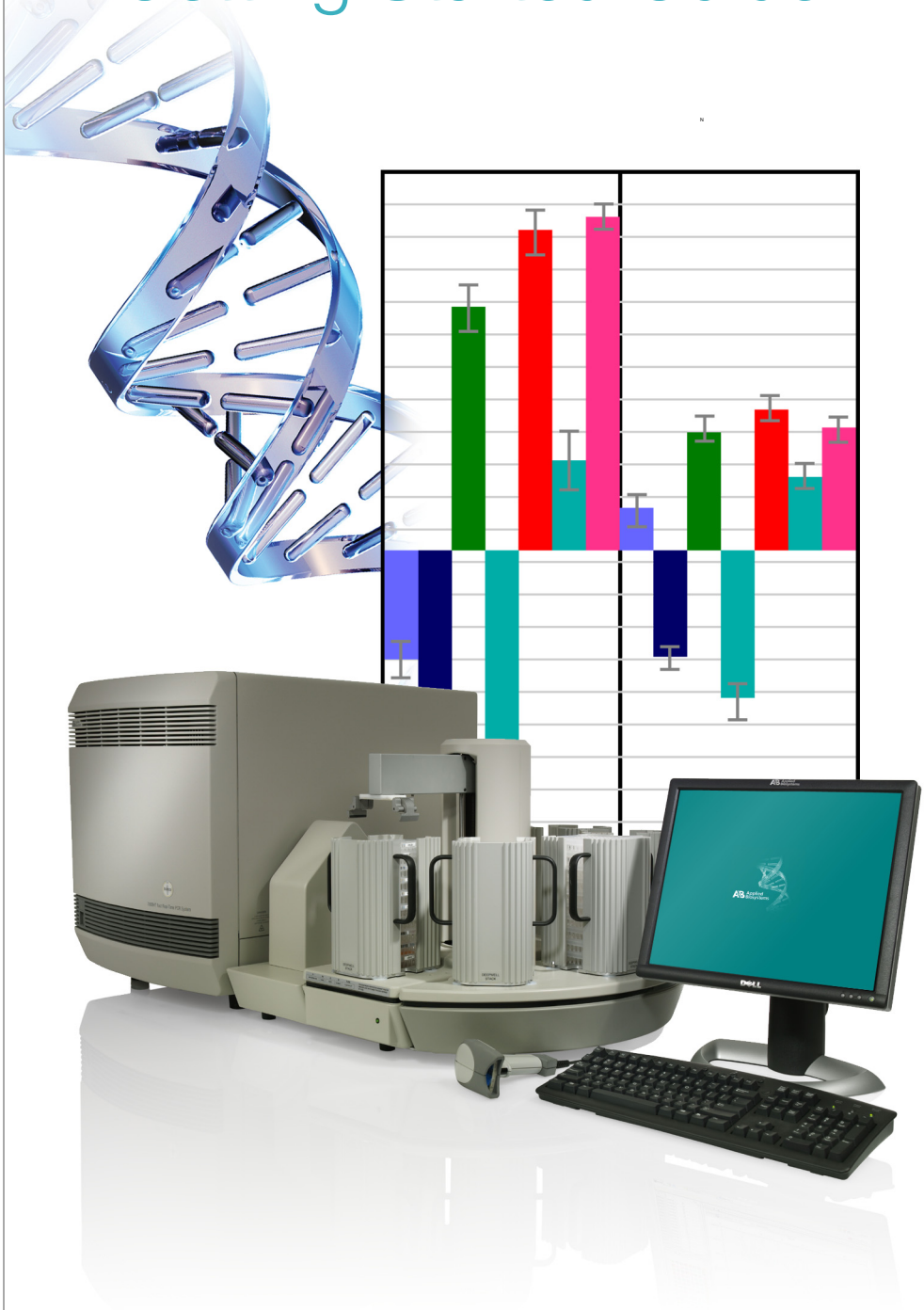
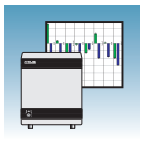
Applied Biosystems

Relative Quantitation Using Comparative C_T Getting Started Guide

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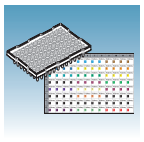
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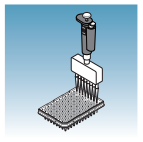
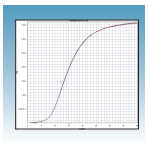
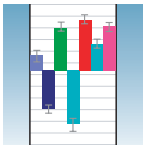

Preparing Samples and Reaction Plates


Creating RQ Plate Documents and Generating Data


Analyzing and Viewing RQ Study Data

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Authorized Thermal Cycler

The Applied Biosystems 7900HT Fast Real-Time PCR System Base Unit, Serial No_____, in combination with its immediately attached sample block modules, comprise an instrument that is an Authorized Thermal Cycler. The purchase price of this Base Unit includes the up-front fee component of a license under the non-U.S. counterparts of U.S. Patents Nos. 4,683,202, 4,683,195 and 4,965,188 owned by F. Hoffmann-La Roche Ltd. (Roche) covering the Polymerase Chain Reaction (PCR) process to practice the PCR process for internal research and development using this instrument. The running royalty component of that license may be purchased from Applied Biosystems or obtained by purchasing Authorized Reagents. This instrument is also an Authorized Thermal Cycler for use with applications licenses available from Applied Biosystems. Its use with Authorized Reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Purchase of this product does not itself convey to the purchaser a complete license or right to perform the PCR process. Further information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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The Applied Biosystems 7900HT Fast Real-Time PCR System is covered by U.S. Patents Nos. 6,563,581 and 6,719,949. This instrument is covered by one or more U.S. Patents Nos. 5,083,852; 5,333,675; 5,475,610; 6,703,236; 6,814,934 and patents pending.

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Contents

Preface v

How to Use This Guide v
How to Obtain More Information vi
How to Obtain Support vii

Chapter 1 **Introduction** 1

About the 7900HT Fast System 2
About Relative Quantitation Assays 3
About RQ Experiments 4
Before You Begin 8

Chapter 2 **Designing an RQ Experiment** 9

Overview 10
Specifying the Components of an RQ Experiment 10
Selecting the PCR Method 12
Selecting the Chemistry 13
Selecting One- or Two-Step RT-PCR 14
Selecting the Probes and Primers 16

Chapter 3 **Preparing the Samples and Reaction Plate** 17

Preparing RNA 18
Converting Total RNA to cDNA 19
Preparing the PCR Master Mix 21
Preparing the Reaction Plate 23

Chapter 4 **Creating RQ Plates and Generating Data** 27

About RQ Plate Documents 28
Performing an RQ Run 35
Analyzing and Viewing the RQ Data 40
Reanalyzing Data 44

Post-Analysis Options 45

Chapter 5 **Analyzing and Viewing RQ Study Data in RQ Manager 1.2** **49**

Creating an RQ Study Document 50
Analyzing RQ Study Data 54
Analyzing and Viewing the Results of the RQ Study 60
Reanalyzing an RQ Study 64
Post-Analysis Options 65

Appendix A **Sample Experiment** **67**

Sample RQ Experiment 67

Appendix B **SDS Automation Controller Software** **73**

Overview 73
Using the Automation Controller Software 73

Appendix C **Flags and Filtering for Relative Quantitation Using Comparative C_T Plate Documents** **81**

Overview 81
Viewing Flags 82

References **85**

Index **87**

Preface

How to Use This Guide

Purpose of This Guide

This guide provides procedures for conducting Relative Quantitation using Comparative C_T for gene expression assays using the Applied Biosystems 7900HT Fast Real-Time PCR System (7900HT Fast System).

Audience

This guide is written for principal investigators and laboratory staff who conduct relative quantitation for gene expression assays using the 7900HT Fast System.

Assumptions

This guide assumes that you have:

- Familiarity with Microsoft® Windows® XP operating system.
- Knowledge of general techniques for handling DNA and RNA samples and preparing them for PCR.
- A general understanding of the theory of relative quantitation using Comparative C_T.
- A general understanding of hard drives and data storage, file transfers, and copying and pasting.

If you want to integrate the 7900HT Fast System into your existing laboratory data flow system, you need networking experience.

Text Conventions

This guide uses the following conventions:

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open**.

User Attention Words

The following user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

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

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

Safety

Follow specific safety practices when using this instrument. For safety guidelines, refer to the "Safety and EMC Compliance" section in the *Applied Biosystems 7900HT Fast Real-Time PCR System Site Preparation Guide* (PN 4351923).

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is available free 24 hours a day. To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**.
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** - To view the document
   - **Print Target** - To print the document
   - **Save Target As** - To download a PDF version of the document to a destination that you choose
4. To have a copy of the document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

How to Obtain More Information

Related Documentation

For more information about using the 7900HT Fast System and performing assays, refer to:

- *Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4364016)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide* (PN 4364014)
- *Applied Biosystems 7900HT Real-Time PCR System Allelic Discrimination Getting Started Guide* (PN 4364015)
- *Applied Biosystems 7900HT Real-Time PCR System Plus/Minus Getting Started Guide* (PN 4364017)
- *Sequence Detection Systems Software version 2.3 Online Help (SDS Online Help)*

- *Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide* (PN 4365542)
- *Applied Biosystems 7900HT Fast Real-Time PCR System Site Preparation Guide* (PN 4351923)
- *ABI PRISM® 6100 Nucleic Acid PrepStation Users Guide* (PN 4326242)
- *DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol* (using BloodPrep™ Chemistry, PN 4343586)
- *High Capacity cDNA Archive Kit Protocol (PN 4312169)*
- *Isolation of Total RNA from Whole Blood and from Cells Isolated from Whole Blood Protocol (*4332809)
- *NucPrep® Chemistry Protocol: Isolation of Genomic DNA from Animal and Plant Tissue* (PN 4333959)
- *PrepMan™ Ultra Sample Preparation Reagent Protocol* (PN 4318925)
- *Primer Express® Software v3.0 Getting Started Guide* (PN 4362460)
- *Real-Time PCR Systems Chemistry Guide* (PN 4363978)
- *TaqMan® Gene Expression Assays Protocol* (PN 4364226)
- *TaqMan® Low Density Array Getting Started Guide* (PN4319399)
- *TaqMan® Universal PCR Master Mix Protocol* (PN 4351891)
- *Tempus™ Blood RNA Tube and Large Volume Consumables Protocol (*4345218)
- *Tissue RNA Isolation: Isolation of Total RNA from Plant and Animal Tissue Protocol (*4330252)
- *TransPrep Chemistry Protocol: Purification of gDNA from Filtrates Obtained After the Isolation of RNA from Homogenized Animal or Plant Tissue Samples* (PN 4326965)

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techpubs@appliedbiosystems.com

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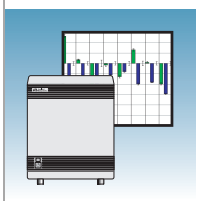
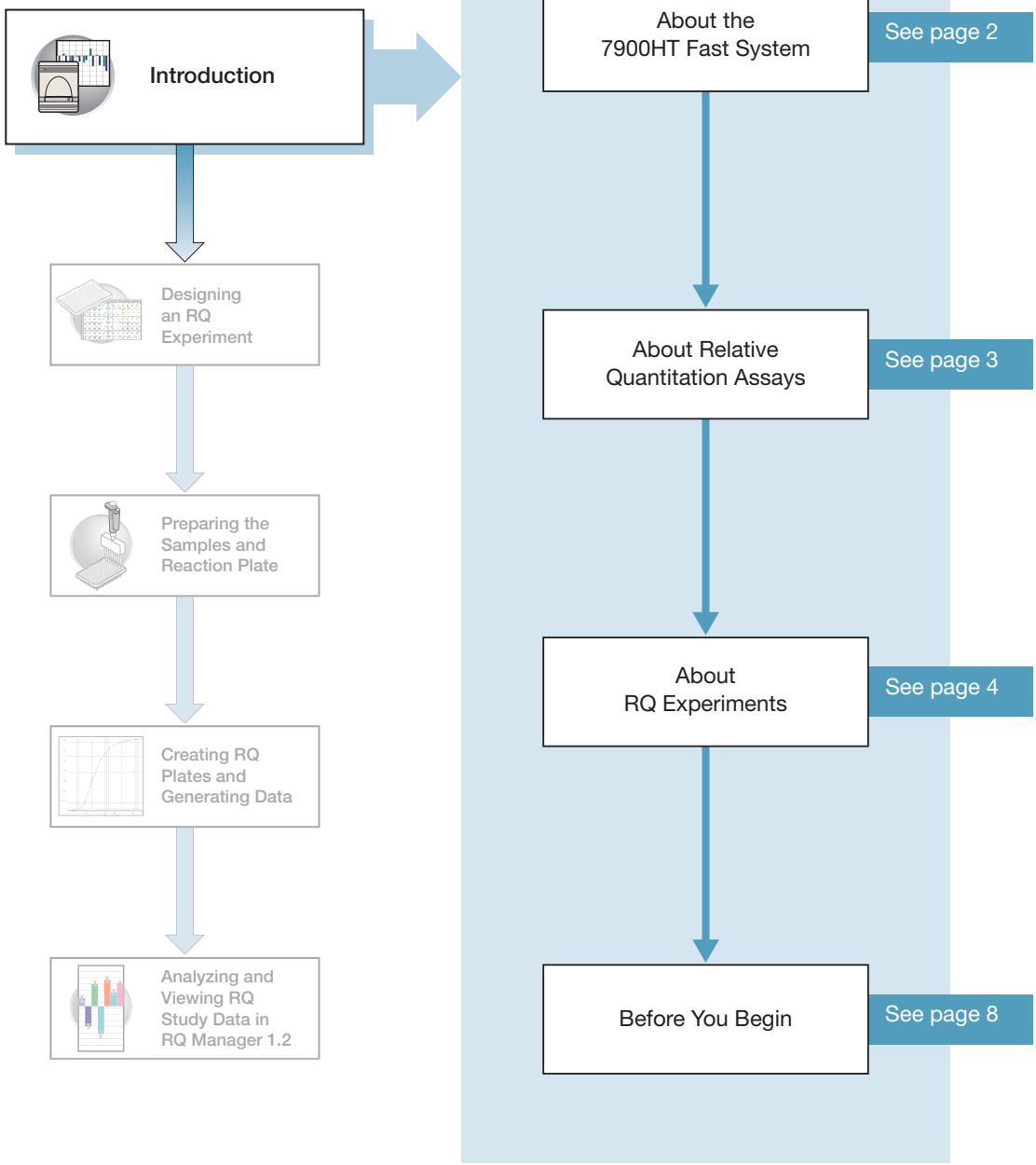
- Obtain worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents

- Obtain information about customer training
- Download software updates and patches



Introduction



Notes

About the 7900HT Fast System

System Description

The Applied Biosystems 7900HT Fast Real-Time PCR system (7900HT Fast System) uses fluorescence-based PCR chemistries to provide:

- Quantitative detection of nucleic acid sequences using real-time analysis
- Qualitative detection of nucleic acid sequences using end-point and dissociation-curve analysis

For more information on the 7900HT Fast System, refer to *7900HT Fast Real-Time PCR System Online Help*.

Note: To access the online help, select *Help* > *SDS Online Help* from the SDS software, RQ Manager software, or Automation Controller software menu bars.

Supported Assays and Consumables

You can perform several assay types on the 7900HT Fast System using reaction plates in the 96-well, 384-well, and TaqMan® Low Density Array formats. This guide describes the relative quantitation (RQ) using comparative C_T assay type.

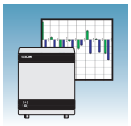
	Reaction Plate and System Block Options			
Assay Type	Fast 96-well	Standard 96-well	Standard 384-well	TaqMan® Low Density Array
Standard curve (AQ)	Yes	Yes	Yes	No
Comparative C_T (RQ)	Yes	Yes	Yes	Yes
Allelic discrimination	No	Yes	Yes	No
Plus/minus	No	Yes	Yes	No

Relative Quantitation (RQ) Using Comparative C_T Assay Configuration

RQ assays are supported on the 7900HT Fast System using reaction plates in the standard 96-well, Fast 96-well, standard 384-well, and TaqMan® Low Density Array formats.

IMPORTANT! A Fast block, Fast reaction plates, and Fast PCR Master Mix are all required for running Fast PCR reactions.

Notes



About Relative Quantitation Assays

Relative Quantitation (RQ) using Comparative C_T determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen, 2001). For example, RQ is commonly used to compare expression levels of wild-type alleles with mutated alleles or the expression levels of a gene in different tissues.

RQ provides accurate comparison between the initial level of template in each sample, without requiring the exact copy number of the template. Also, the relative levels of templates in samples can be determined without the use of standard curves.

Real-Time PCR Assays

RQ assays are performed using Real-Time PCR where you monitor the progress of the PCR as it occurs (Kwok and Higuchi, 1989). Data are collected throughout the PCR process rather than at the end of the PCR process (end-point PCR).

In Real-Time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end of PCR (Saiki *et al*., 1985).

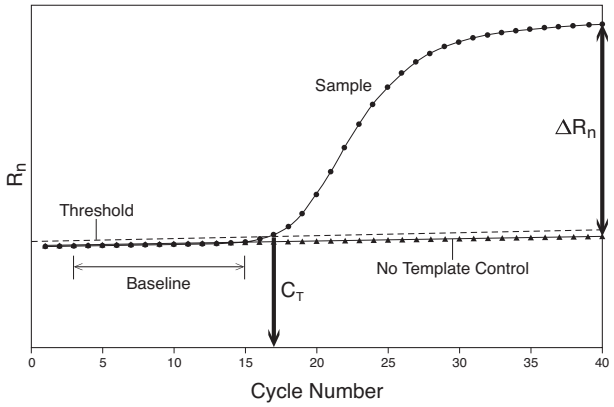
There are two types of quantitative real-time PCR: absolute and relative.

Terms Used in Quantitation Analysis

Term	Definition
Baseline	The initial cycles of PCR in which there is little change in fluorescence signal.
Threshold	A level of ΔR_n—automatically determined (or manually set) by the SDS software—used for C_T determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the Amplification plot defines the C_T.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a fluorescent signal that indicates specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by the specified set of PCR conditions ($\Delta R_n = R_n$ –baseline).

Notes

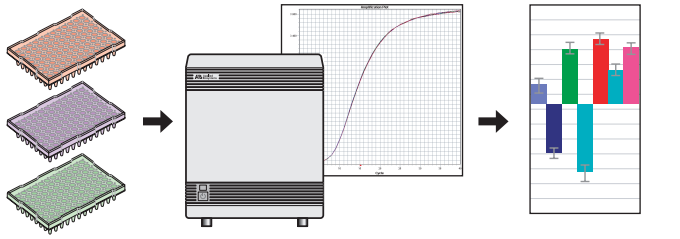
The figure below shows a representative amplification plot that includes some of the terms defined above.



About RQ Experiments

RQ Experiment Workflow

In this document, the term "RQ experiment" refers to the entire process of relative quantitation using comparative C_T experiment, beginning with generating cDNA from RNA (reverse transcription) through analyzing RQ data. The general workflow of an RQ experiment is shown below.



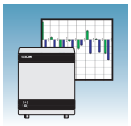
Sample RQ Experiment

A sample experiment, representing a typical relative quantitation experiment, is provided in Appendix A, "Sample Experiment," on page 67. Excerpts of the sample experiment are provided at various points in this guide (as they apply to the RQ process). These excerpts appear in boxes with a blue heading. You can use the summarized procedures of the sample relative quantitation experiment in Appendix A, "Sample Experiment," on page 67 to familiarize yourself with the entire relative quantitation workflow.

RQ Studies with the 7900HT Fast System

The data-collection part of an RQ assay is a in single plate document called the RQ plate. Amplification data from PCR runs are stored with sample setup information on the RQ plate.

Notes



The data analysis part of a single or multiple RQ assay is called an RQ Study. You can analyze up to ten RQ plates in a study. RQ study documents do not control the instrument, nor do they provide tools for setting up or modifying plates. When adding plate results to a study, ensure that you do not combine results from different plate formats, different thermal cycling conditions, or different reaction volumes in the same RQ study.

Note: The 7900HT Fast System software uses the *comparative C_T method ($\Delta\Delta C_T$)* to calculate relative quantities of a nucleic acid sequence. If you want to perform comparative C_T using the *relative standard curve method*, you should use an AQ assay type and refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4363978).

Required User-Supplied Materials

Chemistry/Reagents

Item	Source
Any of the following DNA isolation and purification chemistry systems:	
• ABI PRISM® 6100 Nucleic Acid PrepStation	Applied Biosystems (PN 6100-01)
• BloodPrep™ Chemistry (genomic DNA from fresh or frozen blood or cells)	Applied Biosystems (PN 4346860)
• NucPrep™ Chemistry (DNA from animal and plant tissue)	Applied Biosystems (PN 4330274)
• PrepMan® Ultra Sample Preparation Reagent Kit	Applied Biosystems (PN 4322547
• High Capacity cDNA Archive Kit	Applied Biosystems (PN 4322171)
• ABI PRISM® TransPrep System (purification of gDNA after the isolation of RNA from animal and plant tissue)	Applied Biosystems web site
TaqMan® reagents appropriate for your probes and primers:	
• TaqMan® Fast Universal PCR Master Mix (2X), No AmpErase UNG	Applied Biosystems (PN 4352042)
• TaqMan® Universal PCR Master Mix (2X)	Applied Biosystems (PN 4304437)
• TaqMan® Universal PCR Master Mix (2X), No AmpErase UNG	Applied Biosystems (PN 4324018)
• SYBR® Green Master Mix, 200 reactions (not for use with TaqMan® Low Density Arrays)	Applied Biosystems (PN 4309155)
• SYBR® Green RT-PCR Reagents	Applied Biosystems (PN 4310179)
Labeled primers and probes from one of the following sources:	
• TaqMan® Gene Expression Assays (predesigned primers and probes)	Applied Biosystems Web site (www.appliedbiosystems.com)
• Custom TaqMan® Gene Expression Assays service (predesigned primers and probes)	Applied Biosystems Web site (www.appliedbiosystems.com)
• Primer Express ® Software (custom-designed primers and probes)	Contact your Applied Biosystems Representative

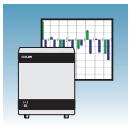
Notes

Reaction Plates and Covers

IMPORTANT! Do not use MicroAmp® caps (domed) or Optical Tubes with the 7900HT Fast System. You can use Optical Caps (PN 4323032) *only* on the standard 96-well plates with the 7900HT Fast System.

Item	Source
Standard 96-well reaction plates	
• MicroAmp® 96-Well Optical Reaction Plate with Barcode (code 128), 20 plates	Applied Biosystems (PN 4306737)
• MicroAmp® 96-Well Optical Reaction Plate with Barcode (code 128), 25-Pack, 500 plates Includes 25 of PN 4306737, MicroAmp® 96-Well Optical Reaction Plates with Barcode	Applied Biosystems (PN 4326659)
• MicroAmp® 96-Well Optical Reaction Plate with Barcode (code 128) and ABI PRISM® Optical Adhesive Cover, 100 plates/100 covers Includes 100 ABI PRISM® Optical Adhesive Covers (PN 4311971) and 5 of PN 4306737, MicroAmp® 96-Well Optical Reaction Plates with Barcode	Applied Biosystems (PN 4314320)
• MicroAmp® Splash Free Support Base for 96-Well Reaction Plates, 10 bases	Applied Biosystems (PN 4312063)
Optical 96-well fast plates	
• Optical 96-Well Fast Thermal Cycling Plate with Barcode (code 128)	Applied Biosystems (PN 4346906)
• MicroAmp® Splash Free Support Base for 96-Well Reaction Plates, 10 bases	Applied Biosystems (PN 4312063
Standard 384-well reaction plates	
• 384-Well Clear Optical Reaction Plate with Barcode (code 128), 50 plates	Applied Biosystems (PN 4309849)
• 384-Well Clear Optical Reaction Plate with Barcode (code 128), 10-Pack, 500 plates Includes 10 of PN 4309849, 384-Well Clear Optical Reaction Plates with Barcode	Applied Biosystems (PN 4326270)
TaqMan® Low Density Arrays	
• TaqMan® Low Density Arrays	Applied Biosystems Web site

Notes



Optical adhesive covers	
• ABI PRISM® Optical Adhesive Cover Starter Kit, 20 covers Includes 20 ABI PRISM® Optical Adhesive Covers, an Applicator, and an ABI PRISM® Optical Cover Compression Pad	Applied Biosystems (PN 4313663)
• ABI PRISM® Optical Adhesive Covers, 100 covers	Applied Biosystems (PN 4311971)
• ABI PRISM® Optical Adhesive Covers, 25 covers	Applied Biosystems (PN 4360954)
• Optical Caps, 8 Caps/Strip, 2400 Caps/300 Strips	Applied Biosystems (PN 4323032)

Other Consumables and Equipment

Item	Source
Centrifuge with adapter for 96-well plates	Major laboratory supplier (MLS)
Centrifuge with adapter for 384-well plates	Major laboratory supplier (MLS)
Centrifuge with adapter for TaqMan® Low Density Arrays	Major laboratory supplier (MLS)
Gloves	MLS
Microcentrifuge	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS
Nuclease-free water	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement	MLS
Safety goggles	MLS
Vortexer	MLS

Notes

Before You Begin

Background and Pure Dye Calibrations

Check that background and pure-dye calibrations have been performed regularly to ensure optimal performance of the 7900HT Fast System. For more information about background and pure dye calibrations, refer to the *SDS Online Help* and the *Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide* (PN 4365542).

Accessing the Online Help

Some steps in this chapter refer you to the *SDS Online Help* for more information. To access the online help, select **Help > SDS Online Help** from the SDS software menu bar.

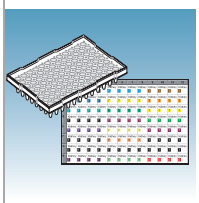
Automation Options

The 7900HT Fast System can run prepared reaction plates individually or in groups using the Automation Accessory with the Zymark Twister Microplate Handler. If you are not using the Automation Accessory, you must run reaction plates individually.

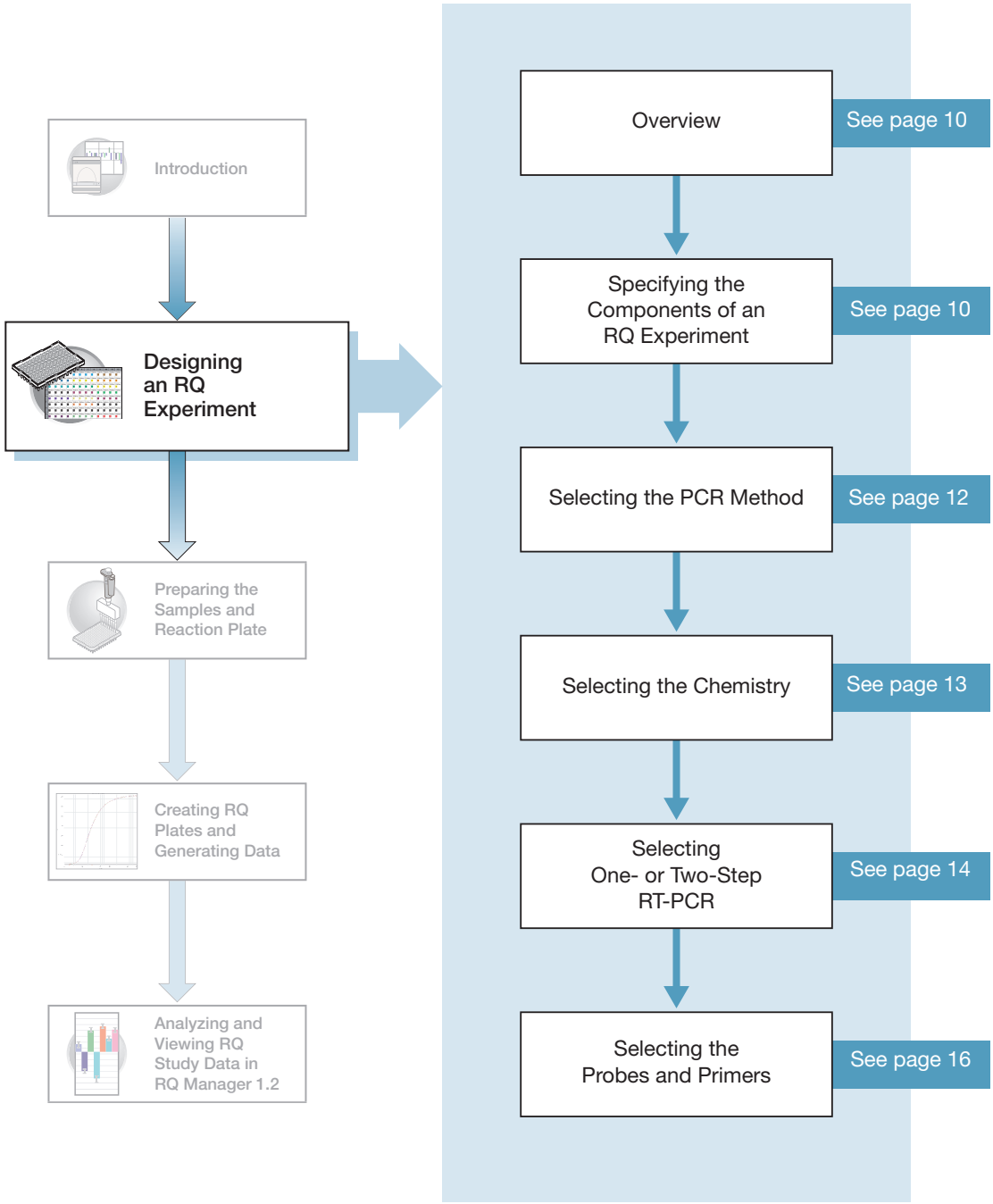
For clarity, this chapter only illustrates running an individual reaction plate. For information on running multiple reaction plates, refer to Appendix B, “SDS Automation Controller Software,” on page 73.

For information on automated operation of the 7900HT Fast System using the Automation Accessory, see the *SDS Online Help*.

Notes



Designing an RQ Experiment



Notes

Overview

Typical RQ experiments are designed for traditional (singleplex) PCR, where a primer pair plus a TaqMan probe or a primer pair plus a SYBR® Green binding dye are present in a reaction. The following sections describe design decisions required for RQ experiments.

Specifying the Components of an RQ Experiment

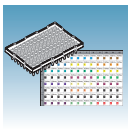
For every sample in an RQ experiment, you must specify:

- **A target** – The nucleic acid sequence that you are studying.
- **A calibrator** – The sample used as the basis for comparative results.
- **An endogenous control** – A gene present at a consistent expression level in all experimental sample sets. By using an endogenous control as an active reference, you can normalize quantitation of a cDNA target for differences in the amount of cDNA added to each reaction. Note that:
  - Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.
  - If samples are spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.

  Typically, housekeeping genes such as β-actin, glyceraldehyde-3-phosphate (GAPDH), and ribosomal RNA (rRNA), are used as endogenous controls because their expression levels tend to be relatively stable (Mullis and Faloona, 1987).
- **Replicate wells** – For Comparative C_T studies, Applied Biosystems recommends using three or more replicate reactions per sample and endogenous control to ensure statistical significance.

For more information about these requirements, refer to the *Real-Time PCR Systems Chemistry Guide.*

Notes



Sample Experiment

In the sample experiment, the objective is to compare the expression levels of seven genes in the adipose, kidney, and muscle tissue of an individual. The 7 genes of interest, including CCKAR, GH, LEPR, CLPIN1, and TGFb, PCSK1, and HL are the targets and the adipose sample serves as the calibrator.

The SDS software sets gene expression levels for the calibrator sample to 1 (log of zero). Consequently, if more gene transcript is in the target sample (kidney or muscle) than in the calibrator sample (adipose), the gene expression level of the target sample is greater than 1. Similarly, if less gene transcript is in the target sample than in the calibrator, the gene expression level in the target sample is less than 1.

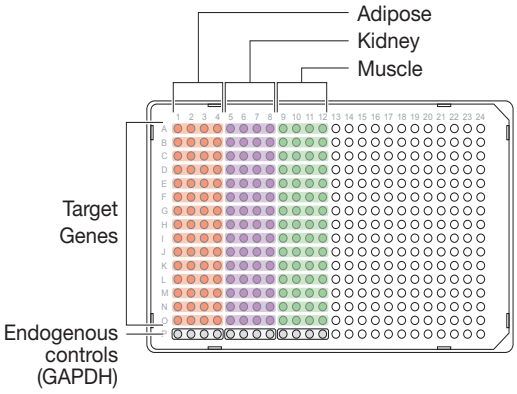
Because RQ is based on PCR, the more template in a reaction, the more the PCR product and the greater the fluorescence. To adjust for possible differences in the amount of template added to the reaction, GAPDH serves as an endogenous control. Thus, expression levels of the endogenous control are subtracted from expression levels of target genes. An endogenous control is required for each tissue sample.

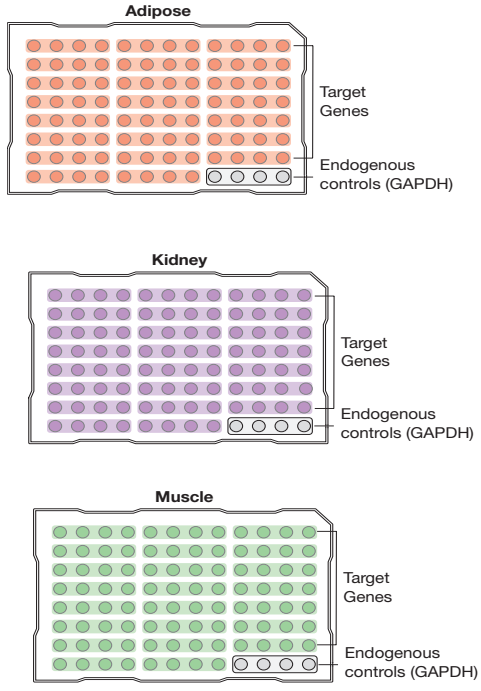
The experiment includes three sets of endogenous controls—one for each tissue. Also, the endogenous control for each tissue must be amplified on the same plate as the target sequences for that tissue. Finally, note that the experiment uses the singleplex PCR format, and therefore, the endogenous controls are amplified in wells different from the target wells.

Four replicates of each target gene and the endogenous control gene are performed to ensure statistical significance (see below).

Note: The sample RQ experiment utilized a single plate for the entire experiment. You can also design experiments so that several samples are amplified on the same plate, as shown in the following table.

Sample Single Plate RQ Study	Optional Multi-plate RQ Study
In the sample RQ experiment, multiple sample types were run on a single plate. An endogenous control for each sample type must also be included on the same plate, as shown here.	A multiple RQ plate setup example where each plate contains a single sample type (tissue). The endogenous control for each tissue should be on the same plate as the targets for that tissue.





Notes

Selecting the PCR Method

Types of PCR Methods

You can perform PCR using either of the following:

- A singleplex reaction, where a single primer pair is present in the reaction tube or well. Only one target sequence or endogenous control can be amplified per reaction.
- A multiplex reaction, where two or more primer pairs are present in the reaction. Each primer pair amplifies either a target sequence or an endogenous control.

Selection Criteria

Both methods give equivalent results for RQ experiments. To select a method for your needs, consider the following:

1. The type of chemistry you use to detect PCR products – Singleplex PCR can use either SYBR® Green or TaqMan reagent-based chemistry. Multiplex PCR can use only TaqMan reagent based chemistry.

   Note: TaqMan Low Density Arrays are designed for singleplex PCR reactions only. For more information on TaqMan Low Density Arrays, refer to the *TaqMan Low Density Array Getting Started Guide* (PN4319399).

2. The amount of time you want to spend optimizing and validating your experiment – Amplifying target sequences and endogenous controls in separate reactions (singleplex PCR) requires less optimization and validation than multiplex PCR.

3. For multiplex PCR methods: the primer limitations, the relative abundance of the target and reference sequences (the endogenous control must be more abundant than the targets), and the number of targets in the study.

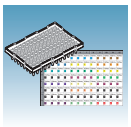
IMPORTANT! As the number of gene targets increases, the singleplex format is typically more effective than the multiplex format because less optimization is required. However, running multiple reactions increases throughput and reduces the effects of pipetting errors.

For more information about multiplex and singleplex PCR, refer to the *Real-Time PCR Systems Chemistry Guide.*

Sample Experiment

The singleplex PCR method is used in the example experiment because the number of targets to be amplified is 7 genes and there is one endogenous control

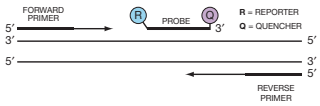
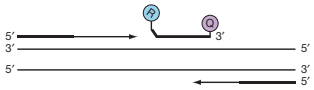
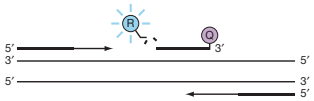
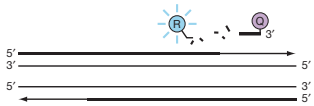
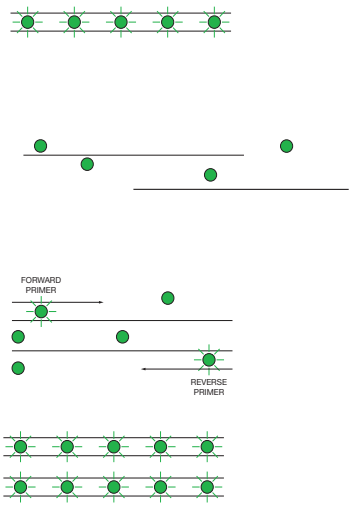
Notes



Selecting the Chemistry

About Chemistries

Applied Biosystems offers two types of chemistries that you can use to detect PCR products on Real-Time instruments, as explained in the following table. Both TaqMan probe-based and SYBR Green I dye chemistries can be used for either one- or two-step RT-PCR. For more information about these chemistries, refer to the *Real-Time PCR Systems Chemistry Guide*.

Chemistry	Process
TaqMan® reagents or kits **Description** TaqMan reagent-based chemistry uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles. **Advantages** • Increases specificity with a probe. Specific hybridization between probe and target generates fluorescence signal. • Provides multiplex capability. • Optimized assays available. • Allows 5′ -nuclease assay to be carried out during PCR. • Improves detection of low-copy target gene in relation to SYBR Green I dye.	**Step 1: Polymerization** A reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe.  **Step 2: Strand Displacement** When both dyes are attached to the probe, reporter dye emission is quenched.  **Step 3: Cleavage** During each extension cycle, the Applied Biosystems hot-start DNA polymerase system cleaves the reporter dye from the probe.  **Step 4: Polymerization Completed** After being separated from the quencher, the reporter dye emits its characteristic fluorescence. 
SYBR® Green I reagents **Description** Uses SYBR Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles. **Advantages** • Economical (no probe needed) • Amplifies all double-stranded DNA • Yields a melting profile of distinct PCR runs • Increases signal fluorescence as amplification product length increases. • Improves detection of low-copy target genes. **Limitations** Binds nonspecifically to all double-stranded DNA sequences. To avoid false positive signals, check for nonspecific product formation using dissociation curve or gel analysis.	 **Step 1: Reaction setup** The SYBR® Green I dye fluoresces when bound to double-stranded DNA. **Step 2: Denaturation** When the DNA is denatured, the SYBR® Green I dye is released and the fluorescence is drastically reduced. **Step 3: Polymerization** During extension, primers anneal and PCR product is generated. **Step 4: Polymerization completed** SYBR® Green I dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the instrument.

Notes

Two Types of TaqMan Probes

Applied Biosystems offers two types of TaqMan probes:

- TaqMan probes with TAMRA dye as a quencher
- TaqMan MGB (minor groove binder) probes with non-fluorescent quencher (NFQ)

For more information about TaqMan probe-based chemistry, refer to the *Real-Time PCR Systems Chemistry Guide.*

Selecting One- or Two-Step RT-PCR

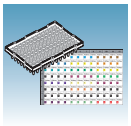
You can perform Real-Time PCR with either reverse transcription (RT) and PCR in a single reaction (one-step) or in separate reactions (two-step).The reagent configuration you use depends on which method you select:

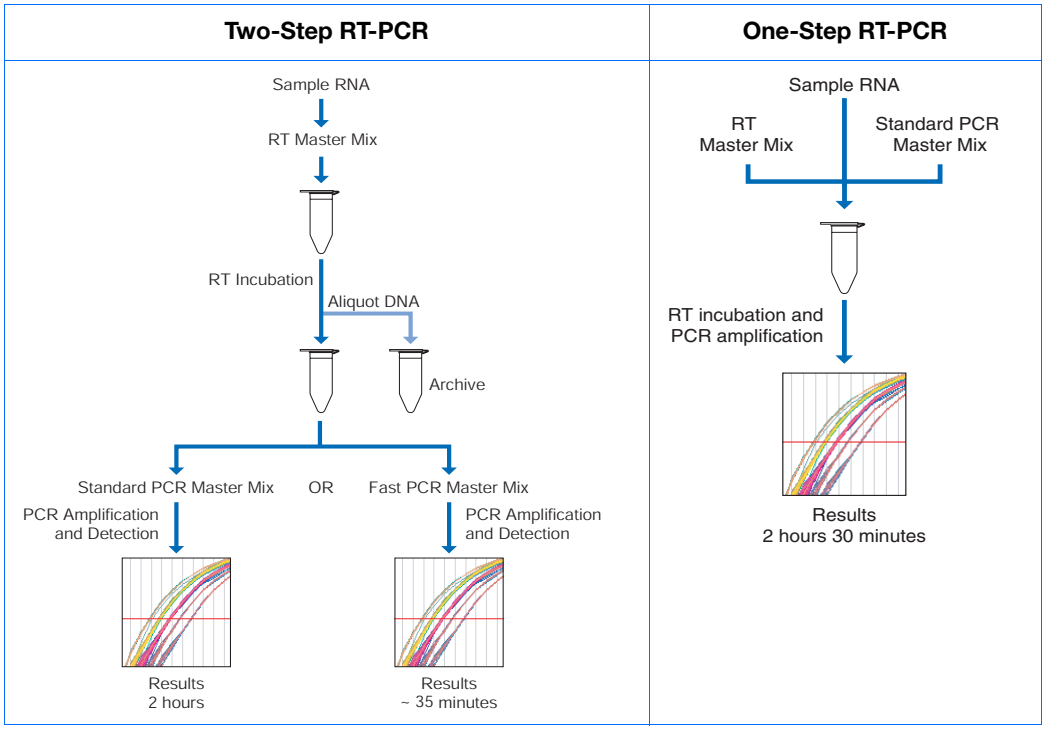
- In one-step RT-PCR, RT and PCR take place in one buffer system, which provides the convenience of a single-tube preparation for RT and PCR amplification. However, Fast PCR Master Mix or the carryover prevention enzyme, AmpErase® UNG cannot be used to perform one-step RT-PCR. For more information about UNG, refer to the *Real-Time PCR Systems Chemistry Guide*.
- Two-step RT-PCR is performed in two separate reactions: First, total RNA is reverse transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. AmpErase® UNG (uracil-N-glycosylase) can be used to prevent carryover contamination.

Note: TaqMan (2✕) Universal PCR Master Mix is available with or without AmpErase UNG. If you are using TaqMan Fast Universal PCR Master Mix (2✕), No Amperase UNG, you must purchase AmpErase UNG separately.

IMPORTANT! This guide provides an RQ experiment which is designed using two-step RT-PCR with TaqMan Universal PCR Master Mix (2✕). For additional options, refer to the *Real-Time PCR Systems Chemistry Guide*.

Notes





Recommended Kits for Two-Step RT-PCR			
Chemistry	**Step**	**Reagent**	**Part Number**
TaqMan reagents or kits	RT	High Capacity cDNA Archive Kit	4322171
	PCR	TaqMan Universal PCR Master Mix (2X)	4304437
		TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG	4352042
SYBR Green I reagents or kits	RT	High Capacity cDNA Archive Kit	4322171
	PCR	SYBR Green Master Mix	4309155
	RT and PCR	SYBR Green RT-PCR Reagents	4310179

Sample Experiment

Pre-made probes and primers for all the genes of interest are obtained from the TaqMan® Gene Expression Assays product line, which uses TaqMan reagent-based chemistry. Two-step RT-PCR is performed using the reagents recommended for TaqMan reagent- or kit-based chemistry shown in the table above.

Notes

Selecting the Probes and Primers

You must select primer/probe sets for your target and endogenous control sequences. Applied Biosystems provides three options for choosing primers and probes:

- **TaqMan® Gene Expression Assays** – Optimized, ready-to-use TaqMan reagent-based 5′ nuclease assays for human, mouse, or rat transcripts. These assays are available as inventoried and non-inventoried assays. Note that TaqMan Low Density Arrays use inventoried assays only. For information on available primer/probe sets or to place an order, go to http://www.allgenes.com.
- **Custom TaqMan® Gene Expression Assays** – Design, synthesize, formulate, and deliver quality-controlled primer and probe sets. Use this service if the assay you need is not currently available. To place an order, go to http://www.allgenes.com.
- **Primer Express® Software** – Helps you design primers and probes for your quantitation experiment. For information about using this software, refer to the *Primer Express® Software Version 3.0 Getting Started Guide* (PN 4362460).

  Applied Biosystems provides quantitation assay design guidelines that provide a reliable system for assay design and optimization. For information about the assay design guidelines, refer to the *Real-Time PCR Systems Chemistry Guide*.

If you ordered TaqMan® Gene Expression Assays or Custom TaqMan® Gene Expression Assays, probes are already labeled with FAM™ as a reporter dye. If you design your own assays, you need to specify a reporter dye for your custom probe(s). For singleplex experiments, you can use the same dye for targets and endogenous control(s). For multiplex experiments, the probe for the target is typically labeled with FAM dye and the endogenous control with VIC® dye.

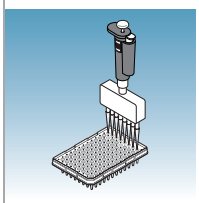
Sample Experiment

Primers and probes for all genes being studied are obtained from Applied Biosystems TaqMan® Gene Expression Assays. Each assay consists of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB probe, provided as a 20× assay mix. All target probes are labeled with FAM™ dye; the endogenous control is also labeled with FAM™ dye.

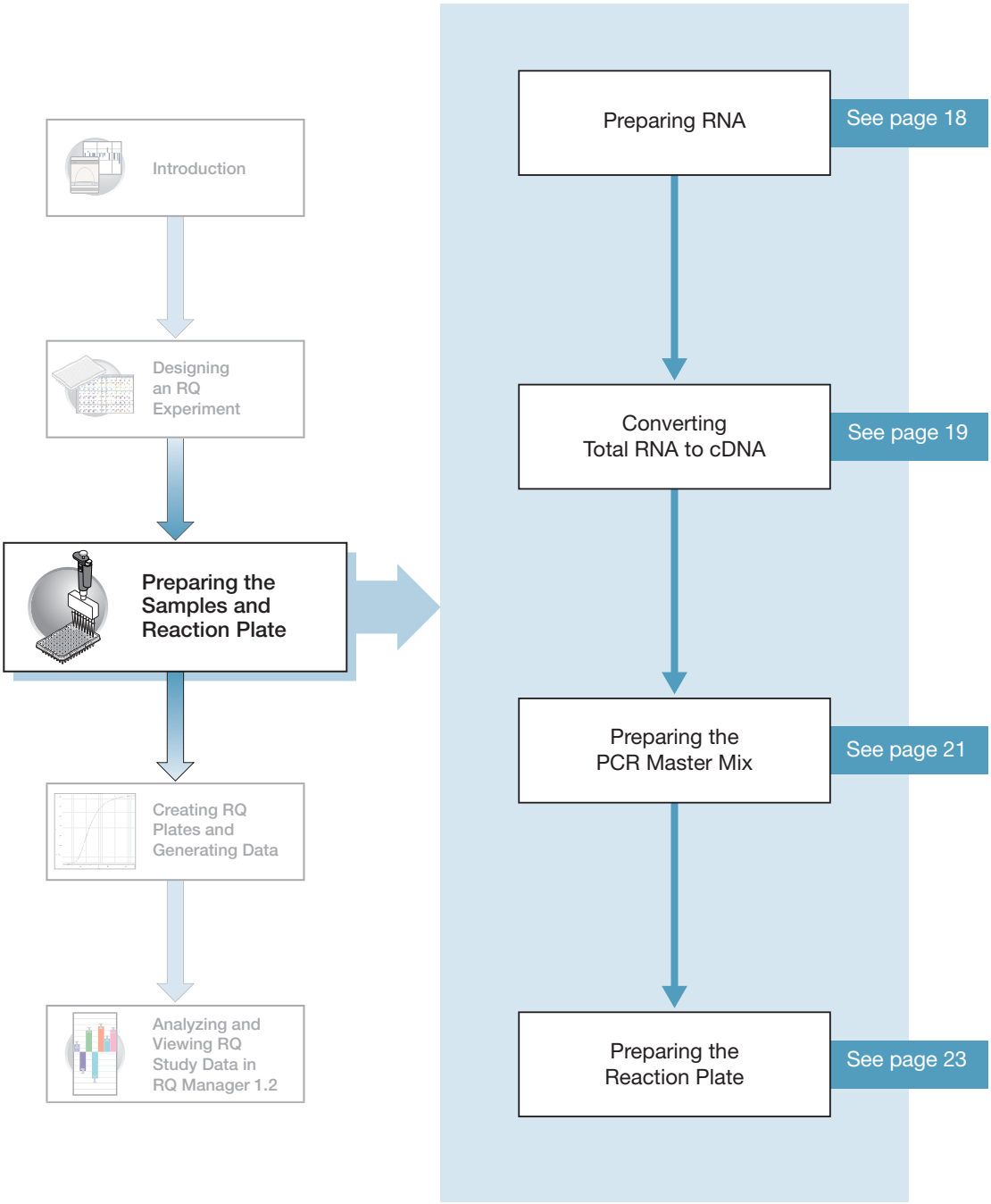
The following table provides the gene symbol, gene name, and Applied Biosystems Assay ID number (provided on the Web site) for the genes studied and the endogenous control.

Gene Symbol	Gene Name	Assay ID #
CCKAR	cholecystokinin A receptor	Hs00167891_m1
GH1	growth hormone 1	Hs00236859_m1
LEPR	leptin receptor	Hs00174492_m1
LPIN1	lipin 1	Hs00299515_m1
TGFB1	transforming growth factor, beta 1	Hs00171257_m1
PCSK1	proprotein convertase subtilisin/kexin type 1	Hs00175619_m1
HL	hepatic lipase	Hs00165106_m1
GAPDH	glyceraldehyde-3-phophate dehydrogenase	Hs99999905_m1

Notes



Preparing the Samples and Reaction Plate



Notes

Preparing RNA

Isolating RNA

Applied Biosystems supplies an instrument system and proprietary chemistries for isolating RNA from a variety of starting materials, such as blood, tissue, cell cultures, and plant material.

System	Part Number
ABI PRISM® 6100 Nucleic Acid PrepStation	6100-01
Total RNA Chemistry Reagents:	
Nucleic Acid Purification Elution Solution	4305893
Nucleic Acid Purification Lysis Solution	4305895
Nucleic Acid Purification Wash Solution I	4305891
Nucleic Acid Purification Wash Solution II	4305890
AbsoluteRNA Wash Solution (DNase treatment)	4305545
Tempus™ Blood RNA Tubes (For collection, stabilization, and isolation of total RNA in whole blood for gene expression analysis using the 6100 PrepStation)	4342972
Isolation of Total RNA from Whole Blood and from Cells Isolated from Whole Blood Protocol	4332809
Tempus™ Blood RNA Tube and Large Volume Consumables Protocol	4345218
Tissue RNA Isolation: Isolation of Total RNA from Plant and Animal Tissue Protocol	4330252

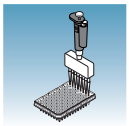
Quality of RNA

The total RNA you use for RQ experiments should:

- Have an $A_{260/280}$ greater than 1.9
- Be intact when visualized by gel electrophoresis
- Be free of RT or PCR inhibitors

Refer to the *High Capacity cDNA Archive Kit Protocol* (4322169) for additional guidelines on preparing the RNA template.

Notes



Converting Total RNA to cDNA

Using the High Capacity cDNA Archive Kit

Use the High Capacity cDNA Archive Kit (PN 4322171) to perform the first step (RT) in the two-step RT-PCR method. Perform the manual method for converting total RNA to cDNA, as described in the *High Capacity cDNA Archive Kit Protocol* (PN 4322169).

Adjusting the Starting Concentration of Total RNA

The High Capacity cDNA Archive Kit is set up to convert 0.1 to 10 µg of total RNA to cDNA. Make sure you convert enough total RNA so that the final concentration of total RNA converted to cDNA is 10 to 100 ng in 5 µL for each 50-µL PCR reaction.

IMPORTANT! The protocol is not shipped with the High Capacity cDNA Archive Kit. To access the protocol, go to **http://docs.appliedbiosystems.com/search.taf**. Select **ABI PRISM® 6100 Nucleic Acid PrepStation** in the Product list box, then click **Search** at the bottom of the page. The protocol is listed under the Protocol heading.

Thermal Profile Parameters for RT

The High Capacity cDNA Archive Kit uses the following thermal profile parameters for the RT step.

Step Type	Time (min)	Temperature (°C)
HOLD	10	25 °C
HOLD	120	37 °C

Note: Thermal cycling conditions for one-step RT-PCR are described on page 35.

Storing cDNA

After RT is complete, store all cDNA samples at –15 °C to –25 °C. To minimize repeated freeze-thaw cycles of cDNA, store cDNA samples in aliquots.

WARNING **CHEMICAL HAZARD. 10 × RT Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Notes

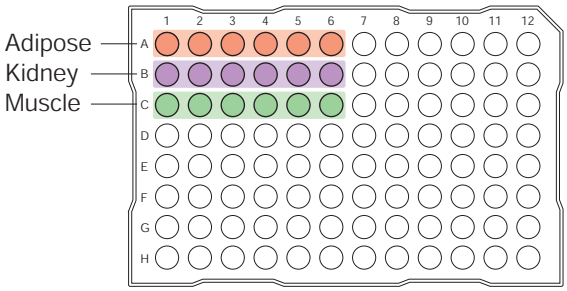
Sample Experiment

For the example experiment, RNA is extracted from the adipose, muscle, and kidney tissues of an individual. RNA concentration is determined spectrophotometrically (using A_{260}), and the RNA is diluted to a final concentration of 50 ng/µL.

The RT reaction mix is prepared as described in the *High Capacity cDNA Archive Kit Protocol*.

Component	µL/Reaction	µL/21 Reactions†
10× Reverse Transcription Buffer	10	210
25× dNTPs	4	84
10× random primers	10	210
MultiScribe™ Reverse Transcriptase, 50 U/µL	5	105
Nuclease-free water	21	441
Total	50	1050

† Each RT reaction is 100 µL (see below). If you need 5 µL cDNA for each of 104 PCR reactions (see "Preparing the Reaction Plate" on page 23), you will need 6 RT reactions. Extra volume (enough for one additional RT reaction) is included to account for pipetting losses, as well as extra cDNA for archiving.



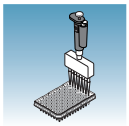
The cDNA archive plate is then prepared by pipetting into each well:

- 50 µL of the RT master mix
- 30 µL of nuclease-free water
- 20 µL of RNA sample (bringing the total starting amount of RNA to 1 µg per 100 µL reaction)

The RNA is then converted to cDNA using the thermal cycling parameters for two-step RT-PCR, as described in "Thermal Profile Parameters for RT" on page 19.

The cDNA is stored at –20 °C until use.

Notes



Preparing the PCR Master Mix

The second step (PCR) in the two-step RT-PCR procedure is amplifying the cDNA. You can use the TaqMan® Universal PCR Master Mix (2×) reagents for a run time of approximately 2 hours, or the TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase UNG for a run time of approximately 35 minutes.

If you design primers and probes using Primer Express software, you must adjust their concentrations for optimal performance, with the universal assay conditions, as specified in the applicable protocols or using the volumes listed in the tables below. All TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assays are formulated so that the final concentration of the primers and probes are within the recommended values.

Note: The reaction volumes for TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assays are included on the CD which accompanies these products.

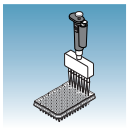
Standard 96-Well or 384-Well Plates Using TaqMan Universal PCR Master Mix (2×)

The following table lists the universal assay conditions (volume and final concentration) for using the TaqMan Universal PCR Master Mix (2×).

CAUTION **CHEMICAL HAZARD. TaqMan Universal PCR Master Mix (2×)** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Reaction Component	µL/ Sample 96-Well Plate	µL/ Sample 384-Well Plate	Final Concentration
TaqMan Universal PCR Master Mix (2×)	25.0	10.0	1×
Forward primer	5.0	2.0	50 to 900 nM
Reverse primer	5.0	2.0	50 to 900 nM
TaqMan probe	5.0	2.0	50 to 250 nM
cDNA sample	5.0	2.0	10 to 100 ng
Nuclease-free water	5.0	2.0	—
Total	50.0	20.0	—

Notes



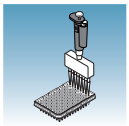
Fast 96-Well Plates Using TaqMan Fast Universal PCR Master Mix (2×), no AmpErase UNG

The following table lists the universal assay conditions (volume and final concentration) for using the TaqMan Fast Universal PCR Master Mix (2×).

CAUTION **CHEMICAL HAZARD**. **TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and glove.

Reaction Component	µL/ Sample Fast 96-Well Plates	Final Concentration
TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG	10.0	1×
Forward primer	2.0	50 to 900 nM
Reverse primer	2.0	50 to 900 nM
TaqMan probe	2.0	50 to 250 nM
cDNA sample	2.0	10 to 100 ng
Nuclease-free water	2.0	—
Total	20.0	—

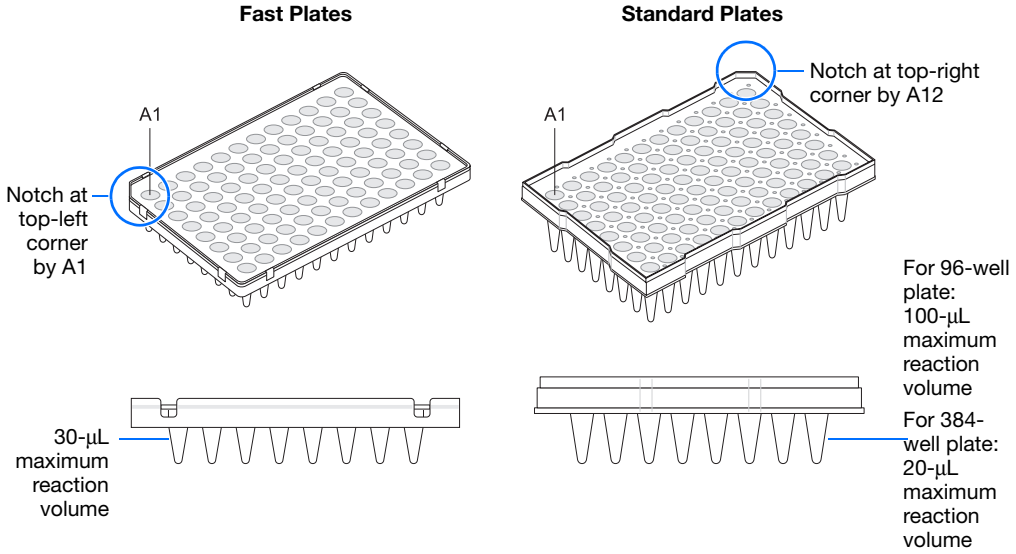
Notes



Preparing the Reaction Plate

Standard and Fast Plates

IMPORTANT! Make sure that the correct block, for the plate being run, is installed in the 7900HT Fast System. Fast plates do not fit into standard blocks and standard plates do fit into fast blocks. Using a plate with the wrong block will result in damage to the plate, loss of data, or both.

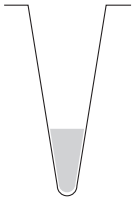
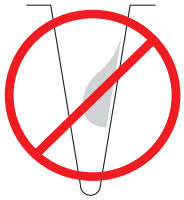
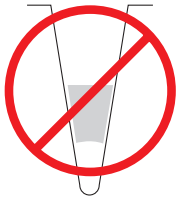


To Prepare the Reaction Plate:

1. Ensure that you have an endogenous control for each sample type (for example, each tissue in a study comparing multiple tissues) on a plate. If samples are spread across multiple plates, each plate must have an endogenous control.
2. Into each well of a:
   - Standard reaction plate, add 50 µL (96-well) or 20 µL (384-well) of the appropriate PCR reaction mix
   - Fast reaction plate, add 20 µL of the appropriate PCR reaction mix
3. Seal the reaction plate with an ABI PRISM® Optical Adhesive Cover.
4. Centrifuge the plate briefly.

Notes

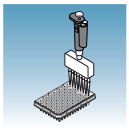
5. Verify that each reaction mix is positioned at the bottom of the well, as shown below.

Correct Position	Incorrect Positions	
		
The reaction mix is at the bottom of the well.	The reaction mix lies on the side wall; the plate was not centrifuged.	An air bubble is at the bottom of the well because the plate was not centrifuged with sufficient force or for sufficient time.

IMPORTANT! If you are using *TaqMan Fast Universal PCR Master Mix* (2×), *No AmpErase UNG*, you must start the run within 2 hours of preparing the reaction plate. The plate can be refrigerated or stored frozen if a run is not started within 2 hours of plate setup.

6. Keep the reaction plates on ice until you are ready to load them into the Zymark® Twister Microplate Handler.

Notes



CAUTION **CHEMICAL HAZARD. TaqMan Universal PCR Master Mix (2×)** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

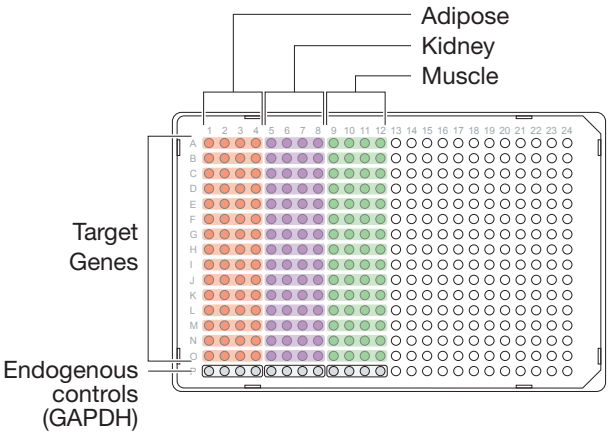
Sample Experiment

Primers and probes for the example RQ experiment are obtained from the TaqMan Gene Expression Assays product line and are provided as a 20× Gene Expression Assay Mix. The PCR reaction mix is prepared as follows:

PCR Reaction Component	µL/ Sample	µL/ 5 Reactions‡	Final Concentration
TaqMan Universal PCR Master Mix (2×)	10.0	50.0	1×
20× Gene Expression Assay Mix†	1.0	5.0	1×
cDNA sample	2.0	10.0	50 ng (for the 50-µL reaction)
Nuclease-free water	7.0	35.0	—
Total	20.0	100.0	—

† Contains forward and reverse primers and labeled probe.
‡ 24 master mixes are prepared, one for each of the 7 target genes (and each of the 3 samples) plus the endogenous control. Volume for five reactions (4 replicates plus extra) to account for pipetting losses.

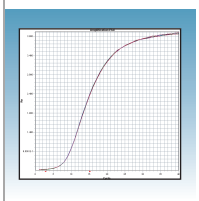
Samples and endogenous controls are arranged on one plate as shown below. 20 µL of PCR master mix containing cDNA are added to each well.



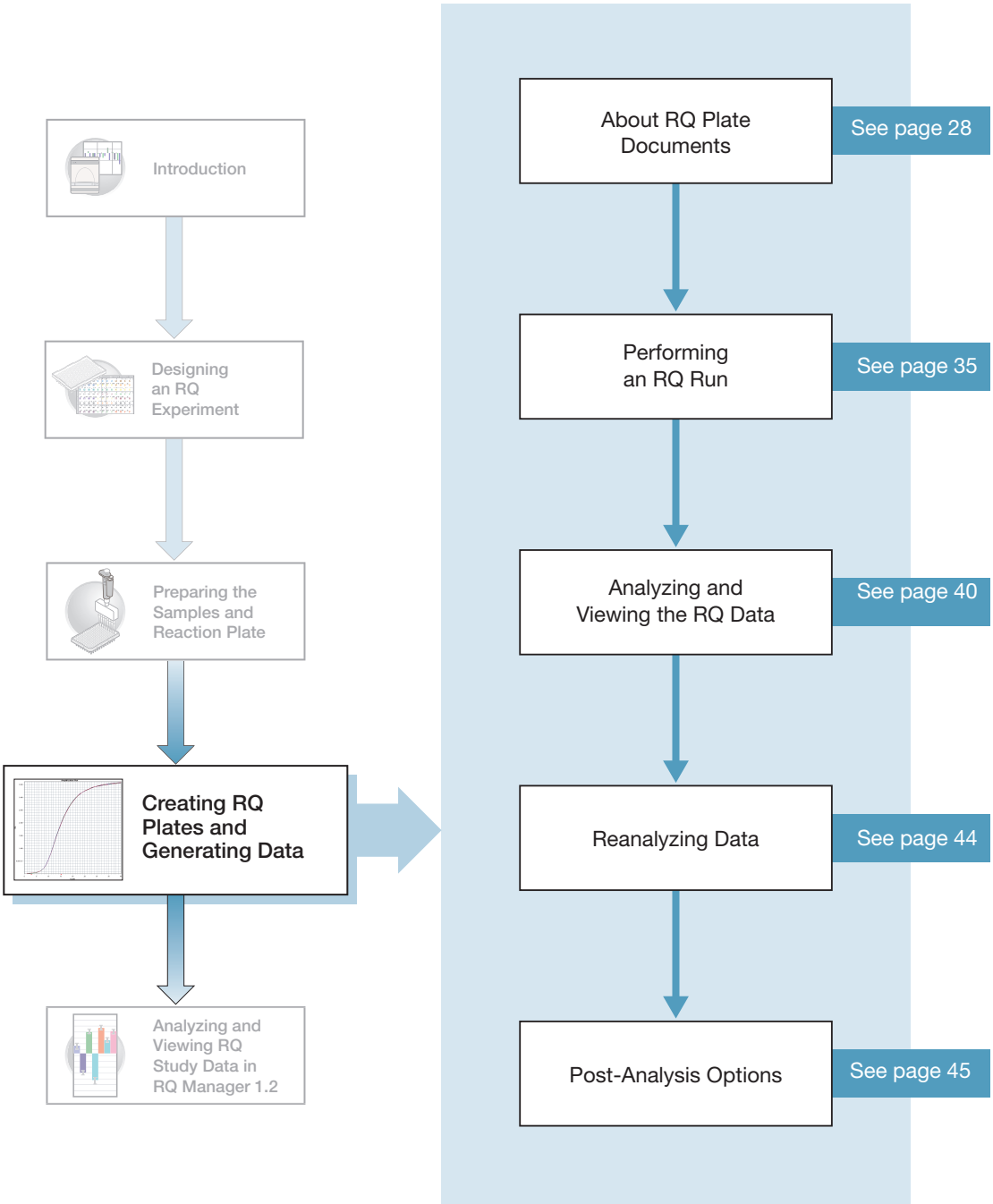
The reactions are kept on ice until the plates are loaded onto the 7900HT Fast System.

Notes

Notes



Creating RQ Plates and Generating Data



Notes

About RQ Plate Documents

Creating Plate Documents

Every RQ reaction plate must have a corresponding RQ Plate document. An RQ Plate document stores data collected from an RQ run (for a single reaction plate) including sample names and detectors.

This chapter describes how to create a new plate document using the New Plate Wizard. Refer to **Help > SDS Online Help** for other methods of creating new plate documents.

Reaction Plate Options

The RQ assay can be run on standard 96-well, fast 96-well, standard 384-well, and TaqMan Low Density Array reaction plates. For clarity, this chapter only illustrates the running of a standard 384-well reaction plate.

Run Setup Requirements

For each RQ plate document that you create, specify detectors, endogenous controls, and detector tasks:

- A detector is a virtual representation of a gene-specific nucleic acid primer and probe combination used in assays. You specify which detector to use for each target sequence.

  IMPORTANT! To compare data from multiple reaction plates in a study, all the plates in the study must contain a common set of detectors.

- An endogenous control(s) is defined in "Selecting the Chemistry" on page 13. If your experiment consists of multiple plates, each plate must have at least one endogenous control with at least three replicates. If your experiment consists of a single plate with multiple samples, you must have an endogenous control for each sample. All plates in an RQ experiment must use the same endogenous control (for example, GAPDH).
- A detector task specifies how the software analyzes the data collected from a well during analysis. You apply one of two tasks to each detector in each well of an RQ plate document.

Detector Task	Symbol	Apply to well that contain...
Target	T	Target sequences.
Endogenous Control	E	Endogenous control sequence.

Notes

Creating an RQ Plate Document

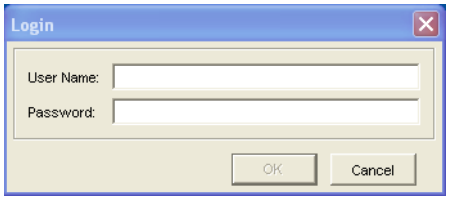
You can enter sample information into a new plate document, import sample information from existing plate documents, or use a template document to set up new plate documents. This section describes setting up new plate documents using the plate wizard to manually enter sample information. Refer to the *SDS Online Help* for information about importing sample information or using template documents.

To create a new plate document:

1. Double-click .

2. If your System Administrator has enabled the login option, the Login dialog box appears. Enter your **User Name** and **Password**, then click **OK**.

   Note: If the login option is not enabled, no Login dialog box appears. Skip to step 3 below.

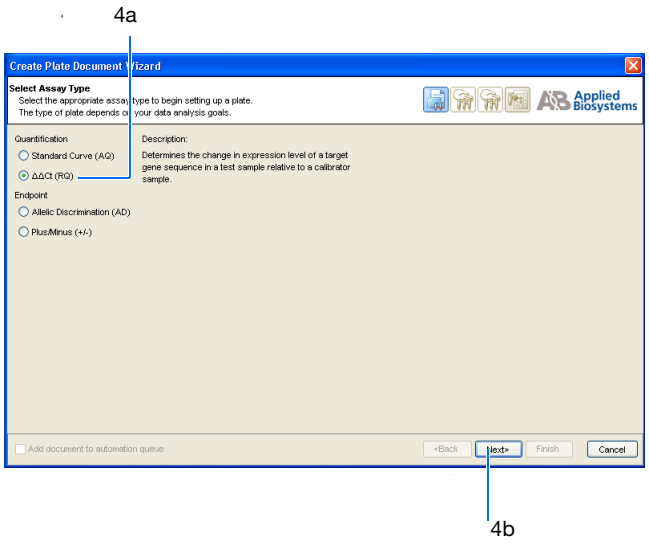


3. Click or select **File** > **New Plate Wizard** to open the Create Plate Document Wizard.

4. Select the assay type:

   a. Select **ΔΔCt (RQ).** Note that you can choose to add the document to the automation queue.

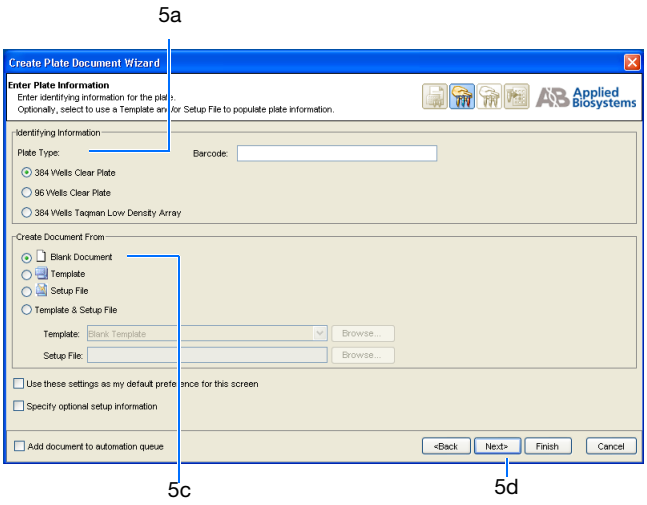
   b. Click **Next >**.



Notes

5. Enter the plate information:

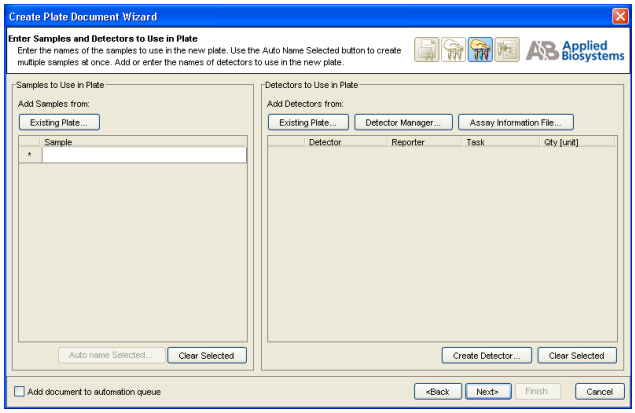
   a. Select your **Plate Type**.



   IMPORTANT! You cannot use RQ Plate documents for AQ assays and vice versa. The information stored in AQ and RQ Plate documents is not interchangeable.

   b. *Optional*. In the barcode field, scan or (or manually enter) the plate barcode. If you are using the Zymark® Twister Microplate Handler and are running more than one plate, click the checkbox box next to **Add document to automation queue**. The Automation Controller software will automatically pull up the plate document information.

   Note: A barcode is also required if you are adding the plate document to the automation queue.



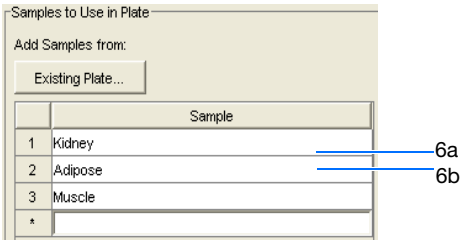
   c. Select to **Create Document From** your preferred source.

   Note: You can create a blank plate document or use existing information from a template or setup file. For more information on other methods of creating plate documents, refer to the *SDS Online Help*.

   d. Click **Next >**.

6. Enter the samples to use in the plate:

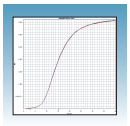
   Note: Samples can be added from an Existing Plate document. To add samples using this option, refer to the *SDS Online Help*.



   a. Double-click in the Sample field, and type a sample name.

   b. *Optional*. Press Enter to activate another row and add more samples.

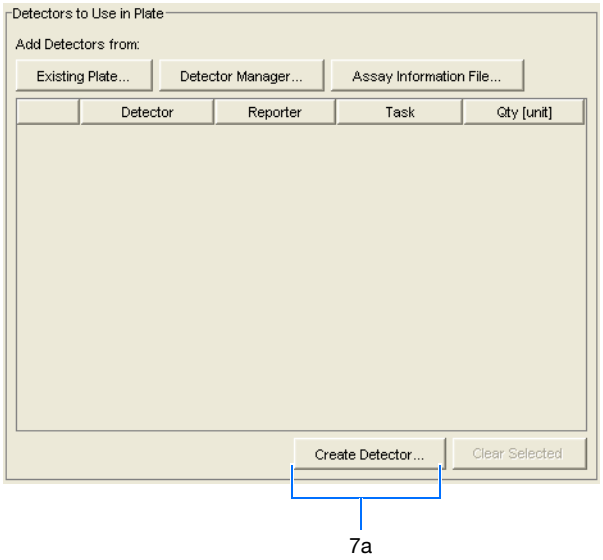
Notes



Note: To remove a sample, select the sample by clicking on it, then click **Clear Selected**.

7. Enter detectors to use in the plate document.

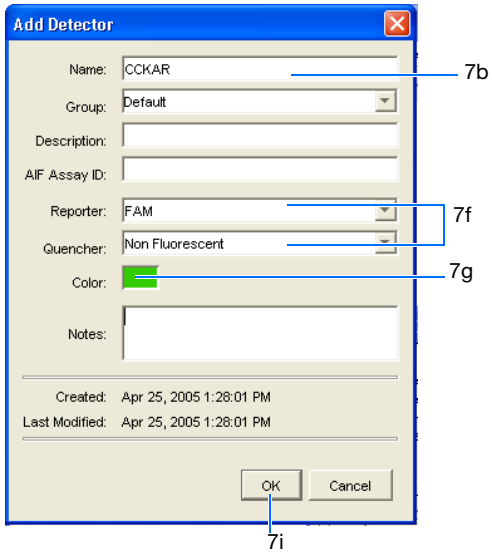
Note: Detectors can be added from an Existing Plate document, the Detector Manager, or an Assay Information File. To add detectors using these options, see *SDS Online Help*.



a. Click **Create Detectors** to open the Add Detector dialog box.

b. In the Add Detector dialog box, enter a name for the detector (for example CCKAR).

IMPORTANT! The name of the detector must be unique and should reflect the target locus of the assay (such as GADPH). Do not use the same name for multiple assays.



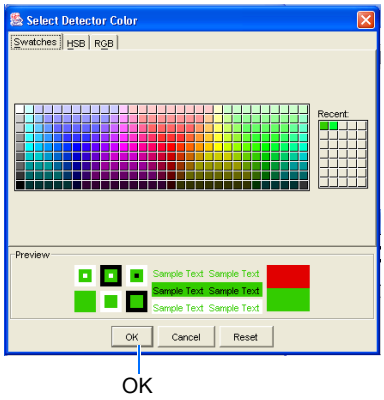
c. *Optional*. Click in the **Group** field, then assign a group name to the detector.

d. *Optional*. Click in the **Description** field, then enter a brief description of the detector.

e. *Optional*. Leave the AIF Assay ID field blank. For more information on Assay Information Files (AIF), refer to *SDS Online Help*.

Notes

f. Select the appropriate dyes for the detector in the Reporter and Quencher drop-downlists. Select **TAMRA** as the quencher for TaqMan probes and **None** for TaqMan MGB probes.



Note: If the dye you want to use does not appear in the list, from the menu bar select **Tools > Dye Manager** to add the dye. Note that your instrument must also be calibrated for that dye. For more information on using the Dye Manager, refer to *SDS Online Help* for more information.

g. Click the **Color** box to open the Select Detector Color dialog box, then select a color to represent the detector. Click **OK** to return to the Add Detector dialog box.

h. *Optional*. Enter any comments for the detector in the Notes field.

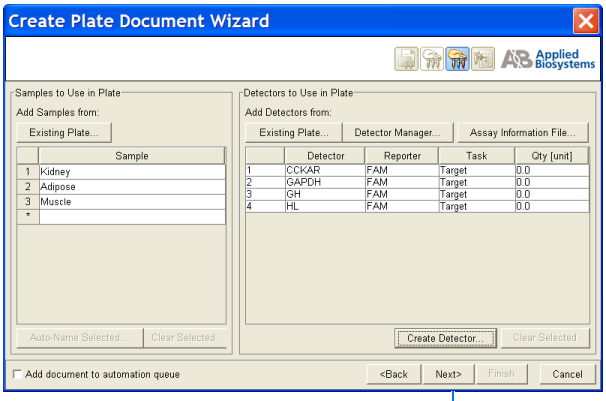
i. Click **OK**. The SDS software saves the new detector and displays it in the Detectors to Use in Plate pane of the wizard.

j. To create additional detectors, repeat steps a through step h.

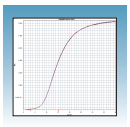
Note: To remove a detector from the Detectors to Use in Plate pane, select the detector, then click **Clear Selected**.

k. Click **Next >**.

8. Specify the samples, detectors, and tasks for each well.

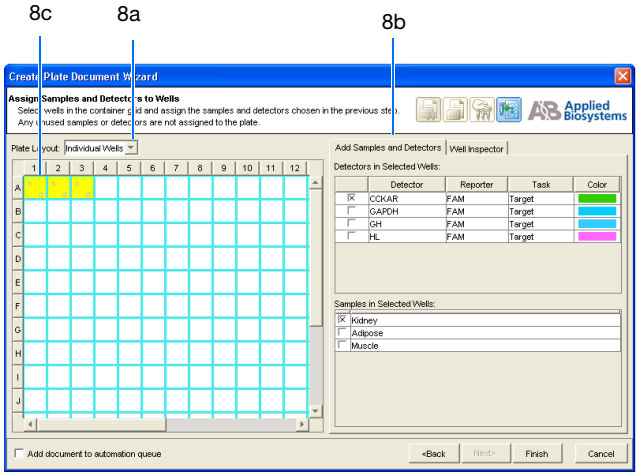


Notes



a. From the Plate Layout drop-down list, select **Individual Wells**. Refer to the *SDS Online Help* for more information on plate layout options.

IMPORTANT! If you change the plate layout after assigning samples or detectors to the wells, all assignment are cleared from the plate grid.



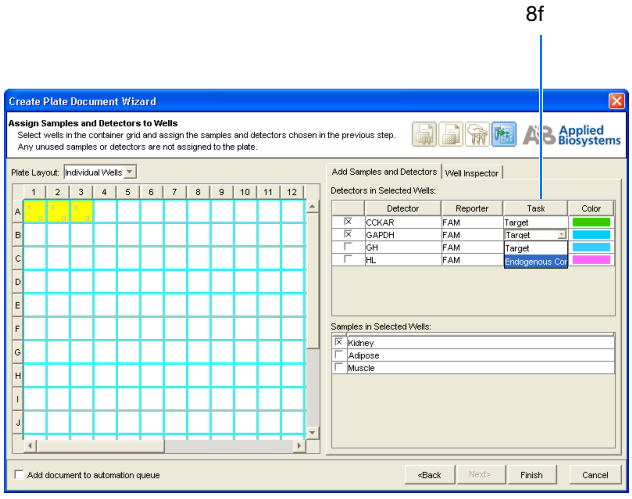
b. Select the **Add Samples and Detectors** tab.

c. Click on a well (or group of wells, for replicates) to select it. To select all wells, click in top left corner of the grid.

d. Check the appropriate detector name(s), for the selected well(s), in the Detectors in Selected Wells pane. Note that a "d" appears in the selected well(s).

e. Check the appropriate sample name for the selected well(s) in the Samples in Selected Wells pane. Note that an "s" appears in the selected well(s).

f. Click into the Task field (next to each detector) to assign the detector task.



g. Assign samples and detectors for the remaining wells (repeat steps 8c through 8f).

h. *Optional*. Click on the Well Inspector tab to review your plate setup.

Note: You can change the sample setup information (sample name, detector, task) after a run is complete.

9. Click **Finish**. The Create Plate Document Wizard closes and a new RQ plate document appears in the main SDS software window. If your experiment does not use all the wells on a

Notes

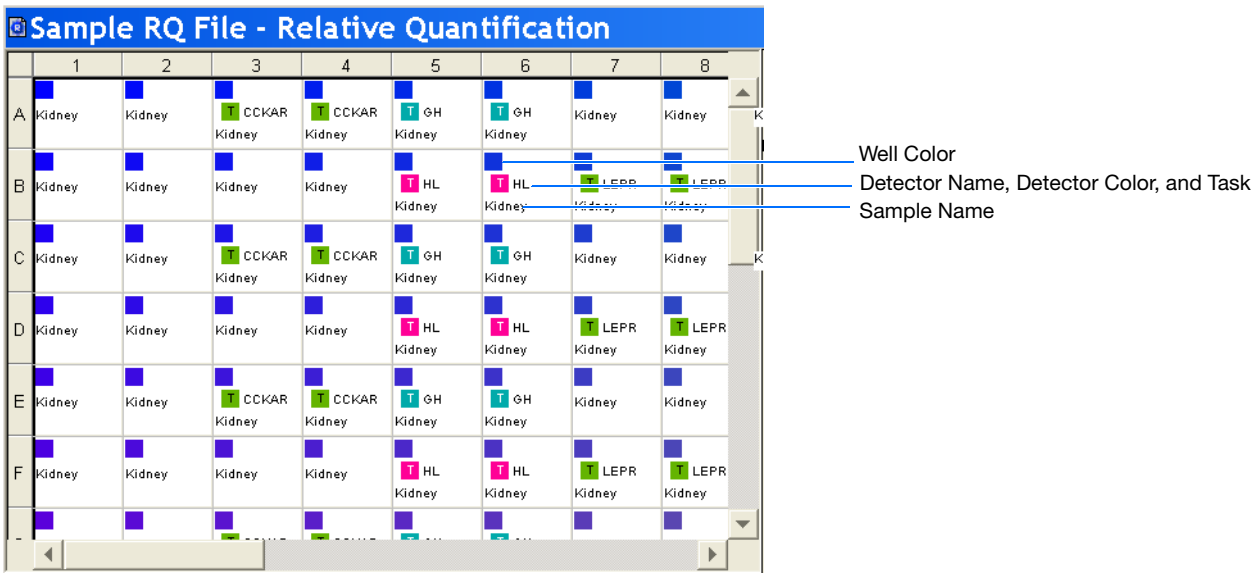
plate, do not omit the wells from use at this point. You can omit unused wells after the run. For information about omitting unused wells, refer to the *SDS Online Help*.

Sample Experiment

In the example RQ experiment, the samples for each of the three tissues (adipose, kidney, and muscle) are loaded on one plate.

Because the experiment is singleplex, the reaction in each well detects only one sequence—either a target sequence or endogenous control—in each well. Each well is associated with a detector (indicated by the colored squares). Additionally, each well is assigned a detector task Target or Endogenous Control.

The figure below shows the example RQ Plate document after sample names, detectors, and detector tasks are assigned for each well in the plate.



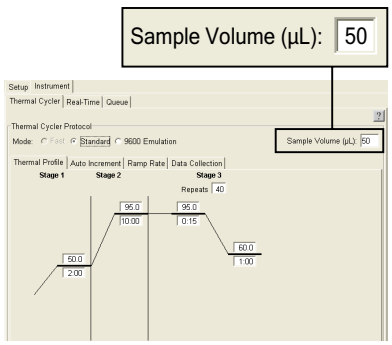
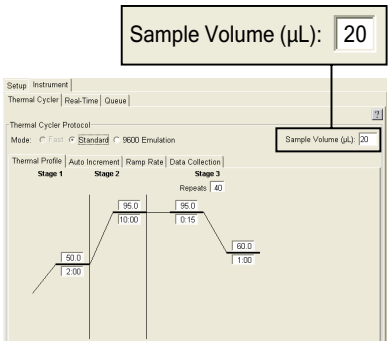
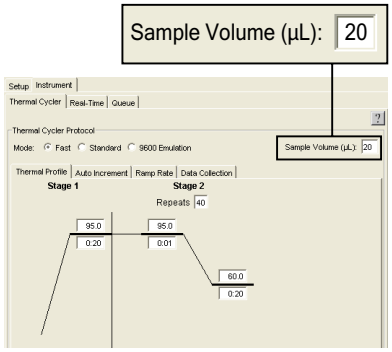
Notes

Performing an RQ Run

Verifying Default Thermal Cycling Conditions for PCR

If you selected the two-step RT-PCR method for your RQ experiment (recommended), you have already completed the RT step. At this point in the workflow, you are ready to PCR amplify cDNA.

The default thermal cycling conditions for the PCR step of the procedure, shown in the following table, should appear in the Instrument > Thermal Profile tab.

Standard 96-Well	Standard 384-Well	Fast 96-Well
		

Note: For information on thermal cycling conditions and parameters for TaqMan Low Density Arrays, refer to *TaqMan® Low Density Array Getting Started Guide* (PN 4319399).

Standard Default Times and Temperatures (Two-step RT-PCR)				
1) RT Step	HOLD 10 min @ 25 °C	HOLD 120 min @ 37 °C	* For reference only. RT is complete at this point.	
Standard Thermal Cycling Conditions				
2) PCR Step	Initial Steps		PCR (Each of 40 cycles)	
	AmpErase® UNG Activation	AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/ Extend
	HOLD	HOLD	CYCLE	
	2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Notes

Fast Default Times and Temperatures (Two-step RT-PCR)			
1) RT Step	HOLD	HOLD	* For reference only. RT is complete at this point.
	10 min @ 25 °C	120 min @ 37 °C	

Fast Thermal Cycling Conditions			
2) PCR Step Fast Conditions	Enzyme Activation	Melt	Anneal/Extend
	HOLD	CYCLE	
	20 sec @ 95 °C	1 sec @ 95 °C	20 sec @ 60 °C

Thermal Cycling Conditions for One-Step RT-PCR (Standard 96-Well Plates)

If you select the one-step RT-PCR method, cDNA generation and amplification take place simultaneously at this point in the workflow.

The following table shows the thermal cycling conditions for one-step RT-PCR experiments.

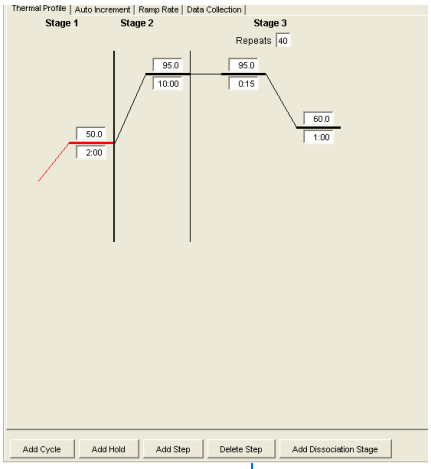
Note: Refer to the *SDS Online Help* for instructions on modifying thermal cycling parameters.

Times and Temperatures (One-step RT-PCR)			
Initial Steps		PCR (Each of 40 Cycles)	
Reverse Transcription	AmpliTaq® Gold DNA Polymerase Activation	Melt	Anneal/Extend
HOLD	HOLD	CYCLE	
30 min @ 48 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Notes

Modifying Settings for TaqMan® Universal PCR Master Mix, No AmpErase® UNG

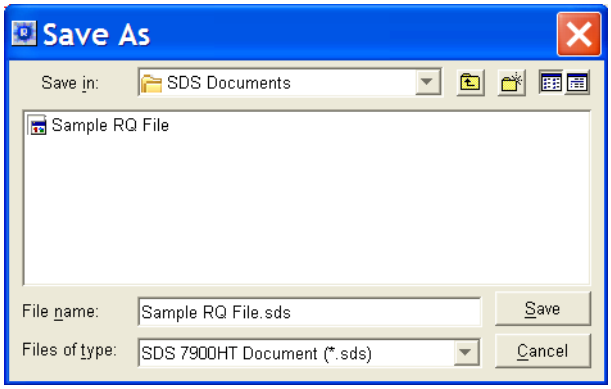
In the Thermal Profile tab, delete the default Stage 1 by clicking inside the stage to select it (the ramp line turns red), then click **Delete Step**.



Saving the Plate Document

To save the new plate document.

1. Select **File > Save As** to open the Save As dialog box.



2. In the Save In field, navigate to and select a directory to save the plate document.
3. In the File name field, enter a file name for the plate document.
4. From the Files of type drop-down list, select SDS 7900HT Document (***.sds**), then click **Save**.
5. *Optional*. If you want to use this plate setup again, you can save it as a template. Select **File > Save As**, type a File name, then select **Files of type: (*.sdt)**.

Note: Refer to the *SDS Online Help* for information about using plate document templates and setup files.

Notes

Starting the Run

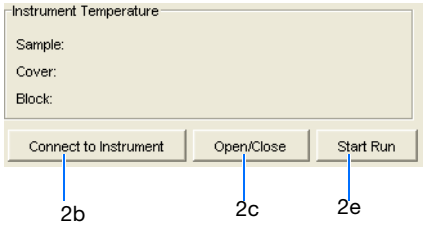
Before starting the run:

1. Verify that:
   - For two-step RT-PCR – The default PCR thermal cycling conditions are set.
   - For one-step RT-PCR, you set the thermal cycling parameters as shown in "Thermal Cycling Conditions for One-Step RT-PCR (Standard 96-Well Plates)" on page 36.
   - Sample volume is correct.
   - Correct run mode is selected: Standard, Fast, or 9600 Emulation.

   Note: When Standard mode is selected, the SDS software uses the 9700 thermal cylcer ramp rate in the 7900HT Fast System for standard PCR reactions. When Fast mode is selected, the SDS software uses the fastest available ramp rate of the 7900HT Fast System for fast PCR reactions. When the 9600 Emulation mode is selected, the SDS software reduces the 7900HT Fast System ramp rate to match that of the 9600 thermal cycler in the ABI PRISM® 7700 Sequence Detection System.

   Note: If you are using SYBR Green I reagent chemistry and you want to determine if there is contamination or if you want to determine the dissociation temperature, create a separate Dissociation assay or template. Refer to the *SDS Online Help* for more information.

2. To run an individual plate:
   a. Select the **Instrument > Real-Time** tab.
   b. Click **Connect to Instrument** to connect the plate document to the instrument.

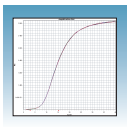


   c. *Optional*. Click **Open/Close**. The instrument tray rotates to the OUT position.
   d. Load the reaction plate into the instrument tray.

   IMPORTANT! Orient the correct A1 position for Fast and Standard plates.

   e. Click **Start Run**. The instrument tray rotates to the IN position. During the run, the instrument, it displays real-time status information in the **Instrument > Real-Time** tab and records the fluorescence emissions.

Notes



f. *Optional*. View the data generated in real-time during the amplification run:

3. To run multiple plates, using the Automation Accessory, see Appendix B, "SDS Automation Controller Software," on page 73.

After the run, the status values and buttons are grayed-out, the Analysis button ( ) is enabled (turns green).

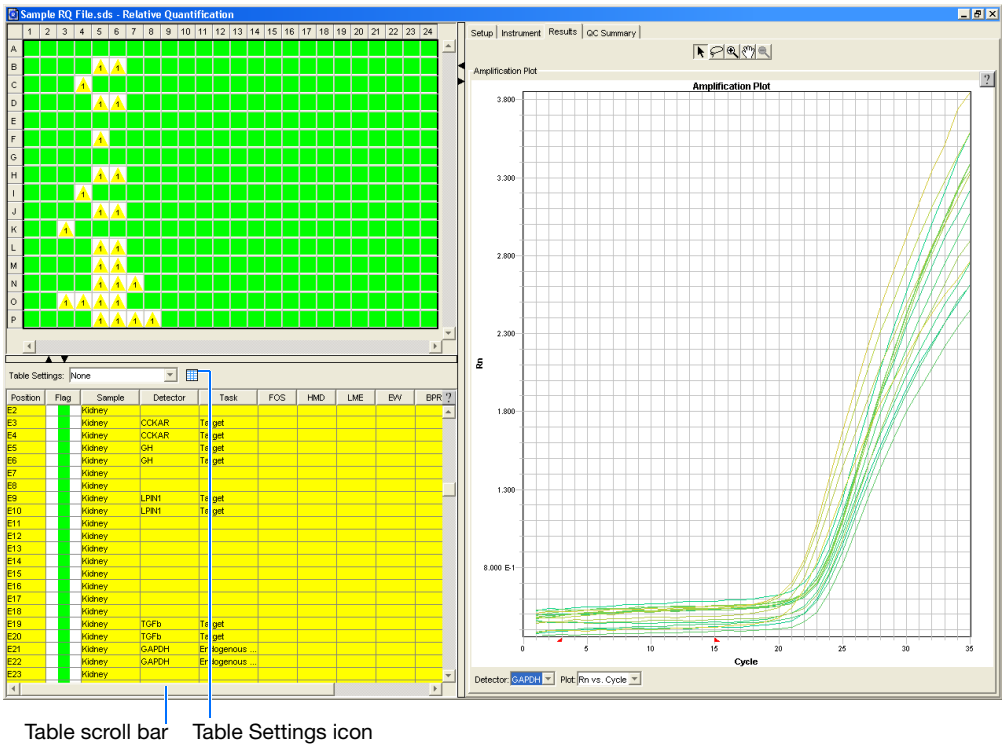
All data generated during the run are saved to the RQ Plate document that you specified in step 2 on page 37.

Notes

Analyzing and Viewing the RQ Data

For the SDS software to analyze RQ Plate data after the run, click or select **Analysis > Analyze**. The SDS software mathematically transforms the raw fluorescence data to establish a comparative relationship between the spectral changes in the passive reference dye and those of the reporter dyes.

Once the plate is analyzed, the SDS software displays the flags associated with each well in the plate grid (for more information on flags, see Appendix C, "Flags and Filtering for Relative Quantitation Using Comparative CT Plate Documents," on page 81. It also displays the resulting plate data in a table underneath the plate grid. The Results tab displays the results data of each well, including an amplification plot with the calculated R_n value.



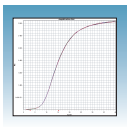
Note: You can configure the Results table entries. Click on (located above the table) or the drop down list (if a profile was previously created). Modify the table display settings accordingly (for more information, see the *SDS Online Help*). You can also can also sort each column by double-clicking on the desired column heading. To view additional columns, scroll to the right using the scroll bar.

About the Results Tab

In the Results tab, you can view the results of the run and manually adjust the baseline and threshold. Details on each tab are provided in the *SDS Online Help*.

- To move between views, click on a tab or the icons on the menu bar.

Notes



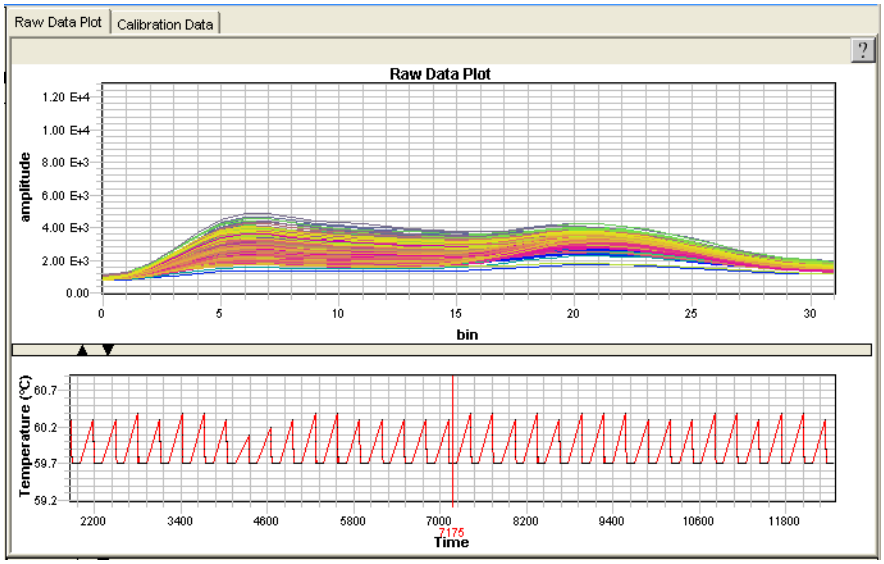
- To select all wells on a plate, click the upper-left corner of the plate well grid.
- To adjust amplification plot settings, double-click the y- or x-axes of a plot to display the Graph Settings dialog (note this option is not available in the Real-Time tab when the instrument is generating plot data). The adjustable settings depend on which plot you are viewing.

In the Results tab, you can see the amplification plot after the run is complete.

Click on (System Raw Data Pane icon) to view the Raw Data tab and Calibration Data tabs.

Raw Data Plot Tab

Here you can view a plot of the raw data.

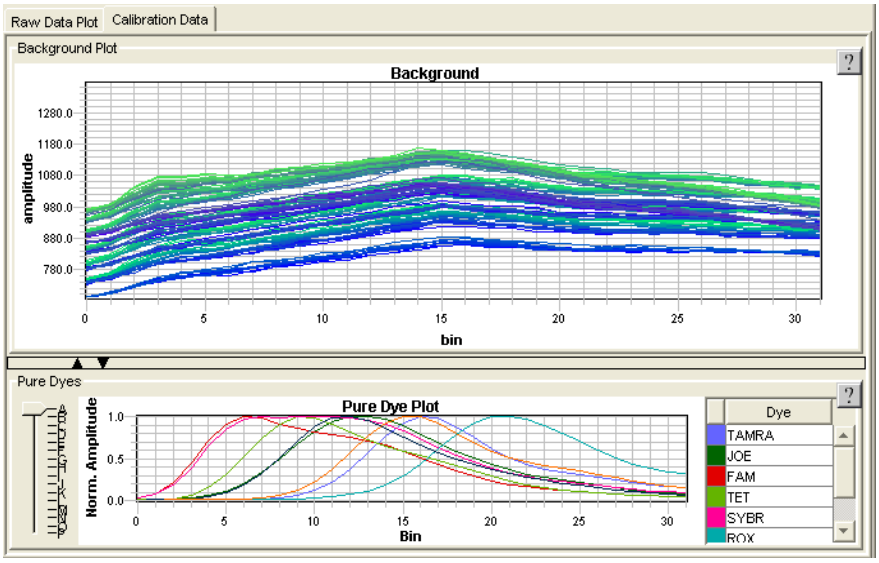


4

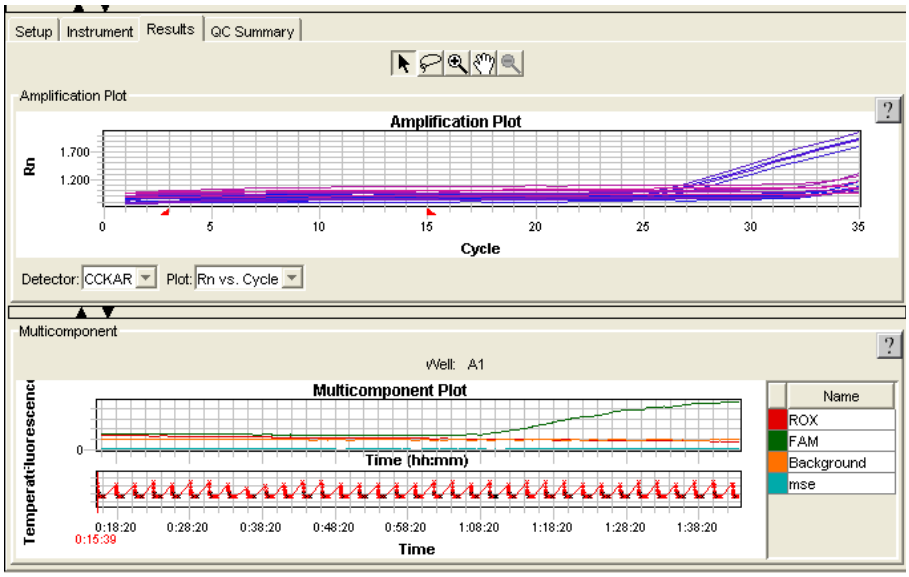
Notes

Calibration Data Tab

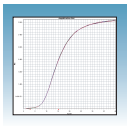
Here you can view background and pure dye data.



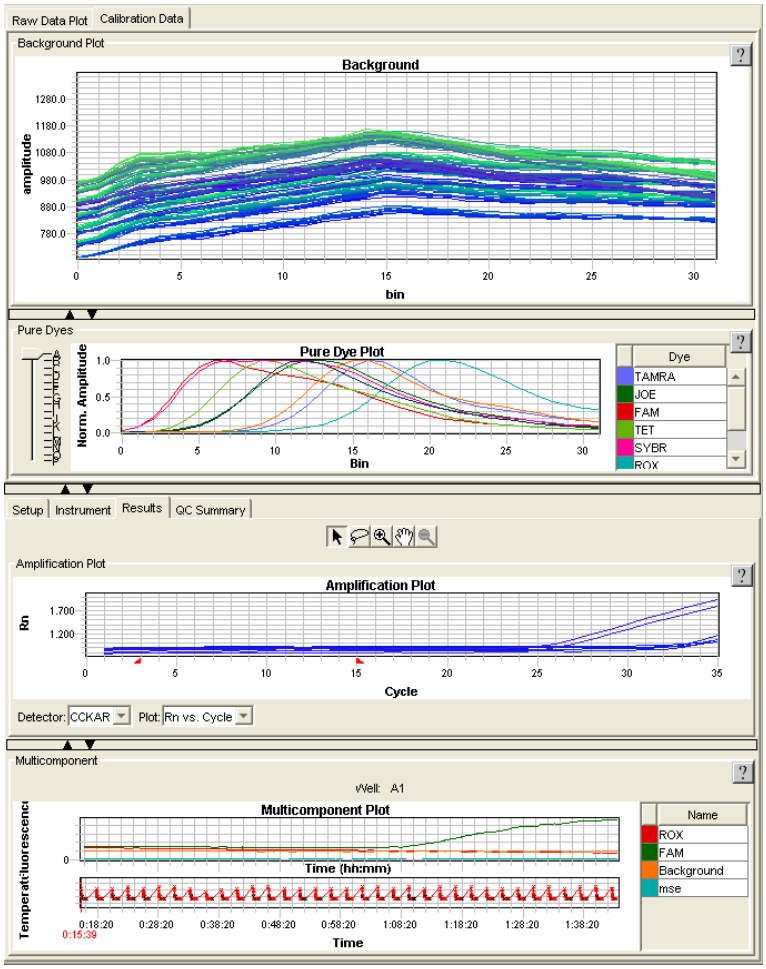
Click on (Multicomponent Data Pane icon) to view the Multicomponent plot.



Notes

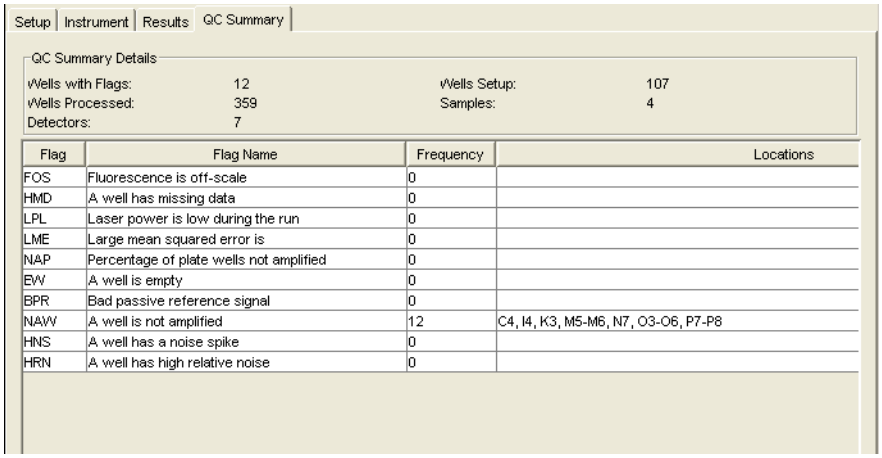


You can view all of this information in multiple pane format. See figure below.



QC Summary Tab

The QC Summary tab details the status of wells that were processed and flagged.



Flag	Flag Name	Frequency	Locations
FOS	Fluorescence is off-scale	0	
HMD	A well has missing data	0	
LPL	Laser power is low during the run	0	
LME	Large mean squared error is	0	
NAP	Percentage of plate wells not amplified	0	
EW	A well is empty	0	
BPR	Bad passive reference signal	0	
NAW	A well is not amplified	12	C4, I4, K3, M5-M6, N7, O3-O6, P7-P8
HNS	A well has a noise spike	0	
HRN	A well has high relative noise	0	

Notes

Reanalyzing Data

Raw fluorescence data, R_n values, and well information (sample name, detector, and detector task) are saved in an RQ plate document.

Note: After the software analyzes data, the Analyze button is disabled ( ). Whenever you change a setting that requires reanalysis, the Analyze button is enabled ( ).

Omitting Samples

Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outlying data (outliers) can result in erroneous measurements.

To ensure precision, carefully view replicate groups for outliers. You can remove outliers manually using the C_T vs. Well Position Amplification Plot. If you decide to omit wells or change well information after a run is complete, you must reanalyze the data.

For more information about omitting samples, refer to the *SDS Online Help*.

Notes

Post-Analysis Options

The following options are available after the analysis:

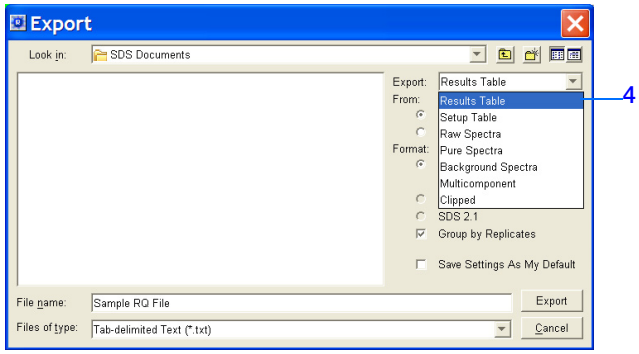
- Exporting Data as Text Files (below)
- Exporting Data as Graphic Files (page 46)
- Printing a Data Report (page 47)

Exporting Data as Text Files

You can export raw or analyzed data from plate documents into tab-delimited text files (*.txt). The text files can then be imported into spreadsheet software, such as Microsoft® Excel software.

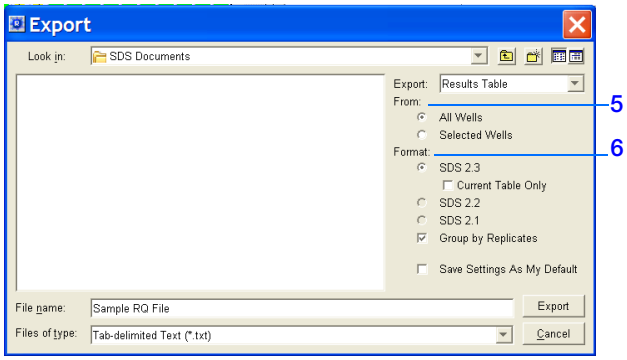
1. Open the plate document from which you wish to export data.
2. Select **File** > **Export** to open the Export dialog box.
3. In the Look in field, navigate to and select a directory for the new file.
4. From the Export drop-down list, select the data type to export.

   Note: For more information about the data type options, see the *SDS Online Help*.



5. Select to export data from **All Wells** or a select group of wells (**Selected Wells**).

   Note: To use the Selected Wells option, you must select a subset of wells in the plate document before opening the Export dialog box.



6. From the Format options, select the appropriate software version.
7. *Optional.* Check **Save Settings As My Default**. Check this option if you want the SDS software to automatically apply the current settings every time you export data as a text file.

Notes

8. In the File name field, type a name for the new file.

   Note: The name of the dialog box depends on the type of data you selected to export.

9. From the Files of type drop-down list, select **Tab-delimited Text (*.txt)**.

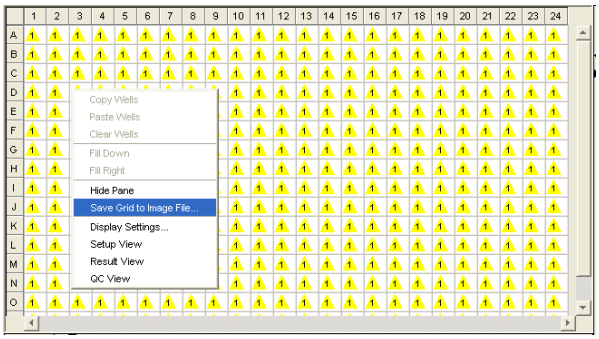
10. Click **Export**. The software exports the new file to the specified directory.

Exporting Data as Graphic Files

You can export the plate grid and plot images from plate documents as Joint Photographic Experts Group (JPEG) graphic files (*.jpeg). The JPEG files can then be viewed in most common word processing, spreadsheet, and HTML-based software.

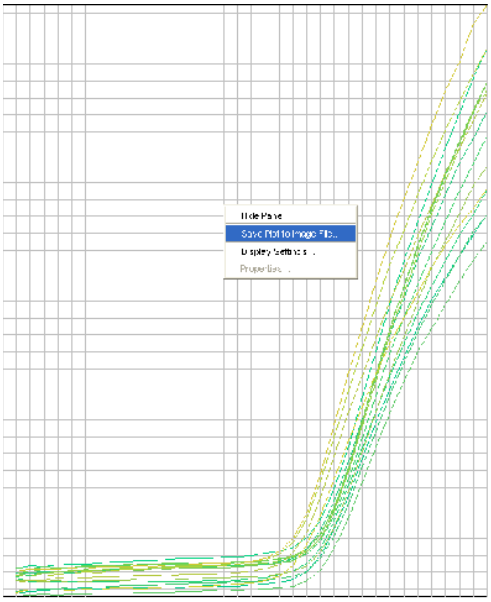
1. Open the plate document from which you wish to export data.

2. To export the:
   - Plate grid, right-click in the plate grid, then select **Save Grid to Image File**. The Save dialog box appears.



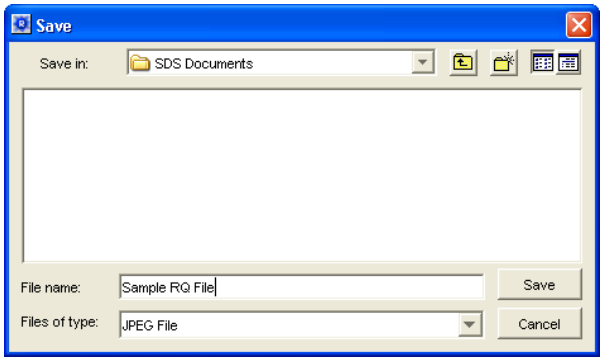
   - Plot, right-click in the plot, then select **Save Plot to Image File**. The Save dialog box appears.

     Note: If desired, adjust the plot dimensions (length and width) as you want them to appear in the exported file. The exported file retains the dimensions of the original screen element.



Notes

3. Complete the Save dialog box:
   a. In the Save in field, navigate to and select a directory for the new file.
   b. In the File name field, type a name for the new file.
   c. From the Files of type drop-down list, select **JPEG File**.
   d. Click **Save**. The software saves the new file to the specified directory.

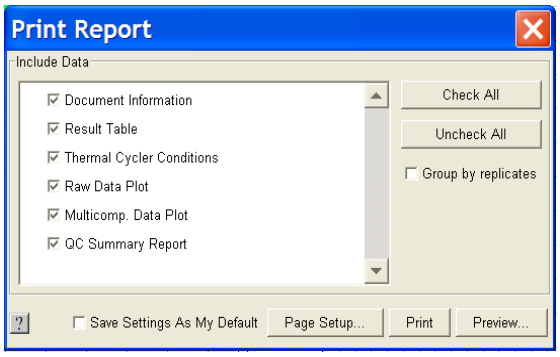


Printing a Data Report

You can use the SDS software to print a report of the analyzed data containing individual or multiple elements of the plate document.

Note: For more information on printing reports, see the *SDS Online Help*.

1. Open the plate document from which you wish to print a report.
2. In the plate grid, select the wells you wish to include in the report.
3. Click (or select **File** > **Print Report**) to open the Print Report dialog box.
4. In the Include Data pane, select the plate document element(s) to print.



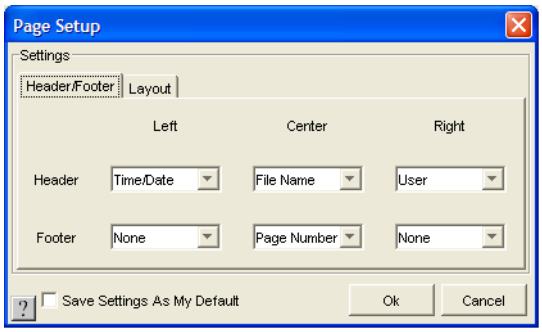
Notes

5. To format the display of the report and how the report is printed, click **Page Setup**.

6. *Optional.* Check **Save Settings As My Default**. Check this option if you want the SDS software to automatically apply the current settings every time you print a report.

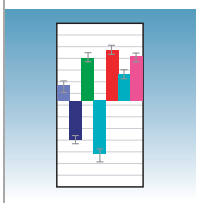
7. *Optional.* Click **Preview** to view the report before printing it.

8. Click **Print**.

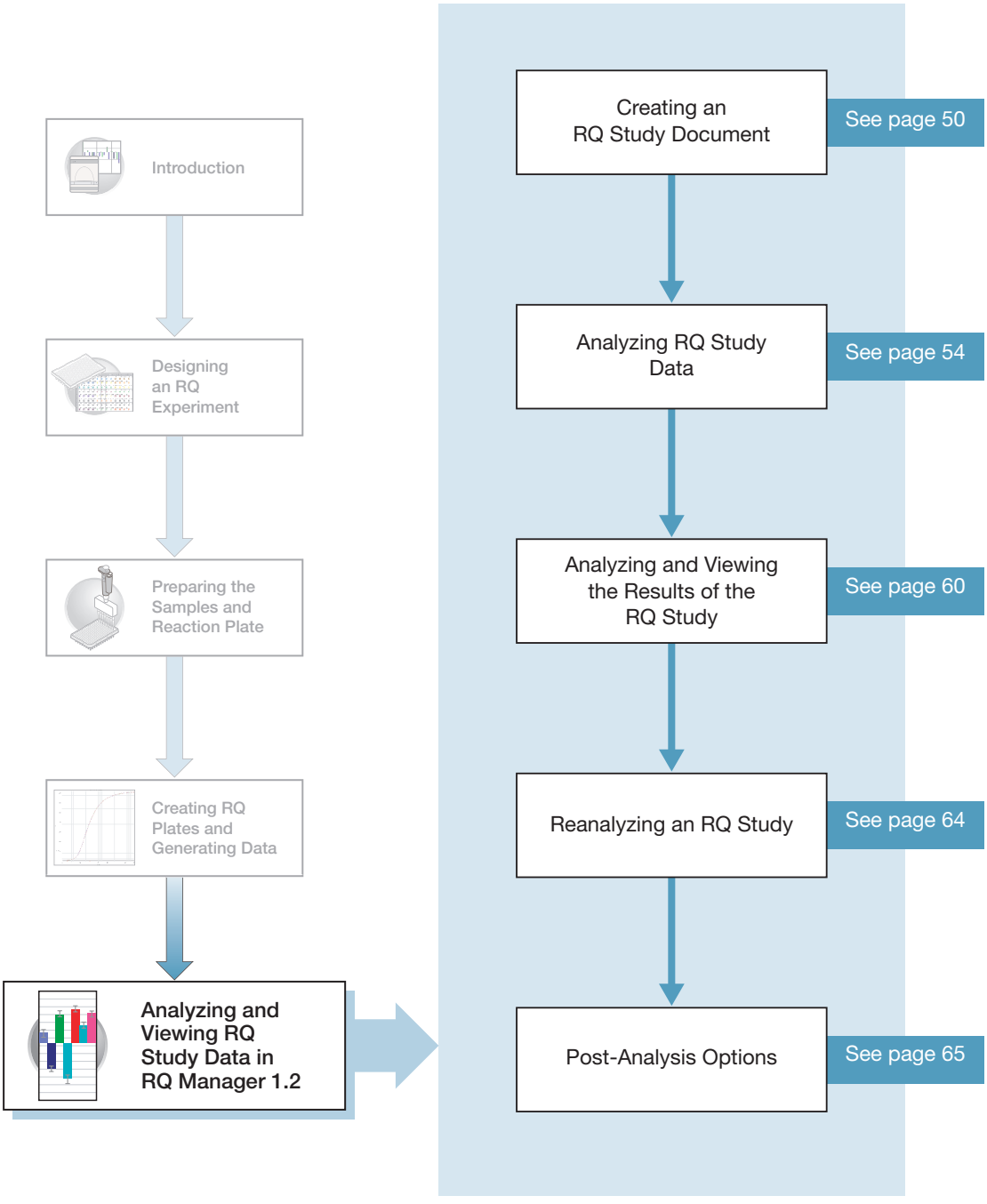


For more information, refer to the *SDS Online Help* for information about exporting files.

Notes



Analyzing and Viewing RQ Study Data in RQ Manager 1.2



Notes

Creating an RQ Study Document

You can conduct a comparative analysis of RQ plates in a study or analyze a single RQ plate experiment, in RQ Manager 1.2 software. This functionality is a separate application from the main SDS software. In order to analyze and view RQ study data, you must first create an RQ Study document.

- The RQ Manager 1.2 software uses the comparative C_T method ($2^{-\Delta\Delta Ct}$) of relative quantitation. For more information about methods of calculating relative quantitation, refer to *Real-Time PCR Systems Chemistry Guide* (PN 4363978).

In an RQ study, you can...	You cannot...
• Select the endogenous control and the calibrator sample. • Select the control type (if your plate contains both multiplexed and non-multiplexed reactions) • Set baseline and threshold values and RQ Min/Max Confidence Levels • Omit individual wells or sample replicates.	• Create, add, or modify samples. • Create, add, or modify detectors. (You can perform these operations in RQ Plate documents.)

Notes

To create an RQ Study document:

1. Double click on .

2. If your System Administrator has enabled the login option, the Login dialog box appears. Enter your **User Name** and **Password**, then click **OK**.

   Note: If the login option is not enabled, no Login dialog box appears. Skip to step 3 below.

3. Select **File**> **New Study** > **From SDS File**.

   Note: To add plates to an existing study, go to "Adding Plates to an Existing Study:" on page 52.

4. In the New Study dialog box:
   a. select the Container type.
   b. Enter a name in the Study Name field.
   c. Click **Add Plates**.

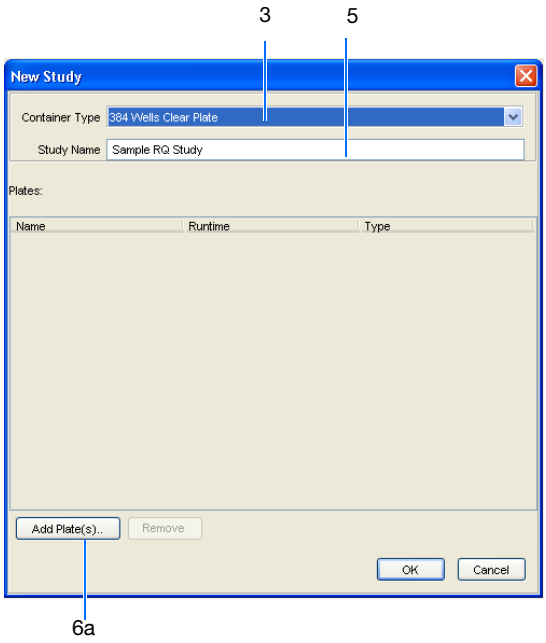
5. In the Add Plates dialog box:
   a. Select the desired plate file(s) that you want to add to the study.
   b. Click **Add** > >.

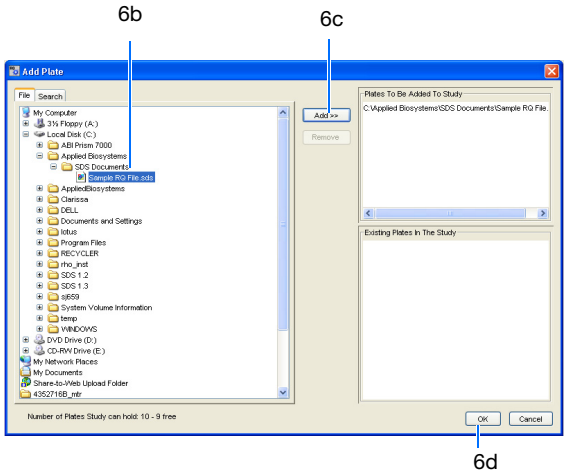
      Note: You can add up to 10 RQ plates to an RQ study.

      IMPORTANT! All plates added to a study must have identical thermal cycling parameters—the same number of steps, cycles, sample volume, and emulation mode.

   c. After you add all the desired plates, click **OK**.

6. In the New Study dialog box, click **OK**.





Notes

Adding Plates to an Existing Study:

1. Select **File > Load Study > From File**.
2. Navigate to the location where your file is stored. Note that the study must have been saved as an *.sdm file. Click **Open**.
3. To add additional plates to the study go to step 5 on page 51.

The RQ Manager software opens a new RQ Study document and displays the RQ Study (detector centric view). Click on the left corner of the detector grid to view the amplification plot for all detectors.

Amplification Tab

In this tab, there are three different menu bar drop-down lists. You can select different formats to view the study information.

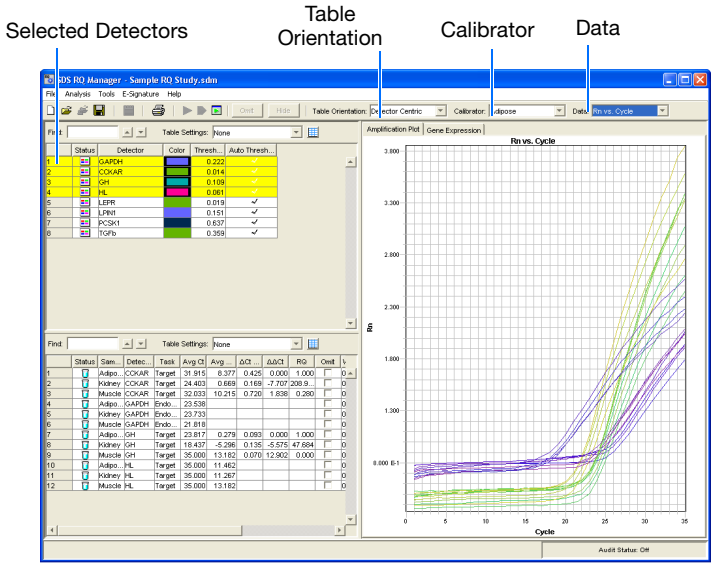


Table Orientation drop-down list allows you to view the information either in Detector Centric (the detectors appear at the top left of the screen), Sample Centric (the samples appear at the top left of the screen), or Plate Centric (the number of plates, associated flags and file name appear at the top left of the screen) formats.

Calibrator drop-down list allows you to select the tissue sample to be used as the calibrator.

Data drop down list allows you to select the data to display (Rn vs Cycle, ΔRn vs. Cycle, C_T vs. Well Position).

Notes

Gene Expression Tab

In this tab, there are three different menu bar drop-down lists. You can select different formats to view the study information.

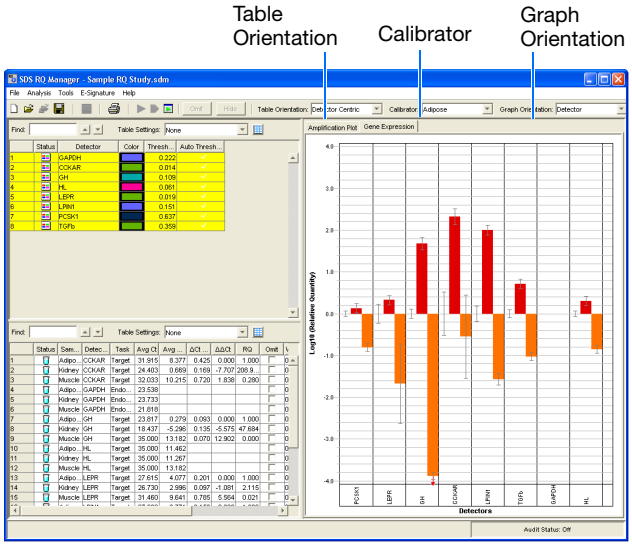


Table Orientation drop-down list allows you to view the information either in Detector Centric (the detectors appear at the top left of the screen), Sample Centric (the samples appear at the top left of the screen), or Plate Centric (the number of plates, associated flags and file name appear at the top left of the screen) formats.

Calibrator drop-down list allows you to select the tissue sample to be used as the calibrator.

Graph Orientation drop-down list allows you to view Detectors or Samples.

Note: If your experiment uses only a single plate, there must be at least two different samples that have different names and have their own endogenous controls. (You can go back to a saved RQ Plate document and change the sample names, if necessary.)

Note: You can save the RQ Study document now, or wait until after specifying analysis settings and analyzing the data. To save the study, go to "Saving the RQ Study" on page 65

Notes

Analyzing RQ Study Data

Starting the Analysis

For RQ Manager to analyze the RQ Study, click or select **Analysis > Analyze All**.

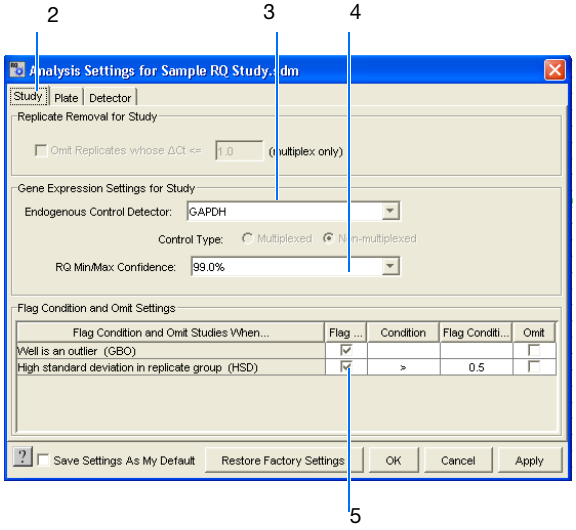
Configuring Analysis Settings

Before analyzing the resulting study data, specify parameter values for the analysis.

Unless you have already determined the optimal baseline and threshold settings for your experiment, use the default automatic baseline and threshold options of the RQ Manager software (Automatic C_T). If the baseline and threshold were called correctly for each well, you can proceed to view the results. Otherwise, you can manually set the baseline and threshold as described on "Manually Setting Baseline and Threshold Settings" on page 56. This section describes how to use the Automatic C_T feature.

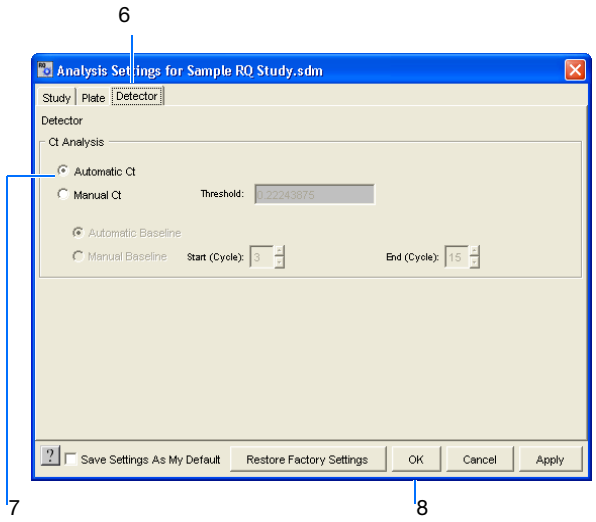
Configuring Automatic Analysis Settings

1. Click or select **Analysis > Analysis Settings**.
2. Select the Study tab.



3. In the Endogenous Control Detector drop-down list, select the desired detector.
4. In the RQ Min/Max Confidence drop down list, select the desired percentage. For more information, see the *SDS Online Help*.
5. In the Flag Condition and Omit Settings panel, select or deselect the appropriate flags. For more information, see the *SDS Online Help*.
6. Select the Detector tab.

Notes



7. Select **Automatic Ct**. The RQ Manager software will automatically generate baseline values for each well and threshold values for the selected detector. Note that, alternatively, you can select Manual Ct and specify the threshold and select Automatic Baseline or Manual Baseline (see "Manually Setting Baseline and Threshold Settings" on page 56).

   IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each well by clicking on the well in the detector grid and viewing the resulting plots.

8. Select **OK**.

9. Select or **Analyze**.

10. Select the Amplification or Gene Expression tab and examine the results.

Notes

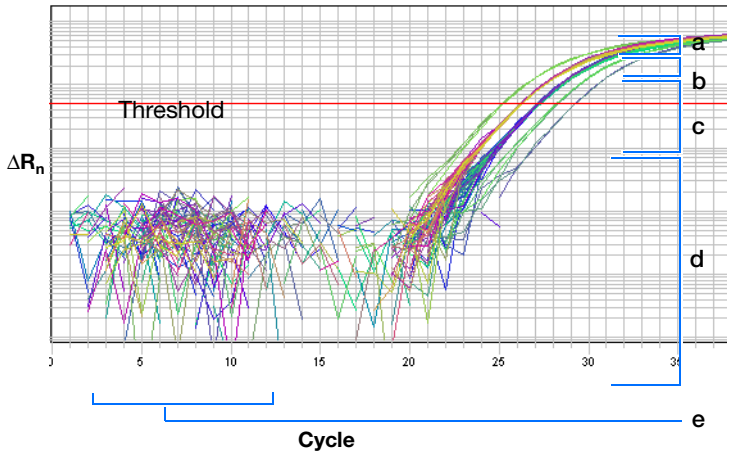
Adjusting the Baseline and Threshold

Automatic Baseline and Threshold Determination

The SDS software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the "typical" amplification curve.

A typical amplification curve has a:

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



Experimental error (such as contamination, pipetting errors, and so on) can produce data that deviate significantly from data for typical amplification curves. Such atypical data cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

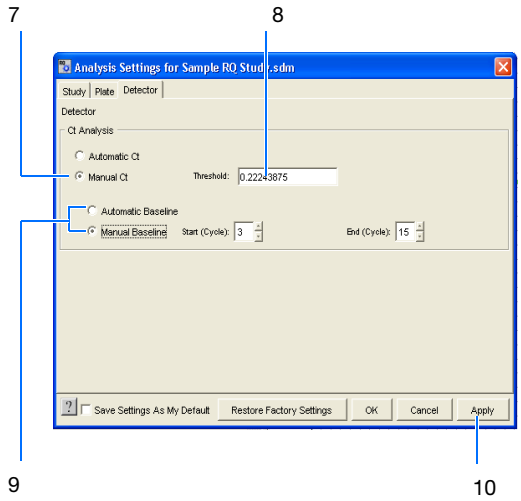
Therefore, Applied Biosystems recommends reviewing all baseline and threshold values after analysis of the study data. If necessary, adjust the values manually as described below.

Manually Setting Baseline and Threshold Settings

1. Click or select **Analysis > Analysis Settings**.
2. Select the Study tab.
3. In the Endogenous Control Detector drop-down list, select the desired detector.
4. In the RQ Min/Max Confidence drop down list, select the desired percentage. For more information, see the *SDS Online Help*.
5. In the Flag Condition and Omit Settings panel, select or deselect the appropriate flags. For more information, see the *SDS Online Help*.

Notes

6. Select the Detector tab.



7. Select **Manual Ct**.

8. Enter a threshold value in the Threshold field.

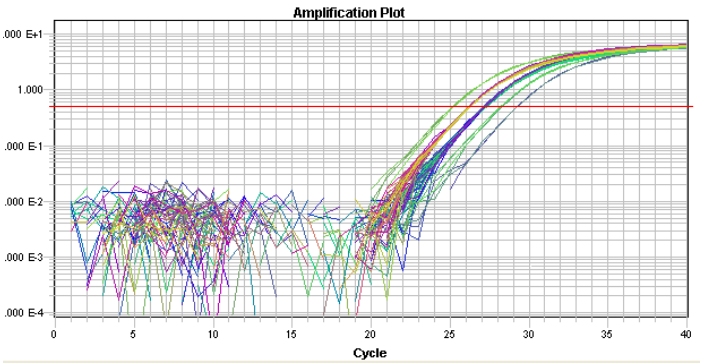
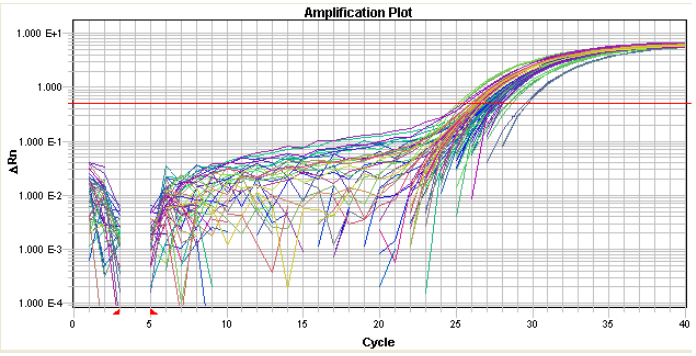
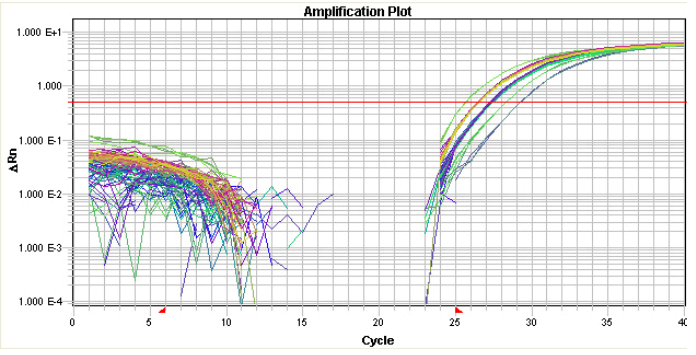
9. Select **Automatic Baseline** or **Manual Baseline**. If setting the baseline manually, specify the Start and End cycles.

10. Click **Apply** to view the new setting adjustments on the plate document but keep the Analysis Settings dialog box open. Alternatively, click **OK** to apply the new settings and close the dialog box.

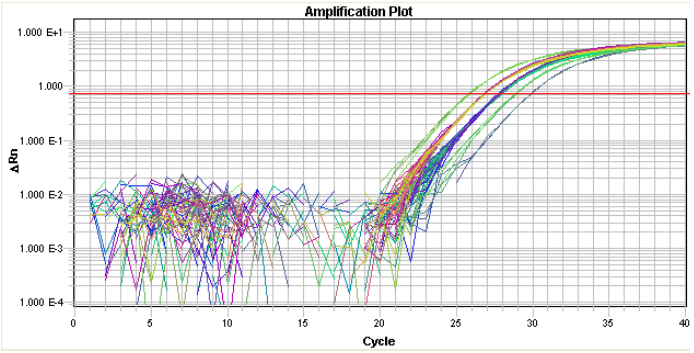
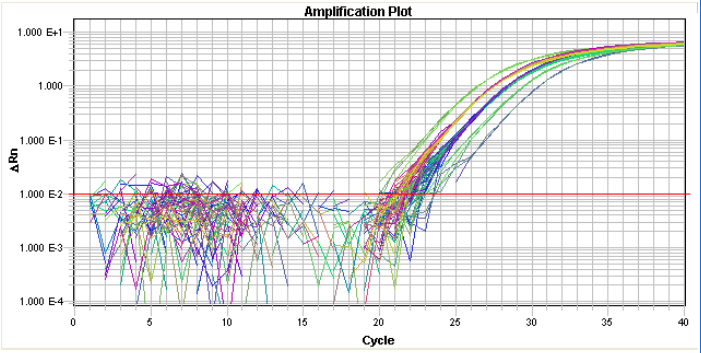
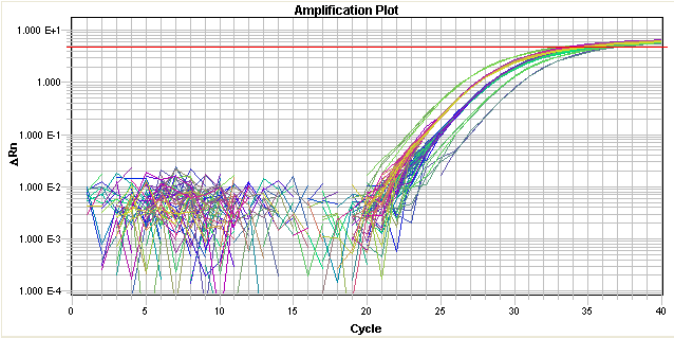
IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each well by clicking on the well in the plate grid and viewing the resulting plots.

Notes

See the sample amplification plots below to determine whether the baseline and threshold settings were correctly set.

Baseline Set Correctly The amplification curve begins after the maximum baseline. No adjustment necessary.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

Notes

Threshold Set Correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold Set Too Low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.	
Threshold Set Too High The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.	

Notes

Analyzing and Viewing the Results of the RQ Study

Selecting Detectors to Include in Results Graphs

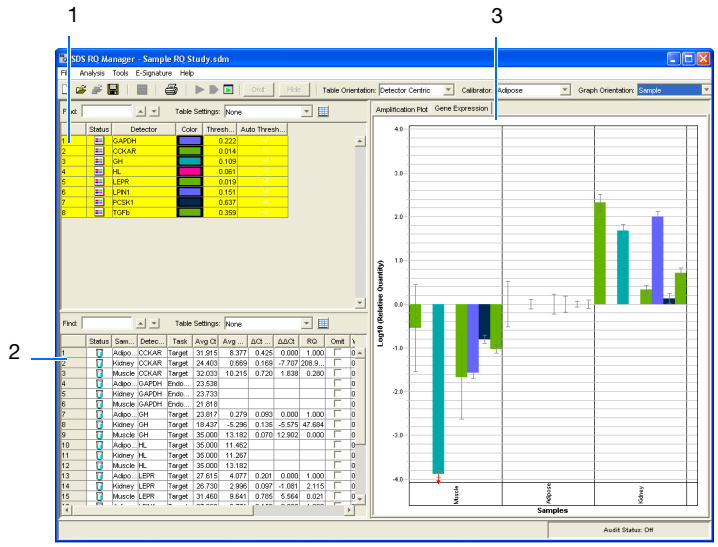
In the Detector table, select detectors to include in the result graphs by clicking a detector. (Ctrl-click to include multiple detectors; Click-drag to include multiple adjacent detectors.)

The corresponding samples appear in the Sample grid. Depending on which tab you select Amplification Plot or Gene Expression analysis results are displayed.

Sample Experiment

Suppose that you want to view the comparative gene expression levels of the following genes when adipose is used as the calibrator: CCKAR, GH, CCR2, LEPR, LPIN1, TGFb, PCSK1, and HL. Selecting the detectors in the RQ Detector grid (1) displays the sample information in the RQ Sample grid (2) and in a result graph in the Gene Expression tab (3). Note that:

- The Gene Expression tab is selected, and the gene expression levels are sorted by detector.
- Gene expression levels for muscle samples are indicated by the green bar; those for kidney samples by the blue bar. These colors also indicate the samples in the RQ Sample Grid and the RQ Results Panel plots.
- Because adipose samples are used as calibrators, the expression levels are set to 1. But because the gene expression levels were plotted as $\log_{10}$ values (and the $\log_{10}$ of 1 is 0), the expression level of the calibrator samples appear as 0 in the graph.
- Because the relative quantities of the targets are normalized against the relative quantities of the endogenous control, the expression level of the endogenous control is 0; there are no bars for GAPDH.
- Fold-expression changes are calculated using the equation $2^{-\Delta\Delta CT}$.



Notes

Amplification Plot

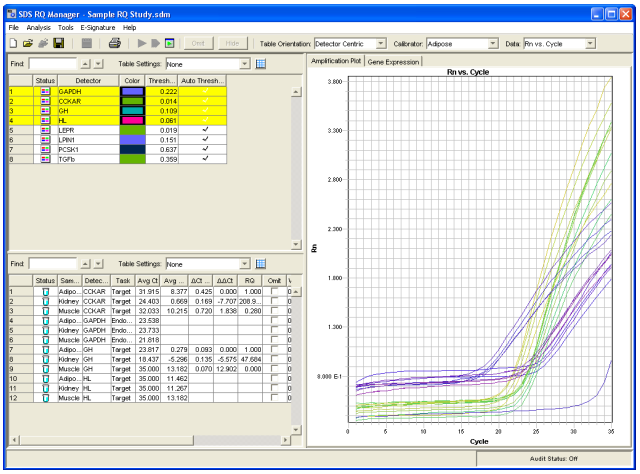
The three Amplification Plots allow you to view post-run amplification of specific samples. The Amplification Plots display all samples for selected detectors.

You can adjust graph settings selecting **Analysis > Graph Settings**.

Rn vs. Cycle View

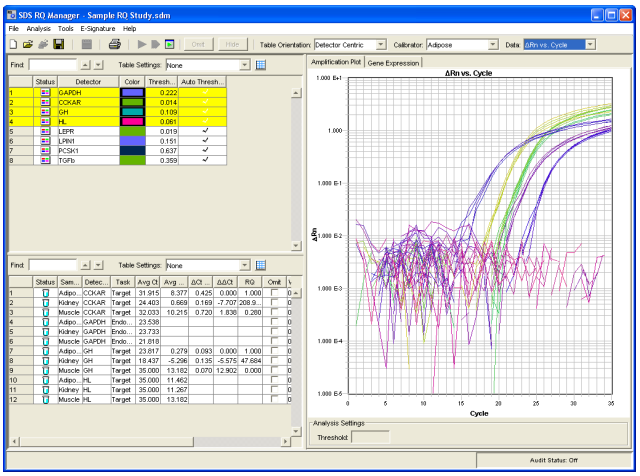
Displays normalized reporter dye fluorescence (R_n) as a function of cycle. You can use this plot to identify and examine irregular amplification.

For more information about R_n, refer to the *Real-Time PCR Systems Chemistry Guide*.



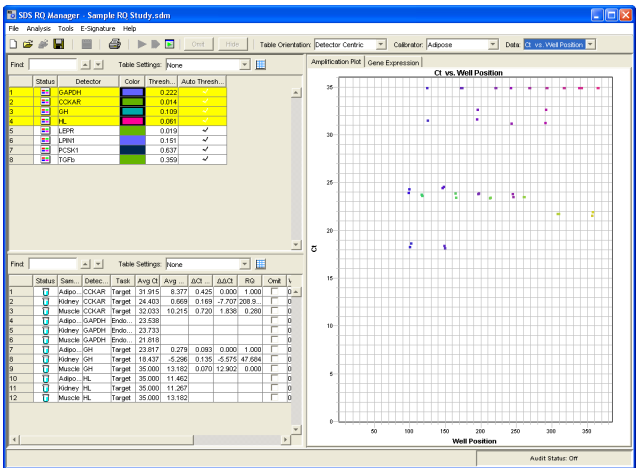
ΔRn vs. Cycle (Log) View

Displays dye fluorescence (ΔR_n) as a function of cycle number. You can use this plot to identify and examine irregular amplification and to manually set the threshold and baseline parameters for the run.



C_T vs. Well Position View

Displays threshold cycle (C_T) as a function of well position. You can use this plot to locate outliers from detector data sets (see "Omitting Samples from a Study" on page 65 for more information).



Notes

Gene Expression Plot

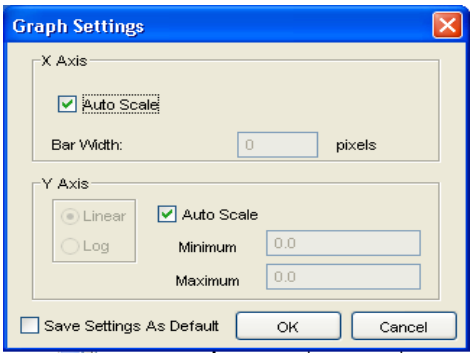
Gene Expression plots show the expression level or fold-difference of the target sample relative to the calibrator.

Because the calibrator is compared to itself, the expression level for the calibrator is always 1.

Adjusting Graph Settings

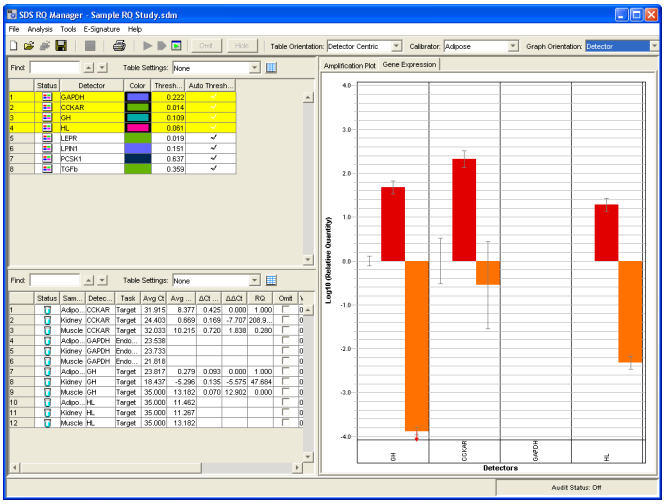
You can adjust graph settings for gene expression plots in the Graph Settings dialog box, including Autoscaling for the X- and Y-Axes and Bar Width.

To access the Graph Settings, select **Analysis > Graph Settings**. Refer to the *SDS Online Help* for more information about adjusting these graph settings.



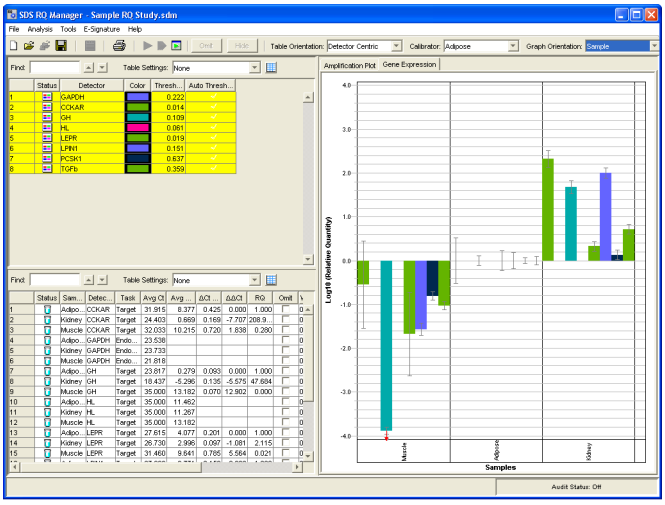
Gene Expression Plot Graph Orientation: Detector

Detectors are plotted on the x-axis, and each bar shows the RQ value of a replicate sample group.



Gene Expression Plot Graph Orientation: Sample

Samples are plotted on the x-axis, and each bar shows the set of RQ values of a replicate group.



Notes

Error Bars for Gene Expression Plots

The RQ Manager software displays error bars for each column in the plot, provided that the associated expression level was calculated from a group of two or more replicates. The error bars display the calculated maximum (RQMax) and minimum (RQMin) expression levels. Collectively, the upper and lower limits define the region of expression within which the true expression level value is likely to occur.

The RQ Manager software calculates the error bars based on the RQMin/Max Confidence Level in the Analysis Settings dialog box.

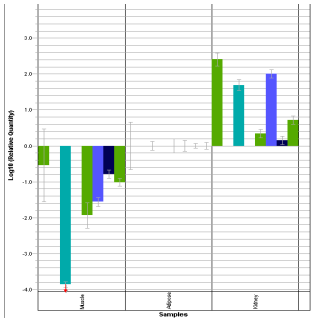
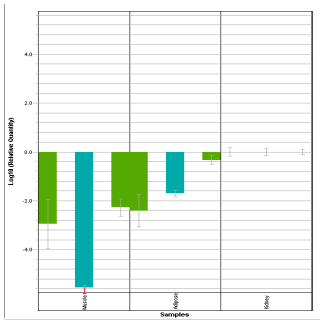
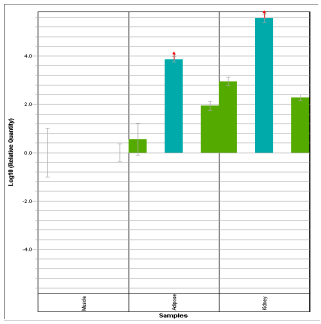
Notes

Reanalyzing an RQ Study

If you change any of the analysis settings, you must reanalyze the data before you can view results. You can switch between the variations of the Amplification and Gene Expression plots without having to reanalyze the data.

Suppose you select Adipose as the calibrator, then perform an analysis. Next, you view the Amplification and Gene Expression plots. If you then want to use Kidney or Muscle as the calibrator, you need to reanalyze the data before viewing results.

Similarly, if you want to change the baseline or threshold values, the endogenous control, the control type, or the RQ Min/Max parameters, you need to reanalyze your data.

Calibrator	Gene Expression Plot
Adipose	
Kidney	
Muscle	

Notes

Omitting Samples from a Study

Experimental error may cause some wells to amplify insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outlying wells (outliers) can result in erroneous measurements.

To ensure precise Comparative C_T, you must carefully view replicate groups for outliers. You can remove outliers manually using the C_T vs. Well Position Amplification Plot. If you decide to omit wells or change well information after a run is complete, you must reanalyze the data.

For more information on how to omit samples, see the *SDS Online Help*.

Post-Analysis Options

Saving the RQ Study

You must save the Study before exporting data.

1. Select **File > Save As**. The Save SDS 2.x RQ Study file dialog box opens.
2. Enter a name in the File name field box.
3. Click **Save**.

Exporting RQ Study Data

You can export numeric data from RQ studies into text files, which can then be imported into spreadsheet applications.

1. Select **File** > **Export**, then select the data type to export:
   - **Amplification Data**
   - **Expression Data**
   - **Clip Data**
   - **Results Data**
2. Enter a file name for the export file.
3. Click **Save**.

For more information, see the *SDS Online Help*.

Notes

Notes

Sample Experiment

Sample RQ Experiment

Sample Experiment

In the example RQ experiment, a detector is created for each target gene and the endogenous control. Eight detectors are created: 7 for the target genes and 1 for the endogenous control, GAPDH.

For example, the detector for the cholecystokinin A receptor gene is named CCKAR and assigned a green color. Because all TaqMan Gene Expression Assays have probes that are labeled with FAM™ dye, FAM was selected for the reporter dye. Additionally, TaqMan Gene Expression Assays use TaqMan MGB probes, which use non fluorescent quenchers. Thus, "None" is selected for the quencher dye.

Note: This experiment is a general example provided by Applied Biosystems and is intended to be used for familiarizing yourself with the software. Note that this procedure will be different for Low Density Arrays.

1. Design the experiment, as explained in Chapter 2 on page 9.
   a. Designate the targets, calibrator, endogenous control, and replicates.
   b. Order the reagents for TaqMan® probe-based chemistry.
   c. Order the appropriate TaqMan® Gene Expression Assays products, which provide predesigned primers and probes for the 7 genes.
2. Isolate total RNA from adipose, kidney, and muscle tissue, as explained in Chapter 3 on page 18.
3. Generate cDNA from total RNA using the High Capacity cDNA Archive Kit.

Notes

a. Prepare the reverse transcription (RT) master mix as indicated in the table to the right.

   Additional guidelines are provided in the *High Capacity cDNA Archive Kit Protocol*.

WARNING **CHEMICAL HAZARD.** **10 × RT Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

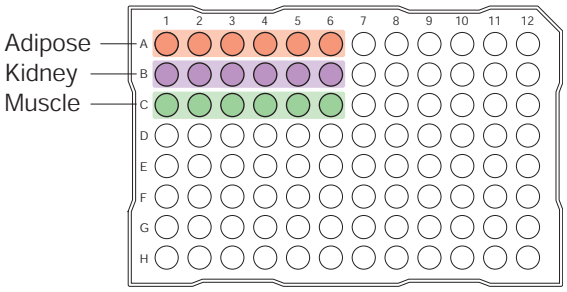
RT Master Mix - Standard Plate		
Component	**µL/Reaction**	**µL/21 reactions†**
10× Reverse Transcription Buffer	10	210
25× dNTPs	4	84
10× random primers	10	210
MultiScribe™ Reverse Transcriptase, 50 U/µL	5	105
Nuclease-free water	21	441
Total	50	1050

† Each RT reaction is 100 µL (see step 3b). If you need 5 µL cDNA for each of 104 PCR reactions per tissue (see step 4), you need 6 RT reactions per tissue. Extra volume (enough for one additional RT reaction per tissue) is included to account for pipetting losses, as well as extra cDNA for archiving.

b. Prepare the cDNA archive plate by pipetting into each well of the plate:

- 50 µL RT master mix
- 30 µL nuclease-free water
- 20 µL RNA sample

Make sure the amount of total RNA converted to cDNA is 10 to 100 ng in 5 µL for each 50-µL PCR reaction.



c. Verify the thermal cycler parameters and conditions (using the indicated parameter values for the RT step of the two-step RT-PCR method).

Note: You have the option to use one-step RT-PCR, as explained in "Selecting One- or Two-Step RT-PCR" on page 14.

Step Type	Time	Temperature
HOLD	10 min	25 °C
HOLD	120 min	37 °C

d. Store the cDNA at –20 °C until use.

Notes

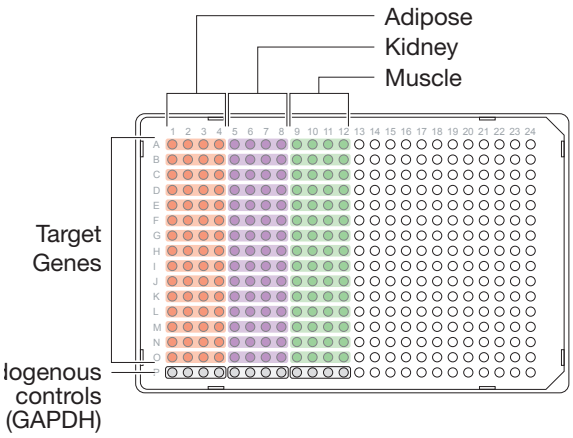
A

4. Prepare the PCR master mix (see Chapter 4 on page 28 for more information).

Note: The reaction volumes for TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assays are included on the CD which accompanies these products. Those for primers and probes designed with Primer Express software follow the universal assay conditions described in Chapter 4.

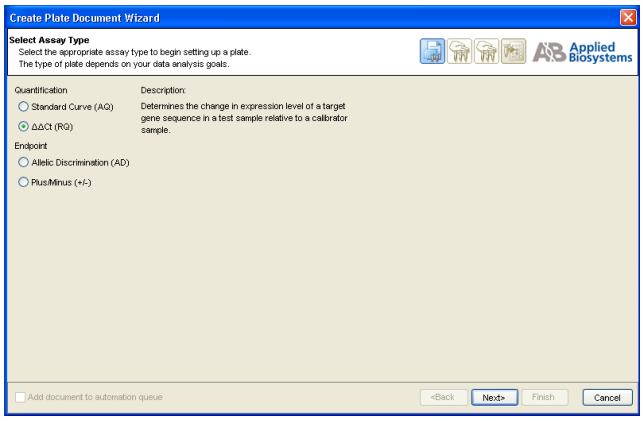
CAUTION **CHEMICAL HAZARD.** **TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

5. Prepare the reaction plate.
   a. Ensure that you include an endogenous control for each sample on the plate.
   b. Pipette 20 µL of the appropriate PCR master mix (containing cDNA) into each well of the standard 384-well plate.
   c. Keep the reaction plate on ice until you are ready to load them into the 7900HT Fast system.



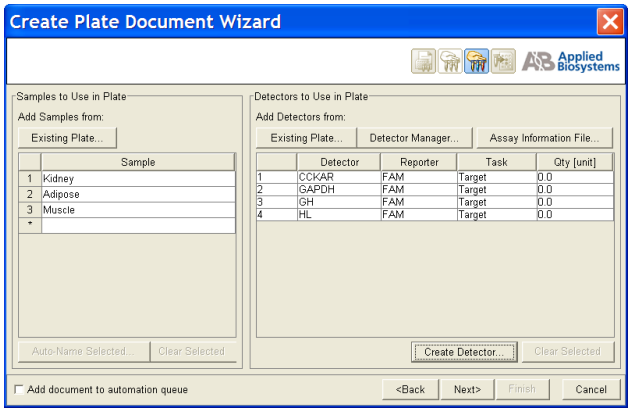
Notes

6. Create an RQ Plate document as described in "Creating an RQ Plate Document" on page 29.

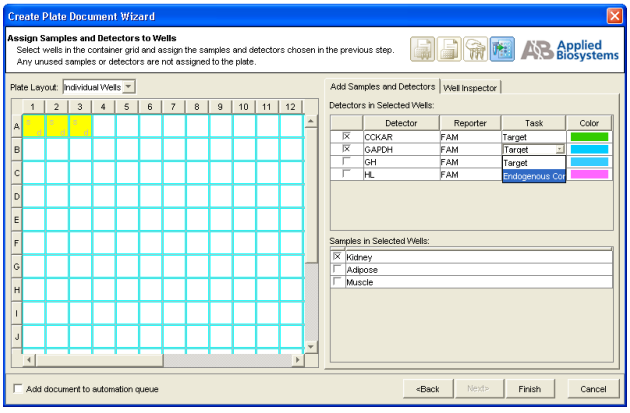


IMPORTANT! All plates added to a study must have identical thermal cycling parameters—the same number of steps, cycles, sample volume, emulation mode.
Also, you cannot use AQ Plate documents for RQ assays and vice versa. The information stored in AQ and RQ Plate documents is not interchangeable.
- You cannot import an AQ plate into an RQ study.
- You cannot analyze your relative expression data directly in an RQ plate setup.
- You cannot run a standard curve or a dissociation curve in an RQ plate setup.
- You can only analyze relative expression data in an RQ study setup.

a. Add sample names and detectors to the plate document.



b. Specify the detectors and tasks for each well, then click **Finish**.



IMPORTANT! If your experiment does not use all the wells on a plate, do not omit the wells from use at this point. You can omit unused wells after the run is completed. For more information about omitting wells, refer to the Online Help.

7. Select **Instrument > Start**. After the run a message displays as to whether the run was successful or whether errors were encountered.

8. Click to analyze the results.

Notes

9. Analyze the RQ data, as explained in "Analyzing and Viewing the RQ Data" on page 40.

10. If desired, save the RQ Plate document.

Notes

Notes

SDS Automation Controller Software

Overview

The Sequence Detection Systems (SDS) Automation Controller Software v2.3 provides an interface between the Zymark® Twister Microplate Handler, the 7900HT Fast System, the fixed-position bar code reader, and the plate documents created in main Sequence Detection Systems Software v2.3.

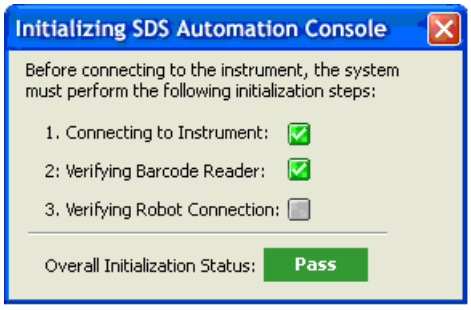
This Automation Controller software controls and coordinates the action of the 7900HT instrument and the automation module. It initiates and controls the sequence detection run and acquires data during the run.

This option allows for plates to be run as part of a group or batch allowing for high-throughput unattended operation. For more information about the Automation Controller, refer to the *SDS Online Help*.

Using the Automation Controller Software

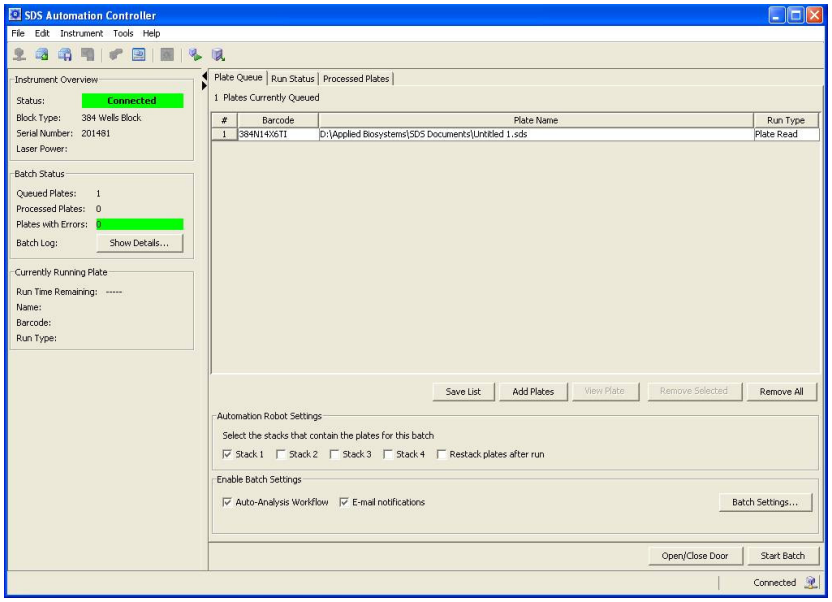
Note: Before launching the Automation Controller software, you must close the main Sequence Detection Systems Software v2.3 application. Failure to do so will result in an instrument connection failure.

1. Double click on the Automation Controller shortcut icon. An initialization pop-up will appear displaying the status of instrument connection and other verifications. If you encounter an error, refer to the *SDS Online Help*.



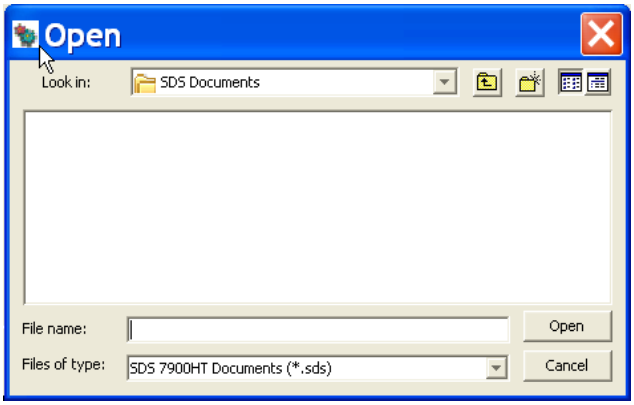
Notes

2. If you have already created and saved the desired plates (sent them to the queue) in the main SDS application, then the plates will appear in the Plate Queue tab. For more information about creating plate documents and sending them to the queue, refer to "Creating Plate Documents" on page 28.



3. You can add plates by clicking **Add Plates** or (or click).

   a. At the Open dialog box, navigate to the location where the plate files are located.

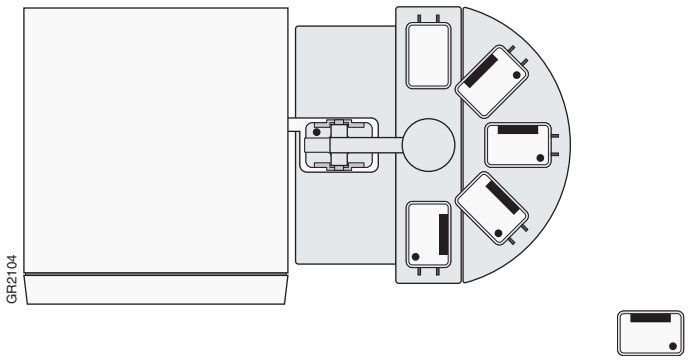


   b. Use the barcode reader to scan the Plate IDs. Note that you can automatically scan the next plate without having to select Add Plates again. You can also save the list of queued plates, by clicking **Save List**.

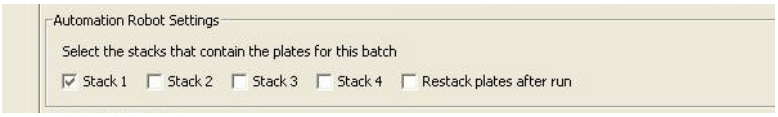
Note: To remove a plate, click on the plate name then click **Remove**. To remove all plates, click **Remove All**.

Notes

4. Place the plates in the input stack of the Zymark® Twister Microplate Handler. The order of the plates may vary from the order they were scanned in (the Handler's fixed barcode reader will verify the location of each plate). The software matches the plate document and the method before it starts the run. Orient the plates inside the stacks so that the well A1 of each plate corresponds to the locations shown in the figure below.

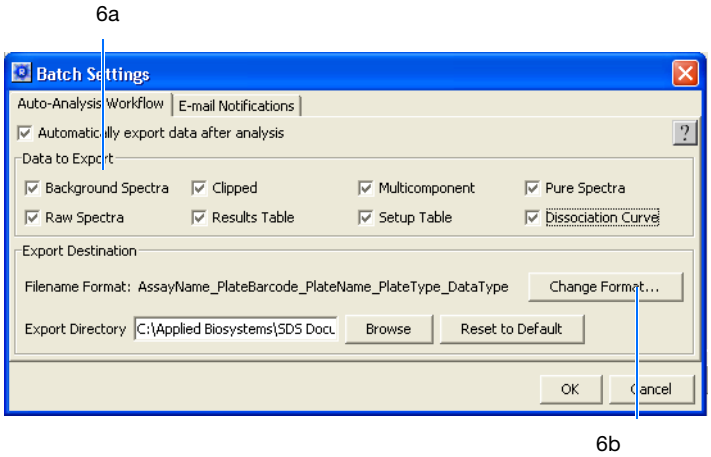


5. Click into the boxes next to each stack location where the plates are located.



6. Select **Tools > Batch Settings** to specify the desired output settings.

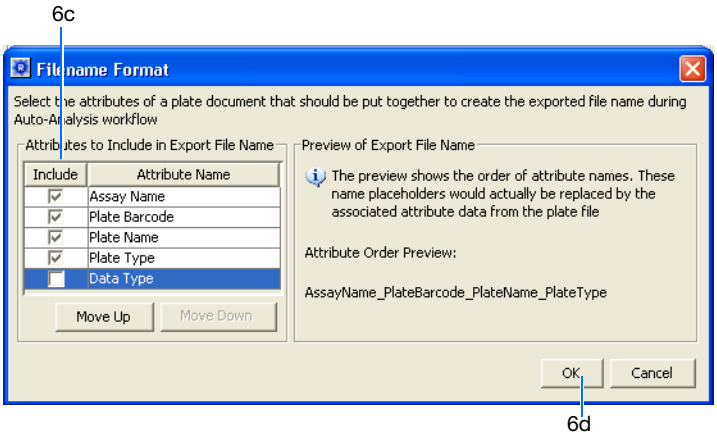
   a. In the Batch Settings dialog box, at the Auto-Analysis Workflow tab, click into a box to select that export option. You can also change the export directory by clicking the Browse button (located in the Export Destination box) and specifying a new location for the file data to be exported to. Note that exported data will be saved as *.txt files.



   b. Click on **Change Format** to assign attributes to be included in the export file name.

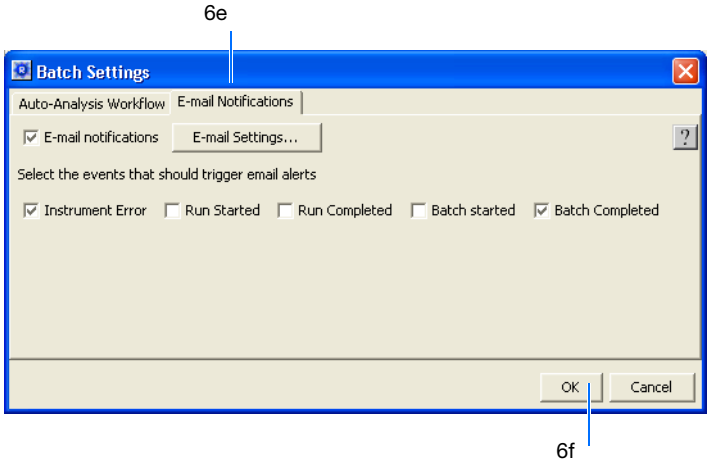
Notes

c. Click into a field box (under the Include column) to deselect an attribute.



d. Click **OK**.

e. Back at the Batch Settings dialog box, click on the **Email Notifications** tab to specify desired email notification options. Form more information on email settings, see your network administrator.

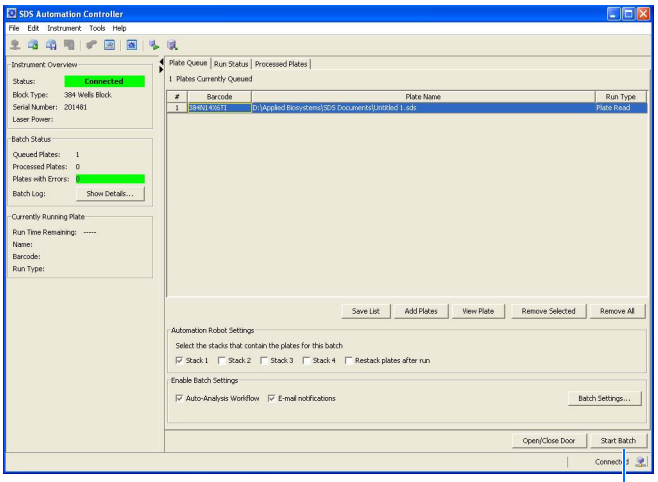


f. Click **OK**.

7. Verify that the plate adapter and the output stack of the Handler are empty.

8. Click **Start Batch**.

Notes

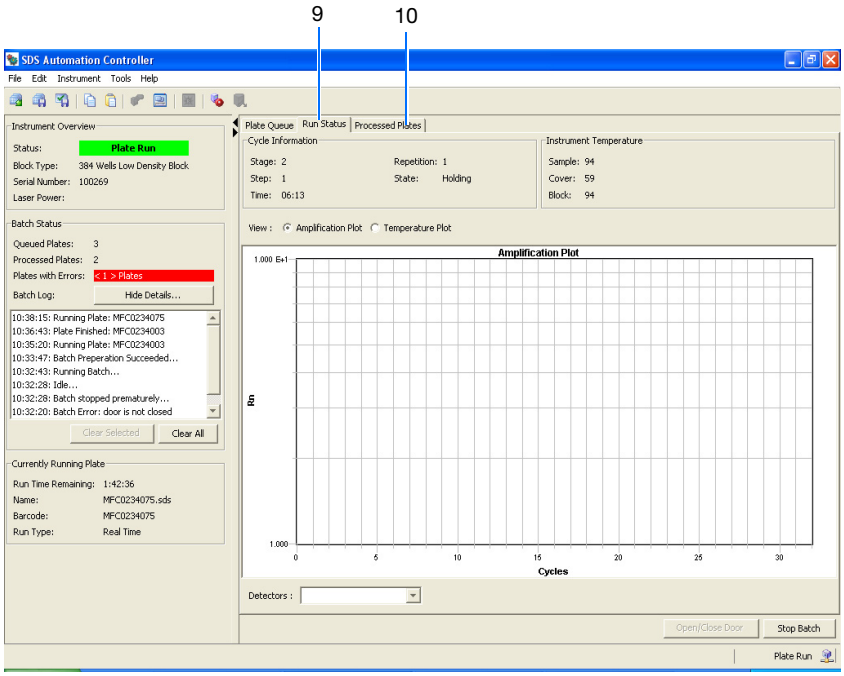


Note: Note that if the plate type does not match the plate block installed on the instrument, the Automation Controller will not start. You can assign plate types by selecting **Tools > Options** and selecting the desired plate format. The handler loads the plates and the instrument starts the run.

Note: To stop the run, click **Stop Batch**. You will see a warning message asking you to verify stopping the run.

9. Click on the **Run Status** tab to view the plate run status. Note that once a plate is being processed, it will not appear in the Processed Plates tab or be listed in the Queued Plates section of the Batch Status box. The plate information will be available under the Currently Running Plate section.

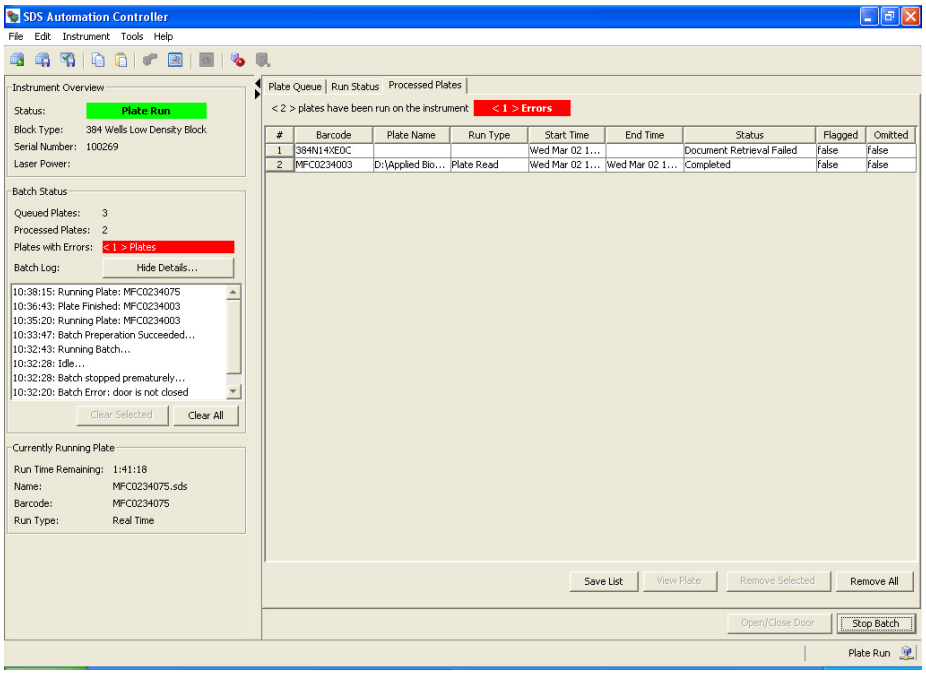
Notes



Note: For standard curve (AQ) and ddCt (RQ) plates, a view of the amplification and temperature plot will be available and can be selected by clicking on the radial button.

10. Click on **Processed Plates** to view information about the processed plates.

Notes


SDS Automation Controller
File Edit Instrument Tools Help
Instrument Overview
Status: Plate Run
Block Type: 384 Wells Low Density Block
Serial Number: 100269
Laser Power:
Batch Status
Queued Plates: 3
Processed Plates: 2
Plates with Errors: < 1 > Plates
Batch Log: Hide Details...
10:38:15: Running Plate: MFC0234075
10:36:43: Plate Finished: MFC0234003
10:35:20: Running Plate: MFC0234003
10:33:47: Batch Preperation Succeeded...
10:32:43: Running Batch...
10:32:28: Idle...
10:32:28: Batch stopped prematurely...
10:32:20: Batch Error: door is not closed
Clear Selected
Clear All
Currently Running Plate
Run Time Remaining: 1:41:18
Name: MFC0234075.sds
Barcode: MFC0234075
Run Type: Real Time
Plate Queue
Run Status
Processed Plates
< 2 > plates have been run on the instrument
< 1 > Errors
Barcode Plate Name Run Type Start Time End Time Status Flagged Omitted
1 384N14XE0C Wed Mar 02 1... Document Retrieval Failed false false
2 MFC0234003 D:\Applied Bio... Plate Read Wed Mar 02 1... Wed Mar 02 1... Completed false false
Save List
View Plate
Remove Selected
Remove All
Open/Close Door
Stop Batch
Plate Run

B

Notes

Notes

Flags and Filtering for Relative Quantitation Using Comparative C_T Plate Documents

Overview

In the SDS Software, you can automatically flag results data to meet specified criteria. Flags are assay specific and are user configured or automatically assigned.

- Automatically assigned flags are not user configurable. These flags are displayed in the Results table and the QC Summary tab.
- User configured flags are set/defined by the user. These flags are also displayed in the Results table and QC Summary tab.

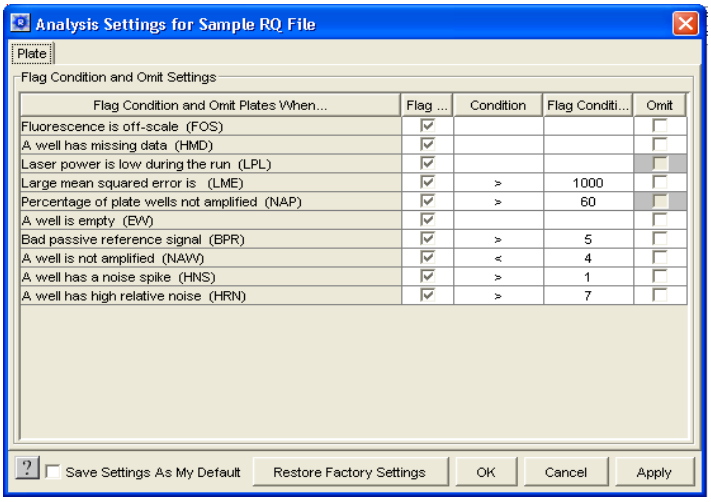
For more information about flags and filters, see the *SDS Online Help*.

Using the Analysis Settings Dialog Box to Assign Flags

You assign flags when you configure the analysis settings for an individual plate document or study. When you open the Analysis Settings dialog box for your current plate document, a list of the flags applicable to that plate document appears in the Plate tab.

1. Click (or select **Analysis** > **Analysis Settings**) to open the Analysis Settings dialog box.

2. Select the **Plate** tab. A list of all applicable flags for the open plate document is displayed. Note that for RQ studies, flags associated with a study will appear in the Study tab.



For more information about assigning flags, see the *SDS Online Help*.

Notes

Plate Tab

The contents of the Plate tab are described below.

No.	Item	Description
1	Flag Condition and Omit Plates When...	Name of the flag setting.
2	Flag column	Indicates whether or not to flag a condition. If the checkbox is checked, the selected condition will be flagged.
3	Condition column	The Condition drop-down lists vary per flag. If conditions are not applicable to a flag, no drop-down list is available.
4	Flag Condition Value column	Allows you to enter a numeric range or threshold for a flag.
5	Omit column	Determines whether or not to omit a well if the flag condition is met. If the checkbox is checked, the well will be omitted if the condition is met. **Note:** Well omission behavior is different depending on whether they are omitted manually or by the flag and filter settings. Wells omitted here will contain result values that were generated until the wells were omitted. You will be able to view the flag data/condition for omitted wells. However, when wells are omitted manually, results data for these wells are lost.

Viewing Flags

In the plate document, you can view flags in the SDS Software:

- Results Table

  View flags, filter the results based on flags, and sort the table by flags
- Well Grid
- QC Summary Tab

  The QC Summary tab provides an overview of the plate document's quality, as shown below.

Flag Icons

The following flag icons are used in both the Results Table and QC view:

Icon	Description
	Passing well (no flags are assigned)

Notes

Icon	Description
	Flagged well
	Well omitted by the algorithm
	Manually omitted wells

For More Information

For more information about flags, see *SDS Online Help*.

Notes

Notes

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Index

Numerics

3D bars 62
5'-nuclease assay 13

A

AmpErase UNG 14, 21, 24
amplification curve 56
amplification plots
  types of amplification plots for RQ studies 60
appearance of graphs 41, 61, 62
Applied Biosystems
  contacting vii
  customer feedback on documentation vii
  Information Development department vii
  Technical Support vii
AQ plates
  omitting samples from 44
assumptions for using this guide v
automatic baseline determination 56
automatic threshold determination 56
autoscaling options 41, 61, 62

B

background in amplification curve 56
bar width 62
baseline
  adjusting 56
  definition 3
  examples 58
baseline, manually setting 56
bold text, when to use v

C

calibrator
  definition 10
CAUTION, description vi
cDNA
  generating 19
  storing 19
  See also reverse transcription
Chemistries 13
comparative method of calculation 5, 50
concentration of RNA 19
conventions
  bold text v
  for describing menu commands v
  IMPORTANTS! v
  in this guide v
  italic text v
  Notes v
  user attention words v
Ct. *See* threshold cycle
curves
  amplification 56
  standard 3
customer feedback, on Applied Biosystems
    documents vii

D

data
  analyzing 40, 60
  exporting 65
  generating PCR data from AQ plates 38
  importing 29
  omitting from a plate 44
  omitting from a study 65
Delta Rn vs.Cycle view 61
designing RQ experiments
  determining reagent configuration 13
  PCR method 12
  primers and probes 16
  selecting the chemistry 13
detector tasks, definition 28
deviation, standard 59
display options 41, 61, 62
documentation, related vi
documents
  exporting 65
  RQ Study 50
  templates 29
dyes
  FAM 16, 67
  reporter 3
  SYBR Green I 13, 15

E

endogenous controls

associating with detectors 28
definition 10
for RQ plates 28
endpoint PCR 3
equipment 5
example RQ experiment
components 11
creating detectors 67
PCR master mix 25
PCR method 12
reverse transcription 20
RQ Plate document, example 34
RQ Study document, example 60
exponential phase of amplification curve 56
exporting data
RQ studies 65
exporting plate data 45, 46

F

FAM dye 16, 67
Fast system
thermal cycling conditions 35, 36

G

Gene Expression plots 62
graph settings 41, 61, 62
guidelines
preparing RNA 18

H

High Capacity cDNA Archive kit 19

I

importing plate setup information 29
Information Development department, contacting vii
italic text, when to use v

L

line width 41, 61, 62
linear phase of amplification curve 56

M

master mix, PCR 21
materials 5
menu commands, conventions for describing v
MSDSs, obtaining vii
multiplex PCR 12

N

normalized reporter 3

O

options, display 41, 61, 62
options, graph 41, 61, 62
outliers 44, 65

P

passive reference 3
PCR
end-point 3
master mix, preparing 21
multiplex 12
real-time 3
selecting a method 12
singleplex 12
phases of amplification curve 56
plateau phase of amplification curve 56
plot appearance 41, 61, 62
Primer Express Software 16
primers 16
probes 16

R

reagent configurations 14
real-time PCR assays 3
reference sample.*See* calibrator
reference, passive 3
relative quantification
comparative method of calculation 5
experiments.See also RQ experiments 4
real-time PCR 3
RQ plates 4
RQ studies 5
replicates 10
Report tab 47
reporter dye 3
Results tab 40
results, RQ Study analysis 60
reverse transcription
guidelines for preparing RNA 18
High Capacity cDNA Archive kit 19
thermal cycling parameters 19
Rn vs. Cycle view 61
Rn. *See* normalized reporter
RNA
guidelines for preparing 18
isolation 18
starting concentration 19

RQ experiments
chemistries 13
components 10
probes and primers 16
reagent configuration 13
requirements 10
workflow 4
RQ Main Study view 52
RQ plates
adding to RQ studies 51
analyzing 40
definition 4
reanalyzing data 44
results 40
RQ Results panel 60
RQ Sample grid 60
RQ studies
adding RQ plates 51
Amplification Plots 60
data types 65
definition 5
exporting data 65
Gene Expression plots 62
omitting samples from 65
orientation 62
reanalyzing data 64
results 60
RQ Study documents 50
RT-PCR
one-step 14, 36
two-step 14, 35

S

safety vi
settings, graph 41, 61, 62
Setup tab 34
singleplex PCR 12
standard curves 3
standard deviation, effect of threshold on 59
standard vs. Fast plates 23
study, RQ. *See* RQ studies
SYBR Green I dye chemistry 13

T

TaqMan Assays 16
TaqMan Custom Gene Expression Assays 16
TaqMan MGB probes 16
TaqMan reagent chemistry 13
TaqMan Universal PCR Master Mix vii
TaqMan® Gene Expression Assays 16
target
associating with detectors 28
definition 10
tasks. *See* detector tasks
Technical Support, contacting vii
template documents 29
text conventions v
thermal cycling conditions
default for PCR 35
one-step RT-PCR 36
specifying 38
two-step RT-PCR 35
thermal cycling parameters
for plates added to a study 51, 70
RT using High Capacity cDNA Archive kit 19
threshold
adjusting 56
definition 3
examples 59
threshold cycle
definition 3
setting for AQ studies 55
threshold, manual settings 56
training, information on viii

U

uracil-N-glycosylase 14
user attention words, described v

W

WARNING, description vi
wells, replicate 10
width, bars 62
width, lines 41, 61, 62
workflow, RQ experiment overview 4

X

x-axis 41, 61, 62

Y

y-axis 41, 61, 62

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