

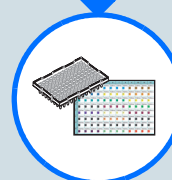
Applied Biosystems

Relative Quantification

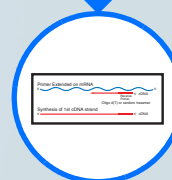
ABI PRISM® 7000 Sequence Detection System



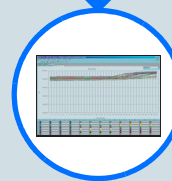
Introduction



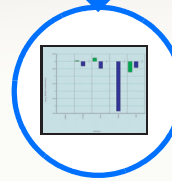
Designing an RQ Experiment



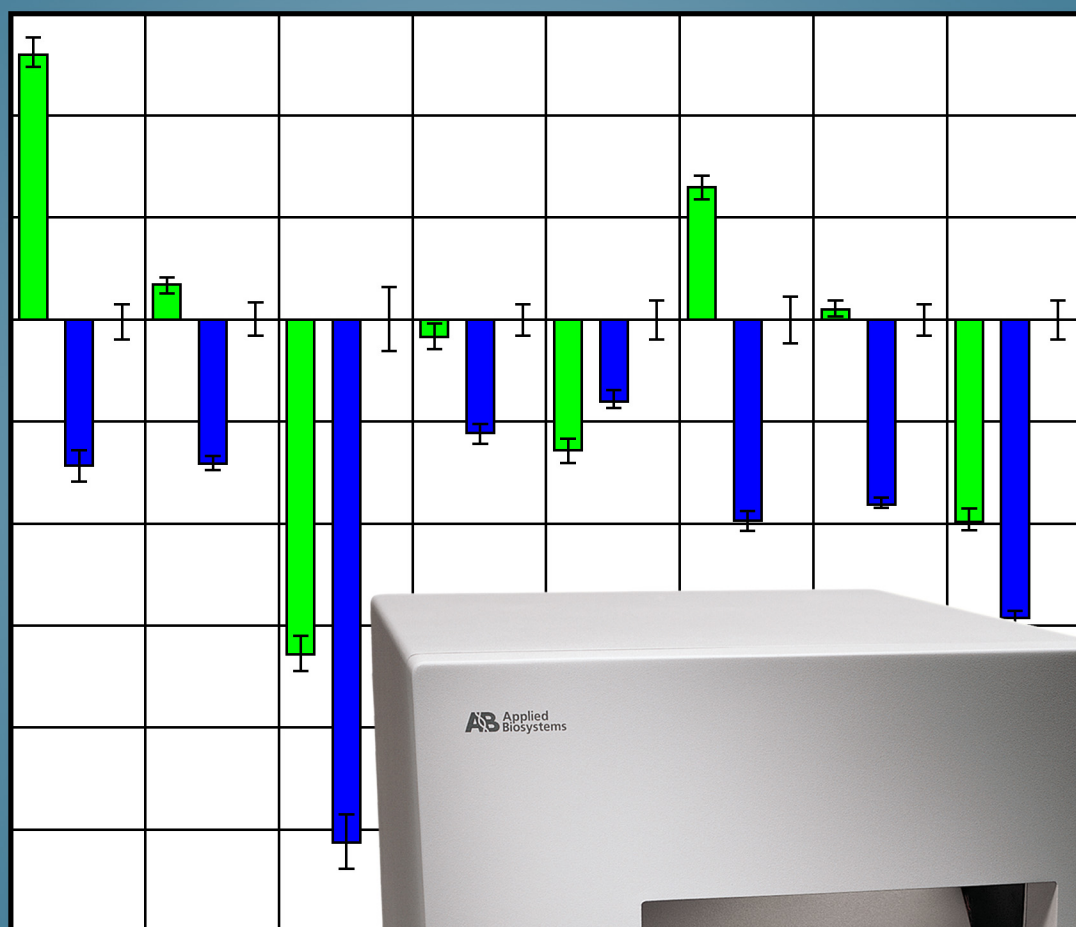
Performing Reverse Transcription



Generating Data from RQ Plates



Performing an RQ Study



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For Research Use Only. Not for use in diagnostic procedures.

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9/2003

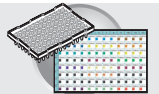
Introduction



1

Introduces concepts related to relative quantification (RQ) experiments using the ABI PRISM® 7000 Sequence Detection System and provides an overview of the RQ Study workflow.

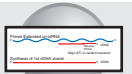
Designing an RQ Experiment



2

Describes the required components of an RQ experiment and describes the set up of a sample RQ experiment. Also provides a list of the materials and equipment required for RQ studies.

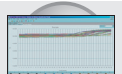
Performing Reverse Transcription



3

Outlines the preferred methodology for reverse transcribing total RNA to cDNA and provides guidelines for RNA quality and starting amounts of total RNA.

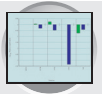
Generating Data from RQ Plates



4

Explains how data generated during the PCR process is captured in RQ Plate documents and provides information about analyzing RQ Plate data.

Performing an RQ Study



5

Explains how to analyze data from one or more RQ Plate documents and to save the results in an RQ Study document.

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Preface

How to Use This Guide

Purpose of This Guide This manual is written for principal investigators and laboratory staff who conduct relative quantification studies for gene expression using the ABI PRISM® 7000 Sequence Detection System (7000 SDS instrument).

Assumptions This guide assumes that you have:

- Familiarity with Microsoft® Windows® 2000 operating system.
- Knowledge of general techniques for handling DNA samples and preparing them for electrophoresis.
- A general understanding of hard drives and data storage, file transfers, and copying and pasting.

If you want to integrate the ABI PRISM® 7000 Sequence Detection 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 > Spot Set**.
Right-click the sample row, then select **View Filter > View All Runs**.

User Attention Words Two 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.

Examples of the user attention words appear below:

Note: The size of the column affects the run time.

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection to the database, you need a valid Oracle user ID and password.

IMPORTANT! You must create a separate Sample Entry Spreadsheet for each 96-well microtiter plate.

Safety Alert Words

Safety alert words also appear in user documentation. For more information, see [“Safety Alert Words” on page x](#).

Related Documentation

For more information about using the instrument and relative quantification, refer to:

- ABI PRISM® 7000 Sequence Detection System Online Help
- Livak, K.J., and Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* 25:402-408.
- *Sequence Detection Systems Chemistry Guide Chemistry Guide* (PN 4330019)
- *ABI PRISM® 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression* (PN 4303859)

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

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Safety and EMC Compliance Information



This section includes the following topics:

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▶ Safety Labels on Instruments	xii
▶ General Instrument Safety	xii
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Safety Conventions Used in This Document


Safety Alert Words


Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT**, **CAUTION**, **WARNING**, **DANGER**—implies a particular level of observation or action, as defined below:

Definitions

IMPORTANT! – Indicates 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.


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


Except for Important, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard icons that are affixed to Applied Biosystems instruments* (see “[Safety Symbols](#)” on [page xi](#)).

Examples

The following examples show the use of safety alert words:

IMPORTANT! You must create a separate Sample Entry Spreadsheet for each 96-well microtiter plate.

 **CAUTION** The lamp is extremely hot. Do not touch the lamp until it has cooled to room temperature.

 **WARNING** **CHEMICAL HAZARD. Formamide.** Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.








 **DANGER** **ELECTRICAL HAZARD.** Failure to ground the instrument properly can lead to an electrical shock. Ground the instrument according to the provided instructions.



Symbols on Instruments






Electrical Symbols on Instruments

The following table describes the electrical symbols that may be displayed on Applied Biosystems instruments.

Symbol	Description
	Indicates the On position of the main power switch.
	Indicates the Off position of the main power switch.
	Indicates the On/Off position of a push-push main power switch.
	Indicates a terminal that may be connected to the signal ground reference of another instrument. This is not a protected ground terminal.
	Indicates a protective grounding terminal that must be connected to earth ground before any other electrical connections are made to the instrument.
	Indicates a terminal that can receive or supply alternating current or voltage.
	Indicates a terminal that can receive or supply alternating or direct current or voltage.

Safety Symbols

The following table describes the safety symbols that may be displayed on Applied Biosystems instruments. Each symbol may appear by itself or in combination with text that explains the relevant hazard (see [“Safety Labels on Instruments”](#) on [page xii](#)). These safety symbols may also appear next to DANGERS, WARNINGS, and CAUTIONS that occur in the text of this and other product-support documents.

Symbol	Description
	Indicates that you should consult the manual for further information and to proceed with appropriate caution.
	Indicates the presence of an electrical shock hazard and to proceed with appropriate caution.
	Indicates the presence of a hot surface or other high-temperature hazard and to proceed with appropriate caution.
	Indicates the presence of a laser inside the instrument and to proceed with appropriate caution.
	Indicates the presence of moving parts and to proceed with appropriate caution.



Safety Labels on Instruments

The following CAUTION, WARNING, and DANGER statements may be displayed on Applied Biosystems instruments in combination with the safety symbols described in the preceding section.

English	Francais
CAUTION Hazardous chemicals. Read the Material Safety Data Sheets (MSDSs) before handling.	ATTENTION Produits chimiques dangereux. Lire les fiches techniques de sûreté de matériels avant la manipulation des produits.
CAUTION Hot surface.	ATTENTION Surface brûlante.
DANGER High voltage.	DANGER Haute tension.
WARNING To reduce the chance of electrical shock, do not remove covers that require tool access. No user-serviceable parts are inside. Refer servicing to Applied Biosystems qualified service personnel.	AVERTISSEMENT Pour éviter les risques d'électrocution, ne pas retirer les capots dont l'ouverture nécessite l'utilisation d'outils. L'instrument ne contient aucune pièce réparable par l'utilisateur. Toute intervention doit être effectuée par le personnel de service qualifié de Applied Biosystems.
CAUTION Moving parts.	ATTENTION Parties mobiles.

General Instrument Safety

Moving and Lifting the Instrument



CAUTION PHYSICAL INJURY HAZARD. The instrument is to be moved and positioned only by the personnel or vendor specified in the applicable site preparation guide. If you decide to lift or move the instrument after it has been installed, do not attempt to lift or move the instrument without the assistance of others, the use of appropriate moving equipment, and proper lifting techniques. Improper lifting can cause painful and permanent back injury. Depending on the weight, moving or lifting an instrument may require two or more persons.

Operating the Instrument

Ensure that anyone who operates the instrument has:

- Received instructions in both general safety practices for laboratories and specific safety practices for the instrument.
- Read and understood all applicable Material Safety Data Sheets (MSDSs). See [“About MSDSs”](#) on [page xiii](#).



WARNING PHYSICAL INJURY HAZARD. Use this instrument as specified by Applied Biosystems. Using this instrument in a manner not specified by Applied Biosystems may result in personal injury or damage to the instrument.



Chemical Safety

Chemical Hazard Warning



WARNING CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 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 a 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**.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDS) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “[About MSDSs](#)” on [page xiii](#).)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.



- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Safety

Chemical Waste Hazard



CAUTION HAZARDOUS WASTE. Refer to Material Safety Data Sheets and local regulations for handling and disposal.



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



WARNING CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.



Physical Hazard Safety

Ultraviolet Light



WARNING

ULTRAVIOLET LIGHT HAZARD. Looking directly at a UV light source can cause serious eye damage. Never look directly at a UV light source and always prevent others from UV exposure. Follow the manufacturer's recommendations for appropriate protective eyewear and clothing.

Biological Hazard Safety

General Biohazard



WARNING

BIOHAZARD. Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; <http://bmbi.od.nih.gov>)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; http://www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html).

Additional information about biohazard guidelines is available at: <http://www.cdc.gov>

Workstation Safety

Correct ergonomic configuration of your workstation can reduce or prevent effects such as fatigue, pain, and strain. Minimize or eliminate these effects by configuring your workstation to promote neutral or relaxed working positions.



CAUTION

MUSCULOSKELETAL AND REPETITIVE MOTION HAZARD. These hazards are caused by potential risk factors that include but are not limited to repetitive motion, awkward posture, forceful exertion, holding static unhealthy positions, contact pressure, and other workstation environmental factors.

To minimize musculoskeletal and repetitive motion risks:

- Use equipment that comfortably supports you in neutral working positions and allows adequate accessibility to the keyboard, monitor, and mouse.
- Position the keyboard, mouse, and monitor to promote relaxed body and head postures.



Safety and Electromagnetic Compatibility (EMC) Standards

This section provides information on:

- [U.S. and Canadian Safety Standards](#)
- [Canadian EMC Standard](#)
- [European Safety and EMC Standards](#)
- [Australian EMC Standards](#)

U.S. and Canadian Safety Standards



This instrument has been tested to and complies with standard UL 3101-1, “Safety Requirements for Electrical Equipment for Laboratory Use, Part 1: General Requirements.”

This instrument has been tested to and complies with standard CSA 1010.1, “Safety Requirements for Electrical Equipment for Measurement, Control, and Laboratory Use, Part 1: General Requirements.”

Canadian EMC Standard

This instrument has been tested to and complies with ICES-001, Issue 3: Industrial, Scientific, and Medical Radio Frequency Generators.

European Safety and EMC Standards



Safety

This instrument meets European requirements for safety (Low Voltage Directive 73/23/EEC). This instrument has been tested to and complies with standards EN 61010-1:2001, “Safety Requirements for Electrical Equipment for Measurement, Control and Laboratory Use, Part 1: General Requirements” and EN 61010-2-010, “Particular Requirements for Laboratory Equipment for the Heating of Materials.”

EMC

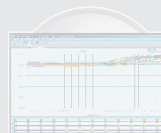
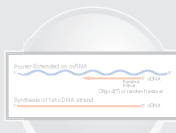
This instrument meets European requirements for emission and immunity (EMC Directive 89/336/EEC). This instrument has been tested to and complies with standard EN 61326 (Group 1, Class B), “Electrical Equipment for Measurement, Control and Laboratory Use – EMC Requirements.”

Australian EMC Standards



This instrument has been tested to and complies with standard AS/NZS 2064, “Limits and Methods Measurement of Electromagnetic Disturbance Characteristics of Industrial, Scientific, and Medical (ISM) Radio-frequency Equipment.”





1

Are you familiar with the 7000 instrument and relative quantification?



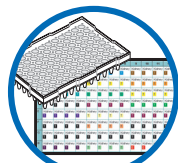
No

Chapter 1

Read the introduction for an overview

Yes

Have you designed your experiment?



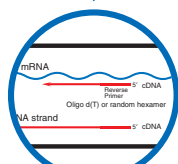
Yes

Chapter 2

Design an RQ experiment.

No

Have you converted total RNA to cDNA?



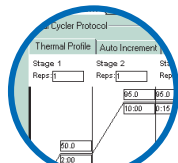
No

Chapter 3

Reverse transcribe total RNA to cDNA.

Yes

Have you collected PCR data in an RQ plate document?



No

Chapter 4

PCR amplify the cDNA and collect data in RQ Plate documents.

Yes

Have you analyzed data in an RQ study?



No

Chapter 5

Analyze one or more RQ Plates in an RQ study.

Introduction



About the 7000 SDS Instrument

Description The ABI PRISM® 7000 Sequence Detection System (7000 SDS instrument) is a second-generation sequence detection instrument capable of quantitative and qualitative detection with fluorescent-based PCR chemistries. The instrument is capable of quantitative detection using real-time analysis, and qualitative detection using end-point and dissociation-curve analysis.

The 7000 SDS instrument combines thermal cycling, fluorescence detection, and application-specific software. It detects accumulated polymerase chain reaction (PCR) product cycle-by-cycle, thus making quantification available immediately after completion of PCR, without the need for further process analysis.

Supported Assay Types The 7000 SDS instrument allows you to perform the following assays with plates or tubes in the 96-well format:

- Relative Quantification (RQ) – Determines the quantity of a single nucleic acid target sequence within an unknown sample, relative to the same sequence within a calibrator sample
- Absolute Quantification (AQ) – Determines the absolute quantity of a single nucleic acid target sequence within a sample
- Allelic Discrimination (AD) – Indicates the genotype of samples.
- Plus/Minus – Indicates the presence or absence of a specific target sequence in a sample

For more information about the assay types, refer to the *Sequence Detection Systems Chemistry Guide* and the Online Help for the 7000 SDS instrument.

About Relative Quantification

Real-time PCR Assays Real-time PCR is the ability to monitor the progress of the PCR as it occurs. Data is 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 the amount of target accumulated at the end of PCR.

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

Notes



Definition of Relative Quantification

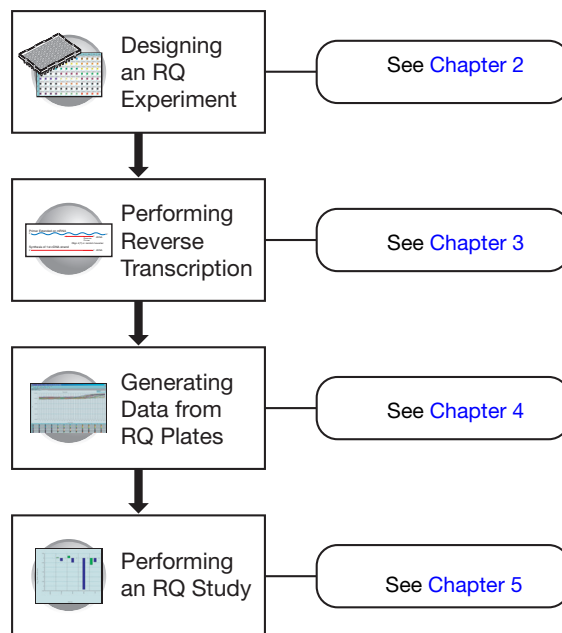
Relative quantification describes the change in expression of the target gene in a test sample relative to a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen, 2001). For example, relative quantification is commonly used to compare expression levels of wild-type versus mutated alleles or the expression levels of a gene in different tissues.

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

About RQ Experiments

RQ Experiment Workflow

This document uses the term “RQ experiments” to refer to the entire process of generating cDNA from RNA (reverse transcription) through analyzing RQ studies. The RQ experiment workflow has several steps, as shown in the following figure.



RQ Studies with the 7000 v1.1 Instrument

The RQ Study Add On software for the 7000 v1.1 SDS instrument enables you to perform RQ assays, which calculate the relative quantification values from data generated during real-time PCR. (Without the add-on software, the 7000 v1.1 SDS instrument can perform AQ, AD, and Plus/Minus but not RQ assays.)

Note: For instructions on installing the RQ Study Software, refer to [Appendix B](#).

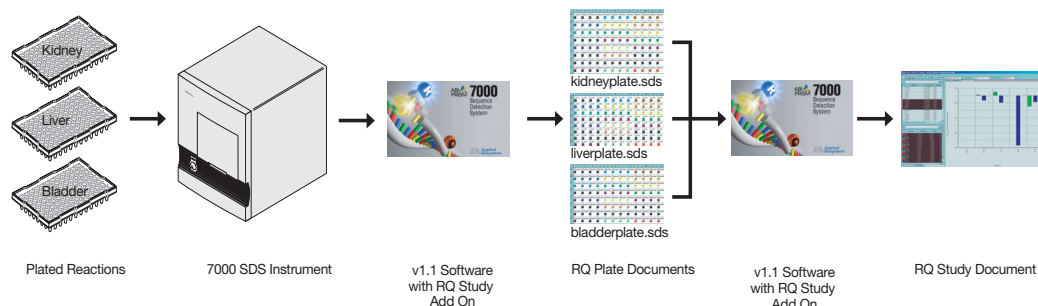
The data-collection part of the assay is a single-plate document, called the RQ Plate. Amplification data from PCR runs is stored with sample setup information on the plate.

Notes



The data-analysis part of the assay is a multi-plate document, called the RQ Study. You can analyze up to ten RQ plates in a study. RQ Study documents neither control the instrument, nor do they provide tools for setting up or modifying plates.

The following figure illustrates the RQ Study process.



IMPORTANT! The 7000 SDS instrument software (v1.1) with the RQ Study Add On uses only the comparative method ($\Delta\Delta Ct$) to calculate relative quantities of a nucleic acid sequence.

About the Sample RQ Experiment

Description of the Sample RQ Experiment

This guide uses a sample RQ experiment to help you understand the workflow.

Example

In the sample RQ experiment, levels of expression of 23 genes were compared in the liver, kidney, and bladder tissue of an individual.

The experiment was designed for singleplex PCR—samples and endogenous controls were amplified in separate wells. Glyceraldehyde-3-phosphate (GAPDH) served as the endogenous control. Four replicates of each sample and endogenous control were amplified. (In this experiment, an entire 96-well-plate is devoted to each tissue because the four replicates of each of the 23 genes plus the endogenous control take up all 96 wells.)

Predesigned and labeled primer/probe sets were selected from the Applied Biosystems Assays-on-Demand™ product line.

Reactions were set up for two-step RT-PCR, where the High Capacity cDNA Archive Kit and the TaqMan® Universal PCR Master Mix were used for reverse transcription and PCR, respectively.

Data was generated by running three RQ plates, one for each tissue.

All three plates were analyzed in an RQ study, with the Liver samples serving as the calibrator.

The implementation details of this sample RQ experiment will be discussed throughout this guide in Example boxes like this one.

Notes



Materials and Equipment

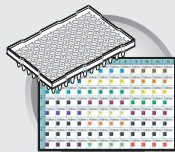
You need to supply the following items to complete an RQ study.

Item	Source
High Capacity cDNA Archive Kit	Applied Biosystems (PN 4322171)
TaqMan® Universal PCR Master Mix	Applied Biosystems (PN 4304437)
MicroAmp® Optical 96-Well Reaction Plate and Optical Caps Note: Reaction plates and caps may also be purchased separately. Reaction plates can also be sealed with ABI PRISM™ Optical Adhesive Covers.	Applied Biosystems (PN 403012)
Labeled primers and probes from one of the following sources: <ul style="list-style-type: none">Assays-on-Demand™ Gene Expression Products (predesigned primers and probes)Assays-by-Design™ service (predesigned primers and probes)Primer Express Software (custom-designed primers and probes)	<ul style="list-style-type: none">Applied Biosystems Web siteContact your Applied Biosystems Sales RepresentativePN 4330710 (1-user license) PN 4330709 (10-user license) PN 4330708 (50-user license)
Reagent tubes with caps, 10-mL	Applied Biosystems (PN 4305932)
Centrifuge with adapter for 96-well plates	Major laboratory supplier (MLS)
Gloves	Major laboratory supplier (MLS)
Microcentrifuge	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS
Nuclease-free water	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement	MLS
Tris-EDTA (TE) Buffer, pH 8.0	MLS
Vortexer	MLS

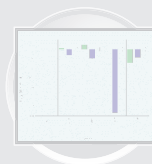
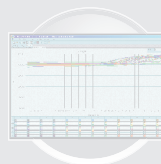
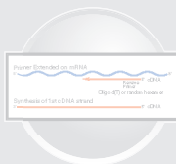
Notes _____



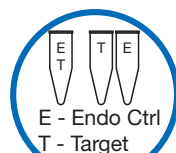
Notes _____



2



**Have you selected a PCR method?
(Single or Multiplex)**



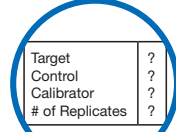
No

[page 7](#)

Select a PCR method.

Yes

Have you designated the targets, calibrator, endogenous control, and replicates for the experiment?



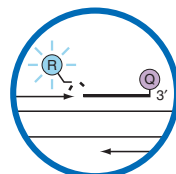
No

[page 9](#)

Designate the four essential components of RQ experiments.

Yes

Have you selected the SDS chemistry (SYBR Green 1 or TaqMan®) and reagent configuration?



No

[page 11](#)

Select the SDS chemistry for your experiment.

Yes

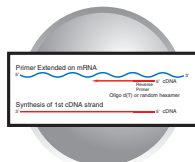
Have you designed your primers and probes?



No

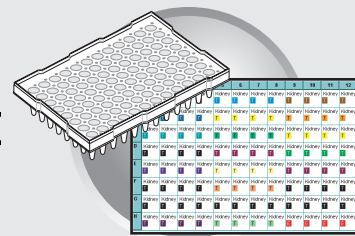
[page 13](#)

Choose primers and probes for your experiment.

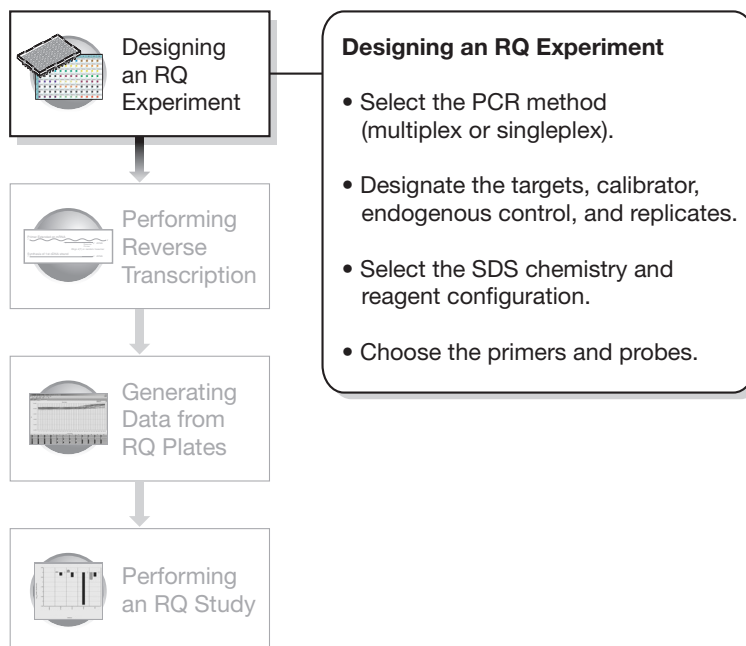


**Go to chapter 3,
Performing Reverse
Transcription**

Designing an RQ Experiment



Workflow

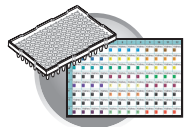


Selecting the PCR Method

Traditional PCR is performed as 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—target sequences and endogenous controls cannot be amplified in the same tube.

In multiplex PCR, two or more primer pairs are present in the reaction. Each primer pair amplifies either a target sequence or an endogenous control. The availability of multiple reporter dyes for TaqMan® probes, each with different emission wavelength maxima, makes multiplex PCR possible.

Notes



Both methods give equivalent results for relative quantification experiments. Which method to use depends on the

- Type of chemistry you will be using to detect PCR products – Singleplex PCR can use either SYBR[®] Green or TaqMan reagent-based chemistry. Multiplex PCR can use only TaqMan chemistry.
- 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. Among the factors to consider when doing multiplex PCR are primer limitation, 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.

- Requirement for high throughput performance – Running multiple reactions in the same tube in a multiplex experiment increases throughput and reduces the effects of pipetting errors.

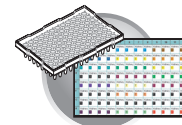
For more information about multiplex and singleplex PCR, refer to the *Sequence Detection Systems Chemistry Guide* (PN 430019).

Example

The singleplex PCR method was used in the sample experiment for the following reasons:

- Large number of targets to be amplified (23 genes, plus one endogenous control)
- No requirement for optimization and validation for singleplex experiments

Notes _____



Specifying the Components of an RQ Experiment

RQ experiments require:

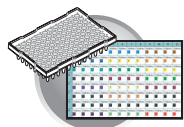
- A target – The nucleic acid sequence that you are studying.
- A calibrator – The sample used as the basis for comparative results.
- An endogenous control – A gene present at a consistent expression level in all experimental samples. By using an endogenous control as an active reference, you can normalize quantification 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.

Typically, housekeeping genes such as β -actin, glyceraldehyde-3-phosphate (GAPDH), and ribosomal RNA (rRNA), are used as endogenous controls.

- Replicate wells – For relative quantification studies, Applied Biosystems recommends the use of three or more replicate reactions per sample and endogenous control to ensure statistical significance. Replicates allow you to preserve data and remove outliers.

For more information about these concepts, refer to the *Sequence Detection Systems Chemistry Guide*.

Notes _____



Example

The objective of the sample experiment is to compare the expression levels of several genes in the liver, kidney, and bladder tissue of an individual. There are 23 genes of interest, including ACVR1, ACVR2, CCR2, CD3D, and FLT4. These genes are the targets.

Once targets are identified, you need to determine which samples will serve as the basis of comparison for the other samples in the study. In this experiment, the liver samples served as the calibrator. The 7000 SDS instrument software sets gene expression levels for the calibrator samples to 1. Consequently, if there is more ACVR1 in the kidney than in the liver, the gene expression level of ACVR1 in the kidney is greater than 1. Similarly, if there is less CD3D in the bladder than in the liver, the gene expression level of CD3D in the bladder is less than 1.

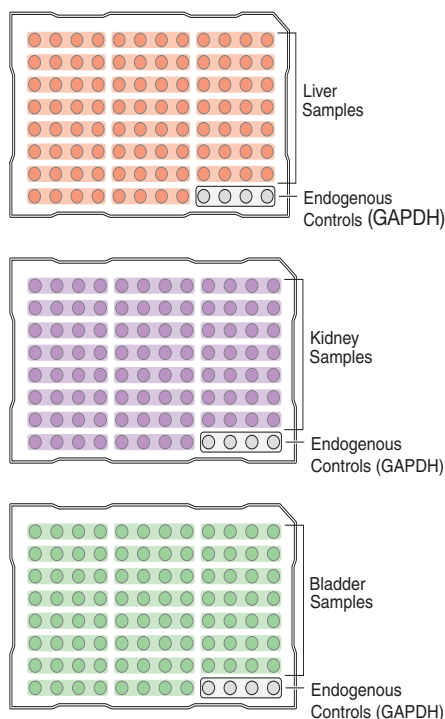
Because RQ is based on PCR, the more template there is in a reaction, the more the PCR product and the greater the fluorescence. To account for possible differences in the amount of template added to the reaction, GAPDH serves as an endogenous control. (Expression levels of the endogenous control are subtracted from expression levels of target genes.) An endogenous control was prepared for each tissue.

The experiment includes three sets of endogenous controls—one for each tissue. Further, 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 different wells from the targets.

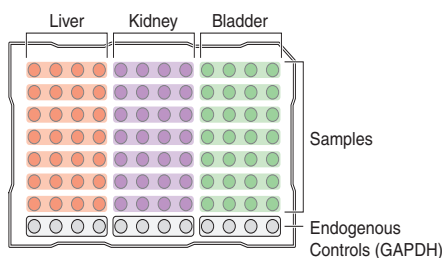
Four replicates of each sample and endogenous control are performed to ensure statistical significance.

Note: The sample RQ experiment requires a separate plate for each of the three tissues because of the number of genes being studied. Experiments can also be designed so that several samples are amplified on the same plate, as shown in the following table.

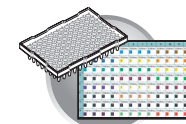
In the sample RQ experiment, each plate contains a single sample type (tissue). The endogenous control for each tissue is on the same plate as the targets for that tissue.



In experiments where multiple sample types are on the same plate, an endogenous control for each sample type must also be included on the same plate.



Notes



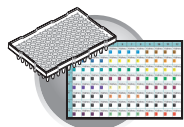
Selecting the Sequence Detection Chemistry and Reagent Configuration

About SDS Chemistries

Applied Biosystems offers two types of chemistries that can be used to detect PCR products on SDS instruments, as explained in the following table. For more information about SDS chemistries, refer to the *Sequence Detection 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 <ul style="list-style-type: none"> Increases specificity with a probe. Specific hybridization between probe and target generates fluorescent signal. Provides multiplex capability Optimized assays available Allows 5' -nuclease assay to be carried out during PCR 	<div> <div> <p>Polymerization</p> <p>5' 3' 5' 3' 5' 3'</p> <p>FORWARD PRIMER PROBE R = REPORTER Q = QUENCHER REVERSE PRIMER</p> <p>Step 1: A reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe.</p> </div> <div> <p>Strand Displacement</p> <p>5' 3' 5' 3' 5' 3'</p> <p>Step 1 continued: when both dyes are attached to the probe, reporter dye emission is quenched.</p> </div> </div> <div> <div> <p>Cleavage</p> <p>5' 3' 5' 3' 5' 3'</p> <p>Step 2: During each extension cycle, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the probe.</p> </div> <div> <p>Polymerization Completed</p> <p>5' 3' 5' 3' 5' 3'</p> <p>Step 3: Once separated from the quencher, the reporter dye emits its characteristic fluorescence.</p> </div> </div>
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 <ul style="list-style-type: none"> Reduces cost (no probe needed) Amplifies all double-stranded DNA Yields a melting profile of distinct PCR runs Increases sensitivity for detecting amplification products relative to product length 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.	<div> <div> <p>Step 1</p> <p>The SYBR Green I dye within the SYBR Master Mix immediately binds with all double-stranded DNA present in the sample.</p> </div> <div> <p>Step 2</p> <p>During PCR, AmpliTaq Gold® DNA Polymerase amplifies each target.</p> </div> <div> <p>Step 3</p> <p>The SYBR Green I dye then binds to each new copy of double-stranded DNA.</p> </div> </div>

Notes



About Reagent Configurations for RT-PCR

There are several TaqMan kits and SYBR Green I dye chemistry kits available for quantitative experiments. The reagent configuration you use depends on whether you are performing one-step or two-step RT-PCR:

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

IMPORTANT! Applied Biosystems recommends that you use the two-step RT-PCR method for RQ experiments. This guide assumes that RQ experiments are designed using two-step RT-PCR. Further, only the recommended reagent configurations are documented. For additional options, refer to the *Sequence Detection Systems Chemistry Guide*.

The following table lists the recommended kits for two-step RT-PCR. For information about the available reagents for one-step RT-PCR, refer to the *Sequence Detection Systems Chemistry Guide*.

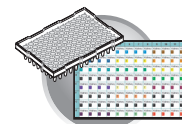
Chemistry	Step	Reagent	Part Number
TaqMan reagents or kits	RT	High Capacity cDNA Archive Kit	4322171
	PCR	TaqMan Universal PCR Master Mix	4304437
SYBR Green I reagents or kits	PCR	SYBR Green Master Mix	4309155
	RT and PCR	SYBR Green RT-PCR Reagents	4310179

Example

The sample experiment seeks to determine the expression levels of specific genes. It requires probes that can bind to specific DNA sequences. Premade probes and primers for all the genes of interest were available from the Assays-on-Demand product line, which uses TaqMan chemistry.

Two-step RT-PCR was performed, using the reagents recommended for TaqMan reagent- or kit-based chemistry in the table above.

Notes



Choosing the Probes and Primers

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

- Assays-on-Demand™ Gene Expression Products – Provide you with optimized, ready-to-use TaqMan 5′ -nuclease assays for human, mouse, or rat transcripts. For information on available primer/probe sets, go to:
<http://www.appliedbiosystems.com> and click the **Assays-on-Demand Gene Expression Products** link on the right-hand column.
- Assays-by-DesignSM Service - Designs, synthesizes, formulates, and delivers quality-controlled primer and probe sets. Use this service if the assay you need is not currently available. To place an order, contact your Applied Biosystems representative.
- Primer Express® Software – Helps you design primers and probes for your own quantification assays. For more information about using this software, refer to the *Primer Express Software v2.0 User's Manual* (PN 4329500).

Applied Biosystems provides Assay Design Guidelines, which have been developed specifically for quantification assays. When used in their entirety, these steps provide a rapid and reliable system for assay design and optimization. For information about the Assay Design Guidelines, refer to the *Sequence Detection Systems Chemistry Guide*.

Example

Premade assays from Assays-on-Demand products are available for all the genes included in the sample experiment; primers and probes were obtained from Applied Biosystems. Each assay consists of two unlabeled PCR primers (forward and reverse) and a FAM™ dye-labeled TaqMan® MGB probe, provided as a 20X assay mix.

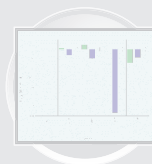
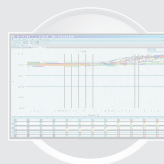
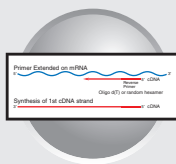
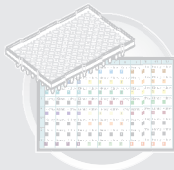
If you order Assays-on-Demand or Assays-by-Design products, probes are already labeled with a reporter dye. (If you design your own assays, you would need to specify a reporter dye for your custom probe(s).) For singleplex experiments, where the targets and endogenous controls are amplified in separate wells, you can use the same dye for targets and endogenous control(s). For multiplex experiments, where the targets and endogenous controls are amplified in the same well, the probe for the target is typically labeled with FAM dye and that for the endogenous control with VIC® dye.

In the sample experiment, all target probes were labeled with FAM dye; the endogenous control was also labeled with FAM dye (GAPDH-FAM).

The following table lists the gene symbol, gene name, and Applied Biosystems Assay ID number (provided on the Web site) for five of the genes studied in the sample experiment, plus the endogenous control.

Gene Symbol	Gene Name	Assay ID #
ACVR1	acrosomal vesicle protein I	Hs00153836 m1
ACVR2	activin A receptor, type II	Hs00155658_m1
CCR2	chemokine (C-C motif) receptor 2	Hs00174150_m1
CD3D	CD3D antigen, delta polypeptide (TiT3 complex)	Hs00174158_m1
FLT4	fms-related tyrosine kinase 4	Hs00176607 m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905 m1

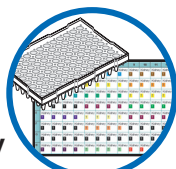
Notes



3

Have you:

- Selected the PCR method?
- Designated the four essential components?
- Selected an SDS chemistry & reagent configuration?
- Designed primers and probes?



No

Chapter 2

Design an RQ experiment according to guidelines in chapter 2.

Yes

Do you have purified total RNA?



No

page 16

Isolate total RNA using these guidelines.

Yes

Is the concentration of RNA within the recommended range?



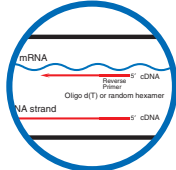
No

page 16

Adjust RNA concentration.

Yes

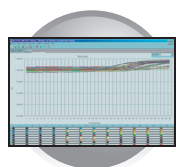
Have you converted the total RNA to cDNA?



No

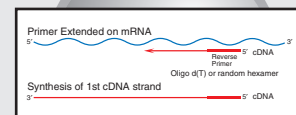
page 16

Convert total RNA to cDNA using the High Capacity cDNA Archive Kit.

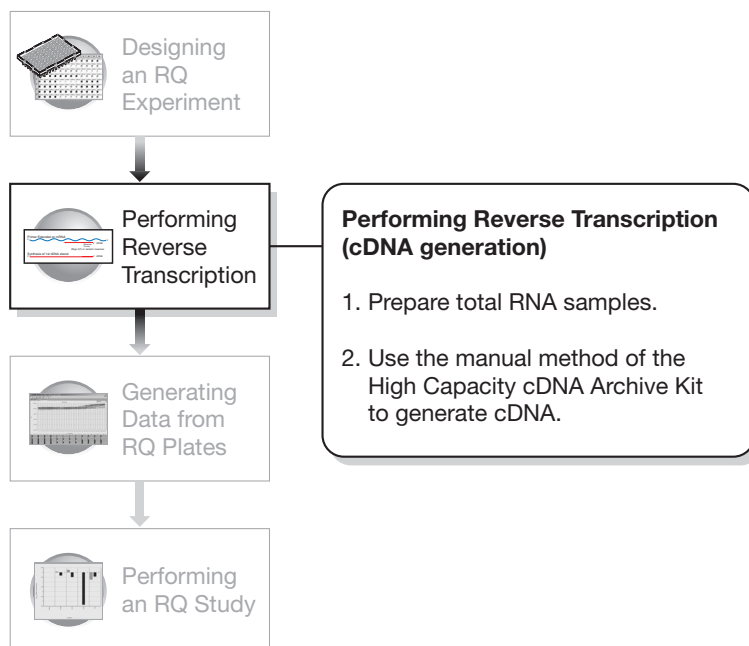


Go to chapter 4, Generating Data from RQ Plates

Performing Reverse Transcription



Workflow

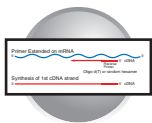


RT-PCR Methods

As discussed in [“Selecting the PCR Method” on page 7](#), there are two RT-PCR methods that you can choose from when performing RQ experiments: one-step RT-PCR or two-step RT-PCR.

IMPORTANT! Applied Biosystems recommends that you use the two-step RT-PCR method for RQ experiments. This guide assumes that RQ experiments are designed using two-step RT-PCR. Further, only the recommended reagent configurations are documented. For additional options, refer to the *Sequence Detection Systems Chemistry Guide*.

Notes



Guidelines for Preparing RNA

Quality of RNA Ensure that the total RNA you use for RQ experiments is of reasonable quality:

- Its $A_{260/280}$ ratio should be greater than 1.9
- It should be intact when visualized by gel electrophoresis
- It should not contain RT or PCR inhibitors

The *High Capacity cDNA Archive Kit Protocol* (4312169) contains additional guidelines for preparing the RNA template.

Starting Amount of Total RNA The High Capacity cDNA Archive Kit is optimized to convert 0.1 to 10 μg of total RNA to cDNA. 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.

Generating cDNA

Using the High Capacity cDNA Archive Kit As mentioned in “[About Reagent Configurations for RT-PCR](#)” on page 12, Applied Biosystems recommends that you use the two-step RT-PCR method for RQ experiments. Synthesis of cDNA from total RNA samples using the High Capacity cDNA Archive Kit (PN 4322171) is the first step in the two-step RT-PCR procedure. Use the manual method for converting total RNA into cDNA, as specified in the *High Capacity cDNA Archive Kit Protocol* (PN 4322169).

Note: The protocol is not shipped with the High Capacity cDNA Archive Kit. You must download the protocol from

<http://docs.appliedbiosystems.com/search.taf>

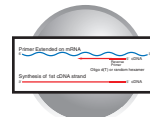
To search for the document, select **ABI PRISM™ 6100 Nucleic Acid PrepStation** under Products, then click **Search**. The protocol is listed under the Protocols heading.

Thermal Cycling Parameters for RT The High Capacity cDNA Archive Kit uses the following thermal cycling parameters for the RT step.


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

Note: Thermal cycling conditions for one-step RT-PCR are described in “[Thermal Cycling Conditions for One-Step RT-PCR](#)” on page 25.

Notes



Storing cDNA After thermal cycling, store all cDNA samples at -15 to -25 °C. To minimize repeated freeze-thaw cycles of cDNA, Applied Biosystems recommends that you store your cDNA samples in aliquots.

 **WARNING** **CHEMICAL HAZARD.** $10\times$ 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.

Example

For the sample experiment, RNA was extracted from the liver, bladder, and kidney tissues of an individual. RNA concentration was determined through spectrophotometry (using A_{260}) and the RNA was diluted to a final concentration of 50 ng/ μ L.

The RT master mix was prepared as follows, using guidelines from the High Capacity cDNA Archive Kit Protocol:

Component	Volume (μ L)/Reaction
10X Reverse Transcription Buffer	10
25X dNTPs	4
10X random primers	10
MultiScribe™ Reverse Transcriptase, 50 U/ μ L	5
Nuclease-free water	21
Total per reaction	50

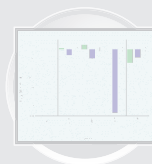
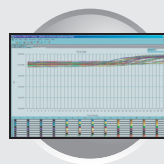
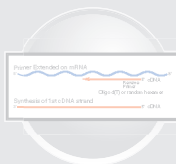
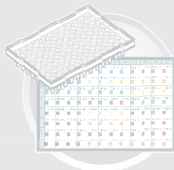
The cDNA archive plate was then prepared by pipetting

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

The RNA was then converted to cDNA using the universal thermal cycling parameters for two-step RT-PCR, as described in [“Thermal Cycling Parameters for RT” on page 16](#).

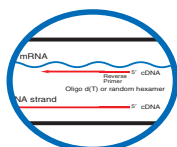
The cDNA was stored at -20 °C for 24 hours.

Notes



4

Do you have cDNA?



No

[Chapter 3](#)

Reverse transcribe total RNA to cDNA.

Yes

Have you prepared the PCR master mix?



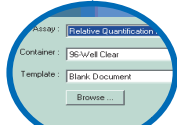
No

[page 20](#)

Prepare the PCR master mix as directed in the TaqMan[®] Universal PCR Master Mix Protocol.

Yes

Have you created an RQ plate document?



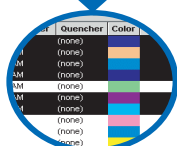
No

[page 22](#)

Create a new RQ plate document.

Yes

Have the detectors for the experiment been added to the software?



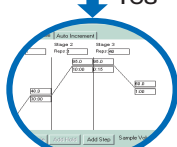
No

[page 23](#)

Create detectors.

Yes

Are the default thermal cycling conditions for PCR set?



No

[page 25](#)

Program the default PCR conditions or enter the thermal cycling conditions for one-step RT-PCR

Yes

Have you saved the data from the PCR run?



No

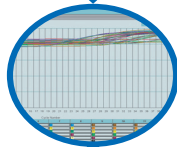
[page 26](#)

Save the data in the RQ plate document.

Yes

Start the run.

Have you viewed the RQ plate data?

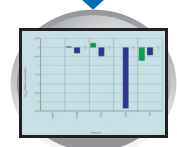


No

[page 28](#)

View the RQ plate data to confirm that the run was successful.

Yes

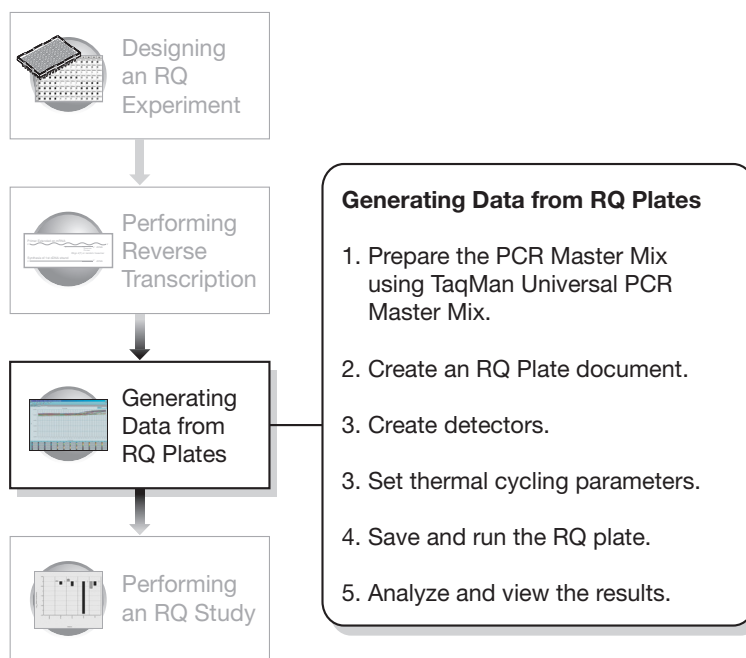


Go to chapter 5, Performing an RQ Study

Generating Data from RQ Plates



Workflow



Before You Begin

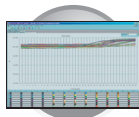
Calibrating the 7000 SDS instrument

Check that background and pure-dye runs have been performed regularly to ensure optimal performance of the 7000 SDS instrument. For more information about calibrating the 7000 SDS instrument, refer to the Online Help for the 7000 SDS instrument.

Preventing Contamination

PCR techniques require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these techniques can lead to amplification of a single DNA molecule (Saiki et al., 1985; Mullis and Faloona, 1987).

Notes




Follow these recommended general PCR practices:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
 - Sample preparation and PCR setup
 - PCR amplification and post-PCR analysis
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Try not to splash or spray PCR samples.
- Quick-spin PCR samples whenever residual sample is present on the inside lid (such as after dropping a tube or when there is condensation on the tube from heating or thawing)
- Keep reactions and components capped as much as possible.
- Use aerosol-resistant or positive-displacement pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% chlorine bleach.

Preparing the PCR Master Mix

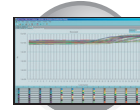
The second step in the two-step RT-PCR procedure is amplifying the cDNA. TaqMan[®] Universal Master Mix reagents provide a PCR mix that may be used with any appropriately designed primer and probe to detect any DNA or cDNA sequence.

The *TaqMan Universal PCR Master Mix Protocol* (PN 4304449) explains how to use the reagents provided in the kit. The following table lists the universal assay conditions (volume and final concentration) for using the master mix.

 **CAUTION** **CHEMICAL HAZARD.** TaqMan Universal PCR Master Mix 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	Volume (μL) Per Sample	Final Concentration
TaqMan Universal PCR Master Mix (2X)	25.0	1X
Forward primer	5.0	50 to 900 nM
Reverse primer	5.0	50 to 900 nM
TaqMan probe	5.0	50 to 250 nM
cDNA sample	5.0	10 to 100 ng
Nuclease-free water	5.0	—
Total	50.0	—

Notes



For most TaqMan reagent- or kit-based assays that are designed and run following Applied Biosystems assay development guidelines (refer to the *Sequence Detection Systems Chemistry Guide*), using a concentration of 900-nM primers and a 250-nM probe provides a highly reproducible and sensitive assay when using cDNA or DNA as a substrate in a singleplex assay.

Probes and primers that you design using Primer Express software must be optimized to work with the universal assay conditions, using the volumes listed in the table on [page 20](#).

All Assays-by-Design (ABD) and Assays-on-Demand (AOD) products are formulated so that the final concentration of the primers and probes falls within the recommended parameters. But because the primers and probes are supplied as a 20X assay mix, the volumes are slightly different from the universal assay conditions, as explained in the following Example.



CAUTION CHEMICAL HAZARD. TaqMan Universal PCR Master Mix 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.

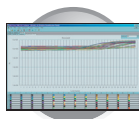
Example

Primers and probes for the sample RQ experiment were obtained from the Assays-on-Demand™ product line and were provided as a 20X Gene Expression Assay Mix. The PCR master mix was prepared as follows (according to guidelines specified in the product insert that comes with all AOD and ABD products):

Reaction Component	Volume (μL) Per Sample	Final Concentration
TaqMan Universal PCR Master Mix (2X)	25.0	1X
20X Assays-on-Demand™ Gene Expression Assay Mix (contains forward and reverse primers and labeled probe)	2.5	1X
cDNA sample	5.0	50 ng (for the 50-μL reaction)
Nuclease-free water	17.5	—
Total	50.0	—

The reactions were kept on ice until the plate was loaded on the 7000 SDS instrument.

Notes



Creating an RQ Plate Document

Summary An RQ Plate document is an SDS document that stores data collected from an RQ run for a single plate; there must be one RQ Plate document for every RQ plate. RQ Plate documents also store other information about the run, including sample names and detectors.

IMPORTANT! You cannot change the data collected during the run. However, the 7000 SDS instrument software allows you to change sample names and detectors even after a run has been completed.

A plate document appears as a three-tabbed pane in the software window. Each tab relates to a step in the analysis process: Setup, Instrument, and Results. The Results tab has several subtabs for the various viewers associated with an assay.

Run Setup Requirements Before creating an RQ Plate document, you must define several parameters for each RQ plate:

- Detectors – Determine which detector you will use for each sample and endogenous control. [Appendix A, “Creating Detectors,”](#) explains how to create detectors.

IMPORTANT! To conduct a comparative analysis of the data in a study, all the plates in the study must contain a common set of detectors.

- Endogenous control(s) – 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, there must be an endogenous control for each sample. [“Specifying the Components of an RQ Experiment” on page 9](#) explains the concept of an endogenous control. All plates must use the same endogenous control (for example, GAPDH).

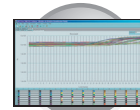
IMPORTANT! In order to open RQ Plate or RQ Study documents, you must have the RQ Study Software installed, as explained in [Appendix B](#).

Detector Tasks A task is a setting that you apply to the detectors within a well of a plate document and that determines the way the software uses the data collected from the well during analysis.



For RQ Plate documents, there are two types of tasks:

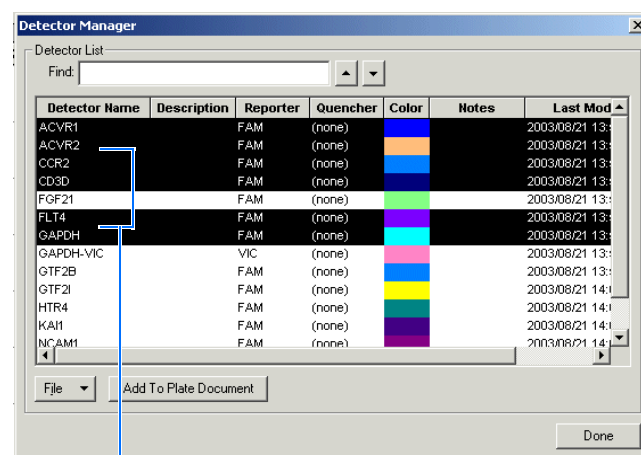
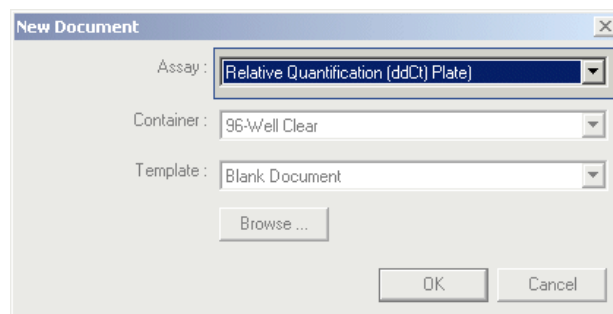
- Target – All detectors of wells that contain PCR reagents for the amplification of target sequences. The instrument software indicates targets by a **T**.
- Endogenous Control – All detectors of wells that contain reagents for the amplification of the endogenous control sequence. The instrument software indicates endogenous controls by an **E**.

Notes



To create a new plate document:

1. Select **Start > Programs > ABI Prism 7000 > ABI Prism 7000 SDS Software** () to start the 7000 SDS instrument software.
2. Select **File > New**. The New Document dialog box opens.
3. In the Assay drop-down list, select **Relative Quantification (ddCt) Plate**. Accept the default settings for the Container and Template fields (**96-Well Clear** and **Blank Document**).
4. Click **OK**. The 7000 SDS instrument software opens a new RQ Plate document.
5. Specify the detectors for the plate.
 - a. Select **Tools > Detector Manager**. The Detector Manager dialog box lists all detectors that have been created for the system. If no detectors are listed in the Detector Manager dialog box, create detectors as explained in [Appendix A, “Creating Detectors.”](#)
 - b. Click on the detector name/s to select the appropriate detector/s.
 - c. Click **Add to Plate Document**. The detectors are added to the plate document.
 - d. Click **Done** or  to close the Detector Manager.

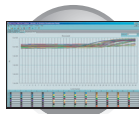



Selected detectors

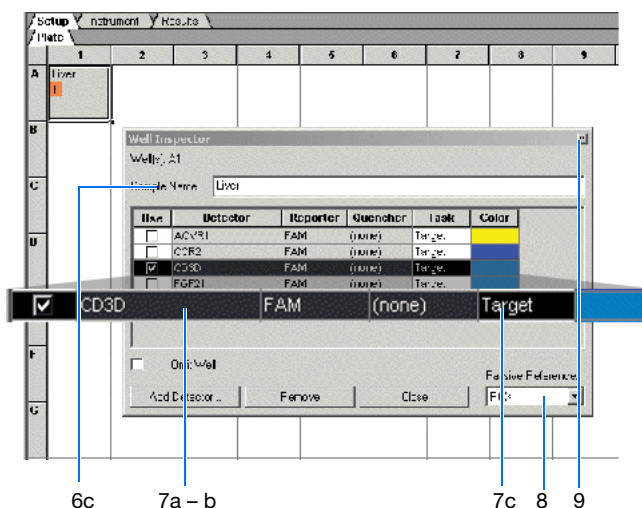
Note: After adding detectors to a document, the Detector Manager remains open until you close the window.

6. Label the wells of the plate.
 - a. Select **View > Well Inspector**. The Well Inspector dialog box lists the detectors that you added in [step 5](#).
 - b. Click a well to select it. If there are replicate wells for a sample, click on all the wells to select them.
 - c. In the Well Inspector, enter the sample name.

Notes



7. In the Well Inspector, select the detector/s and detector tasks for each sample.
 - a. Click on a detector to select it.
 - b. Click the Use check box.
 - c. Click under the Task column to assign the detector task.
 - d. Repeat steps a to c until all detectors and detector tasks have been specified.
8. Accept the default setting for the Passive Reference (ROX™ dye).
9. Click  to close the Well Inspector.
10. Verify the information on each well in the Setup tab.



Note: You can import sample information from spreadsheets or use template documents to set up plate documents. Refer to the Online Help for the 7000 SDS instrument for more information about these tasks.

Example

In the sample RQ experiment, the samples for each of the three tissues (liver, kidney, and bladder) were plated on three separate plates. Consequently, there were three RQ Plate documents created, one for each of the sample plates.

Because it is a singleplex experiment, there is only one sample—either a target or endogenous control, but not both—in each well. Each well is associated with a detector (indicated by the colored squares). Additionally, each well has also been assigned a detector task—T (target) or E (endogenous control).

The figure below shows a sample RQ Plate document after sample names, detectors, and detector tasks have been assigned for each well in the liver plate.

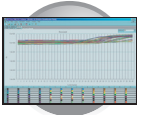
	1	2	3	4	5	6	7	8	9	10	11	12
A	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
B	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
C	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
D	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
E	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
F	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
G	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T
H	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T	Liver T

Sample Name

Detector Task and Color

For a sample illustration of how a multiplexed plate would appear, refer to the “Comparative Method” topic of the Online Help for the 7000 SDS instrument.

Notes

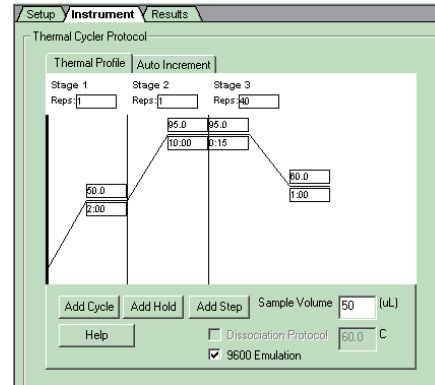


Specifying Thermal Cycling Conditions and Starting the Run

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 on the Instrument tab.

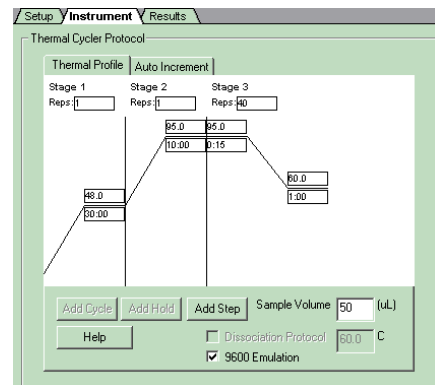


Times and Temperatures (Two-step RT-PCR)				
1) RT Step	HOLD	HOLD	* For reference only. RT is complete at this point.	
	10 min @ 25 °C	120 min @ 37 °C		
2) PCR Step	Initial Steps		PCR (Each of 40 cycles)	
	AmpErase® UNG Activation	AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/Extend
	HOLD	HOLD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

Thermal Cycling Conditions for One-Step RT-PCR

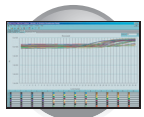
If you selected the one-step RT-PCR method, cDNA generation and amplification take place simultaneously at this point in the workflow.

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



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

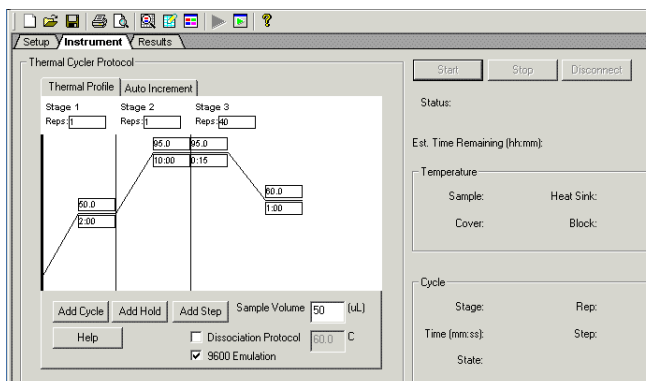
Notes



To specify thermal cycling conditions and start the run:

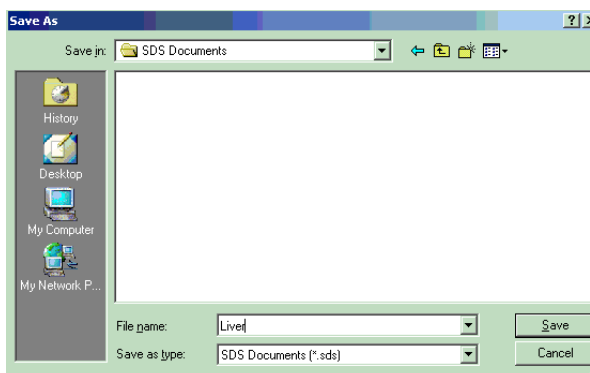
1. Select the *Instrument* tab.

By default, the standard PCR conditions for the PCR step of the two-step RT-PCR method are displayed.



2. Verify that:

- If you are using two-step RT-PCR – The default thermal cycling conditions are set.
- If you are using one-step RT-PCR – You set the thermal cycling parameters as shown in “[Thermal Cycling Conditions for One-Step RT-PCR](#)” on page 25.
- The sample volume is set to 50 µL.
- The 9600 Emulation check box is selected.



3. Select *File > Save As*, enter a name for the RQ Plate document, then click *Save*.

4. Load the plate into the instrument.

5. Click *Start*.

As the instrument performs the PCR run, it displays real-time status information in the *Instrument* tab. When the run is finished, the status values and the buttons are grayed-out. Additionally, the *Analysis* button is enabled (▶).

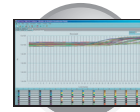
During the run, the instrument records the fluorescent emissions resulting from cleavage of TaqMan probes in the presence of the target and reference sequences.

All data generated during the run are saved to the RQ Plate document that you specified in [step 3](#).



Position plate with well A1 at the upper left-hand corner.

Notes



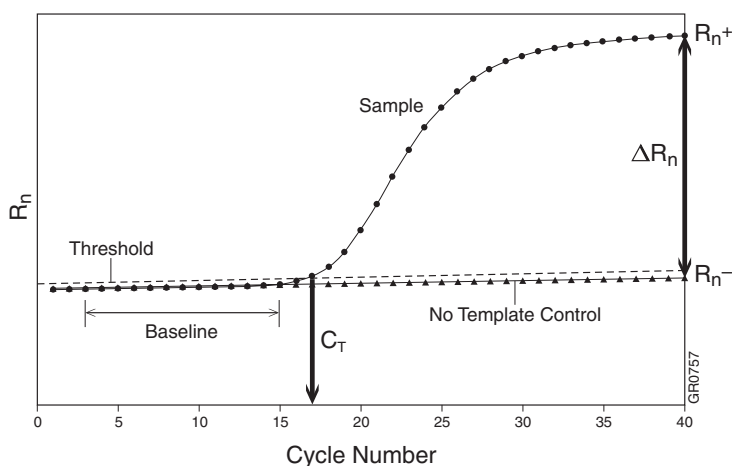
Analyzing and Viewing RQ Plate Data

Terms Used in Quantification Analysis

Following are terms commonly used in quantification analysis.

Term	Definition
Baseline	A line fit to the initial cycles of PCR, in which there is little change in fluorescence signal. For information about setting the baseline, refer to the Online Help for the 7000 SDS instrument.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold. For information about setting the threshold, refer to the Online Help for the 7000 SDS instrument.
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 fluorescent fluctuations caused by changes in concentration or of volume.
Reporter dye	The dye attached to the 5' end of a TaqMan probe. The dye provides a signal that is an indicator of 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 given set of PCR conditions. ($\Delta R_n = R_n - \text{baseline}$)

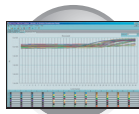
The figure below shows a representative amplification plot and includes some of the terms defined above.



Starting the Analysis

To analyze RQ Plate data after the run, 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. Based on that comparison, the software generates several types of result views, as described in the following section.

Notes

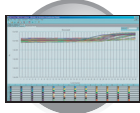


Types of Result Views

Viewing the analysis results helps you verify that the cDNA was correctly amplified. The four types of result views are shown in the following table.

View/Description	To view the plot...	Example
Plate Displays the sample name, detector task and color, and R_n value.	In the Results tab, click the Plate subtab. The plate appears on the screen.	
Spectra Displays a plot of spectra (raw fluorescence data) for the selected cycle number and well/s. The plot varies depending on the reporter dye used in the assay. The example on the right shows a successful amplification when the FAM reporter dye is used.	<ol style="list-style-type: none"> In the Results tab, click the Spectra subtab. The Cycle Number slider is at 1 and the plot is empty until you select wells. Click a well to include it in the plot. (Ctrl-click to include multiple wells; Click-drag to include multiple adjacent wells.) Move the slider to indicate the cycle number. 	
Component Displays a plot of fluorescence level of each dye against the cycle number for selected well/s.	<ol style="list-style-type: none"> In the Results tab, click the Component subtab. The graph is blank until you select wells. Click on a well to include it in the plot. (Ctrl-click to include multiple wells; Click-drag to include multiple adjacent wells.) 	

Notes



View/Description	To view the plot...	Example
Amplification Plot This view displays a plot of R_n against cycle number for the selected detector and well/s.	<ol style="list-style-type: none">1. In the Results tab, click the Amplification Plot subtab. The plot is empty until you select a detector (or all detectors) and well/s.2. Click on a well to include it in the plot. (Ctrl + click to include multiple wells; Click and drag to include multiple adjacent wells.)3. Select an individual detector or All (all detectors) in the Detector drop-down list.	

Note: You can modify the graph settings for several of the result views, as explained in [“Modifying Graph Settings” on page 39](#).

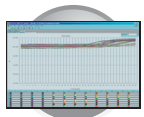
Reanalyzing Data

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

If you decide to omit wells or change well information after a run has been completed, you must reanalyze the data.

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

Notes _____

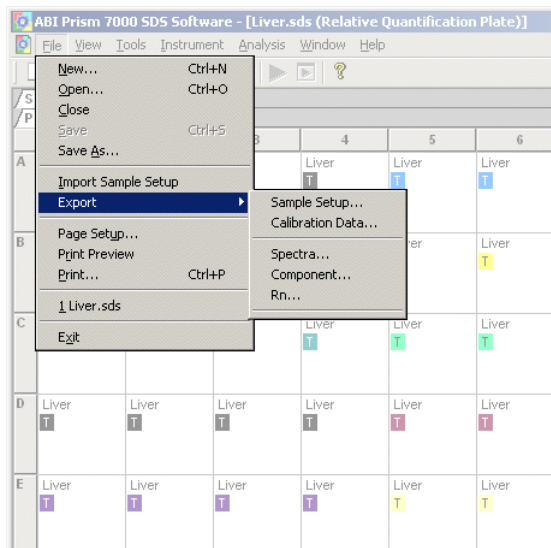


Exporting RQ Plate Documents

1. Select **File > Export**, then select the data type to export:

- Sample Setup (*.txt)
- Calibration Data (*.csv)
- Background Spectra (*.csv)
- Component (*.csv)
- Rn (*.csv)

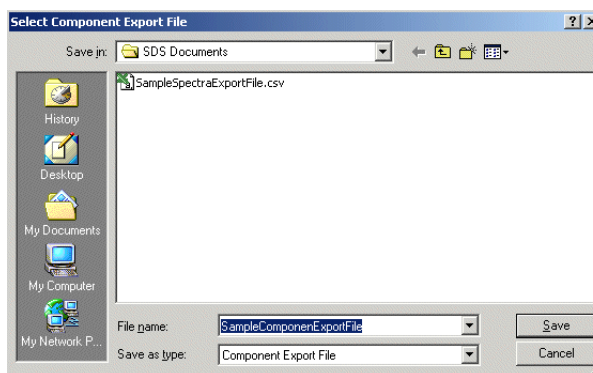
Typically, you export sample setup data for newly created and newly run plates; other data types are exported for existing plates.



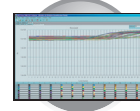
2. Enter a file name for the export file.

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

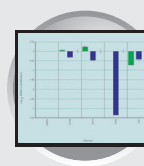
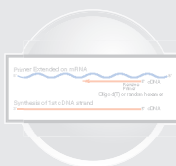
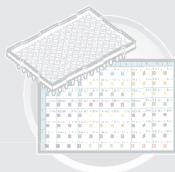
3. Click **Save**.



Notes

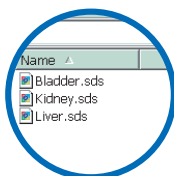


Notes _____



5

Have you have one or more RQ plate documents?



No

Chapter 4

Generate data and save it in RQ plate documents.

Yes

Have you created an RQ Study document?



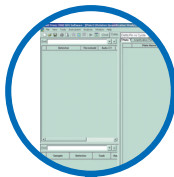
No

page 33

Create a new RQ study document.

Yes

Have you added plates to the study?



No

page 34

Add one or more plates to the study.

Yes

Have you configured the analysis settings for the study?



No

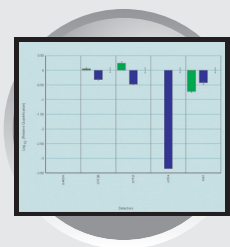
page 35

Specify the analysis settings for the study.

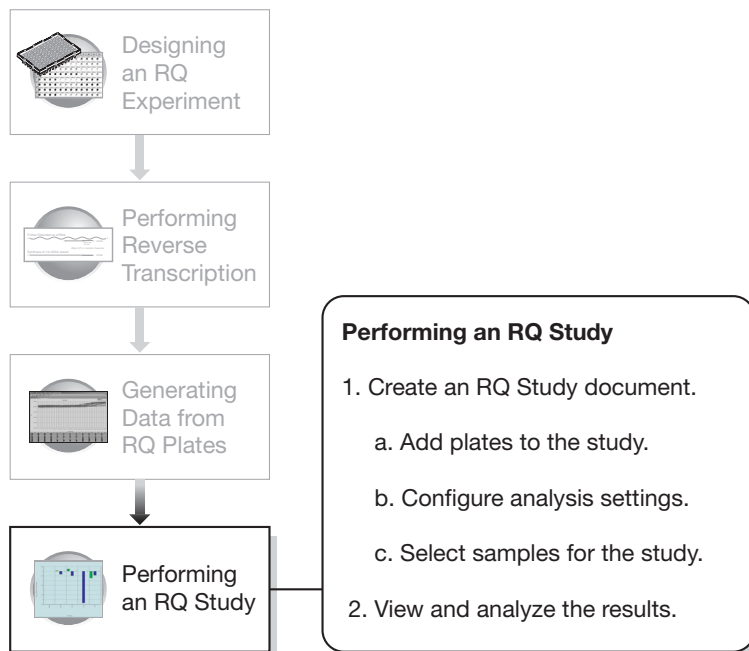
Yes

Analyze the results of the RQ study by viewing the amplification or gene expression plates.

Performing an RQ Study



Workflow

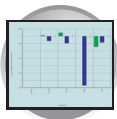


Creating an RQ Study Document

To conduct a comparative analysis of RQ plates in a study, you must first create an RQ Study document. The 7000 v1.1 software with the RQ Study Add On uses the comparative method ($2^{-\Delta\Delta C_t}$) of relative quantification. For more information about methods of calculating relative quantification, refer to *ABI PRISM® 7700 Sequence Detection System User Bulletin #2*.

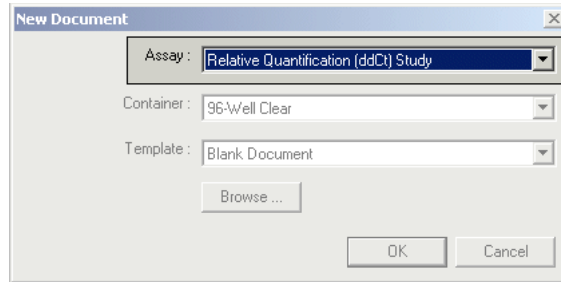
In an RQ study, you can...	You cannot
<ul style="list-style-type: none"> • Select the endogenous control and the calibrator sample • Select the control type (The 7000 SDS instrument software assigns the control type when applicable.) • Set baseline and threshold parameters and RQ Min/Max Confidence Levels • Omit individual wells or sample replicates 	<ul style="list-style-type: none"> • Create, add, or modify samples • Create, add, or modify detectors • Change detector tasks

Notes



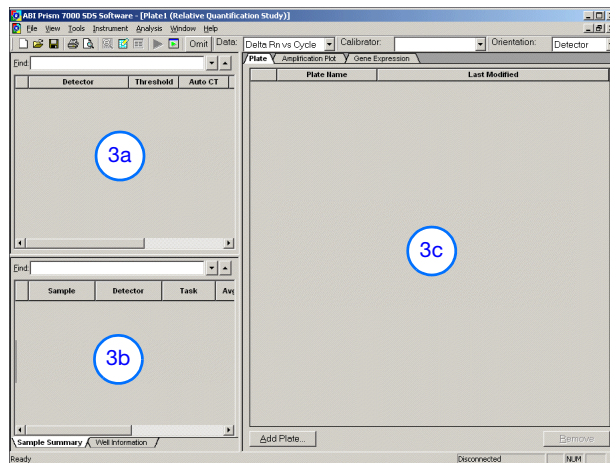
To create a new RQ Study document:

1. Select **File> New**. The New Document dialog box opens.
2. In the Assay drop-down list, select **Relative Quantification (ddCt) Study**. Accept the default settings for the Container and Template fields (**96-Well Clear** and **Blank Document**).
3. Click **OK**. The SDS software opens a new RQ Study document and displays the RQ Study Main View.

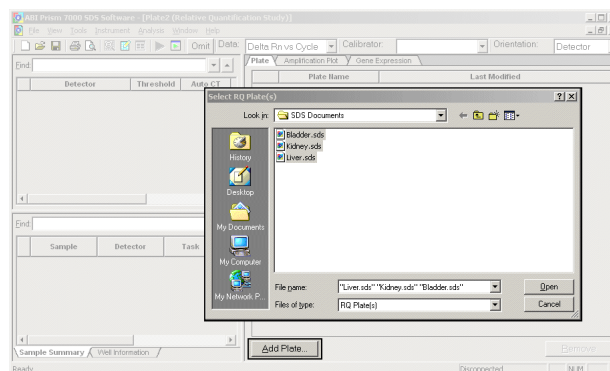


The RQ Study Main View has three frames:

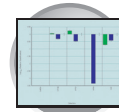
- a. RQ Detector Grid – Allows you to select detectors to associate with the loaded study. The following information is displayed for each detector: Color, Detector name, Threshold Value, Auto-Ct, and Baseline.
 - b. RQ Sample Grid – Displays the samples associated with the selected detector(s). The Sample Grid displays numerical results of RQ computations and has two subtabs: Sample Summary or Well Information.
 - c. RQ Results Panel – Contains the three results-based tabs (Plate, Amplification Plot, and Gene Expression).
4. Add plates to the study.
 - a. Click **Add Plate**. The Select RQ Plate(s) dialog box opens.
 - b. Select the plate(s) that you want to add to the study, then click **Open**. The selected plates are displayed in the Plate tab.



IMPORTANT! All plates added to a study must have identical thermal cycling parameters—the same number of steps, cycles, sample volume, emulation mode. The SDS software will reject a plate if it detects any differences.



Notes



5. Configure analysis settings.

- Select **Analysis > Analysis Settings**. The Analysis Settings dialog box opens.
- In the Detectors drop-down list, select **All**.
- Select **AutoCt** or **Manual Ct**.
 - If you select AutoCt, the SDS software automatically calculates both the threshold and baseline. After analysis, you must verify that the baseline and threshold were called correctly for each detector. Refer to the Online Help for the 7000 SDS instrument.
 - If you select ManualCt, specify both the threshold and the baseline for the selected detector(s).
- Select the calibrator sample.

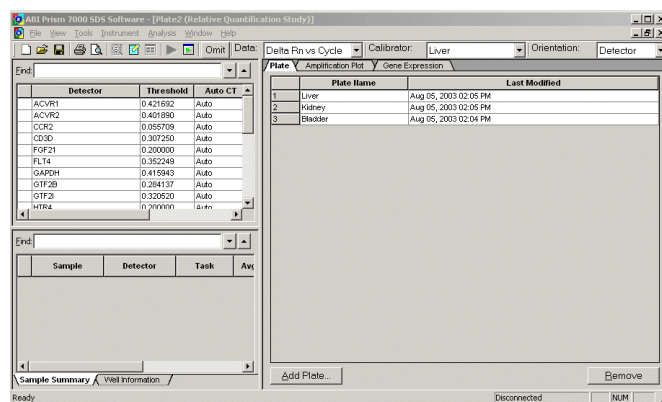
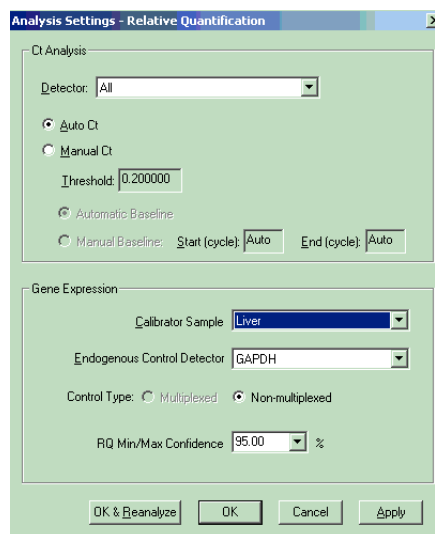
Note: As discussed in “[Specifying the Components of an RQ Experiment](#)” on [page 9](#), if your experiment uses only a single plate, there must be at least two different samples that have different names and have their own endogenous controls. (You can go back to a saved RQ Plate document and change the sample names, if necessary.)

- Select the Endogenous Control Detector.
- Select the Control Type if the study contains both multiplex and nonmultiplex reactions.

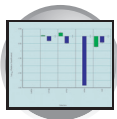
Note: If the study contains only singleplex reactions, Nonmultiplexed is automatically selected; if it contains only multiplex reactions, Multiplexed is automatically selected. Only when a study contains both types of reactions are you asked to indicate the control type.

- Select the RQ Min/Max Confidence level.
- Click **OK & Reanalyze**. The detector information appears in the RQ Detector grid.

For more information about the settings in the Analysis Settings dialog box, refer to the online help for the 7000 SDS instrument.



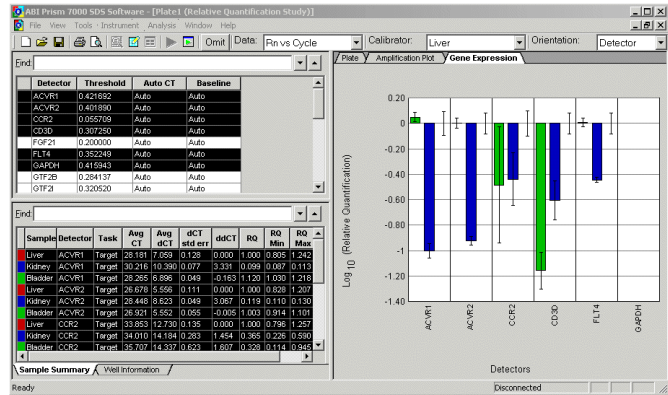
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6. In the RQ Detector Grid, select detectors to include in the result graphs by clicking a detector. (Ctrl-click to include multiple detectors; Click-drag to include multiple adjacent detectors.)

The corresponding samples appear in the RQ Sample Grid. Depending on which tab you select in the RQ Results Panel (Plate, Amplification Plot, or Gene Expression), analysis results are displayed.

To see information about a specific well, select the **Well Information** tab.

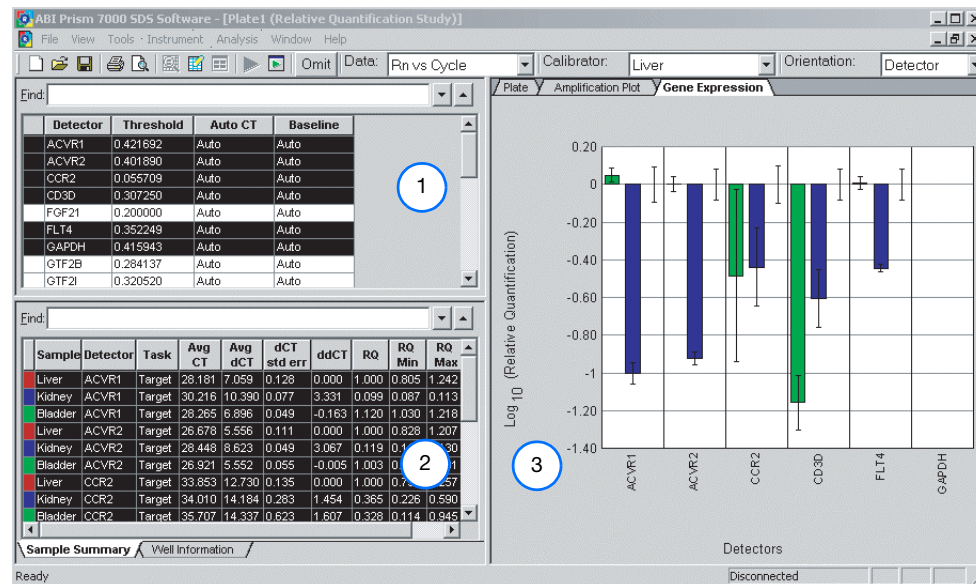


Example

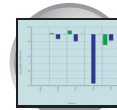
Suppose that you wanted to view the comparative gene expression levels of the following genes when the liver tissue was used as the calibrator: ACVR1, ACVR2, CCR2, CD3D, and FLT4.

Selecting the detectors in the RQ Detector grid (1) displays the sample information in the RQ Sample grid (2) and a result graph in the RQ Results panel (3). Note that

- The Gene Expression tab is selected.
- The gene expression levels are sorted by detector.
- Gene expression levels for bladder samples are denoted by the green bar; those for kidney samples by the blue bar. These colors are used to denote the samples in the RQ Sample Grid and the RQ Results Panel plots.
- Liver samples were used as calibrators; consequently, the expression level of these samples is set to 1. But because the graph plots gene expression levels as log₁₀ values (and the log₁₀ of 1 is 0), the expression level of the calibrator sample appears 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 in for GAPDH.
- The value in the RQ column in the Sample Summary tab is $2^{-\Delta\Delta CT}$.



Notes



Analyzing and Viewing the Results of the RQ Study

Viewing Amplification Plots

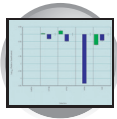
Amplification Plots show either of the following:

- The fluorescence of each detector as a function of cycle number
- The threshold cycle (C_T) as a function of well position.

View/Description	To view the plot...	Example
<p>Rn vs. Cycle (Linear)</p> <p>The Rn vs. Cycle plot displays normalized reporter (R_n) dye fluorescence as a function of cycle. You can use this plot to identify and examine irregular amplification.</p> <p>For more information about R_n, refer to the online help for the 7000 SDS instrument.</p>	<ol style="list-style-type: none"> 1. In the RQ Results panel, select the Amplification Plot tab. 2. In the Data drop-down list, select Rn vs. Cycle. 	
<p>ΔR_n vs. Cycle (Log)</p> <p>The ΔR_n vs. Cycle plot displays Rn dye fluorescence 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.</p>	<ol style="list-style-type: none"> 1. In the RQ Results panel, select the Amplification Plot tab. 2. In the Data drop-down list, select Delta Rn vs. Cycle. 	
<p>Ct vs. Well Position</p> <p>The Ct vs. Well Position plot 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 41 for more information).</p>	<ol style="list-style-type: none"> 1. In the RQ Results panel, select the Amplification Plot tab. 2. In the Data drop-down list, select Ct vs. Well Position. 	

Refer to [“Terms Used in Quantification Analysis”](#) on page 27 for definitions of R_n , ΔR_n , C_T , and other terms used in quantification analysis.

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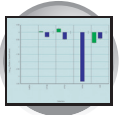
Viewing Gene Expression Plots

Gene Expression plots show the expression level or fold-difference of the target sample relative to the calibrator as \log_{10} of the $2^{-\Delta\Delta Ct}$ value. For example, if the bladder has a \log_{10} relative quantification value of 5 relative to the kidney, then that gene (detector) is expressed at a level five times higher in the bladder than in the kidney.

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

View/Description	To view the plot...	Example
<p>Gene Expression Plot Orientation: Detector</p> <p>Detectors are plotted on the x-axis, and each bar shows the detector value of a given sample.</p>	<p>1. In the RQ Results panel, select the Gene Expression tab.</p> <p>2. In the Orientation drop-down list, select Detector.</p>	
<p>Gene Expression Plot Orientation: Sample</p> <p>Samples are plotted on the x-axis, and each bar shows the set of sample values of a given detector.</p>	<p>1. In the RQ Results panel, select the Gene Expression tab.</p> <p>2. In the Orientation drop-down list, select Sample.</p>	

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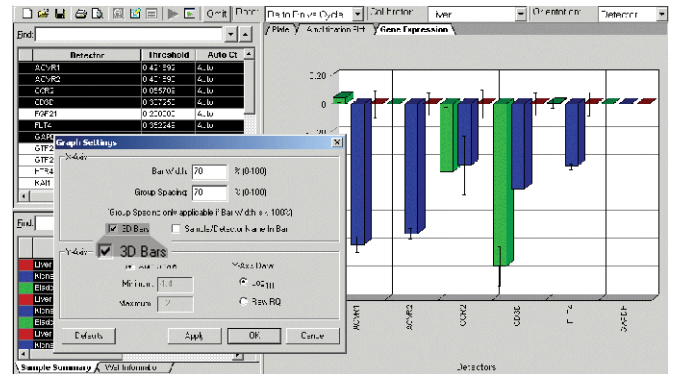


Modifying Graph Settings

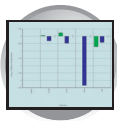
You can modify the default graph settings for several graphs, including Amplification and Gene Expression plots. For example, you can use labeled 3-D bars in the Gene Expression plot.

1. In a graph, double-click an axis of a plot or select **Analysis > Graphical Settings** to display the Graph Settings dialog box.
 - To set real-time settings, change them before you start the run. Auto Scale is the default.
 - To set the graph scaling for post-run data, set the y-axis of the graph to linear or log.
2. Select **Apply**. The system applies the changes made in the graphical settings box to the active plot.
3. Select **OK** to close the Graph Settings dialog.

For more information about graph settings, refer to the Online Help for the 7000 SDS instrument.



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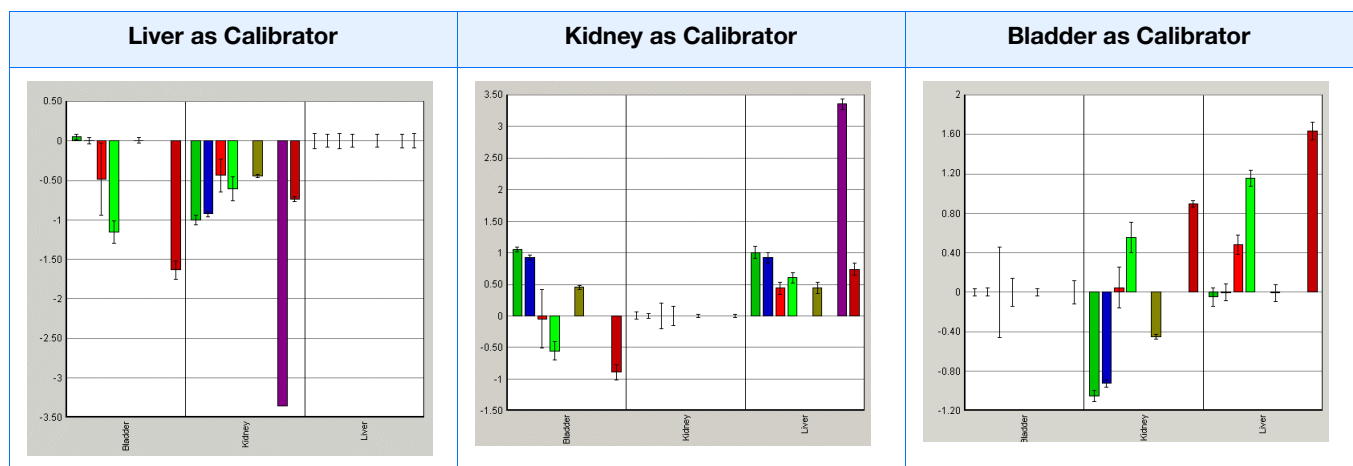


Reanalyzing an RQ Study

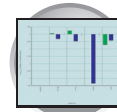
If you change any of the analysis settings, you must reanalyze the data before you can view any results. (You can switch between the variations of the Amplification and Gene Expression plots without having to reanalyze the data.)

Suppose you select Liver as the calibrator, then perform an analysis. Next, you view the Amplification and Gene Expression plots. If you then want to use Kidney or Bladder 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.



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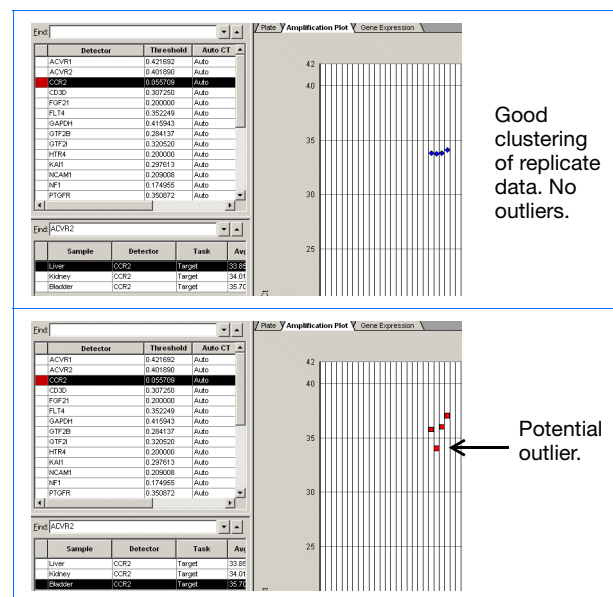
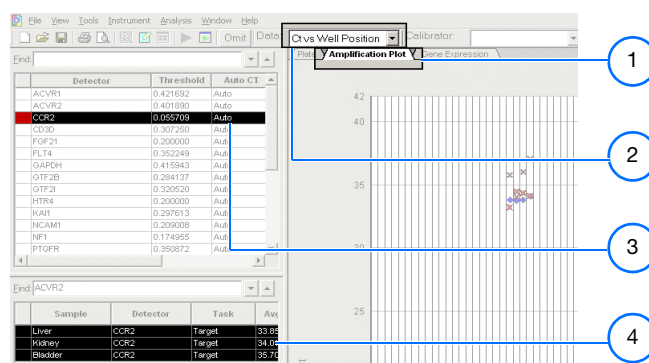
Omitting Samples from a Study

For any PCR, 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 outliers can result in erroneous measurements.

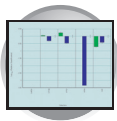
To ensure precise relative quantification, you must carefully view replicate groups for outlying wells. You can remove outliers manually using the C_T vs. Well Position Amplification Plot.

To remove samples from an RQ Study:


1. Select the **Amplification Plot** tab.
2. In the Data drop-down list, select **Ct vs. Well Position**.
3. In the RQ Detector grid, select a detector to examine. All samples that use this detector are displayed in the RQ Samples grid.
4. In the RQ Samples grid, click to select the samples to display in the Amplification Plot.
5. Verify the uniformity of each replicate population by comparing the groupings of C_T values for the wells that make up the set.

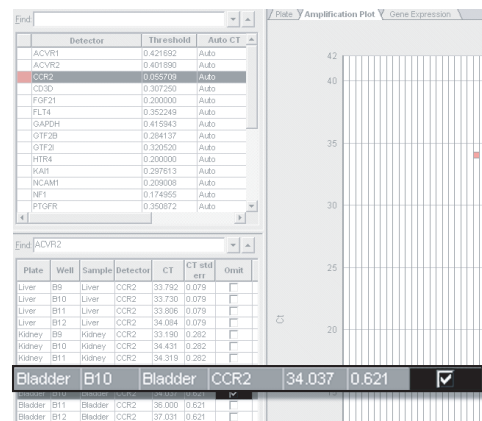
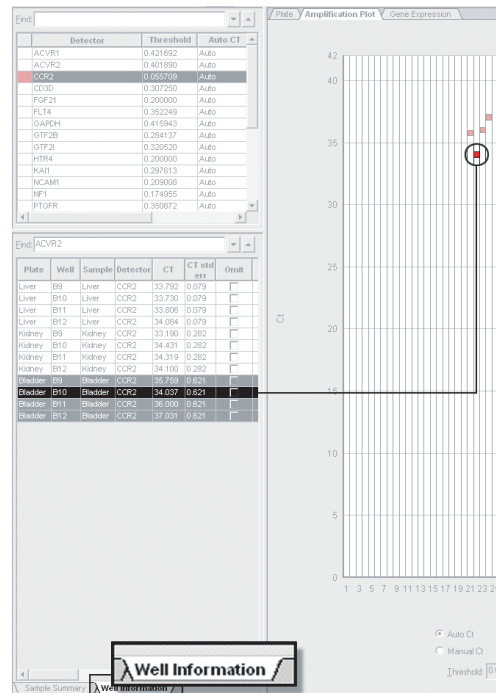


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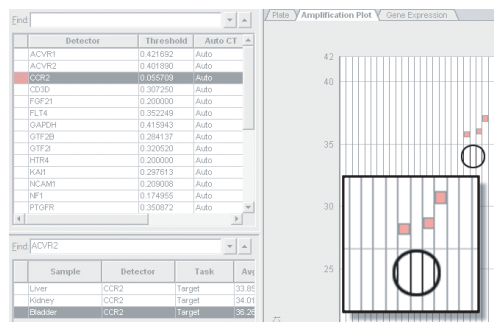


Chapter 5 Performing an RQ Study Omitting Samples from a Study

6. Do one of the following:
 - If outliers are present, select the **Well Information** tab, find the outlying sample, and select the **Omit** check box for the sample.
 - If outliers are not present, go to step 7.
7. Repeat steps 5 and 6 to screen the remaining replicate groups.
8. Select **Analysis > Analyze** () to reanalyze the run without the outlying data.
9. Repeat steps 3 to 8 for other detectors you want to screen.

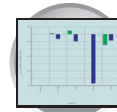


Select the Omit check box.



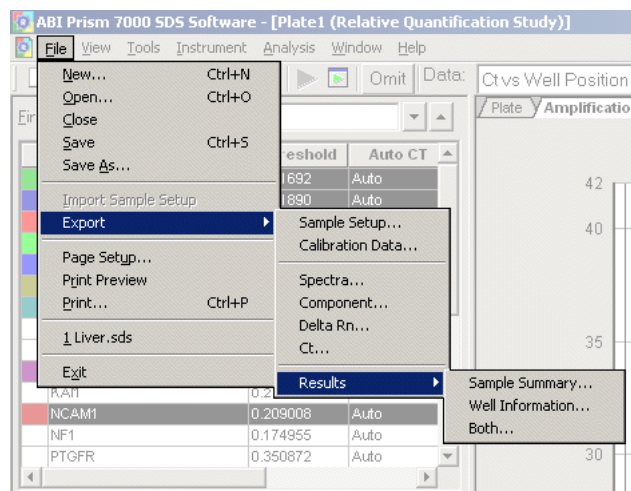
The outlier is removed during analysis.

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Exporting RQ Study Results

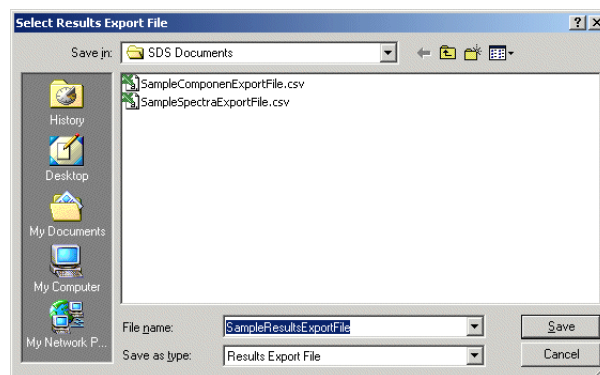
1. Select **File > Export > Results**, then select the data type to export:
 - Sample Summary (*.csv)
 - Well Information (*.csv)
 - Both (*.csv)



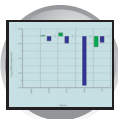
2. Enter a file name for the export file.

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

3. Click **Save**.



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Chapter 5 Performing an RQ Study

Exporting RQ Study Results

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References

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* 25:402–408.

Saiki, R.K., Scharf, S., Faloona, F., *et al.* 1985. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

Creating Detectors

A

Before you can use a plate document to run a plate, it must be configured with detector information for the experiment. A detector is a virtual representation of a gene- or allele-specific nucleic acid probe reagent used for analyses performed on SDS instruments. Examples of reagents represented as detectors include TaqMan® probes and the SYBR® Green 1 dsDNA binding dye.

You must create and apply detectors for all assays present on the plate before running it.

To create a detector:

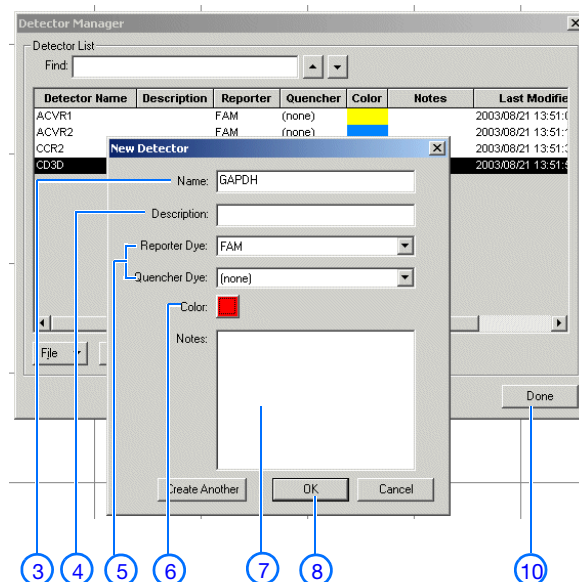
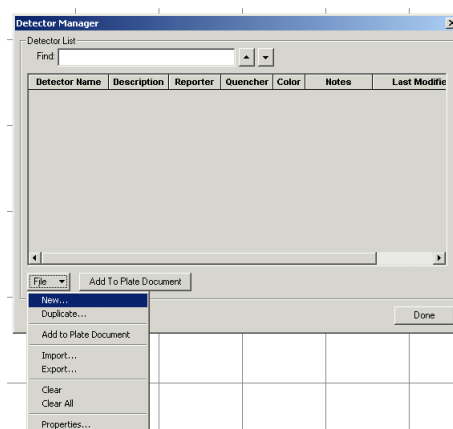
1. Select **Tools > Detector Manager**. The Detector Manager dialog box opens.

Note: A plate document (any type) must be open before you can access the Tools menu.

2. In the Detector Manager, select **File > New**. The New Detector dialog box opens.
3. In the New Detector dialog box, enter a name for the detector.

IMPORTANT! The name of the detector must be unique and should reflect the target locus of the assay (such as GAPDH or RNase P). Do not use the same name for multiple detectors.

4. Optionally, click the **Description** field, then enter a brief description of the detector.



Notes

5. In the Reporter Dye and Quencher Dye drop-down lists, select the appropriate dyes for the detector.

Note: The dyes that appear on the Reporter and Quencher Dye lists are those that have been previously entered using the Dye Manager. If the dye that you want to use does not appear in a list, use the Dye Manager to add the dye and then return to this step in this procedure. Refer to the Online Help for the 7000 SDS instrument for more information.

6. Click the **Color** box, select a color to represent the detector using the Color dialog box, then click OK.
7. Optionally, click the **Notes** field, then enter any additional comments for the detector.
8. Click **OK** to save the detector and return to the Detector Manager dialog box.
9. Repeat [steps 2 through 8](#) for the remaining detectors.
10. In the Detector Manager dialog box, click **Done** when you have finished adding detectors.

Example

In the sample RQ experiment, a detector was created for each target gene and endogenous control. 24 detectors were created: 23 for the target genes and 1 for the endogenous control, GAPDH.

For example, the detector for the ACVR1 gene was named ACVR1 and assigned a yellow color. Because all Assays-on-Demand™ products have probes that are labeled with FAM™ dye, FAM was selected for the reporter dye. Additionally, Assays-on-Demand products use TaqMan MGB probes, which do not need quenchers; no quencher dye was selected for the detector.

Assays-on-Demand products are shipped with an assay information file (AIF). This text-based file contains information about the assays that you ordered, including the Applied Biosystems Assay ID number, well-location of each assay, primer concentration, and primer sequence. The file also indicates the reporter dyes and quenchers (if applicable) that are used for each assay. When creating detectors, you would use the reporter dye and quencher information (and optionally, the gene name or symbol for the sample name).

You can view the contents of AIFs in a spreadsheet program, such as Microsoft Excel.

Notes

Upgrading the 7000 Software with RQ Study

B

Installing the RQ Study Software

This section describes how to upgrade ABI PRISM® 7000 SDS Software v1.1 to add the RQ Study v1.0 software. The new software is installed in the ABI PRISM 7000 folder.

For a new installation, you can install the RQ Study v1.0 software when you install the ABI PRISM® 7000 SDS Software v1.1. Follow the instructions in the *ABI PRISM 7000 SDS Installation Guide*.

Before you begin installation, check that you have:

- The RQ Study v1.0 Installer CD.
- Installed the current ABI PRISM® 7000 SDS Software v1.1.
- Administrator privileges.

To install the RQ Study software:

1. Insert the RQ Study v1.0 Installer CD into the CD-ROM drive. The installer on the CD starts automatically. Follow the onscreen instructions as the software installs the files.
2. When the Welcome window opens, click **Next**. The New Installation page opens to indicate that the installer is ready to perform a new installation.
3. Click **Next** again. The wizard loads all the necessary files, creates the ABI PRISM 7000 SDS Software shortcut on the desktop, and registers your software.
4. When the installation is complete, click **Finish**.
5. When the software opens, enter the Registration code on the CD jewel case cover. Cancel if you do not have a registration code.
6. Remove the CD, place it in its holder, and store it in a safe place.

Checking Program File Location

After the software is installed, check that the program files are in the following locations:

- The default location for the 7000 SDS software program:
C:\Program Files\ABI Prism 7000\Prism7000.exe

Notes

- The Start menu location for the 7000 SDS software shortcut:

Start\Programs\ABI Prism 7000\ABI Prism 7000 SDS Software

IMPORTANT! The installer program creates a link to the ABI PRISM 7000 SDS Software by placing a shortcut icon on the desktop during the installation. Clicking the icon opens the program from the desktop without having to go through the Start menu.

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