRNA	18S ribosomal RNA makes up 80% of total RNA and its level is a good indicator for the relative amount of total RNA. It is transcribed by a different polymerase from mRNAs and its level is less likely to fluctuate with the test sample. The 18S rRNA endogenous reference is the most abundant target on the TaqMan Human Endogenous Control Plate.

Endogenous Control	Role
Acidic ribosomal protein (huPO)	Acidic ribosomal protein is moderately abundant (Rich *et al.*, 1987) and found in most tissue types. Because huPO gene expression level seems to remain relatively constant (Okubo *et al.*, 1997), some researchers select it as their standard when studying samples that are affected by estrogen treatment.
Beta-actin (huβA)	The beta-actin gene is ubiquitously expressed in all eukaryotic cells and one of the most frequently used as an internal standard. It is a moderately abundant gene, constituting 0.1% of mRNA and 0.003% of total RNA. Its level fluctuates in some cells and tissues (Greenberg *et al.*, 1985; Dodge *et al.*, 1990). Actins are highly conserved proteins involved in various types of cell motility.
Cyclophilin (huCYC)	Cyclophilin is a major cellular component, comprising 0.1–0.4% of total cellular protein. It is found in all cells of wide phylogenetic distribution (Koletsky *et al.*, 1986). It was originally isolated as the main cyclosporin A binding protein.
Glyceraldehyde-3-phosphate dehydrogenase (huGAPDH)	GAPDH is a key enzyme involved in glycolysis and is moderately abundant (Allen *et al.*, 1987). Its expression changes with insulin treatment and shows fluctuation through cell cycles and among different cell lines and tissue types.
Phosphoglycero-kinase (huPGK)	PGK is a key enzyme involved in glycolysis following GAPDH. Because typical concentrations of glycolytic intermediates are 1 µM for 1,3-bisphosphoglycerate and 118 µM for 3-phosphoglycerate, the regulation may be different.
β_2-Microglobulin (huβ2m)	β_2-microglobulin is involved with immune response. It is moderately abundant and expressed in most tissue types (Güssow *et al.*, 1987). The level of β_2-microglobulin expression may vary in different tissues (Okubo *et al.*, 1997).
β-Glucronidase (huGUS)	β-glucronidase is a relatively abundant glycoprotein that is expressed constitutively in many tissues. It acts as an exoglycosidase in lysomes (Oshima *et al.*, 1988).
Hypoxanthine ribosyl transferase (huHPRT)	Hypoxanthine ribosyl transferase is located on the X chromosome and is constitutively expressed at low levels (Patel *et al.*, 1986). It plays an important role in the metabolic salvage of purines in mammalian cells.
Transcription Factor IID, TATA Binding Protein (huTBP)	The TATA binding protein is constitutively expressed in many tissues and cells at low levels. It is required for transcription directed by RNA polymerases I, II, and III (Chalut *et al.*, 1995).
Transferrin Receptor (huTfR)	Transferrin receptor mediates cellular iron uptake and is expressed at low levels in both tissues and cells. The expression of the receptor on the cell surface correlates with cellular proliferation, being highest on rapidly dividing cells and much lower on resting cells and more terminally differentiated cell types (McClelland *et al.*,1984). As shown in the figure in “Demonstrating Performance with TaqMan Human Control Total RNA” on page 5-20, transferrin receptor exhibits the lowest level of gene expression when evaluating TaqMan Human Control Total RNA using the TaqMan Human Endogenous Control Plate.

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Glossary

calibrator A sample used as a basis for comparison with the other samples on the TaqMan Human Endogenous Control Plate.

endogenous control RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantification of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.

exogenous control Characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an *in vitro* construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase.

reference A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification. The active reference has its own set of primers and probe.

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

R_n^- The R_n value of an unreacted sample. This value can be obtained from the early cycles of a Real Time run (the cycles prior to a detectable increase in fluorescence) or from a reaction not containing template.

ΔR_n 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.

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

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