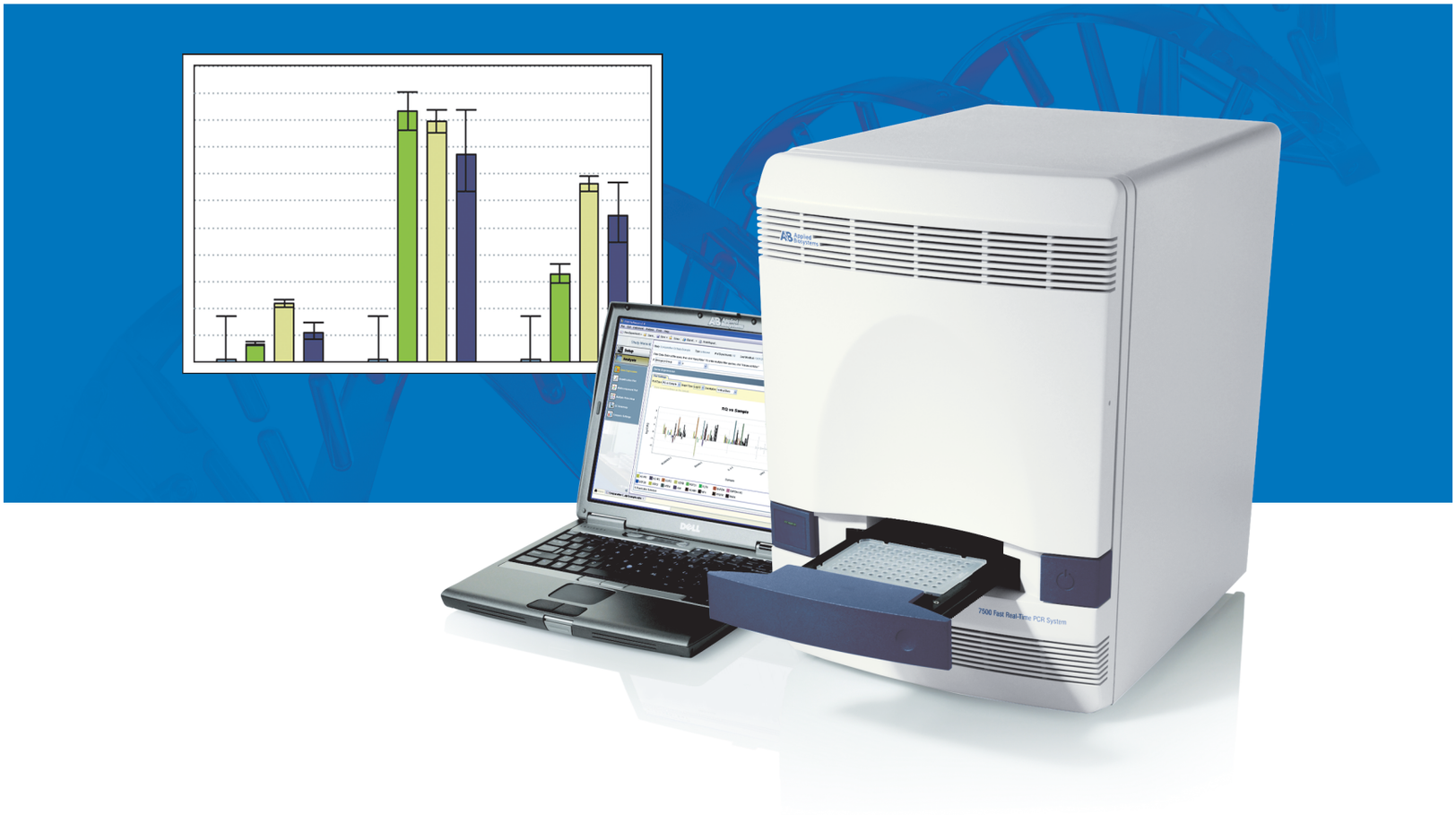
Applied Biosystems

Applied Biosystems 7500/7500 Fast

Real-Time PCR System

Relative Standard Curve and Comparative C_T Experiments



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Real-Time PCR System

Relative Standard Curve and Comparative C_T Experiments

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Preface

How to Use This Guide

About the System Documentation

The guides listed below are shipped with the Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems.

Guide	Purpose and Audience	PN
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Genotyping Experiments	Explains how to perform experiments on the 7500/7500 Fast system. Each Getting Started Guide functions as both a: • Tutorial, using example experiment data provided with the Applied Biosystems 7500/7500 Fast Real-Time PCR Software (7500/7500 Fast software). • Guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the 7500/7500 Fast system.	4387784
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Presence/Absence Experiments		4387785
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments		4387783
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments		4387779
Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide	Explains how to install and maintain the 7500/7500 Fast system. Intended for laboratory staff responsible for the installation and maintenance of the 7500/7500 Fast system.	4387777
Applied Biosystems 7500/7500 Fast Real-Time PCR System Installation Quick Reference Card		4387778
Real Time PCR System Reagent Guide	Provides information about the reagents you can use on the 7500/7500 Fast system, including: • An introduction to TaqMan® and SYBR® Green reagents. • Descriptions and design guidelines for the following experiment types: – Quantitation experiments – Genotyping experiments – Presence/absence experiments Intended for laboratory staff and principal investigators who perform experiments using the 7500/7500 Fast system.	4387787
Applied Biosystems 7500/7500 Fast Real-Time PCR System Site Preparation Guide	Explains how to prepare your site to receive and install the 7500/7500 Fast system. Intended for personnel who schedule, manage, and perform the tasks required to prepare your site for installation of the 7500/7500 Fast system.	4387776

Guide	Purpose and Audience	PN
Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 Help	Explains how to use the 7500/7500 Fast software to: • Set up, run, and analyze experiments using the 7500/7500 Fast system. • Calibrate a 7500/7500 Fast instrument. • Verify the performance of a 7500/7500 Fast instrument with an RNase P run. Intended for: • Laboratory staff and principal investigators who perform experiments using the 7500/7500 Fast system. • Laboratory staff responsible for the installation and maintenance of the 7500/7500 Fast system.	NA

Assumptions

This guide assumes that you:

- Are familiar with the Microsoft Windows® XP operating system.
- Are familiar with the Internet and Internet browsers.
- Know how to handle DNA and/or RNA samples and prepare them for PCR.
- Understand data storage, file transfer, and copying and pasting.
- Have networking experience, if you plan to integrate the 7500/7500 Fast system into your existing laboratory data flow.

Text Conventions

This guide uses the following conventions:

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

  Enter **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 symbol (▸) separates successive commands you select from a drop-down list or shortcut menu. For example:

  Select **File ▸ Open**.

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 reagent kit use, or safe use of a chemical.

Examples of the user attention words appear below:

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

IMPORTANT! To verify your client connection, you need a valid user ID.

Safety Alert Words

Safety alert words also appear in user documentation. For more information, see "Safety Alert Words" on page xii.

How to Obtain More Information

Related Documentation

Documents Related to Genotyping Experiments

Document	PN
Allelic Discrimination Pre-Developed TaqMan® Assay Reagents Quick Reference Card	4312212
Custom TaqMan® Genomic Assays Protocol Submission Guidelines	4367671
Custom TaqMan® SNP Genotyping Assays Protocol	4334431
Ordering TaqMan® SNP Genotyping Assays Quick Reference Card	4374204
Performing a Custom TaqMan® SNP Genotyping Assay for 96-Well Plates Quick Reference Card	4371394
Performing a TaqMan® Drug Metabolism Genotyping Assay for 96-Well Plates Quick Reference Card	4367636
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol	4312214
TaqMan® Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan® SNP Genotyping Assays Protocol	4332856

Documents Related to Presence/Absence Experiments

Document	PN
DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs Protocol	4343586
NucPrep® Chemistry: Isolation of Genomic DNA from Animal and Plant Tissue Protocol	4333959

Documents Related to Relative Standard Curve and Comparative C_T Experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

Documents Related to Standard Curve Experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
TaqMan® Gene Expression Assays Protocol	4333458
User Bulletin #2: Relative Quantitation of Gene Expression	4303859

Documents Related to the Reagent Guide

Document	PN
Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
Custom TaqMan® Gene Expression Assays Protocol	4334429
Custom TaqMan® Genomic Assays Protocol: Submission Guidelines	4367671
Custom TaqMan® SNP Genotyping Assays Protocol	4334431
Power SYBR® Green PCR Master Mix and RT-PCR Protocol	4367218
Pre-Developed TaqMan® Assay Reagents Allelic Discrimination Protocol	4312214
Primer Express® Software Version 3.0 Getting Started Guide	4362460
SYBR® Green PCR and RT-PCR Reagents Protocol	4304965
SYBR® Green PCR Master Mix and RT-PCR Reagents Protocol	4310251
TaqMan® Drug Metabolism Genotyping Assays Protocol	4362038
TaqMan® Exogenous Internal Positive Control Reagents Protocol	4308335
TaqMan® Fast Universal PCR Master Mix (2X) Protocol	4351891
TaqMan® Gene Expression Assays Protocol	4333458
TaqMan® Gene Expression Master Mix Protocol	4371135
TaqMan® Genotyping Master Mix Protocol	4371131
TaqMan® SNP Genotyping Assays Protocol	4332856
TaqMan® Universal PCR Master Mix Protocol	4304449
User Bulletin #2: Relative Quantitation of Gene Expression	4303859
Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies Application Note	127AP08

Note: For more documentation, see "How to Obtain Support" on page xi.

Obtaining Information from the Software Help

The 7500 Software Help describes how to use each feature of the touchscreen. Open the Help from within the software by doing one of the following:

- Press **F1**.
- Click in the toolbar.
- Select **Help ▸ 7500 Software Help**.

To find topics of interest in the Help:

- Review the table of contents.
- Search for a specific topic.
- Search the alphabetized index.

Send Us Your Comments

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

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. (See "How to Obtain Support" on page xi).

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

IMPORTANT! When directed to do so by this guide, or when you need to schedule maintenance for your 7500/7500 Fast instrument (such as annual planned maintenance or temperature verification/calibration), contact the Applied Biosystems Care Center. To obtain a phone number for or to send an e-mail to the center, go to **http://www.appliedbiosystems.com/support/contact**.

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 IMPORTANTs, 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 xiii).

Examples

The following examples show the use of safety alert words:

IMPORTANT! Wear powder-free gloves when you handle the halogen lamp.

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

WARNING **CHEMICAL HAZARD.** Ethanol is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. 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	Symbol	Description
	Indicates the **On** position of the 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 the **Off** position of the main power switch.		Indicates a protective grounding terminal that must be connected to earth ground before any other electrical connections are made to the instrument.
	Indicates a standby switch by which the instrument is switched on to the **Standby** condition. Hazardous voltage may be present if this switch is on standby.		Indicates a terminal that can receive or supply alternating current or voltage.
	Indicates the **On/Off** position of a push-push main power switch.		Indicates a terminal that can receive or supply alternating or direct current or voltage.

Safety Symbols

The following table describes the safety *s*ymbols 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 xiv). 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.

Environmental Symbols on Instruments

The following symbol applies to all Applied Biosystems electrical and electronic products placed on the European market after August 13, 2005.

Symbol	Description
	Do not dispose of this product as unsorted municipal waste. Follow local municipal waste ordinances for proper disposal provisions to reduce the environmental impact of waste electrical and electronic equipment (WEEE). **European Union customers:** Call your local Applied Biosystems Customer Service office for equipment pick-up and recycling. See **http://www.appliedbiosystems.com** for a list of customer service offices in the European Union.

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	Français
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 Hazardous waste. Refer to MSDS(s) and local regulations for handling and disposal.	**ATTENTION** Déchets dangereux. Lire les fiches techniques de sûreté de matériels et la régulation locale associées à la manipulation et l'élimination des déchets.
WARNING Hot lamp.	**AVERTISSEMENT** Lampe brûlante.
WARNING Hot. Replace lamp with an Applied Biosystems lamp.	**AVERTISSEMENT** Composants brûlants. Remplacer la lampe par une lampe Applied Biosystems.
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.
WARNING This instrument is designed for 12 V, 75 W Halogen lamps only.	**AVERTISSEMENT** Cet instrument est conçu pour des lampes d'halogène de 12 V et 75 W seulement.

Locations of Warnings

The Applied Biosystems 7500/7500 Fast Real-Time PCR System contains warnings at the locations shown below.

General Instrument Safety

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

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.

Moving and Lifting Stand-Alone Computers and Monitors

WARNING Do not attempt to lift or move the computer or the monitor without the assistance of others. Depending on the weight of the computer and/or the monitor, moving them may require two or more people.

Things to consider before lifting the computer and/or the monitor:

- Make sure that you have a secure, comfortable grip on the computer or the monitor when lifting.
- Make sure that the path from where the object is to where it is being moved is clear of obstructions.
- Do not lift an object and twist your torso at the same time.
- Keep your spine in a good neutral position while lifting with your legs.
- Participants should coordinate lift and move intentions with each other before lifting and carrying.
- Instead of lifting the object from the packing box, carefully tilt the box on its side and hold it stationary while someone slides the contents out of the box.

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 xvii.

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.

Cleaning or Decontaminating the Instrument

CAUTION Before using a cleaning or decontamination method other than those recommended by the manufacturer, verify with the manufacturer that the proposed method will not damage the equipment.

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.

WARNING **CHEMICAL HAZARD.** All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

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.

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

The MSDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain MSDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then click **MSDS Search**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS 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

Note: For the MSDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About MSDSs” on page xvii.)
- 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.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Electrical Safety

ELECTRICAL SHOCK HAZARD. Severe electrical shock can result from operating the Applied Biosystems 7500/7500 Fast Real-Time PCR System without its instrument panels in place. Do not remove instrument panels. High-voltage contacts are exposed when instrument panels are removed from the instrument.

Fuses

FIRE HAZARD. Improper fuses or high-voltage supply can damage the instrument wiring system and cause a fire. Before turning on the instrument, verify that the fuses are properly installed and that the instrument voltage matches the power supply in your laboratory.

FIRE HAZARD. For continued protection against the risk of fire, replace fuses only with fuses of the type and rating specified for the instrument.

Power

ELECTRICAL HAZARD. Grounding circuit continuity is vital for the safe operation of equipment. Never operate equipment with the grounding conductor disconnected.

ELECTRICAL HAZARD. Use properly configured and approved line cords for the voltage supply in your facility.

ELECTRICAL HAZARD. Plug the system into a properly grounded receptacle with adequate current capacity.

Overvoltage Rating

The Applied Biosystems 7500/7500 Fast Real-Time PCR System has an installation (overvoltage) category of II, and is classified as portable equipment.

Physical Hazard Safety

Moving Parts

PHYSICAL INJURY HAZARD. Moving parts can crush and cut. Keep hands clear of moving parts while operating the instrument. Disconnect power before servicing the instrument.

Biological Hazard Safety

General Biohazard

WARNING **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, 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 equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **bmbl.od.nih.gov**).
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR §1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

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 61010A-1, "Safety Requirements for Electrical Equipment for Laboratory Use, Part 1: General Requirements" and with standard UL 61010-2-010, "Particular Requirements for Laboratory Equipment for the Heating of Materials."

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 2006/95/EC). 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," and with standard EN 61010-2-081:2002+A1:2003 "Particular Requirements for Automatic and Semi-Automatic Laboratory Equipment for Analysis and Other Purposes."

EMC

This instrument meets European requirements for emission and immunity (EMC Directive 2004/108/EC). 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 Get Started

This chapter covers:

- About the 7500/7500 Fast System 2
- Supported Consumables 4
- About Relative Standard Curve and Comparative C_T Experiments 7
- How to Use This Guide. 12
- About the Example Experiments 14
- Example Experiment Workflow 17

Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

About the 7500/7500 Fast System

The Applied Biosystems 7500/7500 Fast Real-Time PCR System is a 96-well, five-color platform that uses fluorescence-based polymerase chain reaction (PCR) reagents to provide:

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

The Applied Biosystems 7500 Fast Real-Time PCR System enables you to perform high-speed thermal cycling with run times for quantitative real-time PCR applications (such as relative quantitation) of fewer than 40 minutes.

About Data Collection

The 7500/7500 Fast system collects raw fluorescence data at different points during a PCR, depending on the type of run that the instrument performs:

Run Type		Data Collection Point
Real-time runs	Standard curve	The instrument collects data after each extension step of the PCR.
	Relative standard curve	
	Comparative C_T ($\Delta\Delta C_T$)	
Post-PCR (endpoint) runs	Genotyping	The instrument collects data: • Before the PCR. (For presence/absence experiments, data collection before the PCR is optional but recommended.) • (Optional) During the PCR. The instrument can collect data during the run (real-time); collecting data during the run can be helpful for troubleshooting. • After the PCR.
	Presence/absence	

Regardless of the run type, a data collection point or *read* on the 7500/7500 Fast instrument consists of three phases:

1. **Excitation** – The instrument illuminates all wells of the reaction plate within the instrument, exciting the fluorophores in each reaction.
2. **Emission** – The instrument optics collect the residual fluorescence emitted from the wells of the reaction plate. The resulting image collected by the device consists only of light that corresponds to the range of emission wavelengths.
3. **Collection** – The instrument assembles a digital representation of the residual fluorescence collected over a fixed time interval. The 7500 software stores the raw fluorescence image for analysis.

After a run, the 7500 software uses region of interest (ROI), optical, dye, and background calibrations to determine the location and intensity of the fluorescence in each read, the dye associated with each fluorescent signal, and the significance of the signals.

Notes

About the Filters

The 7500/7500 Fast system uses the following filters:

Filter	1	2	3	4	5
Dye	• FAM™ dye • SYBR® Green dye	• JOE™ dye • VIC® dye	• TAMRA™ dye • NED™ dye • Cy3® dye	• ROX™ dye • Texas Red® dye	Cy5® dye

For More Information

For information on:

- The 7500/7500 Fast system – Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR Software Help*.

  Note: To open the Help, select **Help ▸ 7500 Software Help** in the 7500 software.

- Genotyping experiments – Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Genotyping Experiments*.
- Presence/absence experiments – Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Presence/Absence Experiments*.
- Standard curve experiments – Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve and Experiments*.

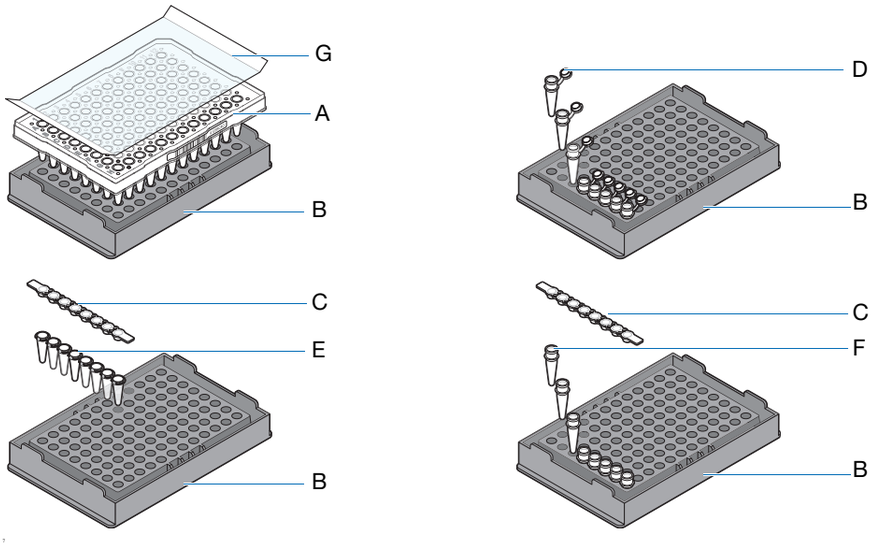
Notes

Supported Consumables

7500 System

The 7500 system supports the consumables listed below.

Consumable	Part Number
• MicroAmp® Optical 96-Well Reaction Plate with Barcode, 0.2-mL • MicroAmp™ Optical Adhesive Film	• 4306737 • 4311971
• MicroAmp™ Optical 8-Tube Strip, 0.2-mL • MicroAmp™ Optical 8-Cap Strip	• 4316567 • 4323032
• MicroAmp® Optical Tube without Cap, 0.2-mL • MicroAmp® Reaction Tube with Cap, 0.2-mL	• N8010933 • N8010540
• Microamp® Splash Free Support Base	• N8010531
• MicroAmp™ Adhesive Film Applicator • MicroAmp® Cap Installing Tool (Handle) • MicroAmp™ Multi-Removal Tool	• 4333183 • 4330015 • 4313950



#	Consumable
A	MicroAmp® Optical 96-Well Reaction Plate, 0.2-mL
B	Microamp® Splash Free Support Base
C	MicroAmp™ Optical 8-Cap Strip, 0.2-mL
D	MicroAmp® Reaction Tube with Cap, 0.2-mL

#	Consumable
E	MicroAmp™ Optical 8-Tube Strip
F	MicroAmp® Optical Tube without Cap
G	MicroAmp™ Optical Adhesive Film

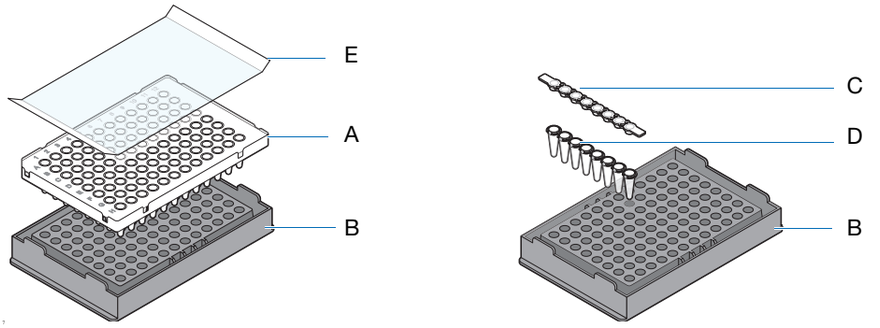
Notes

7500 Fast System

The 7500 Fast system supports the consumables listed below.

IMPORTANT! Use only Fast consumables (reaction plates, tube strips, and tubes) with the 7500 Fast system, even when performing an experiment with standard reagents.

Consumable	Part Number
• MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1-mL • MicroAmp™ Optical Adhesive Film	• 4346906 • 4311971
• MicroAmp™ Fast 8-Tube Strip, 0.1-mL • MicroAmp™ Optical 8-Cap Strip	• 4358293 • 4323032
• Microamp® Splash Free Support Base	• N8010531
• MicroAmp™ Adhesive Film Applicator • MicroAmp® Cap Installing Tool (Handle) • MicroAmp™ Multi-Removal Tool	• 4333183 • 4330015 • 4313950



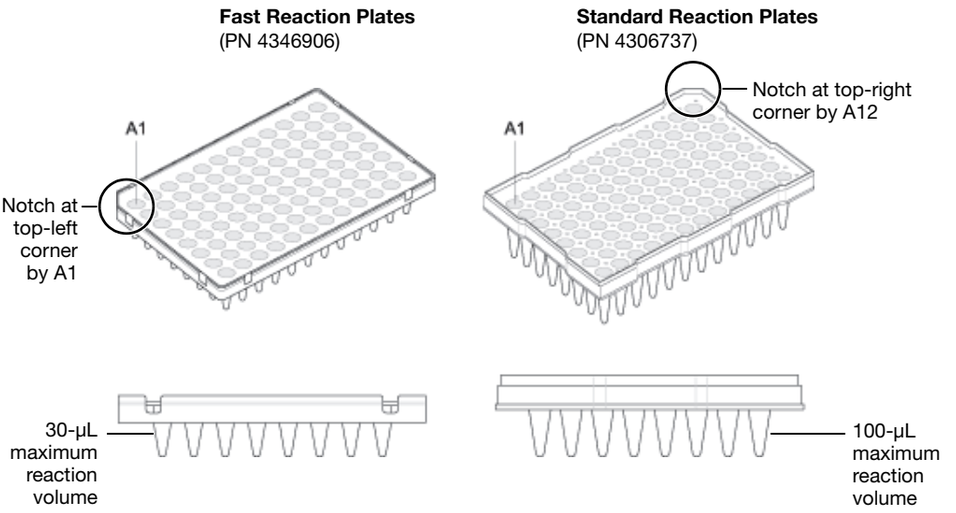
#	Consumable
A	MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1-mL
B	Microamp® Splash Free Support Base
C	MicroAmp™ Optical 8-Cap Strip
D	MicroAmp™ Fast 8-Tube Strip
E	MicroAmp™ Optical Adhesive Film

Notes

Standard versus Fast Reaction Plates and Tubes

Make sure that you use the correct reaction plate for your system:

System	Reaction Plate
7500 system	• MicroAmp® Optical 96-Well Reaction Plate (PN 4306737, also called *standard reaction plates*) • MicroAmp® Optical Tubes without Caps, 0.2-mL (PN N8010933, also called *standard reaction tubes*) • MicroAmp® Reaction Tubes with Caps, 0.2-mL (PN N2070540) • MicroAmp™ Optical 8-Tube Strip, 0.2-mL (PN 4316567, also called *standard reaction tube strips*) **IMPORTANT!** Fast reaction plates and tubes do not fit into the standard sample block correctly and will result in loss of data.
7500 Fast system	• MicroAmp™ Fast Optical 96-Well Reaction Plate, (PN 4346906, also called *Fast reaction plates*) • MicroAmp™ Fast 8-Tube Strip, 0.1-mL (PN 4358293, also called *Fast reaction tube strips*) **IMPORTANT!** Standard reaction plates and tube strips will not function properly and might be crushed when using the Fast sample block.



Notes

About Relative Standard Curve and Comparative C_T Experiments

Real-Time PCR Experiments

Relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments are real-time PCR experiments. In real-time PCR experiments:

- The instrument monitors the progress of the PCR as it occurs.
- Data are collected throughout the PCR process.
- Reactions are characterized by the point in time during cycling when amplification of a target is first detected.

Note: In this guide, the term *experiment* refers to the entire process of performing a run using the 7500/7500 Fast system, including setup, run, and analysis.

About Relative Standard Curve Experiments

The relative standard curve method is used to determine relative target quantity in samples. With the relative standard curve method, the 7500 software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates target quantity in the samples and in the reference sample. The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

Relative standard curve experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample vs. an untreated sample.
- Compare expression levels of wild-type alleles with those of mutated alleles.

Components

The following components are required when setting up PCR reactions for relative standard curve experiments:

- **Sample** – The sample in which the quantity of the target is unknown.
- **Reference sample** – The sample used as the basis for relative quantitation results. For example, in a study of drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called *calibrator*.
- **Standard** – A sample that contains known standard quantities; used in quantitation experiments to generate standard curves.
- **Standard dilution series** – A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
- **Endogenous control** – A target or gene that should be expressed at similar levels in all samples you are testing. The endogenous control is used to normalize fluorescence for the target you are quantifying. Housekeeping genes can be used as endogenous controls.
- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.

Notes

- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

About Comparative C_T Experiments

The comparative C_T ($\Delta\Delta C_T$) method is used to determine the relative target quantity in samples. With the comparative C_T method, the 7500 software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

Comparative C_T experiments are commonly used to:

- Compare expression levels of a gene in different tissues.
- Compare expression levels of a gene in a treated sample vs. an untreated sample.
- Compare expression levels of wild-type alleles with those of mutated alleles.

Components

The following components are required when setting up PCR reactions for comparative C_T experiments:

- **Sample** – The sample in which the quantity of the target is unknown.
- **Reference sample** – The sample used as the basis for relative quantitation results. For example, in a study of drug effects on gene expression, an untreated control would be an appropriate reference sample. Also called *calibrator*.
- **Endogenous control** – A target or gene that should be expressed at similar levels in all samples you are testing. The endogenous control is used to normalize fluorescence for the target you are quantifying. Housekeeping genes can be used as endogenous controls.
- **Replicates** – The total number of identical reactions containing identical samples, components, and volumes.
- **Negative Controls** – Wells that contain water or buffer instead of sample template. No amplification of the target should occur in negative control wells.

Notes

Relative Standard Curve vs. Comparative C_T Experiments

Consider the following when choosing between relative standard curve and comparative C_T experiments:

	Comparative C_T ($\Delta\Delta C_T$) Experiment	**Relative Standard Curve Experiment**
Description	Uses arithmetic formulas to determine the change in expression of a target in a sample relative to the same target in a reference sample. Best for high-throughput measurements of relative gene expression of many genes in many samples.	Uses a standard curve to determine the change in expression of a target in a sample relative to the same target in a reference sample. Best for assays that have suboptimal PCR efficiency.
Advantage	• Relative levels of target in samples can be determined without the use of a standard curve, if the PCR efficiencies of the target and endogenous control are relatively equivalent. • Reduced reagent usage. • More space available in the reaction plate.	Requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not need to be equivalent.
Limitation	• Suboptimal (low PCR efficiency) assays may produce inaccurate results. • Before you use the comparative C_T method, Applied Biosystems recommends that you determine that the PCR efficiencies for the target assay and the endogenous control assay are approximately equal.	A standard curve must be constructed for each target, which requires more reagents and more space in the reaction plate.

Notes

PCR Options

When performing real-time PCR, choose between:

- Singleplex and multiplex PCR (below)

  and

- 1-step and 2-step RT-PCR (below)

Singleplex vs. Multiplex PCR

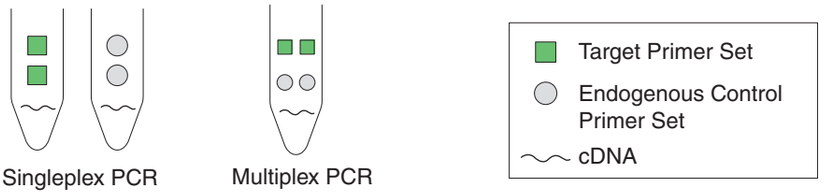
You can perform a PCR reaction using either:

- **Singleplex PCR** – In singleplex PCR, a single primer set is present in the reaction tube or well. Only one target or endogenous control can be amplified per reaction.

  or

- **Multiplex PCR** – In multiplex PCR, two or more primer sets are present in the reaction tube or well. Each set amplifies a specific target or endogenous control. Typically, a probe labeled with FAM™ dye detects the target and a probe labeled with VIC® dye detects the endogenous control.

IMPORTANT! SYBR® Green reagents cannot be used for multiplex PCR.



1- vs. 2-Step RT-PCR

You can perform reverse transcription (RT) and PCR in a single reaction (1-step) or in separate reactions (2-step). The reagent configuration you use depends on whether you are performing 1- or 2-step RT-PCR:

- In 1-step RT-PCR, RT and PCR take place in one buffer system, which provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use Fast PCR Master Mix or the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), to perform 1-step RT-PCR.
- 2-step RT-PCR is performed in two separate reactions: First, total RNA is reverse-transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. The AmpErase® UNG enzyme can be used to prevent carryover contamination.

Note: For more information on AmpErase® UNG, refer to the *Real Time PCR System Reagent Guide*.

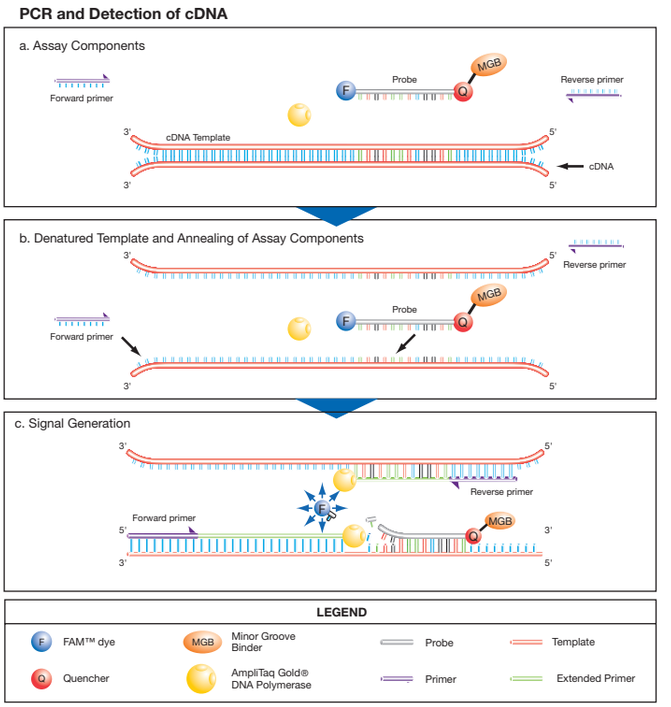
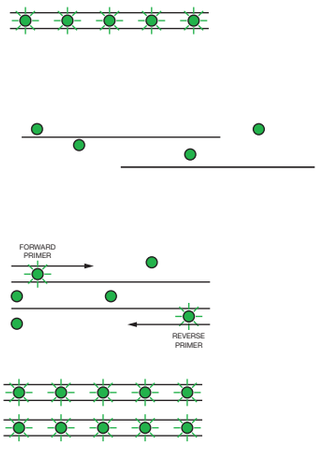
Notes

Supported Reagents

TaqMan® and SYBR® Green Reagents

Applied Biosystems offers TaqMan® and SYBR® Green reagents for use on the 7500/7500 Fast system. Both reagent types are briefly described in the table below.

Note: If you use TaqMan® or SYBR® Green reagents, the 7500 software automatically calculates reaction volumes in the Reaction Setup screen.

Reagent Type	Process
TaqMan® reagents or kits **Description** TaqMan reagents use a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles. **Advantages** • Increased specificity with the addition of a fluorogenic probe. • Provides multiplex capability. • Preformulated assays, optimized to run under universal thermal cycling conditions, are available. • Can be used for either 1- or 2-step RT-PCR. **Limitations** Requires synthesis of a unique fluorogenic probe.	
SYBR® Green reagents **Description** SYBR Green reagents use SYBR® Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles. **Advantages** • Economical (no probe needed). • Allows for melt curve analysis to measure the Tm of all PCR products. • Can be used for either 1- or 2-step RT-PCR. **Limitations** Binds nonspecifically to all double-stranded DNA sequences. To avoid false-positive signals, check for nonspecific product formation using melt curve or gel analysis.	 **Step 1: Reaction setup** The SYBR® Green I dye fluoresces when bound to double-stranded DNA. **Step 2: Denaturation** When the DNA is denatured into single-stranded DNA, the SYBR® Green I dye is released and the fluorescence is drastically reduced. **Step 3: Polymerization** During extension, primers anneal and PCR product is generated. **Step 4: Polymerization completed** SYBR® Green I dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the instrument.

Notes

Other Reagents

If you use fluorescence-based reagents other than TaqMan® reagents, you must design your experiment using Advanced Setup instead of the Design Wizard (see "Advanced Setup Workflow" on page 204).

For More Information

For more information on real-time PCR experiments, PCR options, and reagents, refer to the *Real Time PCR System Reagent Guide*.

How to Use This Guide

This guide functions as both a tutorial and as a guide for performing your own experiments.

Using This Guide as a Tutorial

Using the example experiment data provided with the 7500 software, you can use this guide as a tutorial for performing a relative standard curve or comparative C_T experiment on a 7500/7500 Fast system. Follow the procedures in the appropriate chapters shown in the table below:

Chapter		Procedure
Relative Standard Curve	**Comparative C_T**	
2	6	Design the experiment using the Design Wizard in the 7500 software.
3	7	Prepare the experiment using the reagents and volumes calculated by the Design Wizard in Chapter 2 (relative standard curve experiment) or Chapter 6 (comparative C_T experiment).
4	8	Run the experiment on a 7500/7500 Fast instrument.
5	9	Analyze the results of an example experiment.

For more information, see "About the Example Experiments" on page 14.

Notes

Using This Guide with Your Own Experiments

After completing the tutorial exercises in Chapters 2 through 9, you can use this guide to lead you through your own relative standard curve or comparative C_T experiments. Each procedure in Chapters 2 through 9 includes a set of guidelines that you can use to perform your own experiments.

Additionally, you can use one of the other workflows provided in the 7500 software to perform your experiments. The table below summarizes the available workflows.

Workflow	Description	See...
Design Wizard	Set up a new experiment with guidance from the 7500 software. The Design Wizard guides you through best practices as you create your own experiment. The Design Wizard is recommended for new users. **Note:** Design options are more limited in the Design Wizard than in Advanced Setup.	Chapter 2 or Chapter 6
Advanced Setup	Set up a new experiment using advanced options. Advanced Setup allows design flexibility as you create your own experiment. Advanced Setup is recommended for experienced users.	page 204
QuickStart	Run a new experiment with no reaction plate setup information. If desired, you can add design parameters after the run.	page 206
Template	Set up a new experiment using setup information from a template.	page 208
Export/Import	Import experiment designs from ASCII text files that contain experiment setup information.	page 210

Notes

About the Example Experiments

About the Relative Standard Curve Example Experiment

The objective of the relative standard curve example experiment is to compare the expression levels of the c-myc transcriptional factor (an oncoprotein that activates the transcription of growth-associated genes) in liver and kidney tissues.

In the relative standard curve example experiment:

- The samples are cDNA prepared from total RNA isolated from liver and kidney tissues.
- The target is human c-myc.
- The endogenous control is human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
- The reference sample is RNA isolated from kidney tissue.
- One standard curve is set up for c-myc (target). The standard used for the standard dilution series is a cDNA sample of known quantity prepared from RNA isolated from lung tissue.
- One standard curve is set up for GAPDH (endogenous control). The standard used for the standard dilution series is a cDNA sample of known quantity prepared from RNA isolated from lung tissue.
- The experiment is designed for singleplex PCR, where the target (c-myc) and endogenous control (GAPDH) assays are performed in separate wells.
- Reactions are set up for 2-step RT-PCR. The High-Capacity cDNA Reverse Transcription Kit is used for reverse transcription; the TaqMan® Universal PCR Master Mix is used for PCR.
- Primer/probe sets are selected from the Applied Biosystems TaqMan® Gene Expression Assays product line:
  - For the target assay (c-myc), the assay ID is Hs00153408_m1 (RefSeq NM_002467.3).
  - For the endogenous control assay (GAPDH), the assay ID is Hs99999905_m1 (RefSeq NM_002046.3).

Reaction Plate Layout

The 7500 software displays the 96-well plate layout, as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	N c-myc	N c-myc	N c-myc	N GAPDH	N GAPDH	N GAPDH	Liver U c-myc 7.39	Liver U c-myc 8.13	Liver U c-myc	Liver U GAPDH 0.47	Liver U GAPDH 0.44	Liver U GAPDH 0.43
B	Kidney U c-myc 5.32	Kidney U c-myc 4.1	Kidney U c-myc 3.76	Kidney U GAPDH 0.44	Kidney U GAPDH 0.43	Kidney U GAPDH 0.43	S c-myc 200	S c-myc 200	S c-myc 200	S c-myc 20	S c-myc 20	S c-myc 20
C	S c-myc 2	S c-myc 2	S c-myc 2	S c-myc 0.2	S c-myc 0.2	S c-myc 0.2	S c-myc 0.02	S c-myc 0.02	S c-myc 0.02	S GAPDH 200	S GAPDH 200	S GAPDH 200
D	S GAPDH 20	S GAPDH 20	S GAPDH 20	S GAPDH 2	S GAPDH 2	S GAPDH 2	S GAPDH 0.2	S GAPDH 0.2	S GAPDH 0.2	S GAPDH 0.02	S GAPDH 0.02	S GAPDH 0.02
E												

Notes

About the Comparative C_T ($\Delta\Delta C_T$) Example Experiment

The objective of the comparative C_T example experiment is to compare the expression of EGR3, MAOB, OGDH, OSGEP, and SERPING1 in brain, liver, kidney, and universal tissues.

In the comparative C_T example experiment:

- The samples are cDNA prepared from total RNA isolated from brain, liver, kidney, and universal tissues.
- The targets are EGR3, MAOB, OGDH, OSGEP, and SERPING1.
- The reference sample is brain.
- The endogenous control is human 18S.
- The experiment is designed for singleplex PCR, where the targets (EGR3, MAOB, OGDH, OSGEP, and SERPING1) and endogenous control (18S) assays are performed in separate wells.
- Reactions are set up for 2-step RT-PCR. The High-Capacity cDNA Reverse Transcription Kit is used for reverse transcription; the TaqMan® Universal PCR Master Mix is used for PCR.
- Primer/probe sets are selected from the Applied Biosystems TaqMan® Gene Expression Assays product line:
  - For the target assays:
    - EGR3 Assay Mix: Hs00231780_m1 (RefSeq NM_004430.2)
    - MAOB Assay Mix: Hs00168533_m1 (RefSeq NM_000898.3)
    - OGDH Assay Mix: Mm00803121_m1 (RefSeq NM_010956.3)
    - OSGEP Assay Mix: Hs00215099_m1 (RefSeq NM_017807.2)
    - SERPING1 Assay Mix: Hs00163781_m1 (RefSeq NM_001032295.1/ NM_000062.2)
  - For the endogenous control assay (18S), the 18S Assay Mix: Hs99999901_s1.

Reaction Plate Layout

The 7500 software displays the 96-well plate layout as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Brain U 18S	Brain U MAOB	Brain U OSGEP	Kidney U 18S	Kidney U MAOB	Kidney U OSGEP	Liver U 18S	Liver U MAOB	Liver U OSGEP	Universal U 18S	Universal U MAOB	Universal U OSGEP
B	Brain U 18S	Brain U MAOB	Brain U OSGEP	Kidney U 18S	Kidney U MAOB	Kidney U OSGEP	Liver U 18S	Liver U MAOB	Liver U OSGEP	Universal U 18S	Universal U MAOB	Universal U OSGEP
C	Brain U 18S	Brain U MAOB	Brain U OSGEP	Kidney U 18S	Kidney U MAOB	Kidney U OSGEP	Liver U 18S	Liver U MAOB	Liver U OSGEP	Universal U 18S	Universal U MAOB	Universal U OSGEP
D	Brain U 18S	Brain U MAOB	Brain U OSGEP	Kidney U 18S	Kidney U MAOB	Kidney U OSGEP	Liver U 18S	Liver U MAOB	Liver U OSGEP	Universal U 18S	Universal U MAOB	Universal U OSGEP
E	Brain U EGR3	Brain U OGDH	Brain U SERPI...	Kidney U EGR3	Kidney U OGDH	Kidney U SERPI...	Liver U EGR3	Liver U OGDH	Liver U SERPI...	Universal U EGR3	Universal U OGDH	Universal U SERPI...
F	Brain U EGR3	Brain U OGDH	Brain U SERPI...	Kidney U EGR3	Kidney U OGDH	Kidney U SERPI...	Liver U EGR3	Liver U OGDH	Liver U SERPI...	Universal U EGR3	Universal U OGDH	Universal U SERPI...
G	Brain U EGR3	Brain U OGDH	Brain U SERPI...	Kidney U EGR3	Kidney U OGDH	Kidney U SERPI...	Liver U EGR3	Liver U OGDH	Liver U SERPI...	Universal U EGR3	Universal U OGDH	Universal U SERPI...
H	Brain U EGR3	Brain U OGDH	Brain U SERPI...	Kidney U EGR3	Kidney U OGDH	Kidney U SERPI...	Liver U EGR3	Liver U OGDH	Liver U SERPI...	Universal U EGR3	Universal U OGDH	Universal U SERPI...

Notes

About the Example Experiment Data

In this getting started guide, you use five files:

- In Chapter 2, you create a relative standard curve example experiment that contains setup data, then you save the file to your computer.
- In Chapter 5, you view results in a relative standard curve example experiment file that contains run data. The data file for the example experiments is installed with the 7500 software. You can find the data file for the example experiment on your computer at:

  <*drive*>:\Applied Biosystems\<*software name*>\experiments\
  Relative Standard Curve Example.eds
- In Chapter 6, you create a comparative C_T example experiment that contains setup data, then you save the file to your computer.
- In Chapter 9, you view results in a comparative C_T example experiment file that contains run data and in a comparative C_T example study file that contains the data from multiple comparative C_T experiments. The data files install with the 7500 software, and are on your computer at:
  - <*drive*>:\Applied Biosystems\<*software name*>\experiments\
    Comparative Ct Example.eds
  - <*drive*>:\Applied Biosystems\<*software name*>\experiments\
    Comparative Ct Study Example.edm

  where:
  - <*drive*> is the computer hard drive on which the 7500 software is installed.
  - <*software name*> is the current version of the 7500 software.

Example Files in the Experiments Folder

The experiments folder of the 7500 software contains several example files that you can reference when analyzing your own data. The following example files install with the 7500 software:

- Comparative Ct Example.eds
- Comparative Ct Study Example.edm
- Comparative Ct Study (Biological Groups).edm
- Genotyping Example.eds
- Presence Absence Example.eds
- Relative Standard Curve Example.eds
- Standard Curve Example.eds

Note: Be sure to use the *Comparative Ct Example.eds*, *Relative Standard Curve Example.eds*, and *Comparative Ct Study Example.edm* files when you perform the procedures in this guide.

Notes

Example Experiment Workflow

This section shows the workflow for the relative standard curve and comparative C_T example experiments.

Start Relative Standard Curve Experiment

Design the Experiment (Chapter 2)

1. Create a new experiment.
2. Define the experiment properties.
3. Define the methods and materials.
4. Set up the targets.
5. Set up the standards.
6. Set up the samples.
7. Set up the relative quantitation.
8. Set up the run method.
9. Review the reaction setup.
10. Order materials for the experiment.
11. Finish the Design Wizard.

Prepare the Reactions (Chapter 3)

1. Prepare the template.
2. Prepare the sample dilutions.
3. Prepare the standard dilution series.
4. Prepare the reaction mix for each target assay.
5. Prepare the reaction plate.

Run the Experiment (Chapter 4)

1. Prepare for the run.
2. Enable the notification settings (Optional).
3. Start the run.
4. Monitor the run.
5. Unload the instrument.

(*continued on page 18*)

Start Comparative C_T ($\Delta\Delta C_T$) Experiment

Design the Experiment (Chapter 6)

1. Create a new experiment.
2. Define the experiment properties.
3. Define the methods and materials.
4. Set up the targets.
5. Set up the samples.
6. Set up the relative quantitation.
7. Set up the run method.
8. Review the reaction setup.
9. Order materials for the experiment.
10. Finish the Design Wizard.

Prepare the Reactions (Chapter 7)

1. Prepare the template.
2. Prepare the sample dilutions.
3. Prepare the reaction mix for each target assay.
4. Prepare the reaction plate.

Run the Experiment (Chapter 8)

1. Prepare for the run.
2. Enable the notification settings (Optional).
3. Start the run.
4. Monitor the run.
5. Unload the instrument.

(*continued on page 18*)

Notes

Relative Standard Curve Experiment
(*continued from page 17*)

Analyze the Experiment (Chapter 5)

Section 1, Review Results:

1. Analyze.
2. View the Standard Curve Plot.
3. View the Amplification Plot.
4. View the Gene Expression Plot/well table.
5. Publish the data.

Section 2, Troubleshoot (If Needed):

1. View the analysis settings; adjust the baseline/threshold.
2. View the quality summary.
3. Omit wells.
4. View the Multicomponent Plot.
5. View the Raw Data Plot.

End Experiment

Comparative C_T ($\Delta\Delta C_T$) Experiment
(*continued from page 17*)

Analyze the Experiment (Chapter 9)

Section 1, Review Results:

1. Analyze.
2. View the Gene Expression Plot/well table.
3. View the Amplification Plot.
4. Publish the data.

Section 2, Troubleshoot (If Needed):

1. View the analysis settings; adjust the baseline/threshold.
2. View the quality summary.
3. Omit wells.
4. View the Multicomponent Plot.
5. View the Raw Data Plot.

Section 3, Create a Study:

1. Create a study.
2. Define replicates.
3. Analyze.
4. View the Gene Expression Plot.
5. View the experiment data.
6. View the Multicomponent Data.
7. View multiple plots.
8. View the quality summary.
9. Publish the data.

End Experiment

Notes

2 Design the Relative Standard Curve Experiment

This chapter covers:

- Chapter Overview 20
- Create a New Experiment 21
- Define the Experiment Properties 22
- Define the Methods and Materials 24
- Set Up the Targets 26
- Set Up the Standards 29
- Set Up the Samples 31
- Set Up the Relative Quantitation Settings 34
- Set Up the Run Method 35
- Review the Reaction Setup 36
- Order Materials for the Experiment 43
- Finish the Design Wizard 46

Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

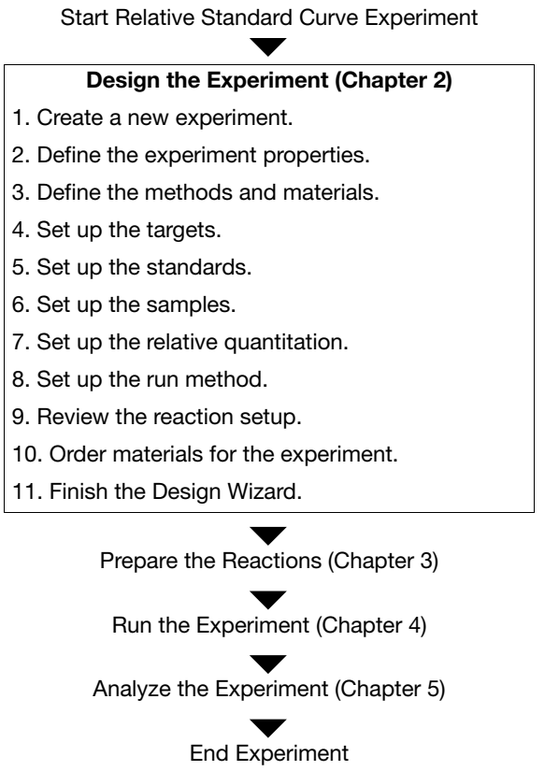
Notes

Chapter Overview

This chapter explains how to use the Design Wizard in the 7500 software to set up the relative standard curve example experiment. The Design Wizard guides you through Applied Biosystems recommended best practices as you enter design parameters for the example experiment.

Note: When you design your own experiments, you can select alternate workflows (see "Using This Guide with Your Own Experiments" on page 13).

Example Experiment Workflow



Notes

Create a New Experiment

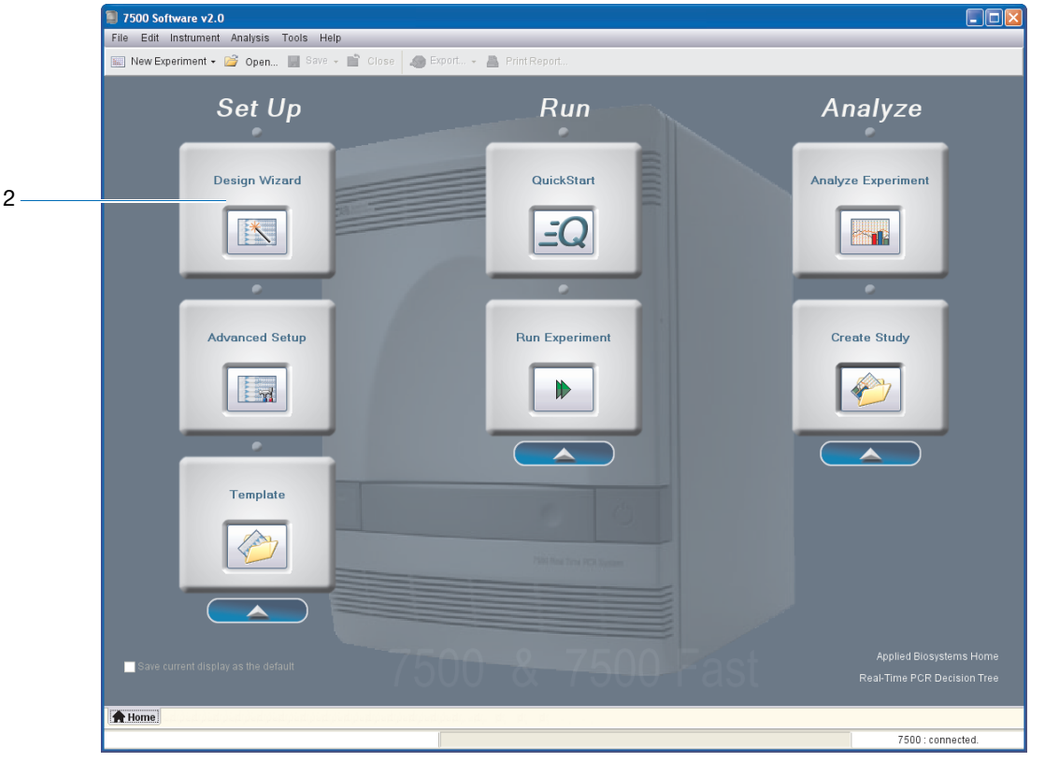
Create a new experiment using the Design Wizard in the 7500 software.

Create an Experiment

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

   where *<software name>* is the current version of the 7500 software.

2. In the Home screen, click **Design Wizard** to open the Design Wizard.



Notes

Define the Experiment Properties

In the Experiment Properties screen, enter identifying information for the experiment, select the instrument type, then select the type of experiment to design.

About the Example Experiment

In the relative standard curve example experiment:

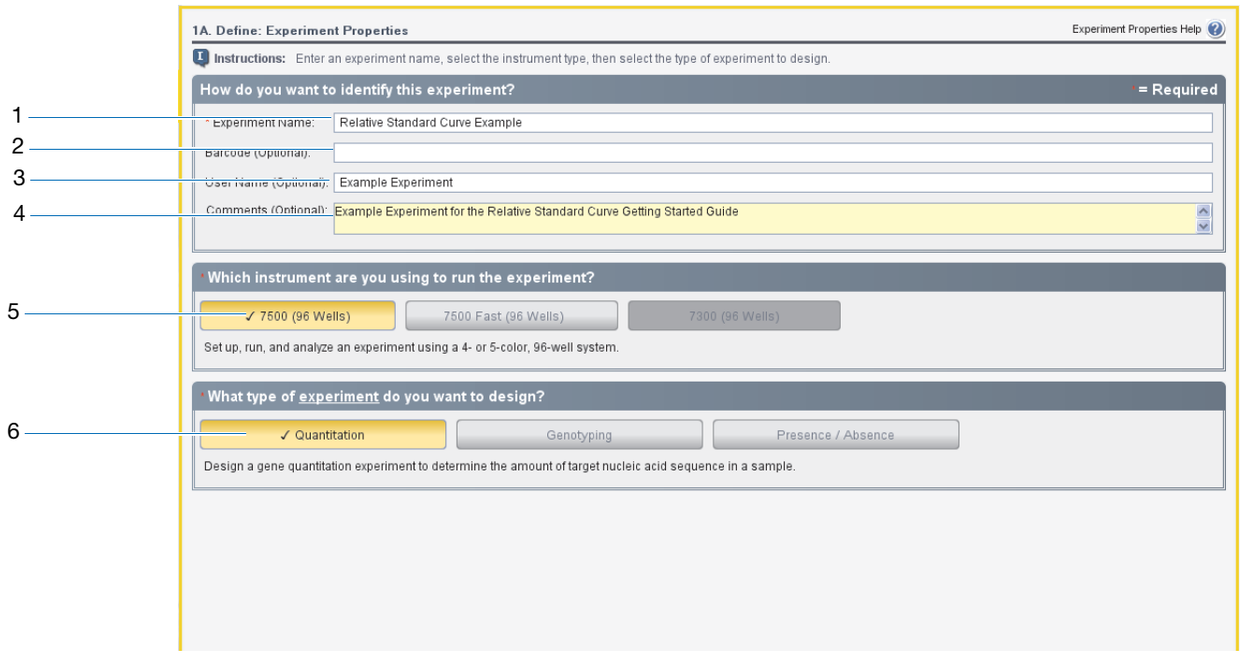
- The experiment is identified as an example.
- The instrument that is selected to run the experiment is the 7500 instrument.
- A MicroAmp® Optical 96-Well Reaction Plate is used.
- The experiment type is quantitation.

Complete the Experiment Properties Screen

1. Click the **Experiment Name** field, then enter **Relative Standard Curve Example**.

   Note: The experiment header is updated with the experiment name that you enter.

2. Leave the Barcode field empty.
3. Click the **User Name** field, then enter **Example User**.
4. Click the **Comments** field, then enter **Example Experiment for the Relative Standard Curve Getting Started Guide**.
5. Select **7500 (96 Wells)**.
6. Select **Quantitation** for the experiment type.
7. Click **Next**.



Notes

Design Guidelines

When you design your own relative standard curve experiment, you:

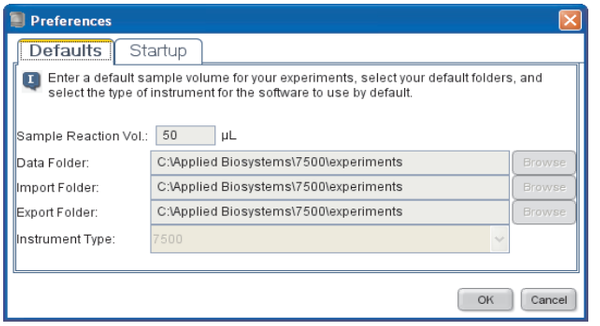
- Enter an experiment name that is descriptive and easy to remember. You can enter up to 100 characters in the Experiment Name field. You cannot use the following characters in the Experiment Name field: / \ > < * ? " | : ;

  Note: The experiment name is used as the default file name.

- (Optional) Enter a barcode to identify the barcode on the PCR reaction plate. You can enter up to 100 characters in the Barcode field.
- (Optional) Enter a user name to identify the owner of the experiment. You can enter up to 100 characters in the User Name field.
- (Optional) Enter comments to describe the experiment. You can enter up to 1000 characters in the Comments field.
- Select the instrument you are using to run the experiment:
  - **7500 (96 Wells)**
  - **7500 Fast (96 Wells)**

  Note: You can use 7500 software v2.0 to design experiments for the 7500/7500 Fast instrument.

  IMPORTANT! To set the default instrument type, select **Tools ▸ Preferences**, then select the **Defaults** tab (default). In the Instrument Type drop-down list, select the appropriate instrument.



For More Information

For more information on:

- Completing the Experiment Properties screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Consumables – See “Supported Consumables” on page 4.
- Quantitation experiments – Refer to the *Real Time PCR System Reagent Guide*.

Notes

Define the Methods and Materials

In the Methods and Materials screen, select the quantitation method, reagents, ramp speed, and PCR template to use for the experiment.

About the Example Experiment

In the relative standard curve example experiment:

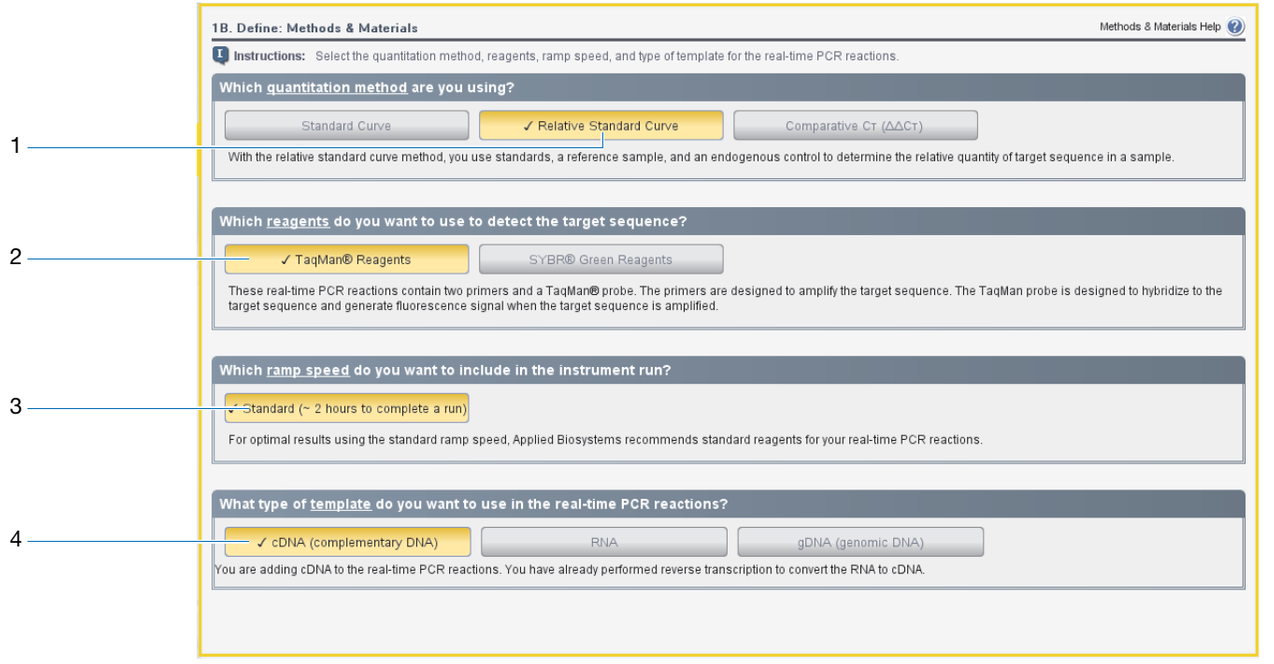
- The relative standard curve quantitation method is used.
- TaqMan® reagents are used.
- The standard ramp speed is used in the instrument run.
- cDNA (prepared from total RNA isolated from liver and kidney tissues) is the template type.You must first perform reverse transcription to convert the RNA to cDNA (see "Prepare the Template" on page 51).

Complete the Methods and Materials Screen

1. In the Methods and Materials screen, select **Relative Standard Curve** as the quantitation method.
2. Select **TaqMan® Reagents** for the reagents.

   IMPORTANT! Fast runs cannot be performed on a 7500 system.

3. Select **Standard (~ 2 hours to complete a run)** for the ramp speed.
4. Select **cDNA (complementary DNA)** for the template type.
5. Click **Next**.



Notes

Design Guidelines

When you design your own relative standard curve experiment, you:

- Select **Relative Standard Curve** as the quantitation method. The relative standard curve method is used to determine relative target quantity in samples. When setting up your reaction plate, the relative standard curve method requires targets, standards, samples, a reference sample, and an endogenous control.
- Select the reagents you want to use:
  - Select **TaqMan® Reagents** if you want to use TaqMan reagents to detect amplification and quantify the amount of target in the samples. TaqMan reagents consist of two primers and a TaqMan® probe. The primers are designed to amplify the target. The TaqMan probe is designed to hybridize to the target and fluoresce when the target is amplified.
  - Select **SYBR® Green Reagents** if you want to use SYBR Green reagents to detect amplification and quantify the amount of target in the samples. SYBR Green reagents consist of two primers and SYBR Green dye. The primers are designed to amplify the target. The SYBR Green dye fluoresces when it binds to double-stranded DNA. SYBR Green dye is often part of the SYBR Green master mix added to the reaction. If you use SYBR Green dye, select the **Include Melt Curve** check box to perform melt-curve analysis of the amplified target.

  IMPORTANT! Although you can use other fluorescence-based reagents on the 7500/7500 Fast system, you must design your experiment using Advanced Setup instead of the Design Wizard.

- Select the appropriate ramp speed for the instrument run:

  IMPORTANT! Select a Fast run only if you use a 7500 Fast system. Fast runs cannot be performed on a 7500 system.

  - Select **Fast (~ 40 minutes to complete a run)** if you are using Fast reagents for the PCR reactions.
  - Select **Standard (~ 2 hours to complete a run)** if you are using standard reagents for the PCR reactions.
- Select the appropriate PCR template:
  - Select **cDNA (complementary DNA)** if you are performing 2-step RT-PCR, and you have already performed reverse transcription to convert the RNA to cDNA. You are adding complementary DNA to the PCR reactions.
  - Select **RNA** if you are performing 1-step RT-PCR. You are adding total RNA or mRNA to the PCR reactions.

    IMPORTANT! To use the Fast ramp speed with RNA templates, you must design your experiment using Advanced Setup instead of the Design Wizard.

  - Select **gDNA (genomic DNA)** if you have already extracted the gDNA from tissue or sample. You are adding purified genomic DNA to the PCR reactions.

Notes

For More Information

For more information on:

- Completing the Methods and Materials screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Using Advanced Setup – See "Advanced Setup Workflow" on page 204.
- Using the comparative C_T quantitation method – See Chapters 6 to 9 of this guide.
- Using the standard curve quantitation method – Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments*.
- TaqMan and SYBR Green reagents – Refer to the *Real Time PCR System Reagent Guide*.
- PCR, including singleplex vs. multiplex PCR and 1-step vs. 2-step RT PCR – Refer to the *Real Time PCR System Reagent Guide*.

Set Up the Targets

In the Targets screen, enter the number of targets that you want to quantify in the PCR reaction plate, then set up the experiment for each target.

About the Example Experiment

In the relative standard curve example experiment:

- Two targets are quantified in the reaction plate.
- The Set Up Standards check box is selected. When this check box is selected, the software automatically displays the Standards screen after you complete the Targets screen. In the Standards screen, you can set up a standard curve for each target assay (see "Set Up the Standards" on page 29).
- The Target 1 experiment is set up for the target you are studying. For the example experiment, the target is human c-myc (an oncoprotein that activates the transcription of growth-associated genes).
- The Target 2 experiment is set up for the endogenous control. For the example experiment, the endogenous control is human glyceraldehyde-3-phosphate (GAPDH). GAPDH is the endogenous control because its expression levels tend to be relatively stable.

Complete the Targets Screen

1. In the Targets screen, click the **How many targets do you want to quantify in the reaction plate?** field, then enter **2**.

   Note: The number of rows in the target assays table is updated with the number you entered.

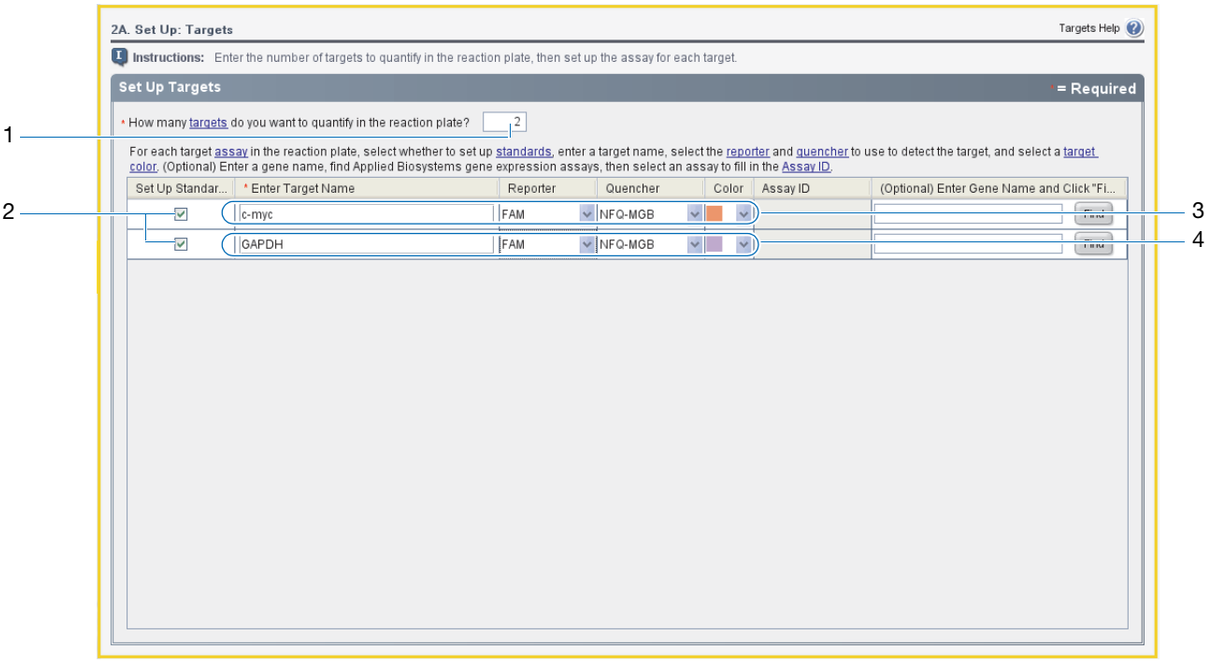
2. Select the **Set Up Standards** check box to set up standards for both target assays.

   Note: The Set Up Standards check box is selected by default.

Notes

3. Set up the Target 1 experiment:
   a. Click the **Enter Target Name** cell, then enter **c-myc**.
   b. In the Reporter drop-down list, select **FAM** (default).
   c. In the Quencher drop-down list, select **NFQ-MGB** (default).
   d. In the Color field, leave the default.
4. Set up the Target 2 experiment:
   a. Click the **Enter Target Name** cell, then enter **GAPDH**.
   b. In the Reporter drop-down list, select **FAM** (default).
   c. In the Quencher drop-down list, select **NFQ-MGB** (default).
   d. In the Color field, leave the default.
5. Click **Next**.

Note: For all targets, leave blank the (Optional) Enter Gene Name field. You can search for the gene/assay ID when you order your materials (see "Order Materials for the Experiment" on page 43).



Notes

Design Guidelines

When you design your own relative standard curve experiment:

- Select the **Set Up Standards** check box. Applied Biosystems recommends that you set up a standard curve for each target assay in the reaction plate.
- Identify each target assay with a unique name and color. You can enter up to 100 characters in the Target Name field.
- Select an endogenous control for each sample. The endogenous control is a target that is in all samples under investigation. It should be expressed equally in all sample types, regardless of treatment or tissue origin (examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA [18S rRNA]). The endogenous control is used to normalize the PCR results; the endogenous control corrects for variable sample mass, nucleic acid extraction efficiency, reverse transcription efficiency, and pipette calibration errors. Note that:
  - Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.
  - If samples are on multiple plates, each reaction plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the reaction plate.
- Select the reporter dye used in the target assay. In the Methods and Materials screen on page 24, if you selected:
  - TaqMan® Reagents – Select the dye attached to the 5′ end of the TaqMan probe.
  - SYBR® Green Reagents – Select **SYBR**.
- Select the quencher used in the target assay. In the Methods and Materials screen on page 24, if you selected:
  - TaqMan® Reagents – Select the quencher attached to the 3′ end of the TaqMan probe.
  - SYBR® Green Reagents – Select **None**.

For More Information

For more information on:

- Completing the Targets screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Selecting an endogenous control – See the Application Note *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies*.

Notes

Set Up the Standards

In the Standards screen, enter the number of points and replicates for all standard curves in the reaction plate. For each standard curve, enter the starting quantity and select the serial factor.

About the Example Experiment

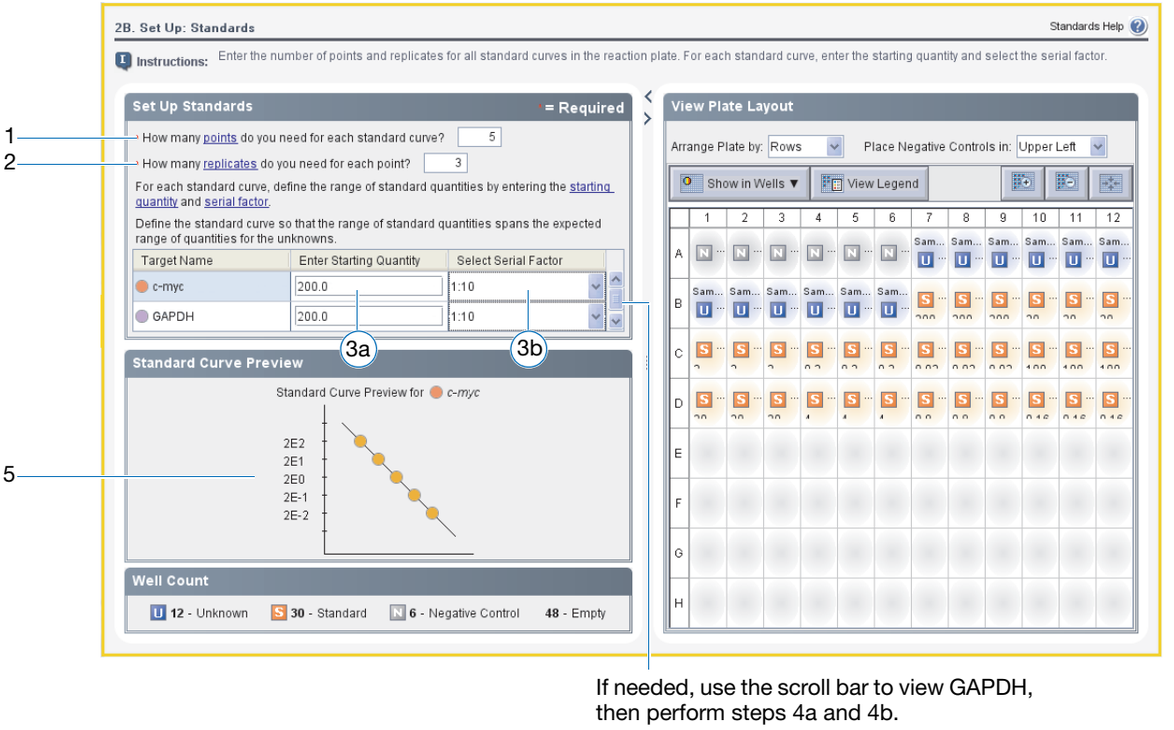
In the relative standard curve example experiment:

- One standard curve is set up for the target (c-myc). The standard used for the standard dilution series is a cDNA sample of known quantity prepared from RNA isolated from lung tissue.
- One standard curve is set up for the endogenous control (GAPDH). The standard used for the standard dilution series is a cDNA sample of known quantity prepared from RNA isolated from lung tissue.
- For each standard curve:
  - Five points are used in the standard curve.
  - Three replicates are used for each point. Replicates are identical reactions, containing identical reaction components and volumes.
  - The starting quantity is 200 ng, and the serial factor is 1:10.

Complete the Standards Screen

1. In the Standards screen, click the **How many points do you need for each standard curve?** field, then enter **5**.
2. Click the **How many replicates do you need for each point?** field, then enter **3**.
3. Define the range of standard quantities for the c-myc assay:
   a. Click the **Enter Starting Quantity** field, then enter **200.0**.
   b. In the Select Serial Factor drop-down list, select **1:10**.
4. Define the range of standard quantities for the GAPDH assay:
   a. Click the **Enter Starting Quantity** field, then enter **200.0**.
   b. In the Select Serial Factor drop-down list, select **1:10**.
5. Review the Standard Curve Preview pane for each assay. The standard curves have the following points: 200, 20, 2, 0.2, and 0.02.
6. Click **Next**.

Notes



If needed, use the scroll bar to view GAPDH, then perform steps 4a and 4b.

Design Guidelines

When you design your own relative standard curve experiment:

- Set up a standard curve for each target in the reaction plate. The targets are previously defined in the Targets screen (“Set Up the Targets” on page 26).
- Enter the number of points for each standard curve in the reaction plate. Applied Biosystems recommends at least five dilution points for each standard curve.
- Enter the number of identical reactions (replicates) for each point in the standard curve. Applied Biosystems recommends three replicates for each point.
- Because the range of standard quantities affects the amplification efficiency calculations, carefully consider the appropriate range of standard quantities for your assay:
  - For more accurate measurements of amplification efficiency, use a broad range of standard quantities, such as between 10^5 and 10^6. If you specify a broad range of quantities for the standards, you need to use a PCR product or a highly concentrated template, such as a cDNA clone.
  - If you have a limited amount of cDNA template and/or if the target is a low-copy number transcript, or known to occur within a specified range, a narrow range of standard quantities may be necessary.
- The serial factor is used to calculate the quantities in all points of the standard curve. If your starting quantity is the highest quantity, select a serial factor such as 1:2, 1:3, and so on. If your starting quantity is the lowest quantity, select a concentration factor such as 2×, 3×, and so on.

Notes

For More Information

For more information on:

- Completing the Standards screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Amplification efficiency – Refer to the *Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.

Set Up the Samples

In the Samples screen, enter the number of samples, replicates, and negative controls to include in the reaction plate, enter the sample names, then select the sample/target reactions to set up.

About the Example Experiment

In the relative standard curve example experiment:

- Two samples are used: cDNA prepared from total RNA isolated from liver and kidney tissues. The samples contain unknown quantities of the c-myc gene (target) and GAPDH gene (endogenous control).
- Three replicates are used. The replicates are identical reactions, containing identical reaction components and volumes.
- Three negative controls are used. The negative control reactions contain water instead of sample and should not amplify.

Complete the Samples Screen

1. In the Samples screen, click the **How many samples do you want to test in the reaction plate?** field, then enter **2**.

   Note: The number of rows in the samples table is updated with the number you entered.

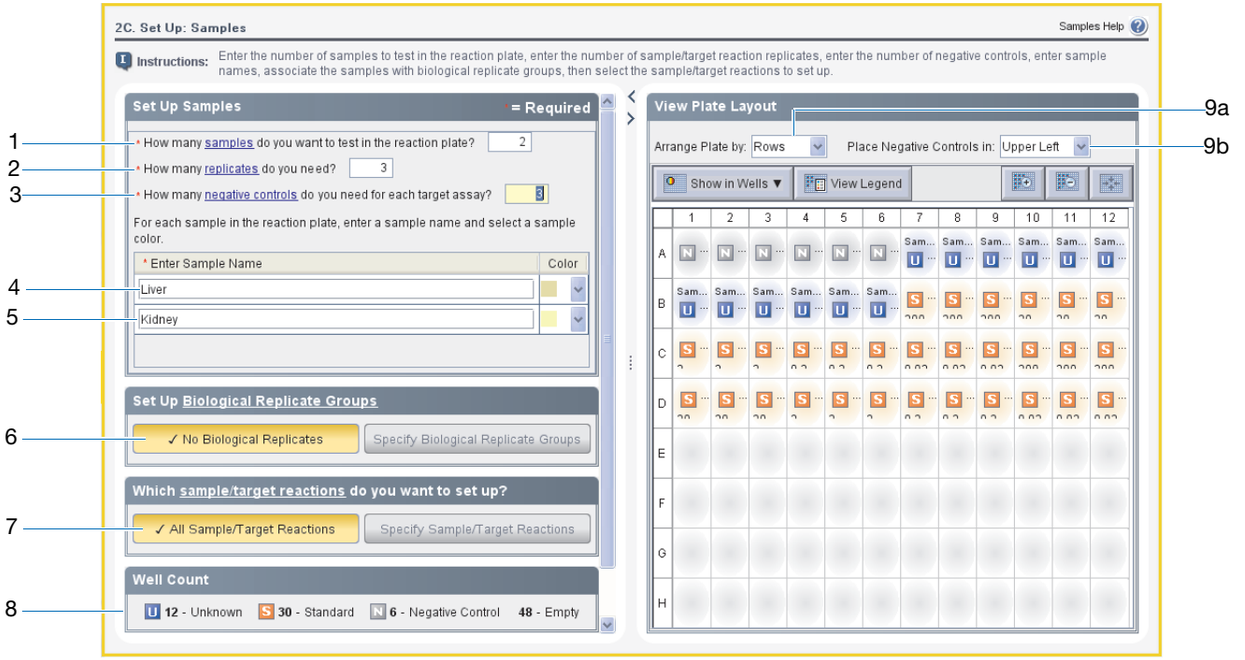
2. Click the **How many replicates do you need?** field, then enter **3**.
3. Click the **How many negative controls do you need for each target assay?** field, then enter **3**.
4. Set up Sample 1:
   a. Click the **Enter Sample Name** field, then enter **Liver**.
   b. In the Color field, leave the default.
5. Set up Sample 2:
   a. Click the **Enter Sample Name** field, then enter **Kidney**.
   b. In the Color field, leave the default.
6. Select **No Biological Replicates**.
7. Select **All Sample/Target Reactions** to test all targets in all samples.

Notes

8. In the Well Count pane, verify that there are:
   - 12 Unknown wells U
   - 30 Standard wells S
   - 6 Negative control wells N
   - 48 Empty wells

9. In the View Plate Layout tab:
   a. In the Arrange Plate by drop-down list, select **Rows** (default).
   b. In the Place Negative Controls in drop-down list, select **Upper Left** (default).

10. Click **Next**.



Design Guidelines

When you design your own relative standard curve experiment:

- Identify each sample with a unique name and color. You can enter up to 100 characters in the Sample Name field.
- Enter the number of replicates (identical reactions) to set up. Applied Biosystems recommends three replicates for each sample reaction.
- Enter the number of negative control reactions to set up. Applied Biosystems recommends three negative control reactions for each target assay.
- Set up biological replicate groups (see "Set Up Biological Replicate Groups" on page 33).

  Biological replicates allow you to assess the representative nature of your results as they relate to the population being studied. Inclusion of biological replicates can give insight into any natural variation that is present within the population.

Notes

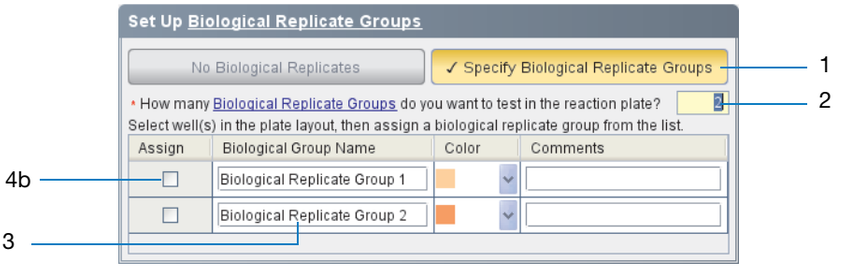
- Select which targets to test in the samples:
  - Select **All Sample/Target Reactions** to test all targets in all samples.
  - Select **Specify Sample/Target Reactions** to specify the targets to test in each sample.

IMPORTANT! When you use the Design Wizard to set up a relative standard curve experiment, you can set up only singleplex reactions (amplification and detection of one target per well). If you want to set up a relative standard curve experiment with multiplex reactions (amplification and detection of two or more targets per well), design your experiment using Advanced Setup instead of the Design Wizard.

Set Up Biological Replicate Groups

1. Select **Specify Biological Replicate Groups**.
2. Enter the number of biological replicate groups that you want to test in the reaction plate.
3. For each biological replicate group, click the cell in the Biological Group Name column, then enter a name for the biological group.
4. Assign the biological replicate group(s) to the reaction plate:
   a. Select wells in the plate layout that contain samples associated with the biological replicate group.
   b. In the Assign column, select the check box of the appropriate biological replicate group.

IMPORTANT! A sample cannot belong to more than one biological group.



For More Information

For more information on:

- Completing the Samples screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Using Advanced Setup – See “Advanced Setup Workflow” on page 204.

Notes

Set Up the Relative Quantitation Settings

In the Relative Quantitation Settings screen, select the reference sample and the endogenous control to perform relative quantitation.

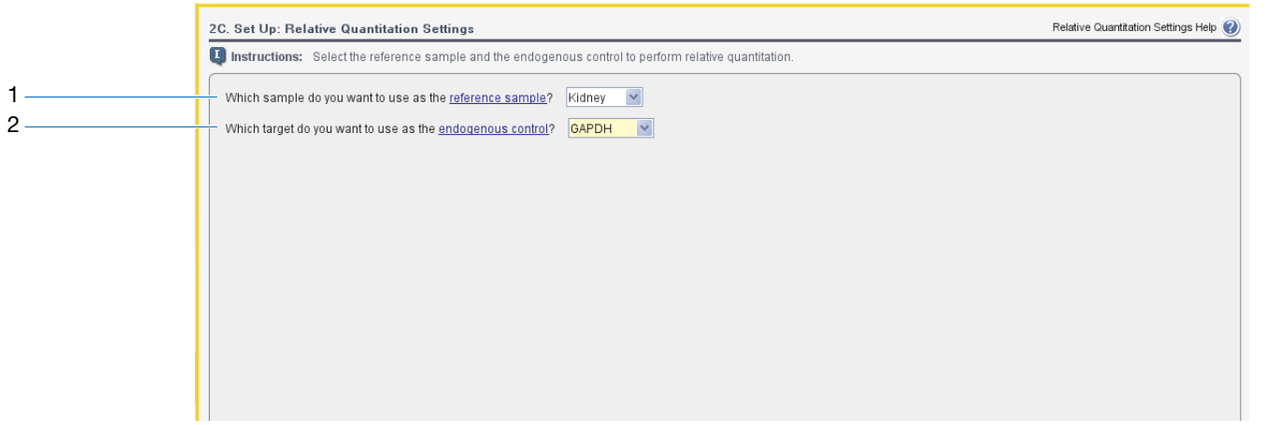
About the Example Experiment

In the relative standard curve example experiment:

- Kidney is used as the reference sample.
- GAPDH is used as the endogenous control.

Complete the Relative Quantitation Settings Screen

1. In the Relative Quantitation Settings screen, select **Kidney** in the Which sample do you want to use as the reference sample? drop-down list.
2. Select **GAPDH** in the Which target do you want to use as the endogenous control? drop-down list.
3. Click **Next**.



Design Guidelines

When you design your own relative standard curve experiment:

- Select a reference sample from your previously created samples (“Set Up the Samples” on page 31). Amplification results from the samples are compared to the amplification results from the reference sample to determine relative expression.
- Select an endogenous control from your previously created target assays (“Set Up the Targets” on page 26). Amplification results from the endogenous control are used to normalize the amplification results from the target for differences in the amount of template added to each reaction.

For More Information

For more information on:

- Completing the Relative Quantitation Settings screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Reference samples (also known as calibrators) and endogenous controls – Refer to *User Bulletin #2: Relative Quantitation of Gene Expression* (PN 4303859B).

Notes

Set Up the Run Method

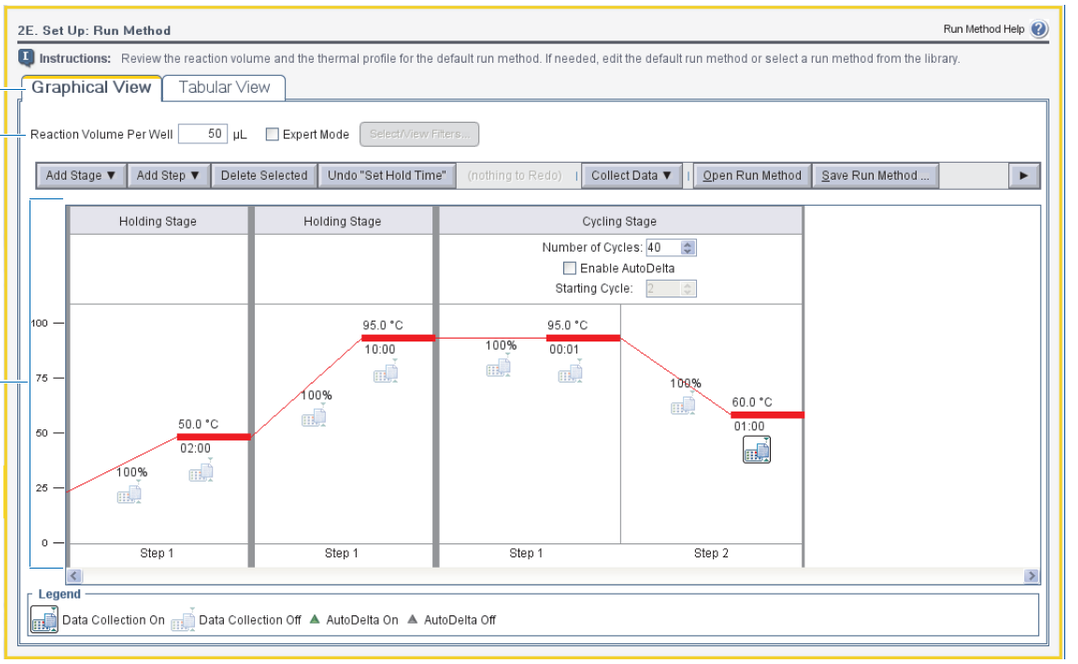
In the Run Method screen, review the reaction volume and the thermal profile for the default run method. If needed, you can edit the default run method or replace it with one from the Run Method library.

About the Example Experiment

In the relative standard curve example experiment, the default run method is used without edits.

Review the Run Method Screen

1. In the Run Method screen, select either the **Graphical View** tab (default) or **Tabular View** tab.
2. Make sure the Reaction Volume Per Well field displays **50 µL**.
3. Make sure the thermal profile displays the holding and cycling stages shown below. If necessary, click a temperature or time setting, then change it.
4. Click **Next**.



Notes

Design Guidelines

When you design your own relative standard curve experiment:

- Enter a number from 10 to 100 for the reaction volume/well. The 7500 system supports reaction volumes from 20 to 100 µL. The 7500 Fast system supports reaction volumes from 10 to 30 µL.
- Review the thermal profile:
  - Make sure the thermal profile is appropriate for your reagents.
  - If you are performing 1-step RT-PCR, include a reverse-transcription step.

  If your experiment requires a different thermal profile, edit the thermal profile or replace the run method with one from the Run Method library. The Run Method library is included in the 7500 software.

For More Information

For more information on:

- The Run Method library or on completing the Run Method screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Using Advanced Setup – See "Advanced Setup Workflow" on page 204.

Review the Reaction Setup

In the Reaction Setup screen, select the assay type (if using TaqMan reagents), then review the calculated volumes for preparing the PCR reactions, standard dilution series, and sample dilutions. If needed, you can edit the reaction volume, excess reaction volume, component concentrations, standard concentration, and/or diluted sample concentration.

About the Example Experiment

In the relative standard curve example experiment:

- Applied Biosystems TaqMan® Gene Expression Assays are used.
- The reaction volume per well is 50 µL.
- The excess reaction volume is 10%.
- The reaction components are:
  - TaqMan® Universal PCR Master Mix (2X)
  - c-myc Assay Mix (20X)
  - GAPDH Assay Mix (20X)
  - Sample or standard
  - Water
- The standard concentration (stock) is 100 ng/µL.
- The diluted sample concentration is 50 ng/µL.
- The sample stock concentration is 100 ng/µL.

Notes

Complete the Reaction Setup Screen

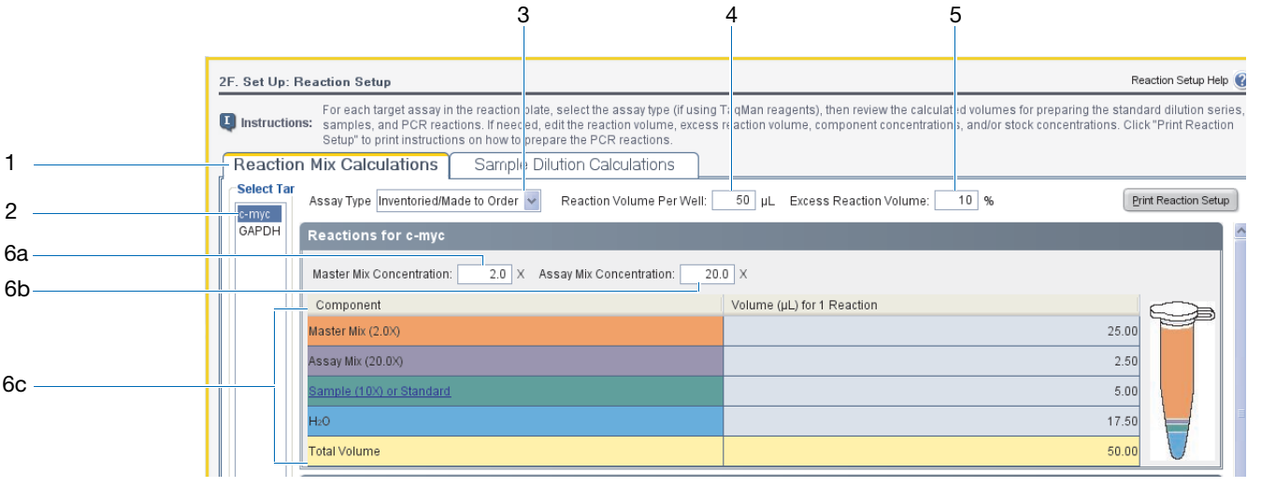
To complete the Reaction Mix Calculations tab *for the c-myc assay*:

IMPORTANT! Perform the following steps for each target assay in the reaction plate.

1. In the Reaction Setup Screen, select the **Reaction Mix Calculations** tab (default).
2. In the Select Target pane, select **c-myc**.
3. In the Assay Type drop-down list, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **50 µL**.
5. Make sure the Excess Reaction Volume field displays **10%**.
6. In the Reactions for c-myc pane:
   a. Make sure the Master Mix Concentration field displays **2.0**X.
   b. Make sure the Assay Mix Concentration field displays **20.0**X.
   c. Review the components and calculated volumes for the PCR reactions:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	25.0
Assay Mix (20.0X)	2.50
Sample (10X) or Standard	5.0‡
H2O	17.50
Total Volume	50.0

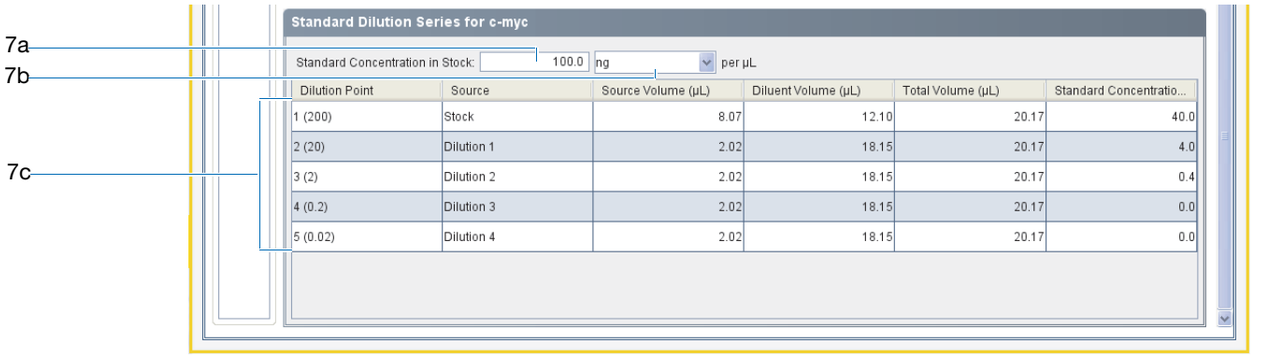
‡ The sample or standard volume is limited to 10% of the total reaction volume.



Notes

7. In the Standard Dilution Series for c-myc pane:
   a. Click the **Standard Concentration in Stock** field, then enter **100.0**.
   b. In the units drop-down list, select **ng** per µL (default).
   c. Review the calculated volumes for preparing the standard dilution series:

Dilution Point	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
1 (200)	Stock	8.07	12.10	20.17	40.0
2 (20)	Dilution 1	2.02	18.15	20.17	4.0
3 (4)	Dilution 2	2.02	18.15	20.17	0.4
4 (0.2)	Dilution 3	2.02	18.15	20.17	0.04
5 (0.02)	Dilution 4	2.02	18.15	20.17	0.004



To complete the Reaction Mix Calculations tab *for the GAPDH assay*:

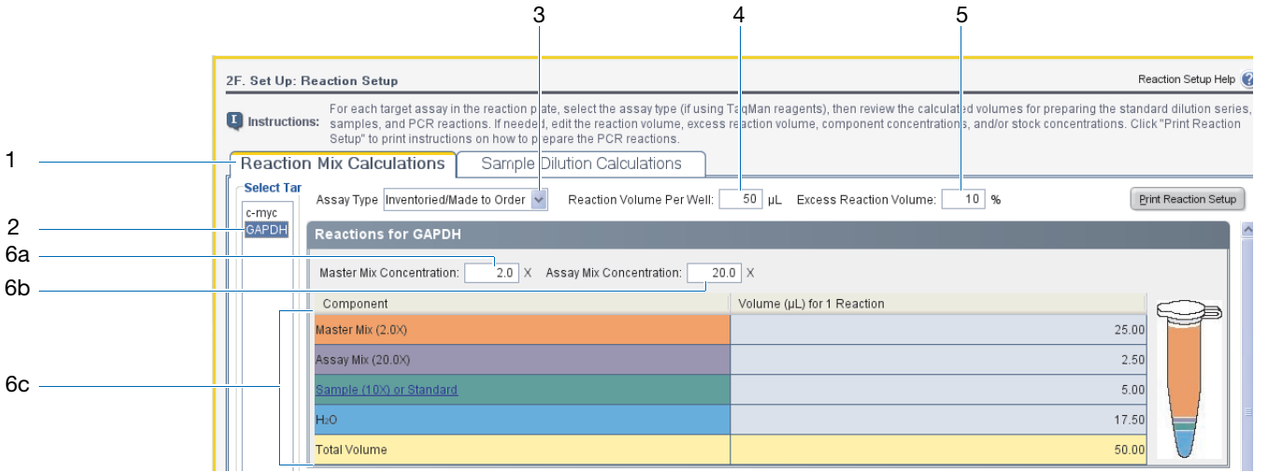
1. In the Reaction Setup screen, select the **Reaction Mix Calculations** tab (default).
2. In the Select Target pane, select **GAPDH**.
3. In the Assay Type drop-down list, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **50 µL**.
5. Make sure the Excess Reaction Volume field displays **10%**.
6. In the Reactions for GAPDH pane:
   a. Make sure the Master Mix Concentration field displays **2.0×**.
   b. Make sure the Assay Mix Concentration field displays **20.0×**.

Notes

c. Review the components and calculated volumes for the PCR reactions:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	25.0
Assay Mix (20.0X)	2.50
Sample (10X) or Standard	5.0‡
H2O	17.50
Total Volume	50.0

‡ The sample or standard volume is limited to 10% of the total reaction volume.



7. In the Standard Dilution Series for GAPDH pane:

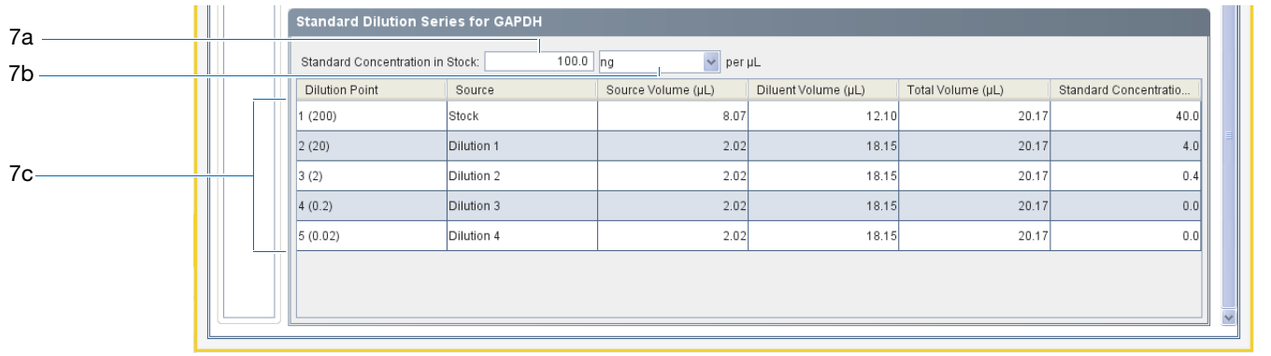
   a. Click the **Standard Concentration in Stock** field, then enter **100.0**.

   b. In the units drop-down list, select **ng** per µL (default).

   c. Review the calculated volumes for preparing the standard dilution series:

Dilution Point	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
1 (200)	Stock	8.07	12.10	20.17	40.0
2 (20)	Dilution 1	2.02	18.15	20.17	4.0
3 (2)	Dilution 2	2.02	18.15	20.17	0.4
4 (0.2)	Dilution 3	2.02	18.15	20.17	0.04
5 (0.02)	Dilution 4	2.02	18.15	20.17	0.004

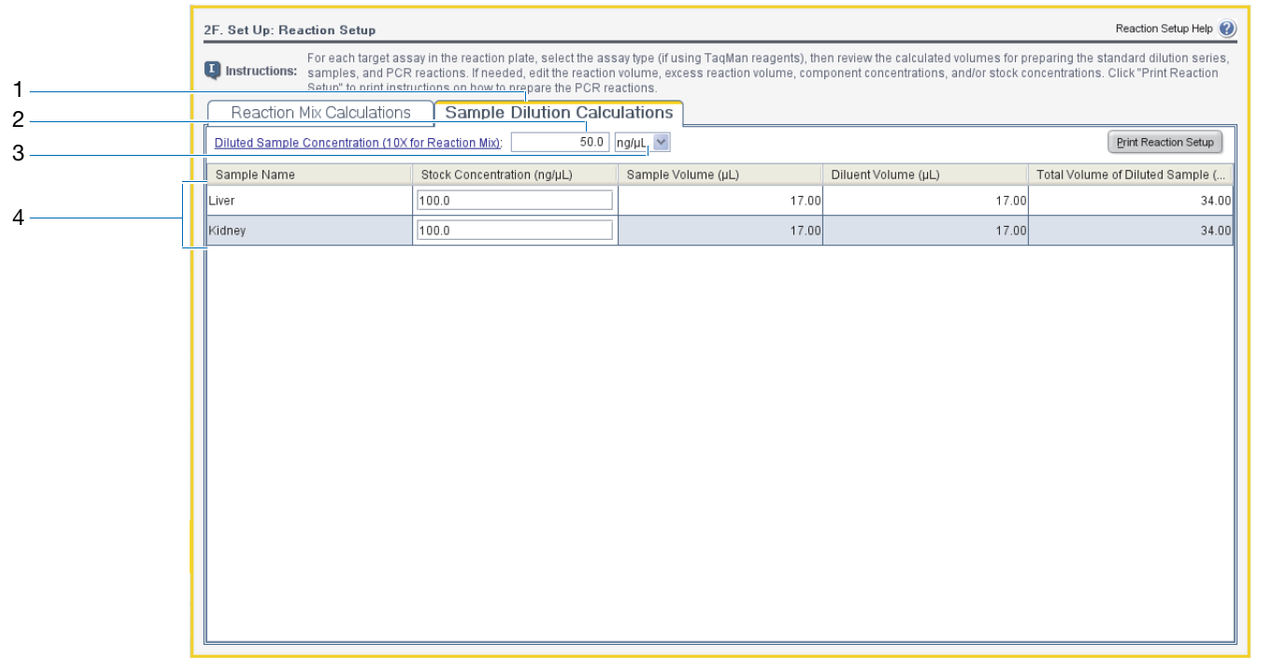
Notes



To complete the Sample Dilution Calculations tab:

1. In the Reaction Setup screen, select the **Sample Dilution Calculations** tab.
2. Click the **Diluted Sample Concentration (10× for Reaction Mix)** field, then enter **50**.
3. In the unit drop-down list, select **ng/µL** (default).
4. Review the calculated volumes for the sample dilutions:

Sample Name	Stock Concentration (ng/µL)	Sample Volume (µL)	Diluent Volume (µL)	Total Volume of Diluted Sample (µL)
Liver	100.0	17.00	17.00	34.00
Kidney	100.0	17.00	17.00	34.00

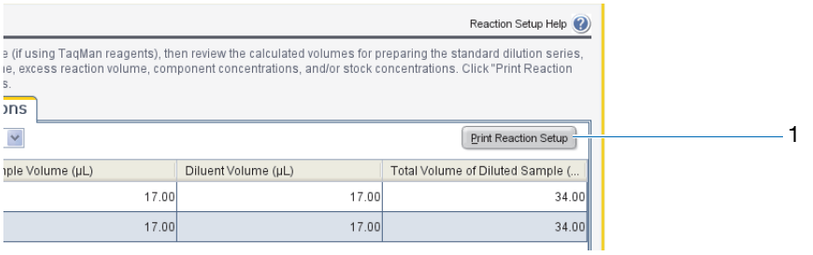


Notes

You can print detailed reaction setup instructions, then save the instructions for Chapter 3, "Prepare the Relative Standard Curve Reactions."

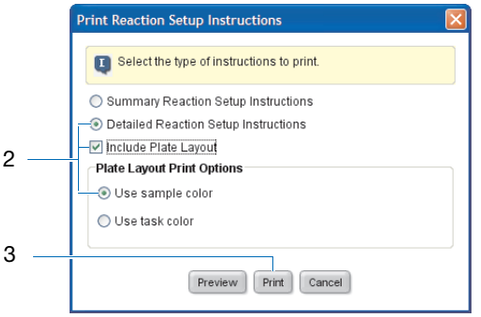
To print Reaction Setup instructions:

1. Click **Print Reaction Setup**.



2. In the Print Reaction Setup Instructions dialog box, select:
   - **Detailed Reaction Setup Instructions**
   - **Include Plate Layout**
   - **Use sample color**

3. Click **Print**.



4. Click **Next**.

Notes

Design Guidelines

When you design your own relative standard curve experiment:

- If you are using TaqMan reagents, select the type of assay you are using:
  - Select **Inventoried/Made to Order** if you are using Applied Biosystems TaqMan® Gene Expression Assays (Inventoried or Made to Order) or Applied Biosystems Custom TaqMan® Gene Expression Assays.
  - Select **Custom** if you are designing your own assays with Primer Express® software.
- Enter a number from 10 to 100 for the reaction volume/well. The 7500 system supports reaction volumes from 20 to 100 µL. The 7500 Fast system supports reaction volumes from 10 to 30 µL.
- Include excess reaction volume to account for the loss that occurs during pipetting. Applied Biosystems recommends an excess reaction volume of at least 10%.
- Review the reaction mix concentrations for each target: If needed:
  - For TaqMan reagents, edit the master mix and assay mix concentrations.
  - For SYBR Green reagents, edit the master mix, forward primer, and reverse primer concentrations.
  - For 1-step RT-PCR, edit the reverse transcriptase concentration.
- Review the reaction mix components for each target:
  - If you are running Fast PCR reactions, make sure you use Fast master mix in the PCR reactions.
  - If you are running standard PCR reactions, make sure you use standard master mix in the PCR reactions.
  - For 1-step RT-PCR, make sure you include reverse transcriptase in the PCR reactions and use a specific buffer.
- Review the standard dilution series calculations for each target. If needed, edit the Standard Concentration in Stock field (including units).

  Note: For the Standard Concentration in Stock field, you can select **ng** or **µg** in the drop-down list or you can enter another unit in the field (for example, **copies**, **IU**, [International Units], **nmol**, **pg**, and so on). The table is updated according to your entry.

- Review the sample dilution calculations for each sample. If needed, edit the diluted sample concentration (including units) and stock concentration.

For More Information

For more information on:

- Completing the Reaction Setup screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Applied Biosystems assays – Refer to the:
  - *TaqMan® Gene Expression Assays Protocol*
  - *Custom TaqMan® Gene Expression Assays Protocol*

Notes

Order Materials for the Experiment

In the Materials List screen, review the list of materials recommended to prepare the PCR reaction plate. You can print the materials list, create a shopping list, then order the recommended materials from the Applied Biosystems Store.

Note: Product availability and pricing might vary according to your region or country. Online ordering through the Applied Biosystems Store is not available in all countries. Contact your local Applied Biosystems representative for help.

Note: The 7500 software recommends the materials to order based on your experiment design. It is assumed that you will design your experiment, order your materials, then prepare (Chapter 3) and run (Chapter 4) the reaction plate when your materials arrive.

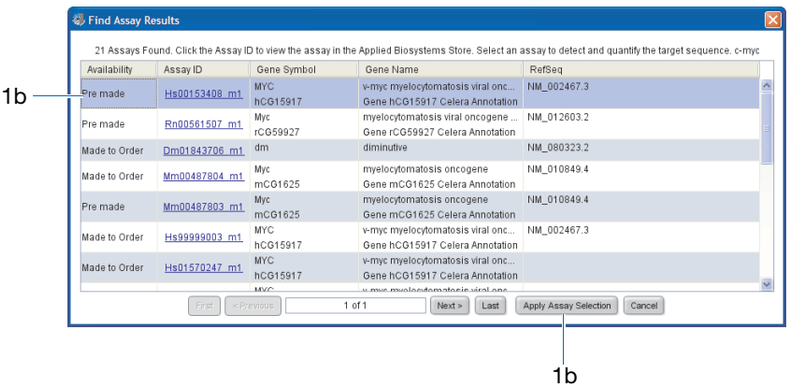
About the Example Experiment

In the relative standard curve example experiment, the recommended materials are:

- MicroAmp® Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- MicroAmp® Splash Free Support Base
- TaqMan® Universal PCR Master Mix (2X), No AmpErase® UNG
- c-myc Assay Mix: Hs00153408_m1 (RefSeq NM_002467.3)
- GAPDH Assay Mix: Hs99999905_m1 (RefSeq NM_002046.3)

Complete the Ordering Materials Screen

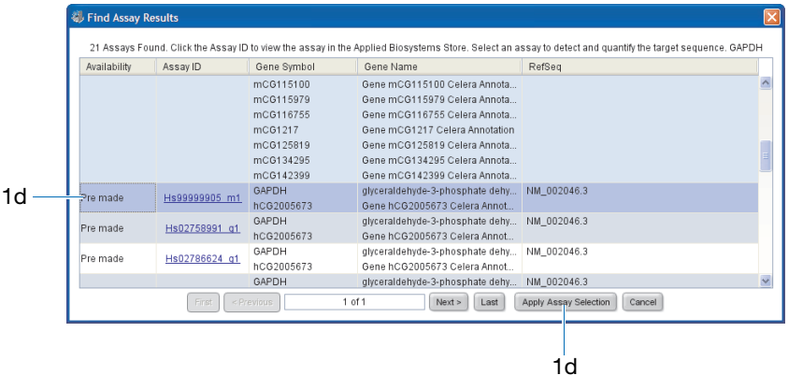
1. Find the target assay in the Applied Biosystems Store:
   a. Click the **Enter Gene Name** field, enter **c-myc**, then click **Find Assay**.
   b. In the Find Assay Results dialog box, select the **Hs00153408_m1** row, then click **Apply Assay Selection**.



   c. Click the **Enter Gene Name** field, enter **GAPDH**, then click **Find Assay**.

Notes

d. In the Find Assay Results dialog box, select the **Hs99999905_m1** row, then click **Apply Assay Selection**.



2. In the Display drop-down list, select **All Items** (default), then review the recommended materials.

Note: For more information on a specific item, click the part number link. You are connected to the product information page at the Applied Biosystems Store.

3. (Optional) Click **Print Materials List** to send the materials list to your printer.

4. (Optional) Create a shopping list:

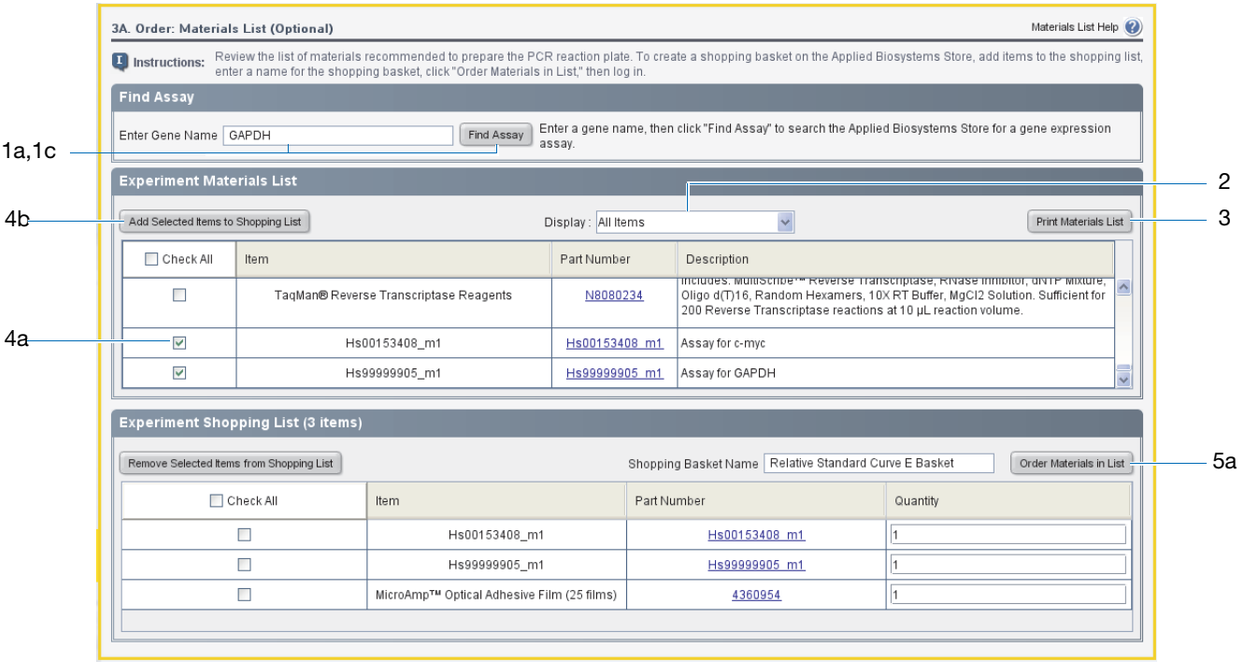
   a. Select the check box next to each of the following items:
      - MicroAmp® Optical 96-Well Reaction Plate
      - MicroAmp™ Optical Adhesive Film
      - MicroAmp® Splash Free Support Base
      - TaqMan® Universal PCR Master Mix (2✕), No AmpErase® UNG
      - Hs00153408_m1 (c-myc Assay Mix)
      - Hs99999905_m1 (GAPDH Assay Mix)

   b. Click **Add Selected Items to Shopping List**.

5. (Optional) Create a shopping basket at the Applied Biosystems Store. Product availability and pricing may vary according to your region or country. Online ordering through the Applied Biosystems Store is not available in all countries. Contact your local Applied Biosystems representative for help.

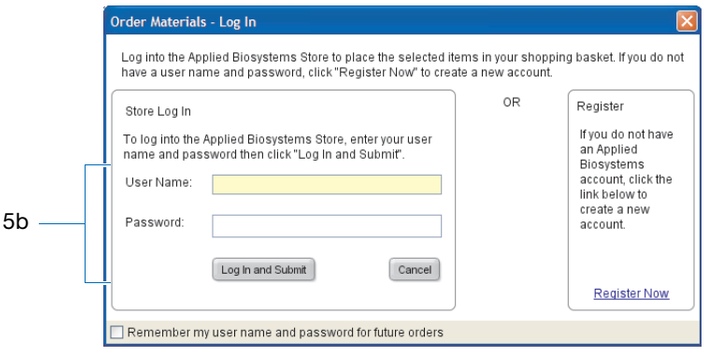
Notes

a. Check that the Experiment Shopping List contains the desired materials and that the quantities are correct, then click **Order Materials in List**.



b. In the Order Materials – Log In dialog box, enter your user name and password for the Applied Biosystems Store, then click **Log In and Submit**.

Note: If you do not have an account with the Applied Biosystems Store, click **Register Now** to create an account.



c. When you are connected to the Applied Biosystems Store, follow the prompts to complete your order.

6. Go to "Finish the Design Wizard" on page 46.

Notes

Design Guidelines

When you design your own relative standard curve experiment:

- Select all the materials that you require for your experiment, then add them to your shopping list.
- To access and use the Applied Biosystems Store:
  - Confirm that your computer has an Internet connection.
  - Use the following Applied Biosystems-recommended browsers and versions of Adobe® Acrobat® Reader:

Desktop Operating System	Netscape® Navigator	Microsoft® Internet Explorer	Macintosh® Safari	Adobe® Acrobat® Reader
Windows® 2000/NT/XP/Vista	v6.x or later	v6.x or later	Not applicable	v4.0 or later
Macintosh® OS 9+ or later	Not supported	Not supported	v2.0.4 or later	v4.0 or later

IMPORTANT! Make sure that cookies and JavaScript are turned on.

For More Information

For more information on completing the Materials List screen, open the 7500 Software Help by clicking or pressing **F1**.

Finish the Design Wizard

Finish the Design Wizard, review the plate layout, then select an exit option.

About the Example Experiment

The 7500 software automatically selects locations for the wells in the reaction plate. In the relative standard curve example experiment:

- The wells are arranged as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	N c-myc	N c-myc	N c-myc	N GAPDH	N GAPDH	N GAPDH	Liver U c-myc 7.39	Liver U c-myc 8.13	Liver U c-myc	Liver U GAPDH 0.47	Liver U GAPDH 0.44	Liver U GAPDH 0.43
B	Kidney U c-myc 5.32	Kidney U c-myc 4.1	Kidney U c-myc 3.76	Kidney U GAPDH 0.44	Kidney U GAPDH 0.43	Kidney U GAPDH 0.43	S c-myc 200	S c-myc 200	S c-myc 200	S c-myc 20	S c-myc 20	S c-myc 20
C	S c-myc 2	S c-myc 2	S c-myc 2	S c-myc 0.2	S c-myc 0.2	S c-myc 0.2	S c-myc 0.02	S c-myc 0.02	S c-myc 0.02	S GAPDH 200	S GAPDH 200	S GAPDH 200
D	S GAPDH 20	S GAPDH 20	S GAPDH 20	S GAPDH 2	S GAPDH 2	S GAPDH 2	S GAPDH 0.2	S GAPDH 0.2	S GAPDH 0.2	S GAPDH 0.02	S GAPDH 0.02	S GAPDH 0.02
E												

- The experiment is saved as is and closed.

Note: For the example experiment, do not perform the run at this time.

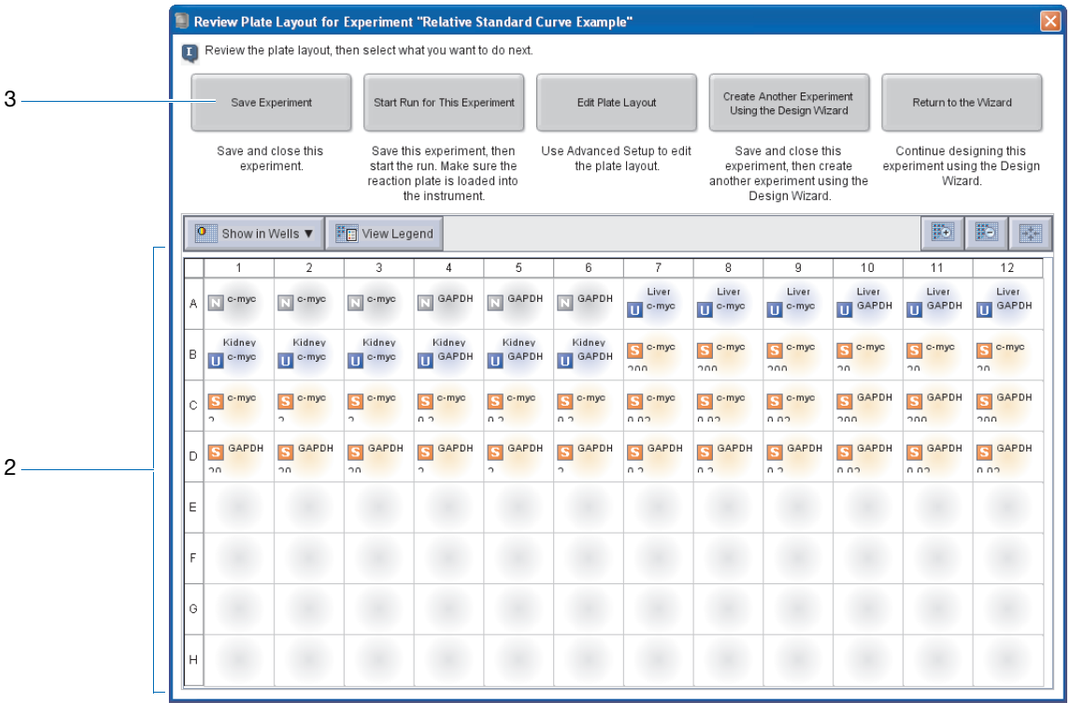
Notes

Finish the Design Wizard

1. At the bottom of the 7500 software screen, click **Finish Designing Experiment**.
2. In the Review Plate Layout for Experiment window, review the plate layout. Make sure there are:
   - 12 Unknown wells
   - 30 Standard wells
   - 6 Negative control wells
   - 48 Empty wells

Note: If the plate layout is incorrect, click **Return to the Wizard** and check your entered values.

3. Click **Save Experiment**.



4. In the Save Experiment dialog box, enter **Relative Standard Curve Example Setup.eds** in the File name drop-down list, then click **Save**. The example experiment is saved and closed, and you are returned to the Home screen.

IMPORTANT! Do not save the experiment using the default file name. Doing so will overwrite the example experiment already present in the experiments folder.

Note: By default, the example experiment is saved to the *<drive>*:\Applied Biosystems*<software name>*\experiments folder.

Notes

Design Guidelines

When you design your own relative standard curve experiment:

- In the Review Plate Layout for Experiment window, select the appropriate exit option:

Click	To...
Save Experiment	Save and close the experiment without making any further changes or starting the run.
Start Run for This Experiment	Save the experiment and start the run. Make sure the reaction plate is loaded in the instrument.
Edit Plate Layout	Use advanced setup to edit the plate layout.
Create Another Experiment Using the Design Wizard	Save and close the current experiment, then create another experiment using the Design Wizard.
Return to the Wizard	Return to the experiment to make changes using the Design Wizard.

- By default, experiments are saved to:

  <*drive*>:\Applied Biosystems\<*software name*>\experiments

  To change the:

  – Save location for a specific experiment – Navigate to the desired location using the Save Experiment dialog box.

  – Default save location – Select **Tools** ▸ **Preferences**, then select the **Defaults** tab. In the Data Folder field, browse to then select the desired location.

For More Information

For more information on using Advanced Setup, see “Advanced Setup Workflow” on page 204.

Notes

3 Prepare the Relative Standard Curve Reactions

This chapter covers:

- Chapter Overview 50
- Prepare the Template 51
- Prepare the Sample Dilutions 53
- Prepare the Standard Dilution Series 55
- Prepare the Reaction Mix 58
- Prepare the Reaction Plate 60

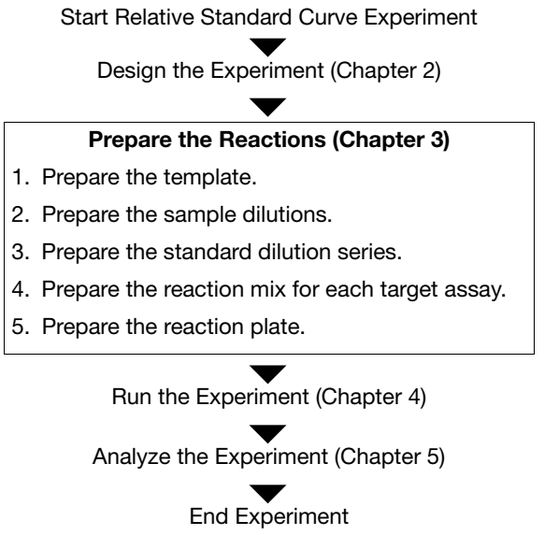
Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

Notes

Chapter Overview

This chapter explains how to prepare the PCR reactions for the relative standard curve example experiment.

Example Experiment Workflow



Notes

Prepare the Template

You prepare the template for the PCR reactions (both samples and standards) using the High-Capacity cDNA Reverse Transcription Kit.

IMPORTANT! Applied Biosystems recommends that you use the High-Capacity cDNA Reverse Transcription Kit to reverse-transcribe cDNA from total RNA. The TaqMan® Gene Expression Assays are compatible with the High-Capacity cDNA Reverse Transcription Kit; other protocols have not been tested for use with the TaqMan Gene Expression Assays.

About the Example Experiment

For the relative standard curve example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples using the High-Capacity cDNA Reverse Transcription Kit.

Required Materials

- One of the following Ambion® starter packs for RNA isolation:

Kit	Contents	Ambion Catalog Number
qRT-PCR Starter Pack	RNA*later*® Tissue Collection: RNA Stabilization Solution	AM7020
	RNase*Zap*® Wipes	AM9786
	RT-PCR Grade Water (nuclease-free)	AM9935
	Silencer® Validated siRNA, Std Purity	AM51331
	One of the following RNA sample preparation products:	
	• RNAqueous®-4PCR Kit **Note:** Recommended if you are isolating RNA from cells or tissues that may be difficult to disrupt or have high RNase activity.	AM1914
	• RiboPure™ Kit **Note:** Recommended if you are: – Isolating RNA from all tissues, including those that may be difficult to disrupt, are rich in lipids, or have high RNase activity *or* – Labeling and amplifying RNA for use on microarrays.	AM1924
	• TURBO DNA-*free*™ **Note:** Recommended if you are using SYBR® Green reagents. If you are using SYBR Green reagents, use TURBO DNA-free with the RiboPure Kit.	AM1907
PCR Starter Pack	RT-PCR Grade Water (nuclease-free)	AM9935
	DNA*Zap*™	AM9890
	Choice of RNA sample preparation products, as listed above under the qRT-PCR Starter Pack.	See above

Notes

Kit	Contents	Ambion Catalog Number
High Capacity cDNA Kit	RNA*later*® Tissue Collection: RNA Stabilization Solution	AM7020
	RNase*Zap*® Wipes	AM9786
	Choice of RNA sample preparation products, as listed above under the qRT-PCR Starter Pack.	See above
miRNA Starter Pack	Pre-miR™ miRNA Starter Kit	AM1540

- For the samples, total RNA isolated from liver and kidney tissues
- For the standards, total RNA isolated from lung tissue

Note: Be sure to prepare template for both your samples and your standards.

- One of the following Applied Biosystems High-Capacity cDNA Reverse Transcription Kits:

Kit	Part Number
High-Capacity cDNA Reverse Transcription Kit (200 reactions)	4368814
High-Capacity cDNA Reverse Transcription Kit (1000 reactions)	4368813
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	4374966
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (1000 reactions)	4374967

Note: The High-Capacity cDNA Reverse Transcription Kit was formerly called the High-Capacity cDNA Archive Kit.

Prepare the Template

Use the High-Capacity cDNA Reverse Transcription Kit to reverse-transcribe cDNA from the total RNA samples. Follow the procedures in the *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol* to:

1. Prepare the RT master mix.

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

2. Prepare the cDNA reactions.
3. Perform reverse transcription on a thermal cycler.

Notes

Preparation Guidelines

When you prepare your own relative standard curve experiment, Applied Biosystems recommends:

- That you first extract DNA or RNA from the tissue or sample.
- The following templates:
  - **Complementary DNA (cDNA)** – cDNA reverse-transcribed from total RNA samples using a High-Capacity cDNA Reverse Transcription Kit.
  - **RNA** – Purified total RNA or mRNA extracted from tissue or sample.
  - **Genomic DNA (gDNA)** – Purified gDNA already extracted from tissue or sample.

For More Information

For more information on:

- Preparing cDNA templates – Refer to the *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol*. The protocol is not shipped with the High-Capacity cDNA Reverse Transcription Kits. You can download the protocol from:

  http://docs.appliedbiosystems.com/search.taf
- Preparing RNA or gDNA templates – Refer to the protocol for the purification reagents that you select. To locate Applied Biosystems purification reagents, visit:

  http://www.appliedbiosystems.com/

Prepare the Sample Dilutions

Perform sample dilutions before adding the samples to the final reaction mix. Dilute the samples using the volumes that were calculated by the 7500 software ("To complete the Sample Dilution Calculations tab:" on page 40).

About the Example Experiment

For the relative standard curve example experiment:

- Sample dilutions are necessary because the sample volume is limited to 10% of the total reaction volume in the 7500 software. Because the total reaction volume is 50 µL/reaction, the sample volume is 5 µL/reaction.
- The stock sample concentration is 100 ng/µL. After diluting the sample according to the Sample Dilutions Calculations table, the sample has a concentration of 50 ng/µL. Adding 17 µL at this concentration to the final reaction mix volume of 34 µL yields a 1× concentration in the final reaction.
- The volumes calculated in the software are:

Sample Name	Stock Concentration (ng/µL)	Sample Volume (µL)	Diluent Volume (µL)	Total Volume of Diluted Sample (µL)
Liver	100.0	17.0	17.0	34.0
Kidney	100.0	17.0	17.0	34.0

Notes

Required Materials

- Water (to dilute the sample)
- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Sample stock
- Vortexer
- Centrifuge

Prepare the Sample Dilutions

1. Label a separate microcentrifuge tube for each diluted sample:
   - **Liver**
   - **Kidney**
2. Add the required volume of water (diluent) to each empty tube:

Tube	Sample Name	Diluent Volume (µL)
1	Liver	17.0
2	Kidney	17.0

3. Add the required volume of sample stock to each tube:

Tube	Sample Name	Sample Volume (µL)
1	Liver	17.0
2	Kidney	17.0

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Preparation Guidelines

When you prepare your own relative standard curve experiment:

- Sample dilutions may be necessary because the sample volume is limited to 10% of the total reaction volume in the 7500 software. You must perform the sample dilutions before adding the samples to the final reaction mix.
- For optimal performance of TaqMan® Gene Expression Assays or Custom TaqMan® Gene Expression Assays, use 10 to 100 ng of cDNA template per 20-µL reaction.
- Use TE buffer or water to dilute the sample.

For More Information

For more information on Applied Biosystems assays, refer to the:

- *TaqMan® Gene Expression Assays Protocol*
- *Custom TaqMan® Gene Expression Assays Protocol*.

Notes

Prepare the Standard Dilution Series

You prepare the standard dilution series using the volumes that were calculated by the 7500 software ("To complete the Reaction Mix Calculations tab for the c-myc assay" on page 37 and "To complete the Reaction Mix Calculations tab for the GAPDH assay" on page 38):

About the Example Experiment

For the relative standard curve example experiment:

- The standard (stock) concentration is 100 ng/µL.
- The volumes calculated in the software for both the c-myc and GAPDH assays are:

Dilution Point (Factor)	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
1 (200)	Stock	8.07	12.10	20.17	40.0
2 (20)	Dilution 1	2.02	18.15	20.17	4.0
3 (2)	Dilution 2	2.02	18.15	20.17	0.4
4 (0.2)	Dilution 3	2.02	18.15	20.17	0.04
5 (0.02)	Dilution 4	2.02	18.15	20.17	0.004

Required Materials

- Water (to dilute the standards)
- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Standard stock
- Vortexer
- Centrifuge

Prepare the Standard Dilution Series for the c-myc Assay

1. Label a separate microcentrifuge tube for each standard:
   - **c-myc Std. 1**
   - **c-myc Std. 2**
   - **c-myc Std. 3**
   - **c-myc Std. 4**
   - **c-myc Std. 5**

Notes

2. Add the required volume of water (diluent) to each empty tube:

Tube	Standard Name	Volume of Diluent to Add (µL)
1	c-myc Std. 1	12.10
2	c-myc Std. 2	18.15
3	c-myc Std. 3	18.15
4	c-myc Std. 4	18.15
5	c-myc Std. 5	18.15

3. In the c-myc Std. 1 tube:
   a. Vortex the stock for 3 to 5 seconds, then centrifuge the tube briefly.
   b. Using a new pipette tip, add 8.07 µL of stock to the c-myc Std. 1 tube.
   c. Vortex Std. 1 for 3 to 5 seconds, then centrifuge the tube briefly.
4. In the c-myc Std. 2 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 1 to the c-myc Std. 2 tube.
   b. Vortex Std. 2 for 3 to 5 seconds, then centrifuge the tube briefly.
5. In the c-myc Std. 3 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 2 to the c-myc Std. 3 tube.
   b. Vortex Std. 3 for 3 to 5 seconds, then centrifuge the tube briefly.
6. In the c-myc Std. 4 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 3 to the c-myc Std. 4 tube.
   b. Vortex Std. 4 for 3 to 5 seconds, then centrifuge the tube briefly.
7. In the c-myc Std. 5 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 4 to the c-myc Std. 5 tube.
   b. Vortex Std. 5 for 3 to 5 seconds, then centrifuge the tube briefly.
8. Place the standards on ice until you prepare the reaction plate.

Prepare the Standard Dilution Series for the GAPDH Assay

1. Label a separate microcentrifuge tube for each standard:
   - **GAPDH Std. 1**
   - **GAPDH Std. 2**
   - **GAPDH Std. 3**
   - **GAPDH Std. 4**
   - **GAPDH Std. 5**

Notes

2. Add the required volume of water (diluent) to each empty tube:

Tube	Standard Name	Volume of Diluent to Add (µL)
1	GAPDH Std. 1	12.10
2	GAPDH Std. 2	18.15
3	GAPDH Std. 3	18.15
4	GAPDH Std. 4	18.15
5	GAPDH Std. 5	18.15

3. In the GAPDH Std. 1 tube:
   a. Vortex the stock for 3 to 5 seconds, then centrifuge the tube briefly.
   b. Using a new pipette tip, add 8.07 µL of stock to the GAPDH Std. 1 tube.
   c. Vortex Std. 1 for 3 to 5 seconds, then centrifuge the tube briefly.
4. In the GAPDH Std. 2 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 1 to GAPDH Std. 2 tube.
   b. Vortex Std. 2 for 3 to 5 seconds, then centrifuge the tube briefly.
5. In the GAPDH Std. 3 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 2 to the GAPDH Std. 3 tube.
   b. Vortex Std. 3 for 3 to 5 seconds, then centrifuge the tube briefly.
6. In the GAPDH Std. 4 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 3 to the GAPDH Std. 4 tube.
   b. Vortex Std. 4 for 3 to 5 seconds, then centrifuge the tube briefly.
7. In the GAPDH Std. 5 tube:
   a. Using a new pipette tip, add 2.02 µL of dilution 4 to the GAPDH Std. 5 tube.
   b. Vortex Std. 5 for 3 to 5 seconds, then centrifuge the tube briefly.
8. Place the standards on ice until you prepare the reaction plate.

Preparation Guidelines

When you prepare your own relative standard curve experiment:

- Standards are critical for accurate analysis of run data.
- Any mistakes or inaccuracies in making the dilutions directly affect the quality of results.
- The quality of pipettors and tips and the care used in measuring and mixing dilutions affect accuracy.
- Use TE buffer or water to dilute the standards.

Notes

Prepare the Reaction Mix

You prepare the reaction mix using the components and volumes that were calculated by the 7500 software ("To complete the Reaction Mix Calculations tab for the c-myc assay" on page 37 and "To complete the Reaction Mix Calculations tab for the GAPDH assay" on page 38).

Note: The software calculates the volumes for all components for the PCR reactions. However, when you prepare the reaction mix in this section, include only the master mix, assay mix, and water. Add the sample or standard when you prepare the reaction plate (see "Prepare the Reaction Plate" on page 60).

About the Example Experiment

For the relative standard curve example experiment:

- The reaction mix components are:
  - TaqMan® Universal PCR Master Mix (2×)
  - c-myc Assay Mix (20×)
  - GAPDH Assay Mix (20×)
  - Water
- The volumes calculated in the software for both target assays are:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0×)	25.0
Assay Mix (20.0×)	2.5
Water	17.5
Total Volume	45.0

Note: The sample or standard is not added at this time.

Required Materials

- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Reaction mix components (listed above)
- Centrifuge

Notes

Prepare the Reaction Mix

IMPORTANT! Prepare the reaction mix for each target assay separately.

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.

1. Label an appropriately sized tube for each reaction mix:
   - **c-myc Reaction Mix**
   - **GAPDH Reaction Mix**
2. For the c-myc assay, add the required volumes of each component to the c-myc Reaction Mix tube:

Component	Volume (µL) for 1 Reaction	Volume (µL) for 24 Reactions Plus 10% Excess
TaqMan® Universal PCR Master Mix (2X)	25.0	660.0
c-myc Assay Mix (20X)	2.5	66.0
Water	17.5	462.0
Total Reaction Mix Volume	45.0	1188.0

3. For the GAPDH assay, add the required volumes of each component to the GAPDH Reaction Mix tube:

Component	Volume (µL) for 1 Reaction	Volume (µL) for 24 Reactions Plus 10% Excess
TaqMan® Universal PCR Master Mix (2X)	25.0	660.0
GAPDH Assay Mix (20X)	2.5	66.0
Water	17.5	462.0
Total Reaction Mix Volume	45.0	1188.0

4. Mix the reaction mix in each tube by gently pipetting up and down, then cap each tube.
5. Centrifuge the tubes briefly to remove air bubbles.
6. Place the reaction mixes on ice until you prepare the reaction plate.

Notes

Preparation Guidelines

When you prepare your own relative standard curve experiment:

- If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.
- Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.
- Include all required components.
- Prepare the reagents according to the manufacturer's instructions.
- Keep the assay mix protected from light (in the freezer) until you are ready to use it. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Mix the master mix thoroughly by swirling the bottle.
  - Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
  - Thaw any frozen samples by placing them on ice. When the samples are thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.

For More Information

For more information on preparing the reaction mix, refer to the protocol appropriate for the reagents you are using in the PCR reactions:

- *TaqMan® Gene Expression Assays Protocol*
- *Custom TaqMan® Gene Expression Assays Protocol*

Prepare the Reaction Plate

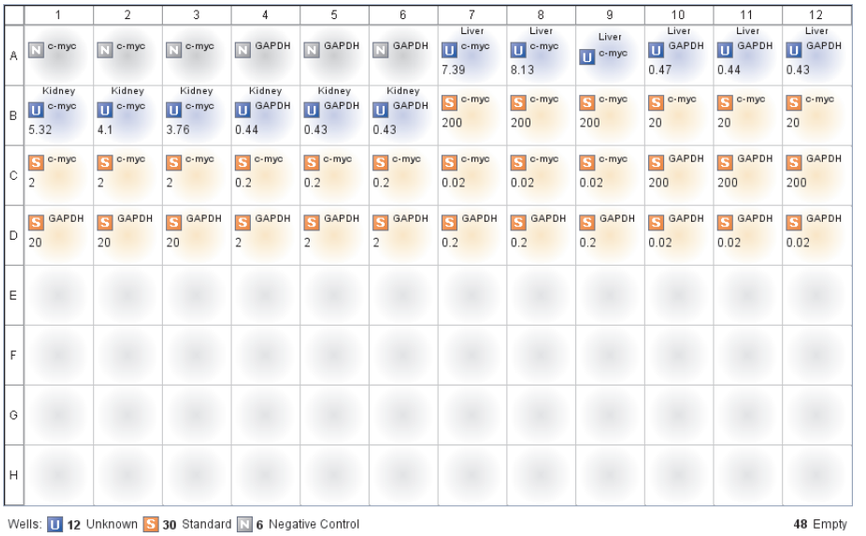
You prepare the reactions for each replicate group, then transfer them to the reaction plate using the plate layout displayed in the 7500 software.

About the Example Experiment

For the relative standard curve example experiment:

- A MicroAmp® Optical 96-Well Reaction Plate is used.
- The reaction volume is 50 µL/well.
- The reaction plate contains:
  - 12 Unknown wells U
  - 30 Standard wells S
  - 6 Negative control wells N
  - 48 Empty wells
- The plate layout that is automatically generated by the 7500 software is used:

Notes



Required Materials

- Microcentrifuge tubes
- Pipettors
- Pipette tips
- c-myc reaction mix (from page 59)
- GAPDH reaction mix (from page 59)
- Water
- c-myc standards (from page 55)
- GAPDH standards (from page 56)
- Samples (from page 54)
- MicroAmp® Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- Centrifuge

Prepare the Reaction Plate

1. For each target, prepare the negative control reactions:

   a. To an appropriately sized tube, add the volumes of reaction mix and water listed below.

Tube	Reaction Mix	Reaction Mix Volume (µL)	Water Volume (µL)
1	c-myc reaction mix	59.4	6.6
2	GAPDH reaction mix	59.4	6.6

   b. Mix the reaction by gently pipetting up and down, then cap the tube.

   c. Centrifuge the tube briefly to remove air bubbles.

   d. Add 50 µL of the negative control reaction to the appropriate wells in the reaction plate.

Notes

2. For each replicate group, prepare the standard reactions:

   a. To appropriately sized tubes, add the volumes of reaction mix and standard listed below.

Tube	Standard Reaction	Reaction Mix	Reaction Mix Volume (µL)	Standard	Standard Volume (µL)
1	c-myc Std 1	c-myc reaction mix	148.5	c-myc Std 1	16.5
2	c-myc Std 2	c-myc reaction mix	148.5	c-myc Std 2	16.5
3	c-myc Std 3	c-myc reaction mix	148.5	c-myc Std 3	16.5
4	c-myc Std 4	c-myc reaction mix	148.5	c-myc Std 4	16.5
5	c-myc Std 5	c-myc reaction mix	148.5	c-myc Std 5	16.5
6	GAPDH Std 1	GAPDH reaction mix	148.5	GAPDH Std 1	16.5
7	GAPDH Std 2	GAPDH reaction mix	148.5	GAPDH Std 2	16.5
8	GAPDH Std 3	GAPDH reaction mix	148.5	GAPDH Std 3	16.5
9	GAPDH Std 4	GAPDH reaction mix	148.5	GAPDH Std 4	16.5
10	GAPDH Std 5	GAPDH reaction mix	148.5	GAPDH Std 5	16.5

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

   c. Centrifuge the tubes briefly to remove air bubbles.

   d. Add 50 µL of the standard reaction to the appropriate wells in the reaction plate.

3. For each replicate group, prepare the reactions for the unknowns:

   a. To appropriately sized tubes, add the volumes of reaction mix and sample listed below.

Tube	Unknown Reaction	Reaction Mix	Reaction Mix Volume (µL)	Sample	Sample Volume (µL)
1	c-myc Liver	c-myc reaction mix	148.5	Liver	16.5
2	c-myc Kidney	c-myc reaction mix	148.5	Kidney	16.5
3	GAPDH Liver	GAPDH reaction mix	148.5	Liver	16.5
4	GAPDH Kidney	GAPDH reaction mix	148.5	Kidney	16.5

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

   c. Centrifuge the tubes briefly to remove air bubbles.

   d. Add 50 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

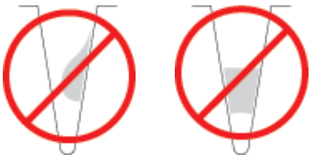
4. Seal the reaction plate with optical adhesive film.

Notes

5. Centrifuge the reaction plate briefly to remove air bubbles.

6. Verify that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.

IMPORTANT! Do not allow the bottom of the reaction plate to become dirty. Fluids and other contaminants that adhere to the bottom of the reaction plate can contaminate the sample block and cause an abnormally high background signal.

Correct	Incorrect
	
Liquid is at the bottom of the well.	• Not centrifuged with enough force, *or* • Not centrifuged for enough time

7. Until you are ready to perform the run, place the reaction plate on ice in the dark.

Preparation Guidelines

When you prepare your own relative standard curve experiment:

- Make sure you use the appropriate consumables.
- Make sure the arrangement of the PCR reactions matches the plate layout displayed in the 7500 software. You can either:
  - Accept the plate layout automatically generated by the software.

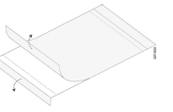
    or
  - Use Advanced Setup to change the plate layout in the software.
- If you use optical adhesive film, seal each reaction plate as follows:

  a. Place the reaction plate onto the center of the 96-well base.

  b. Load the reaction plate as desired.

  c. Remove a single optical adhesive film (film) from the box. Fold back one of the end-tabs. Hold the film with its backing side up.

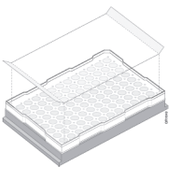
  d. In one continuous movement, peel back the white protective backing from the center sealing surface. Do not touch the center sealing surface.

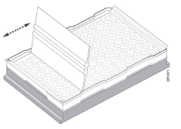
  IMPORTANT! Improper peeling of the optical adhesive film may result in haziness, but does not affect results. Haziness disappears when the film comes into contact with the heated cover in the instrument.

Notes

e. While holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Be sure the film completely covers all wells of the reaction plate.

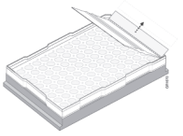


f. While applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good contact between the film and the entire surface of the reaction plate.



g. While using the applicator to hold the edge of the film in place, grasp one end of the end-tab and pull up and away sharply. Repeat for the other end-tab.

h. Repeat step f to ensure a tight, evaporation-free seal. While applying firm pressure, run the edge of the applicator along all four sides of the outside border of the film.



Note: Optical adhesive films do not adhere on contact. The films require the application of pressure to ensure a tight seal.

i. Inspect the reaction plate to be sure all wells are sealed. You should see an imprint of all wells on the surface of the film.

For More Information

For more information on:

- Preparing the reaction plate – Refer to the protocol appropriate for the reagents you are using in the PCR reactions:
  - *TaqMan® Gene Expression Assays Protocol*
  - *Custom TaqMan® Gene Expression Assays Protocol*
- Consumables – See “Supported Consumables” on page 4.
- Using Advanced Setup to change the plate layout – See “Advanced Setup Workflow” on page 204.

Notes

4 Run the Relative Standard Curve Experiment

This chapter covers:

- Chapter Overview 66
- Prepare for the Run 67
- Enable the Notification Settings (Optional) 69
- Start the Run 71
- Monitor the Run 72
- Unload the Instrument 74

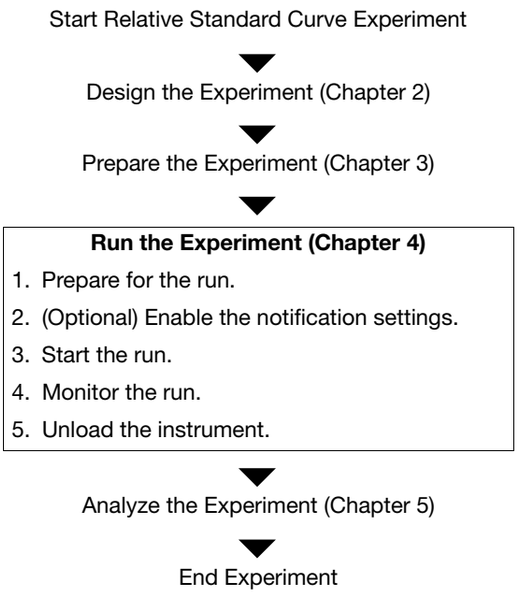
Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

Notes

Chapter Overview

This chapter explains how to perform a run on the Applied Biosystems 7500/7500 Fast Real-Time PCR System.

Example Experiment Workflow



Notes

Prepare for the Run

You prepare for the run by opening the example experiment file you created in Chapter 2, then loading the sealed reaction plate into the 7500/7500 Fast instrument.

Open the Example Experiment

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

   where *<software name>* is the current version of the 7500 software.

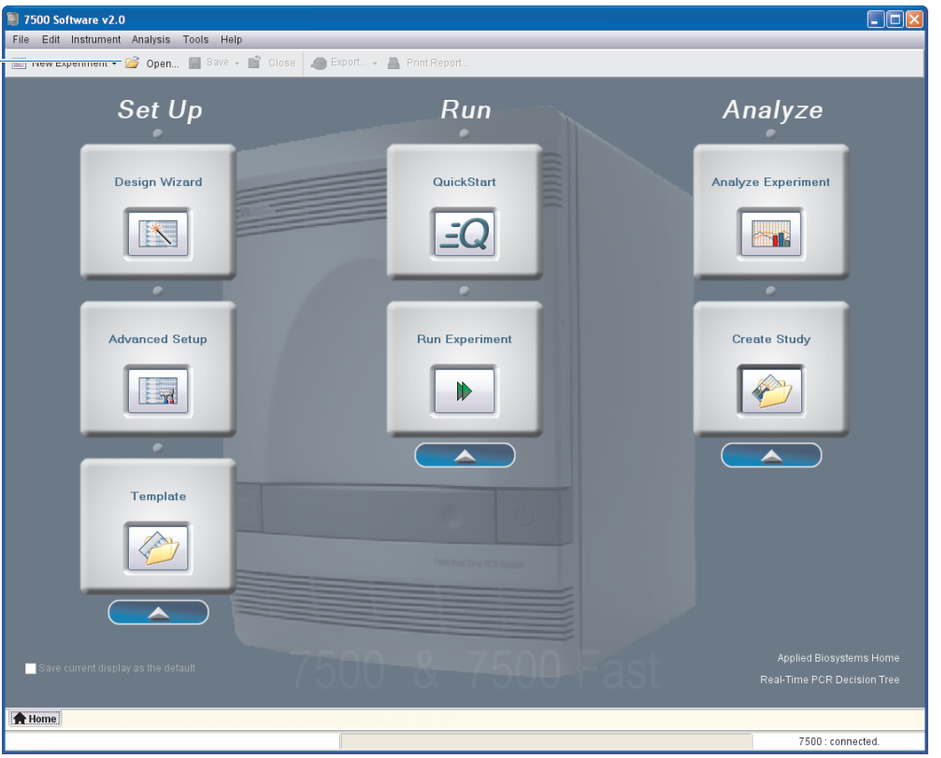
2. In the Home screen, click **Open**.

3. In the Open dialog box, navigate to the **experiments** folder (default) at:

   <drive>:\Applied Biosystems*<software name>*\experiments

4. Double-click the example experiment file that you created in Chapter 2.

2



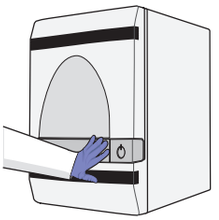
Notes

Load the Reaction Plate into the Instrument

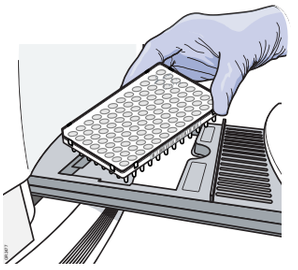
WARNING **PHYSICAL INJURY HAZARD.** During operation, the sample block can be heated to 100 °C. Before performing the following procedure, be sure to wait until the sample block reaches room temperature.

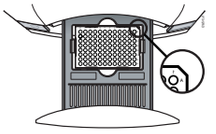
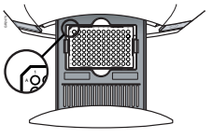
IMPORTANT! Wear powder-free gloves when you handle the reaction plate.

1. Push the tray door to open it.



2. Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.



7500 system	**7500 Fast system**
Load standard plates with the notched A12 position at the top-right of the tray.	Load Fast plates with the notched A1 position at the top-left of the tray.
	

3. Place the reactions in the precision plate holder (PPH). If you use:
   - **A reaction plate** – Place the reaction plate in the PPH with well A1 at the back-left corner.
   - **Reaction tube strips** – Place the tube strips in the PPH for tube strips.

   Note: Fast Reaction 8-tube strips can be used only on the 7500 Fast system. For the 7500 system, use MicroAmp® Optical 8-Tube Strips.

Notes

- **Reaction tubes** – Place the tubes in the PPH.

Note: MicroAmp® Fast Reaction Tubes (PN 4358297) cannot be used in the 7500 Fast system.

IMPORTANT! For optimal performance with partial loads:

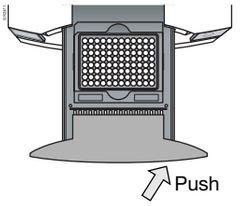
For a 7500 Instrument

Load at least 16 tubes, arranging them first in the center columns of the instrument system (columns 6 and 7). Move outward towards columns 1 and 12 as you add more tubes.

For a 7500 Fast Instrument

- Place empty tube strips in columns 1 and 12 to prevent crushing of tubes containing samples.
- Place tube strips with samples in the PPH vertically, starting in columns 6 and 7 and moving outward.
- A maximum of 6 tube strips can be used in the 7500 Fast system. Leave columns 2, 3, 10, and 11 empty.

4. Close the tray door. Apply pressure to the right side of the tray and at an angle.



Enable the Notification Settings (Optional)

Enable the notification settings so that the 7500 software alerts you by email when the 7500/7500 Fast instrument begins and completes a run, or if an error occurs during a run. Enabling the notifications settings is optional and does not affect the performance of the 7500/7500 Fast system or the duration of the run.

IMPORTANT! The notification settings feature is available only if the computer that you are using is running the 7500/7500 Fast instrument *and* is connected to an Ethernet network.

About the Example Experiment

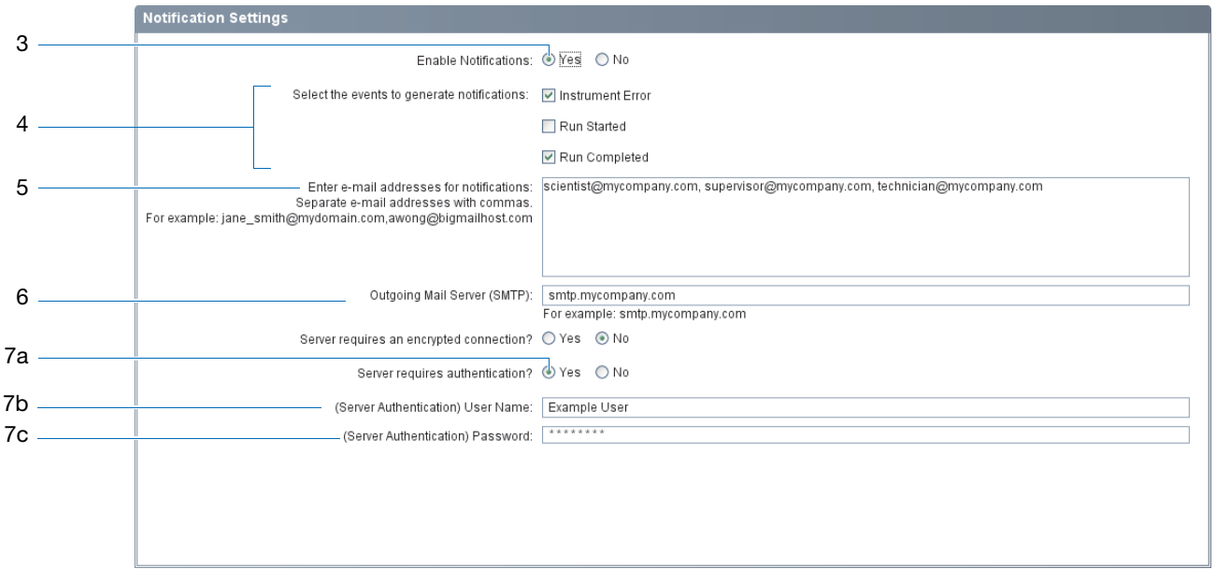
In the example experiment:

- The 7500 software is set up to send notifications to three users (scientist, supervisor, and technician at mycompany.com) when the 7500/7500 Fast system ends the run *and* if it encounters any errors during operation.
- The example SMTP server (smtp.mycompany.com) is set up for secure sockets layer (SSL) encryption and requires authentication for use.

Notes

Set Up the Notification Settings

1. In the 7500 software, click **Run** in the navigation pane.
2. Click **Notification Settings**.
3. Select **Yes** for Enable Notifications.
4. Select the events that will generate notifications:
   a. Select **Instrument Error**.
   b. Select **Run Completed**.
5. In the Enter e-mail addresses for notifications field, enter: **scientist@mycompany.com, supervisor@mycompany.com, technician@mycompany.com**.
6. In the Outgoing Mail Server (SMTP) field, enter **smtp.mycompany.com**.
7. Set the authentication settings:
   a. Select **Yes** for Server requires authentication.
   b. In the User Name field, enter **Example User**.
   c. In the Password field, enter **password**.



Notes

Run Guidelines

When you set up the 7500/7500 Fast system for automatic notification:

- Your system must be set up for network use. Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*.
- Select the events for which you want to receive e-mail notifications. Select:
  - **Instrument Error** – To notify recipients by email of all errors encountered by the instrument during each run.
  - **Run Started** – To notify recipients by email when the instrument starts a run.
  - **Run Completed** – To notify recipients by email when the instrument completes a run.
- Obtain e-mail addresses of those whom you want to be notified.

  IMPORTANT! Separate addresses with a comma (,).

- Contact your systems administrator or information technology department if you need:
  - The e-mail addresses of users who will receive notifications
  - A network address for a simple mail transfer protocol (SMTP) server on the local area network (LAN)
  - A user name and password for the server, if required for access
  - The Secure Sockets Layer (SSL) setting of the server (on or off)

Start the Run

IMPORTANT! While the 7500/7500 Fast instrument is performing a run, do not create experiments, perform maintenance, or allow the computer to run antivirus software or to enter hibernation mode. Performing such activities while the instrument is running an experiment will cause gaps in data collection.

To start your 7500/7500 Fast instrument:

1. In the 7500 software, click **Run** in the navigation pane.
2. Click **START RUN** .

Notes

Monitor the Run

You can view the progress of the run in real time as described below. During the run, periodically view all three available plots from the 7500 software for potential problems.

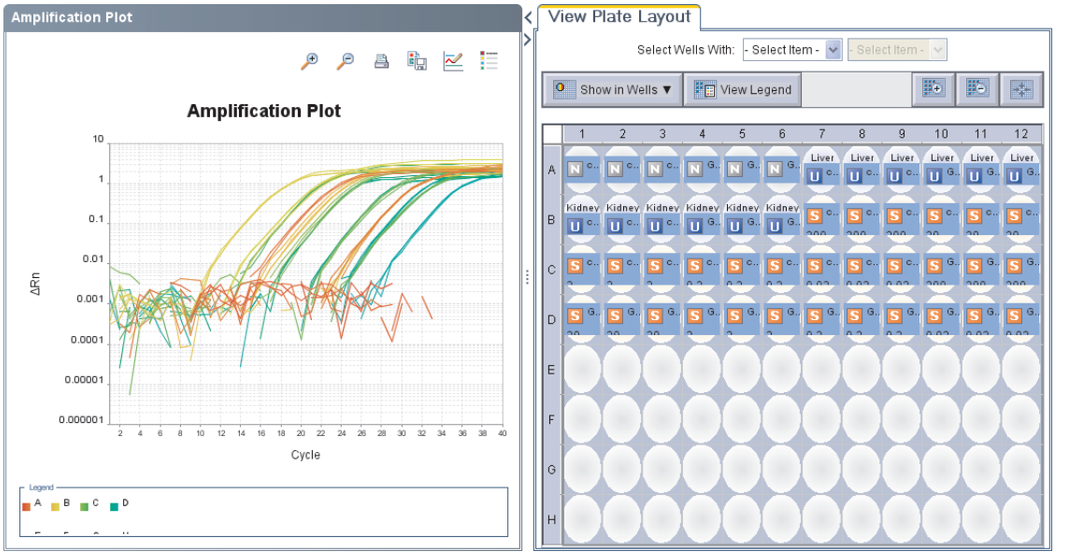
To...	Action
Stop the run	1. In the 7500 software, click **STOP RUN**. 2. In the Stop Run dialog box, click one of the following: – **Stop Immediately** to stop the run immediately. – **Stop after Current Cycle/Hold** to stop the run after the current cycle or hold. – **Cancel** to continue the run.
View amplification data in real time	Select **Amplification Plot**. See “About the Amplification Plot Screen” on page 72.
View progress of the run in the Run Method screen	Select **Run Method**. See “About the Run Method Screen” on page 73.
Enable/disable the Notification Settings	Select or deselect **Enable Notifications**. See “Enable the Notification Settings (Optional)” on page 69.

About the Amplification Plot Screen

This screen displays sample amplification as your instrument collects fluorescence data during a run. If a method is set up to collect real-time data, the Amplification Plot screen displays the data for the wells selected in the View Plate Layout tab. The plot displays normalized dye fluorescence (ΔRn) as a function of cycle number. The figure below shows the Amplification Plot screen as it appears during the example experiment.

To view data in the Amplification Plot screen, select the wells that you want to view in the View Plate Layout tab.

Notes



The Amplification Plot screen helps you identify and examine abnormal amplification. Abnormal amplification can include:

- Increased fluorescence in negative control wells.
- Absence of detectable fluorescence at an expected cycle (determined from previous similar experiments that were run using the same reagents under the same conditions).

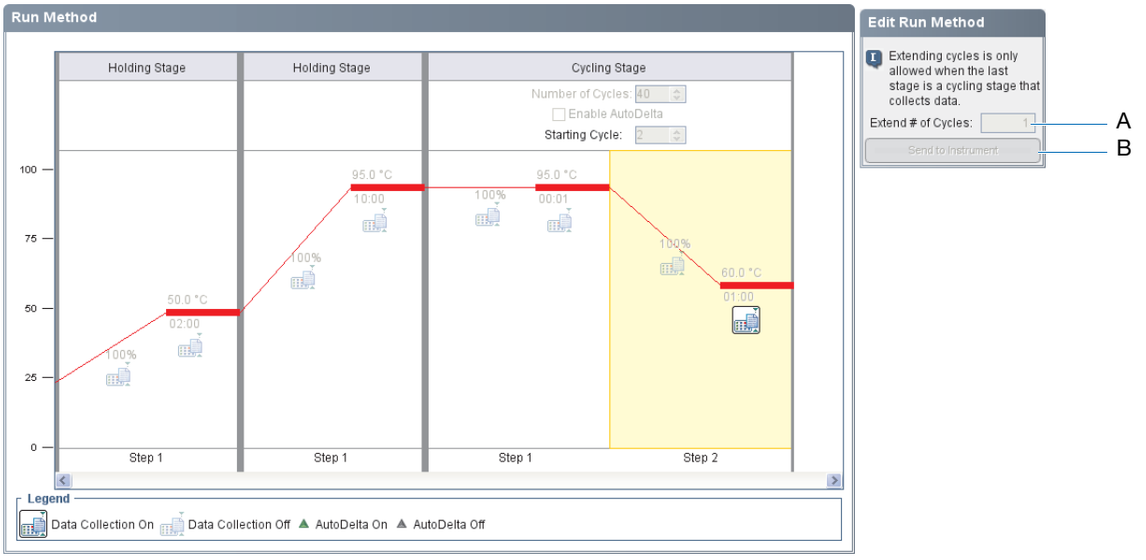
If you notice abnormal amplification or a complete absence of florescence, troubleshoot the error as explained in the 7500 Software Help (click ? or press **F1**).

About the Run Method Screen

This screen displays the method for the run in progress. The software updates the Run Status field throughout the run. The figure below shows the Run Method screen as it appears in the example experiment.

	To…	Action
A	Change the number of cycles	In the **Number of Cycles** field, enter a number.
B	Apply your changes	Click **Save Run Method**.

Notes



If an alert is displayed, click the error for more information, then troubleshoot the problem as explained in the 7500 Software Help (click or press **F1**).

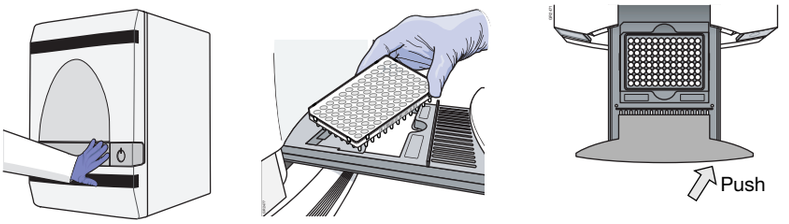
Unload the Instrument

When your 7500/7500 Fast instrument displays the Run Complete, unload the reaction plate from the instrument.

Unload the Reaction Plate

WARNING **PHYSICAL INJURY HAZARD.** During operation, the sample block can be heated to 100 °C. Before performing the following procedure, be sure to wait until the sample block reaches room temperature.

1. Push the tray door to open it.
2. Remove the calibration plate.
3. Push the tray door to close it.



Notes

5 Analyze the Relative Standard Curve Experiment

This chapter covers:

- Chapter Overview 76

Section 5.1 Review Results 77

- Analyze the Experiment 78
- View the Standard Curve 82
- View the Amplification Plot 85
- View the Gene Expression Plot and Well Table 93
- Publish the Data 97

Section 5.2 Troubleshoot (If Needed) 99

- View the Analysis Settings 100
- View the QC Summary 103
- Omit Wells from the Analysis 105
- View the Multicomponent Plot 107
- View the Raw Data Plot 109

Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

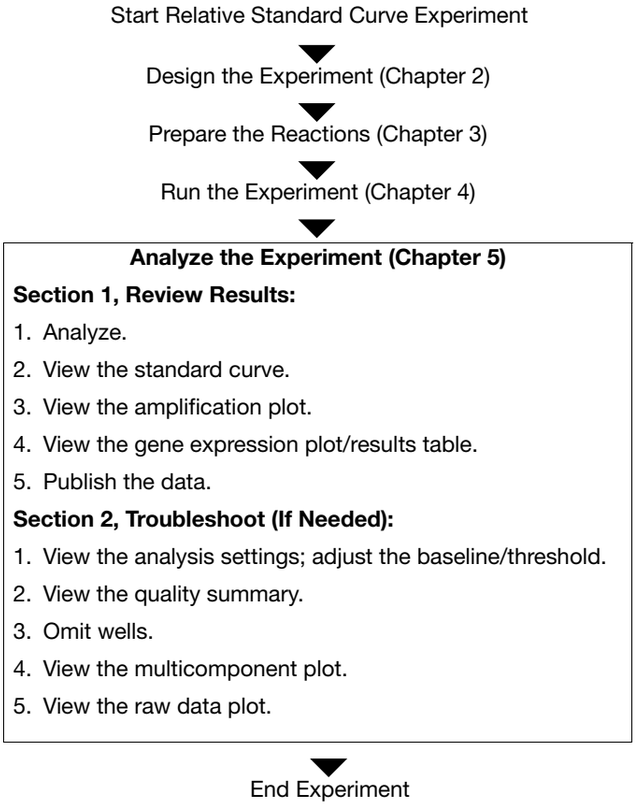
Notes

Chapter Overview

The 7500 software analyzes the data using the relative standard curve quantitation method. Section 1 of this chapter explains how to review the analyzed data using several of the analysis screens and how to publish the data. Section 2. If you obtain questionable results, Section 2 of this chapter explains how to perform some troubleshooting.

Note: The relative standard curve example experiment includes three flagged wells. The flags indicate common problems that you may encounter when performing your own experiments. Procedures are provided for viewing the flags and omitting one of the wells.

Example Experiment Workflow



Notes

Section 5.1 Review Results

This section covers:

- Analyze the Experiment 78
- View the Standard Curve 82
- View the Amplification Plot 85
- View the Gene Expression Plot and Well Table 93
- Publish the Data 97

Notes

Analyze the Experiment

The 7500 software analyzes the experiment and displays results in the analysis screens (for example, the Amplification Plot screen, QC Summary screen, and so on).

About the Example Experiment

For the relative standard curve example experiment, use the data file that is installed with the 7500 software. The data file was created with the same design parameters that are provided in Chapter 2, then run and analyzed on a 7500/7500 Fast instrument.

The data file for the example experiment is on your computer at:

<drive>:\Applied Biosystems*<software name>*\experiments\
Relative Standard Curve Example.eds

where:

- *<drive>* is the computer hard drive on which the 7500 software is installed.
- *<software name>* is the current version of the 7500 software.

Analyze the Example Experiment

1. Double-click (7500 software) or select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **7500 Software** ▸ ***<software name>***

   where *<software name>* is the current version of the 7500 software.

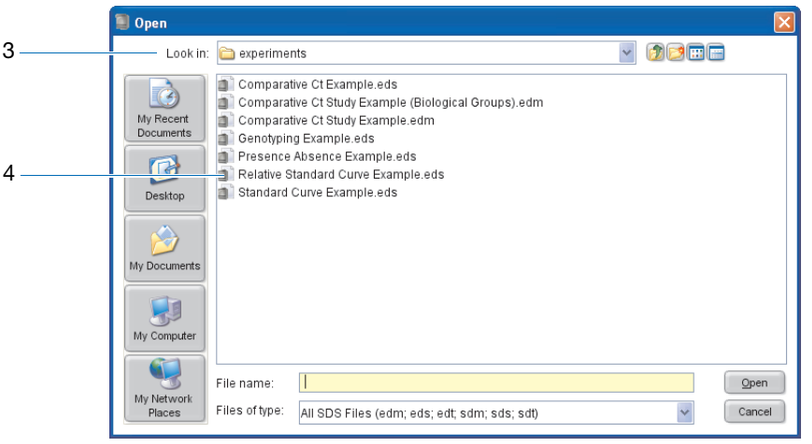
2. In the Home screen, click **Open**.

3. In the Open dialog box, navigate to the **experiments** folder at:

   <drive>:\Applied Biosystems*<software name>*\experiments\

4. Double-click **Relative Standard Curve Example.eds** to open the example experiment.

   Note: The examples folder contains several data files; be sure to select **Relative Standard Curve Example.eds**.



5. Click **Analyze**. The software analyzes the data using the default analysis settings.

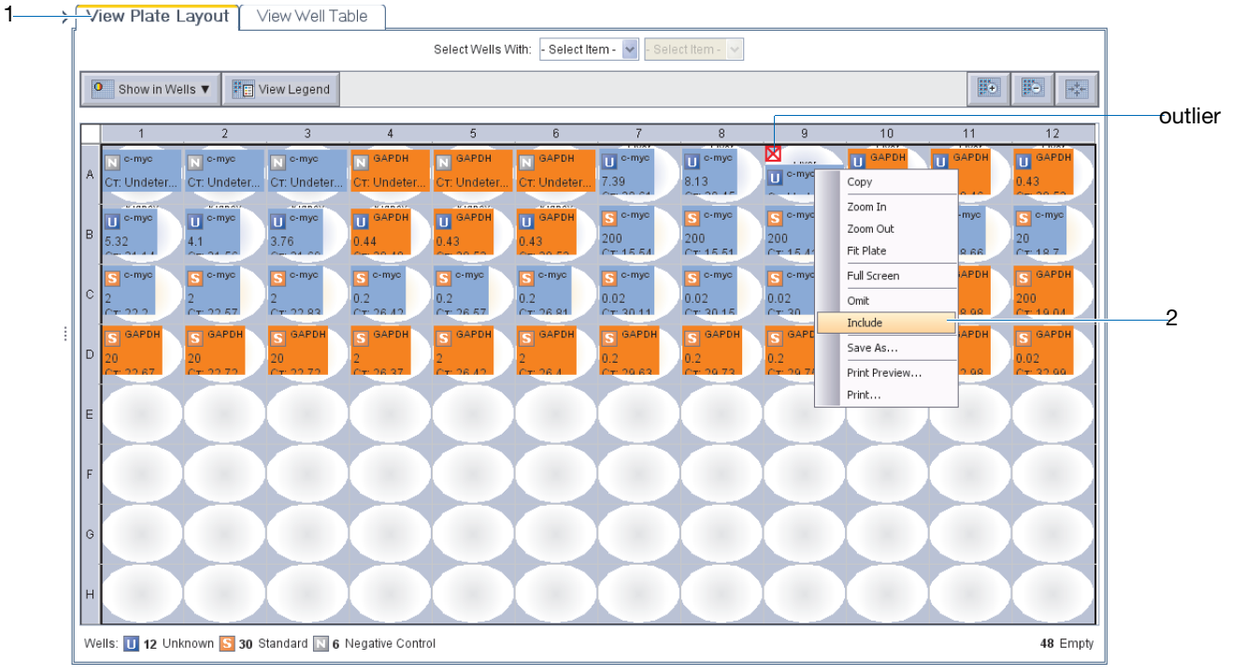
Notes

6. See "Navigation Tips" on page 80 for information on navigating within the analysis screens.

Preparing the Experiment for Review

The example file contains a single outlier (well A9) that has been omitted from the analysis for you. To use the experiment file with this chapter, you must include the well and reanalyze the experiment.

1. Select the **View Plate Layout** tab, then select the outlier well that is marked (well A9).
2. Right-click well A9, then select **Include**.
3. Click **Analyze**. The software analyzes the data using the default analysis settings.



Notes

Guidelines

When you analyze your own relative standard curve experiment:

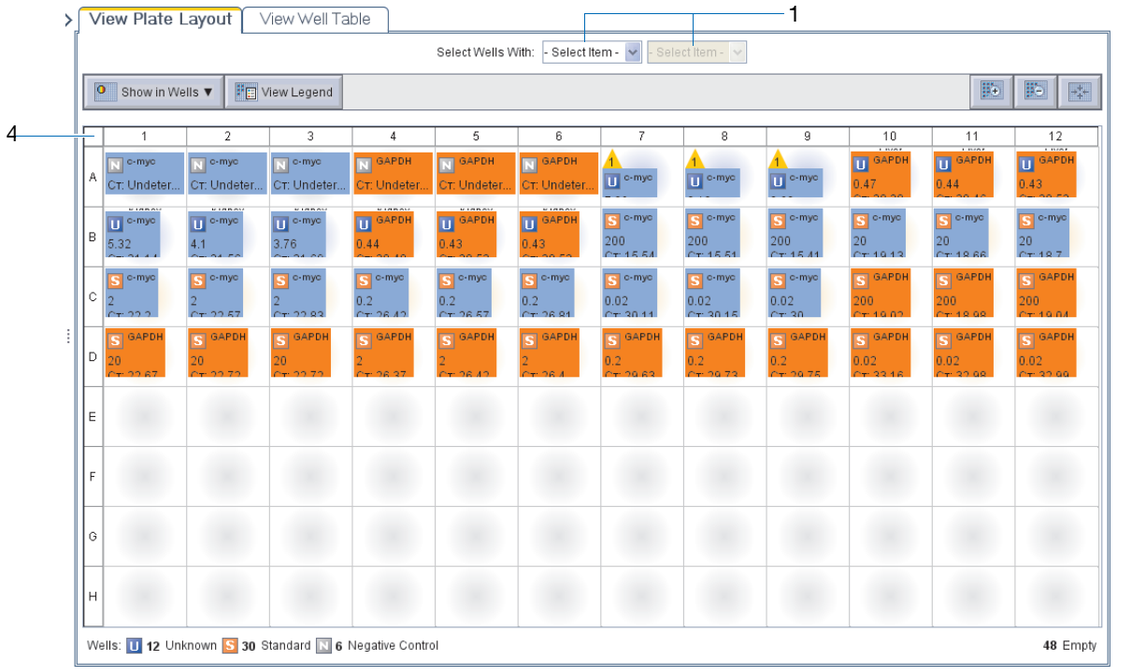
- Immediately after a run, the 7500 software automatically analyzes the data using the default analysis settings, then displays the Amplification Plot screen on your computer.
- To reanalyze the data, select all the wells in the plate layout, then click **Analyze**.

Navigation Tips

How to Select Wells

To display specific wells in the analysis screens, select the wells in the View Plate Layout tab:

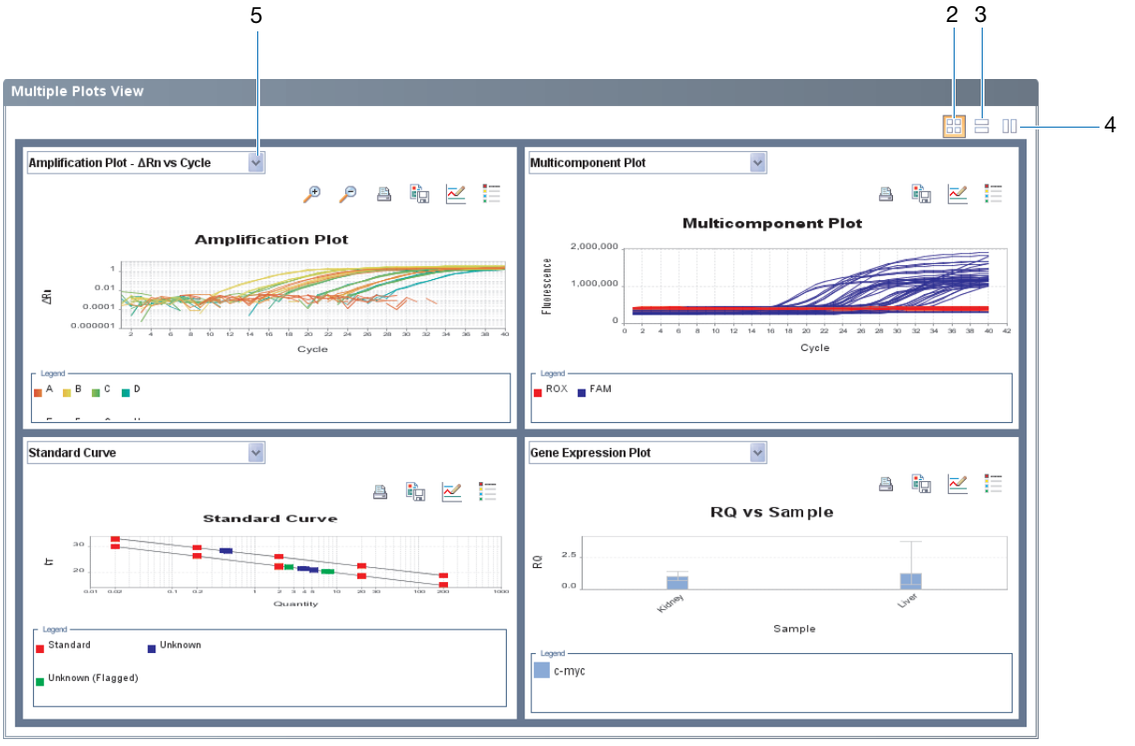
1. To select wells of a specific type in the Select Wells With drop-down list, select **Sample**, **Target**, or **Task**, then select the sample, target, or task name.
2. To select a single well, click the well in the plate layout.
3. To select multiple wells: Click-drag over the desired wells, or **CTRL+click** or **Shift+click** the desired wells in the plate layout.
4. To select all 96 wells, click the upper left corner of the plate layout.



Notes

How to Display Multiple Plots

1. In the navigation pane, select **Analysis** ▸ **Multiple Plots View**.
2. To display four plots, click (Show plots in a 2 × 2 matrix).
3. To display two plots in rows, click (Show plots in two rows).
4. To display two plots in columns, click (Show plots in two columns).
5. To display a specific plot, select the plot in the drop-down list above each plot display.



Notes

View the Standard Curve

The Standard Curve screen displays the standard curve for samples designated as standards. The 7500 software calculates the quantity of an unknown target from the standard curve.

About the Example Experiment

In the relative standard curve example experiment, you review the Standard Curve screen for the following values:

- Slope/amplification efficiency
- R^2 value (correlation coefficient)
- C_T values

View the Standard Curve

1. In the navigation pane, select **Analysis** ▸ **Standard Curve**.

   Note: If no data are displayed, click **Analyze**.

2. Display all 96 wells in the Standard Curve screen by clicking the upper left corner of the plate layout in the View Plate Layout tab.
3. In the Plot Settings tab, select **All** in the Target drop-down list (default).
4. In the Plot Settings tab, select **Default** in the Plot Color drop-down list.
5. Click (Show a legend for the plot).

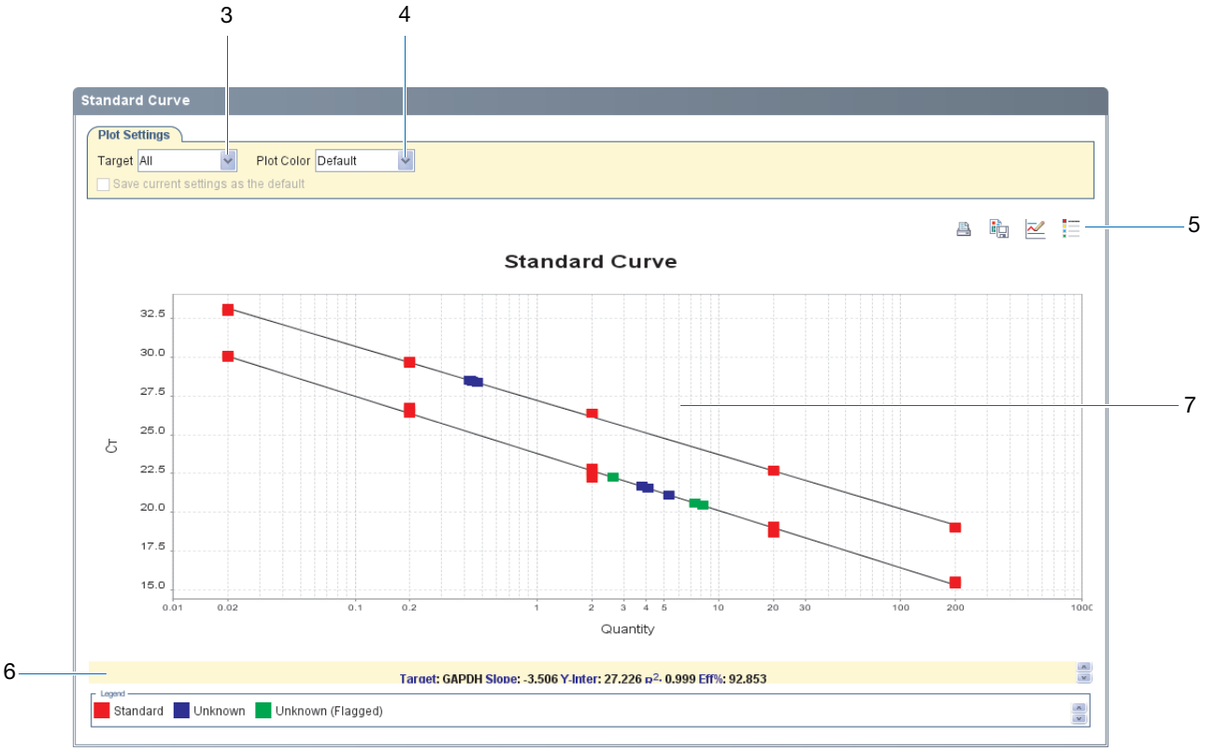
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

6. View the values displayed below the standard curve. In the example experiment, the values for each target are within the acceptable ranges:

Target	Slope	Y-Inter	R^2 Value	Amplification Efficiency (Eff%)
GAPDH	-3.506	27.226	0.999	92.853
c-myc	-3.696	23.82	0.998	86.438

Notes

7. Check that all samples are within the standard curve. In the example experiment, all samples (blue dots) are within the standard curve (red dots).

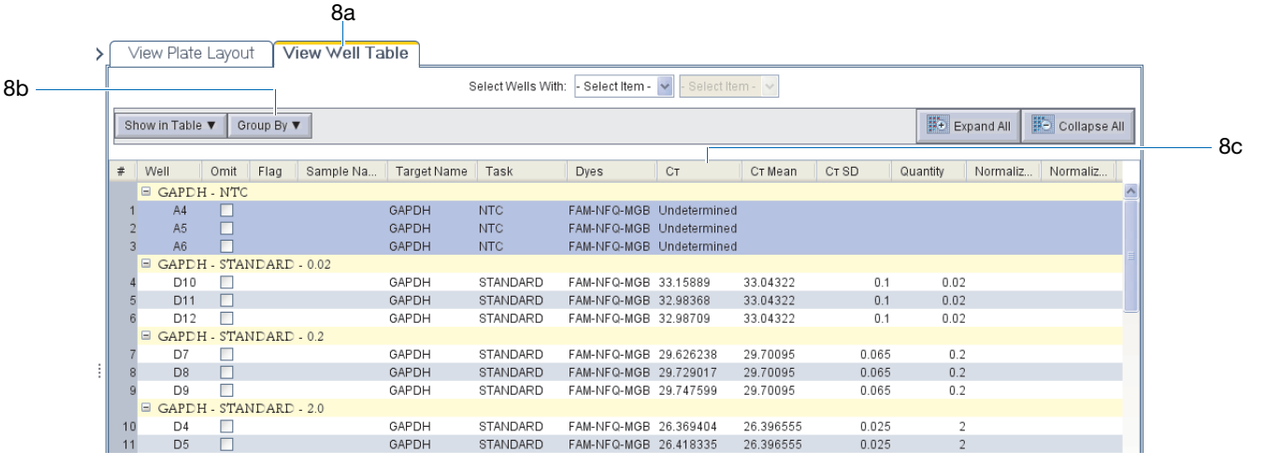


8. Check the C_T values:

   a. Select the **View Well Table** tab.

   b. In the Group By drop-down list, select **Replicate**.

   c. Look at the values in the C_T column. In the example experiment, the C_T values are within the expected range (>8 and <35).



Notes

Analysis Guidelines

When you analyze your own relative standard curve experiment, look for:

- **Slope/amplification efficiency values** – Calculated using the slope of the regression line in the standard curve. A slope close to -3.3 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency are the:
  - Range of standard quantities – For more accurate and precise efficiency measurements, use a broad range (10^5 to 10^6 fold) of standard quantities.
  - Number of standard replicates – For more accurate efficiency measurements, include replicates to decrease the effects of pipetting inaccuracies.
  - PCR inhibitors – PCR inhibitors in the reaction can reduce amplification efficiency.
- **R^2 values (correlation coefficient)** – A measure of the closeness of fit between the regression line and the individual C_T data points of the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points. An R^2 value >0.99 is desirable.
- **C_T values** – The PCR cycle number at which the fluorescence level equals the threshold. A C_T value >8 and <35 is desirable. A C_T value <8 indicates that there is too much template in the reaction. A C_T value >35 indicates a low amount of target in the reaction; for C_T values >35, expect a higher standard deviation.

If your experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Omit Wells from the Analysis" on page 105).

  or
- Rerun the experiment.

For More Information

For more information on:

- The Standard Curve screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Amplification efficiency – Refer to the *Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.

Notes

View the Amplification Plot

The Amplification Plot screen displays amplification of all samples in the selected wells. Three plots are available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – Rn is the fluorescence from the reporter dye normalized to the fluorescence from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

Each plot can be displayed as a linear or log10 graph.

About the Example Experiment

In the relative standard curve example experiment, you review each target in the Amplification Plot for:

- Correct baseline and threshold values
- Outliers

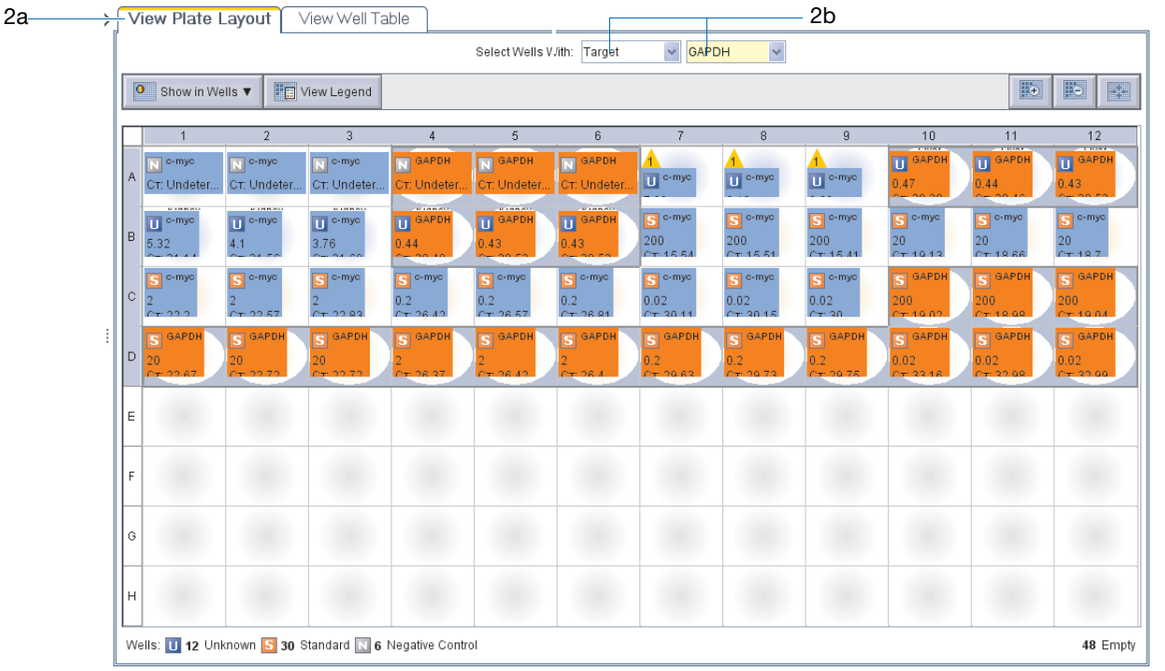
Notes

View the Plot

1. In the navigation pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the GAPDH wells in the Amplification Plot screen:
   a. Select the **View Plate Layout** tab.
   b. In the Select Wells With drop-down lists, select **Target**, then **GAPDH**.

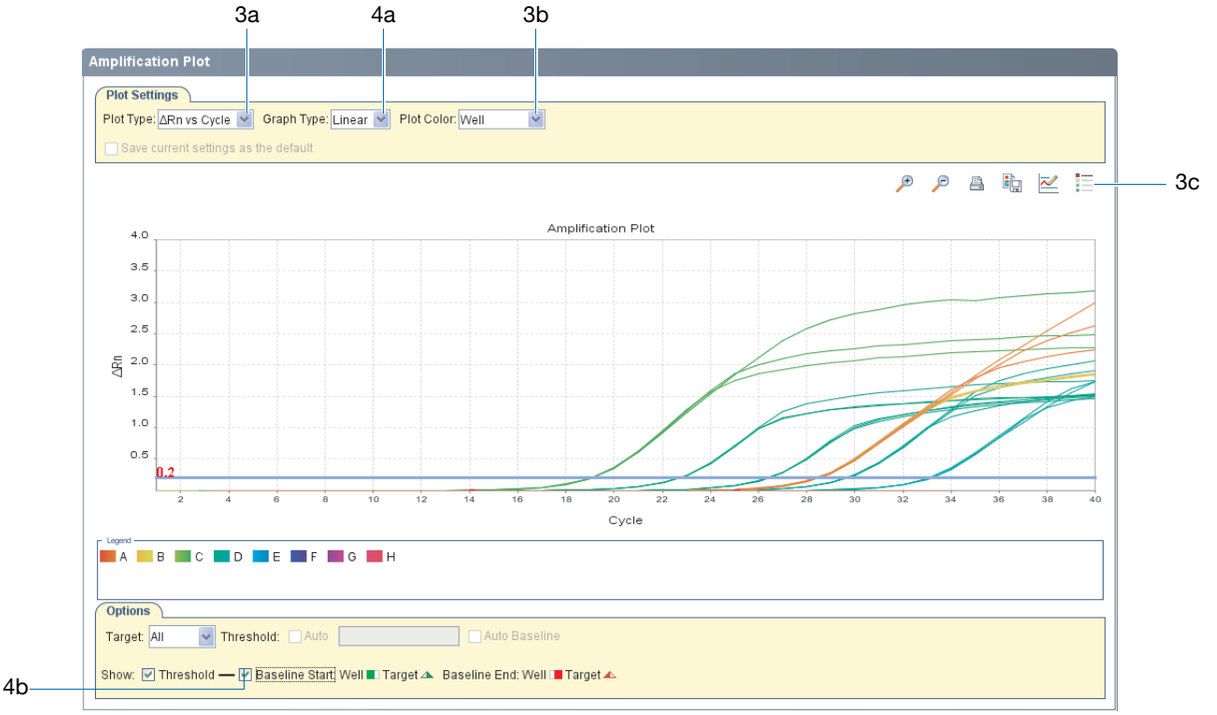


3. In the Plot Settings tab of the Amplification Plot:
   a. In the Plot Type drop-down list, select **ΔRn vs Cycle** (default).
   b. In the Plot Color drop-down list, select **Well**.
   c. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

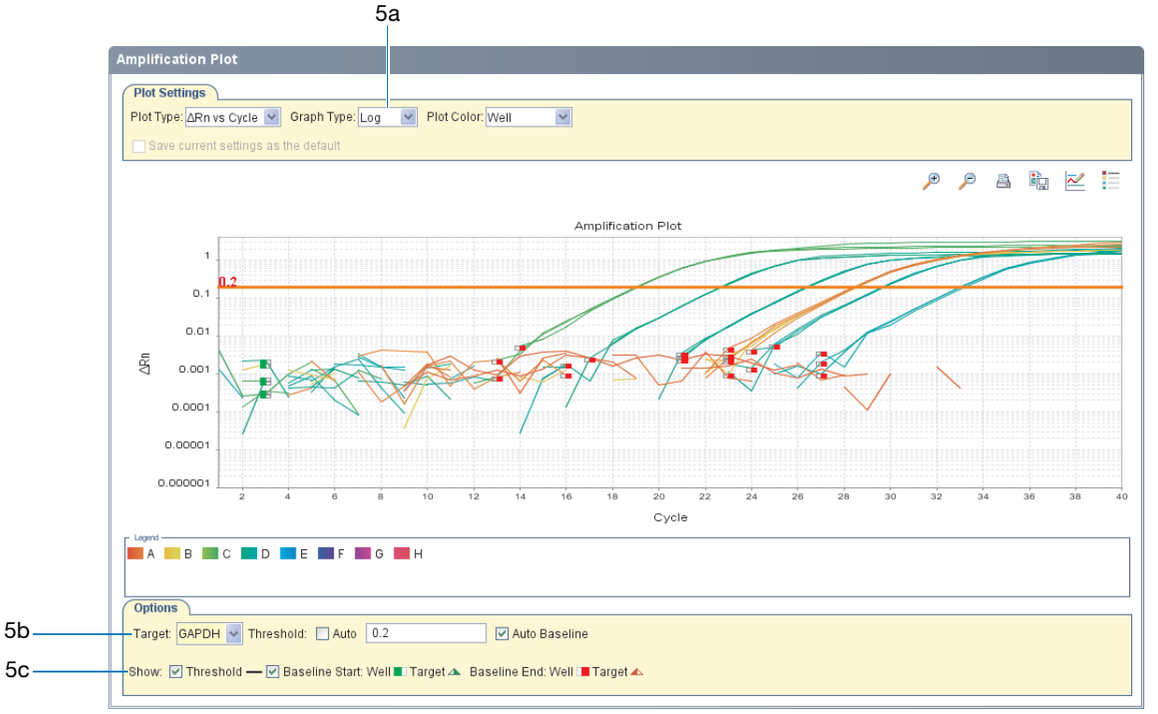
Notes

4. View the baseline values (Plot Settings):
   a. In the Graph Type drop-down list, select **Linear**.
   b. Select the **Baseline** check box to show the start cycle and end cycle.
   c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant fluorescence is detected. In the example experiment, the baseline is set correctly.



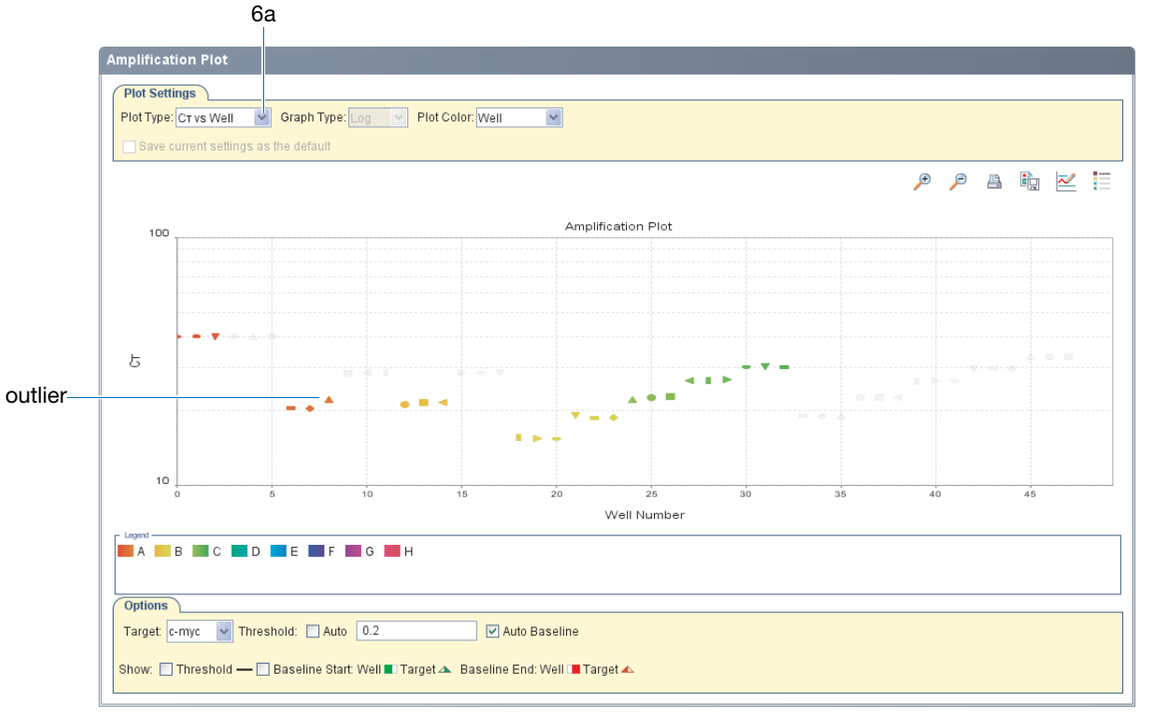
Notes

5. View the threshold values:
   a. In Plot Settings tab, select **Log** in the Graph Type drop-down list.
   b. Select **GAPDH** in the Target drop-down list.
   c. Select the **Threshold** check box to show the threshold (default).
   d. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.



Notes

6. Locate any outliers:
   a. In the Plot Type drop-down list, select **C_T vs Well**.
   b. Look for outliers in the amplification plot. In the example experiment, there are no outliers for GAPDH.
7. Repeat steps 2 through 6 for the c-myc target. In the example experiment, there is one outlier for c-myc (well A9). You omit this well in the troubleshooting section ("Omit Wells from the Analysis" on page 105).



Notes

Analysis Guidelines

When you analyze your own relative standard curve experiment, look for:

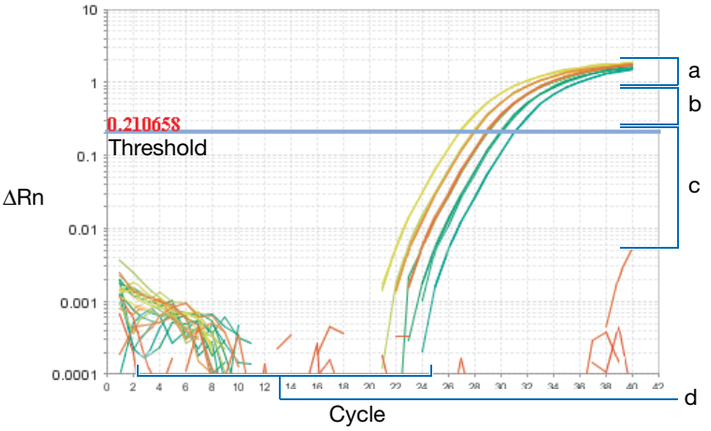
- Outliers
- A typical amplification plot – The 7500 software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:

  a. Plateau phase

  b. Linear phase

  c. Exponential (geometric phase)

  d. Baseline



IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the 7500 software. Therefore, Applied Biosystems recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis is complete.

- Correct baseline and threshold values – See “Threshold Examples” on page 91 and “Baseline Examples” on page 92.

Notes

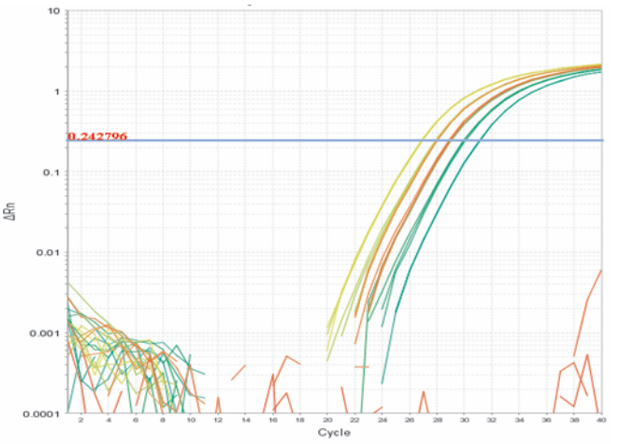
Threshold Examples

Threshold Set Correctly

The threshold is set in the exponential phase of the amplification curve.

Threshold settings above or below the optimum increase the standard deviation of the replicate groups.

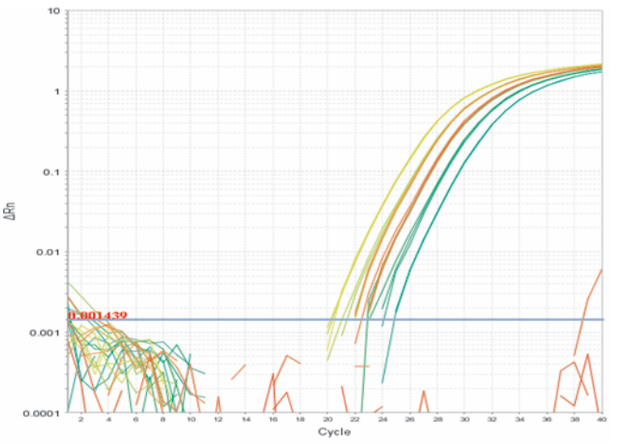
Task	Dyes	Ct	Ct Mean	Ct SD	Quantity	Quantity ...	Quantity ...	Comm
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
UNKNOWN	FAM-NFQ-...	28.96287	28.923796	0.074	2,484.31	2,551.476	126.2	
UNKNOWN	FAM-NFQ-...	28.838797	28.923796	0.074	2,697.054	2,551.476	126.2	
UNKNOWN	FAM-NFQ-...	28.96972	28.923796	0.074	2,473.064	2,551.476	126.2	



Threshold Set Too Low

The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.

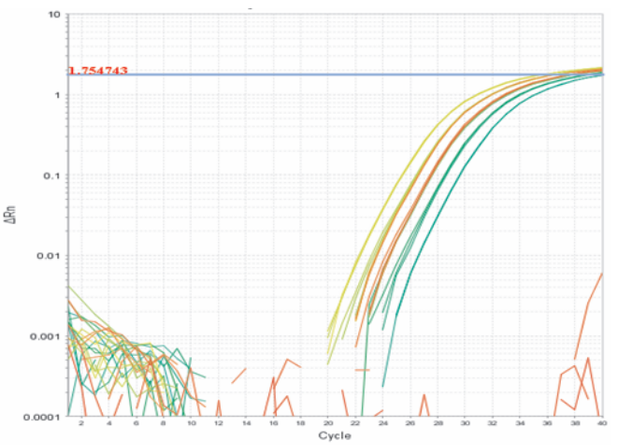
Task	Dyes	Ct	Ct Mean	Ct SD	Quantity	Quantity ...	Quantity ...
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	38.453182					
UNKNOWN	FAM-NFQ-...	22.85404	22.761744	0.252	2,314.852	2,472.463	400.435
UNKNOWN	FAM-NFQ-...	22.476973	22.761744	0.252	2,927.722	2,472.463	400.435
UNKNOWN	FAM-NFQ-...	22.954218	22.761744	0.252	2,174.816	2,472.463	400.435



Threshold Set Too High

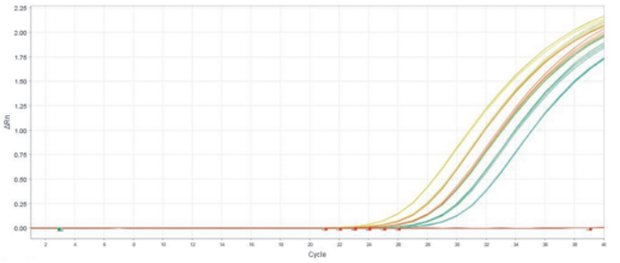
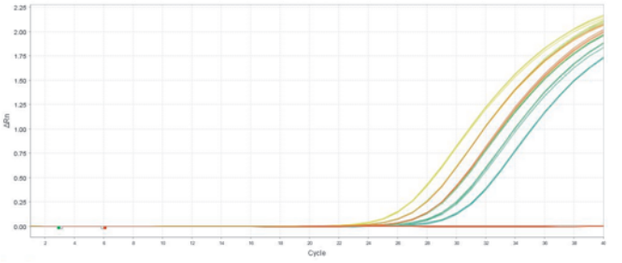
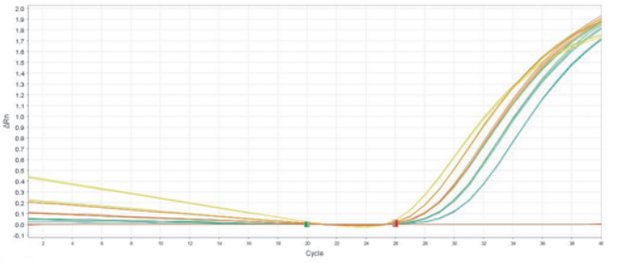
The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.

Task	Dyes	Ct	Ct Mean	Ct SD	Quantity	Quantity ...	Quantity ...	Comme
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
UNKNOWN	FAM-NFQ-...	37.681107	37.49507	0.202	2,571.177	2,888.429	353.775	
UNKNOWN	FAM-NFQ-...	37.27971	37.49507	0.202	3,269.923	2,888.429	353.775	
UNKNOWN	FAM-NFQ-...	37.524395	37.49507	0.202	2,824.187	2,888.429	353.775	



Notes

Baseline Examples

Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

If your experiment does not meet the guidelines above:

- Omit wells (see “Omit Wells from the Analysis” on page 105).

  or

- Manually adjust the baseline and/or threshold (see “View the Analysis Settings” on page 100).

For More Information

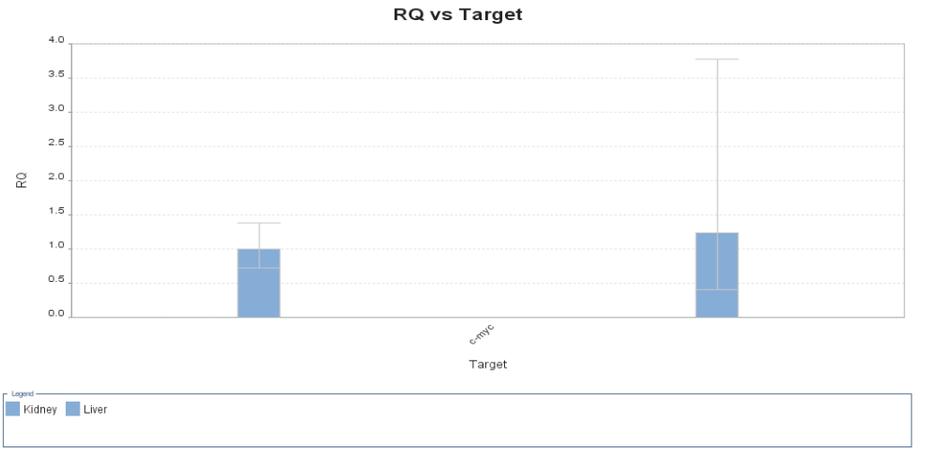
For more information on the Amplification Plot screen, open the 7500 Software Help by clicking or pressing **F1**.

Notes

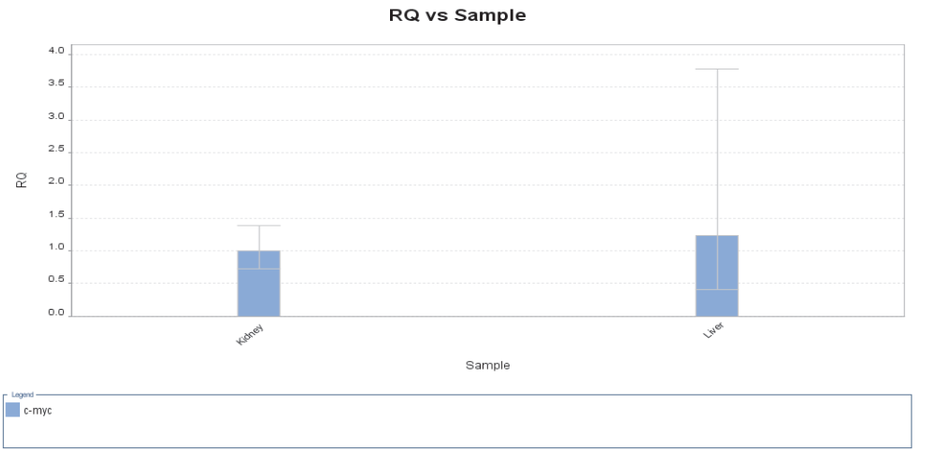
View the Gene Expression Plot and Well Table

The Gene Expression Plot screen displays the results of relative quantitation calculations in the gene expression profile. Two plots are available:

- **RQ vs Target** – Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. You can display the plot on a linear, log10, Ln, or log2 scale.



- **RQ vs Sample** – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample. You can display the plot on a linear, log10, Ln, or log2 scale.



The Well Table displays data for each well in the reaction plate, including:

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

Notes

About the Example Experiment

In the relative standard curve example experiment, you review:

- Each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference sample.
- The Well Table to evaluate the precision of the replicate groups.

View the Gene Expression Plot and Tables

1. In the navigation pane, select **Analysis** ▸ **Gene Expression**.

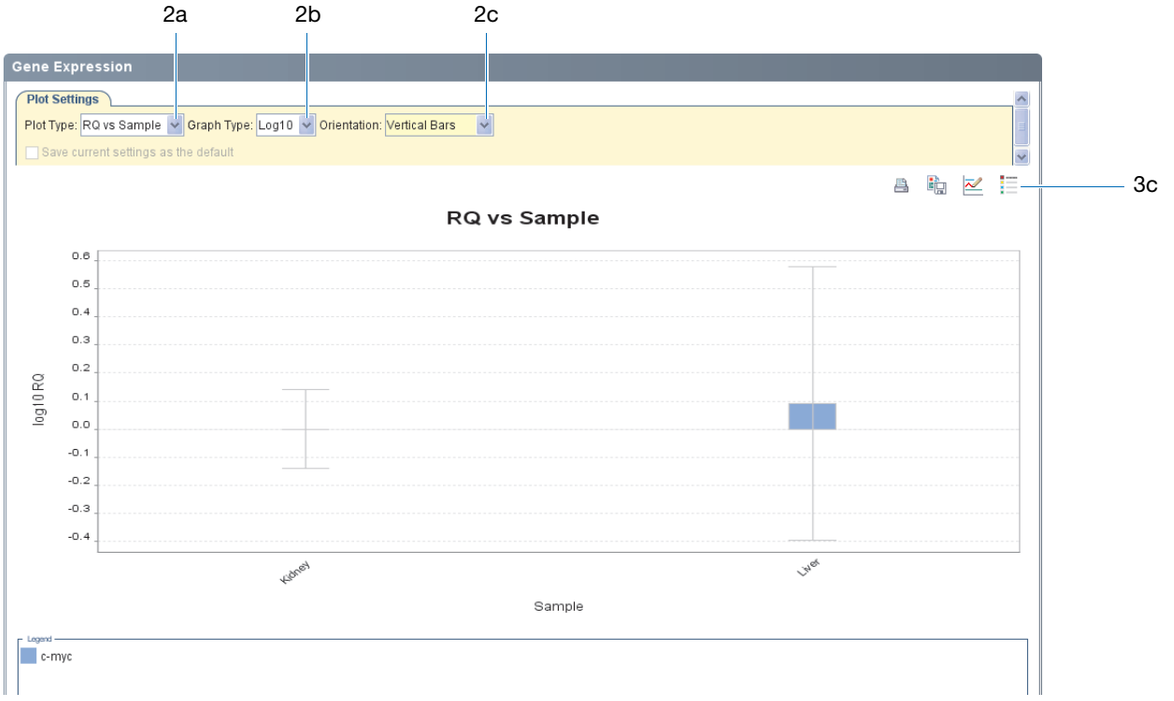
   Note: If no data are displayed, click **Analyze**.

2. In the Gene Expression Plot screen:
   a. In the Plot Type drop-down list, select **RQ vs Sample**.
   b. In the Graph Type drop-down list, select **Log10**.
   c. In the Orientation drop-down list, select **Vertical Bars**.

3. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

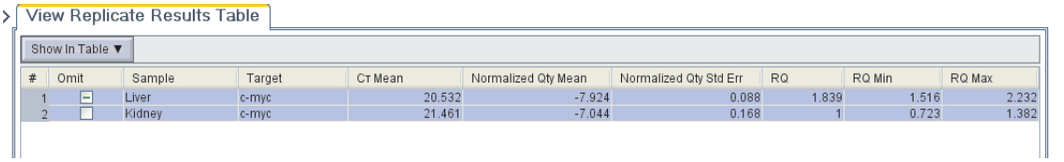
   In the example experiment, the expression level of c-myc in the liver sample is displayed relative to its expression level in the reference sample (kidney). Because the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Notes

4. Click < at the top left of the View Replicate Results Table tab.

   The figure below shows the replicate results table.



Column	Description
Omit	For all technical replicates that evaluate the associated sample/target combination: • A check mark (✓) indicates that all replicates have been removed from the analysis. • A hyphen (–) indicates that one or more replicates have been removed from the analysis.
Sample	The sample associated with the data displayed in the row.
Target	The target associated with the data displayed in the row.
C_T Mean	The arithmetic average of the technical replicate C_T values.
Normalized Qty Mean	The point estimate of the normalized quantities computed at the replicate level as the geometric mean.
Normalized Qty Std Err	The confidence interval based variability associated with the normalized quantities computed at the replicate level as the geometric standard error of the mean.
RQ	The relative level of gene expression for the replicate group computed using normalized quantities.
RQ Min	The minimum relative level of gene expression in the test samples computed using normalized quantities and the confidence level setting. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	The maximum relative level of gene expression in the test samples computed using normalized quantities and the confidence level setting. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

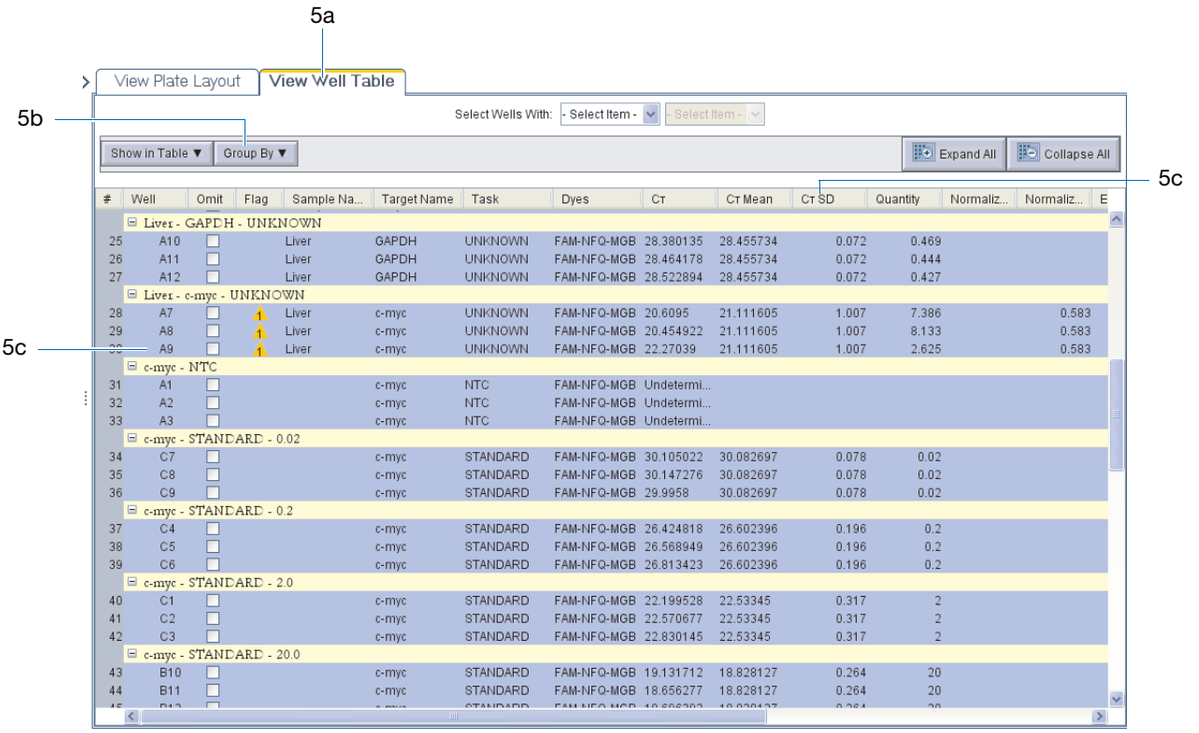
Notes

5. View the Well Table:

   a. In the navigation pane, select **Analysis** ▸ **Amplification Plot**, then select the **View Well Table** tab.

   b. In the Group By drop-down list, select **Replicate**.

   c. Look at the C_T SD column to evaluate the precision of the replicate groups. In the example experiment, there is one outlier (well A9). You will omit this well in the troubleshooting section ("Omit Wells from the Analysis" on page 105).



Note: To show/hide columns in the Well Table, select/deselect the column name in the Show in Table drop-down list.

Analysis Guidelines

When you analyze your own relative standard curve experiment, look for:

- Differences in gene expression (as a fold change) relative to the reference sample.
- Standard deviation in the replicate groups (C_T SD values). If needed, omit outliers ("Omit Wells from the Analysis" on page 105).

For More Information

For more information on the Gene Expression Plot screen or Well Table, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Publish the Data

You can publish the experiment data in several ways:

- Save the plot as an image file
- Print the plot
- Print the reaction plate layout
- Create slides
- Print a report
- Export data to applications such as Microsoft® Excel® and Microsoft® PowerPoint®.

For information on performing these tasks, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Notes

Section 5.2 Troubleshoot (If Needed)

This section covers:

- View the Analysis Settings 100
- View the QC Summary 103
- Omit Wells from the Analysis 105
- View the Multicomponent Plot 107
- View the Raw Data Plot 109

Notes

View the Analysis Settings

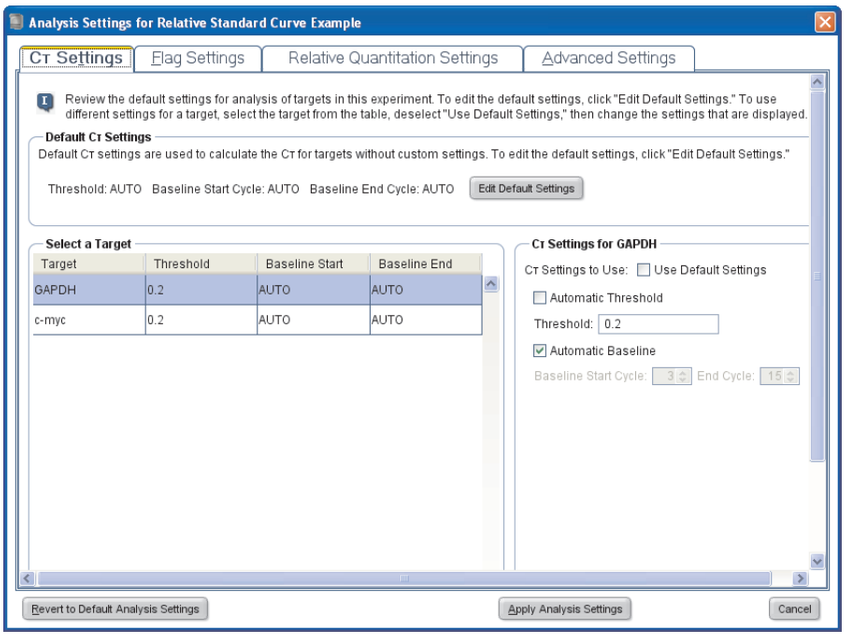
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and advanced options. If the default analysis settings in the 7500 software are not suitable for your experiment, you can change the settings, then reanalyze your experiment.

About the Example Experiment

In the standard curve example experiment, the default analysis settings are used without changes.

View the Settings

1. In the navigation pane, select **Analysis**.
2. Click **Analysis Settings** to open the Analysis Settings dialog box.
3. In the example experiment, view the default analysis settings, which are specified in each of the following tabs:
   - C_T Settings
   - Flag Settings
   - Advanced Settings



4. Click **Apply Analysis Settings**.

Notes

Analysis Guidelines

Unless you have already determined the optimal settings for your experiment, use the default analysis settings in the 7500 software. If the default settings are not suitable for your experiment, you can change the:

- **C_T Settings** – Use this tab to manually set the threshold and baseline. When manually setting the threshold and baseline, Applied Biosystems recommends the following:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescence is detected.

- **Relative Quantitation Settings** – Use this tab to:
  - Select the type of analysis to perform.
  - Select the reference sample(s).
  - Select the endogenous control. You can set more than one endogenous control for relative standard curve experiments. See "Set Multiple Endogenous Controls" on page 101).
  - Reject outliers.
  - Select the algorithm to use to determine RQ Min/Max values (confidence level or standard deviations).
- **Flag Settings** – Use this tab to:
  - Adjust the sensitivity so that more wells or fewer wells are flagged.
  - Change the flags that are applied by the 7500 software.
- **Advanced Settings** – Use this tab to change baseline settings well by well.

Set Multiple Endogenous Controls

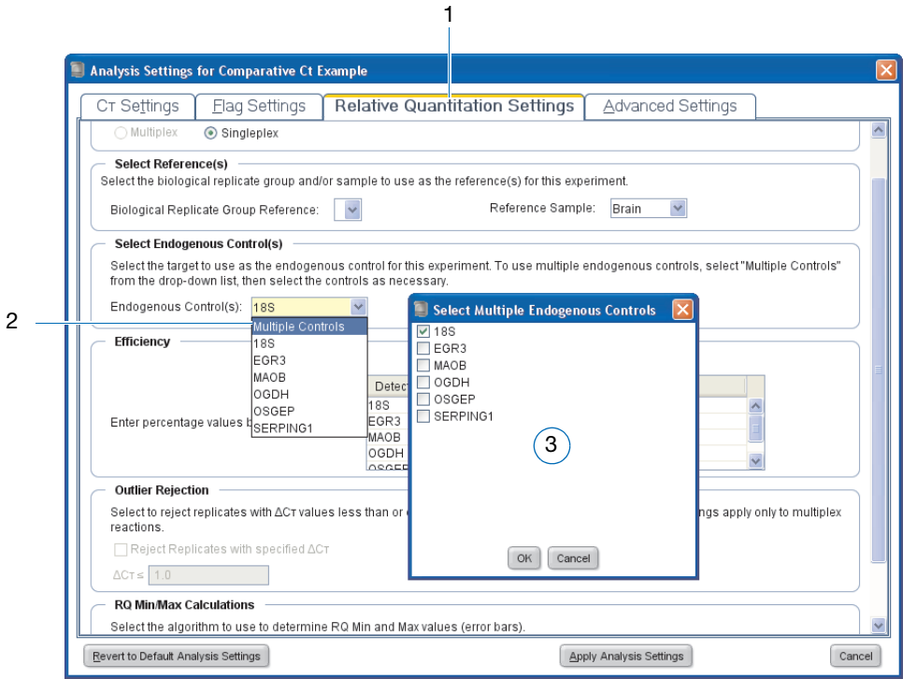
You can select multiple endogenous controls for a relative standard curve experiment.

To select multiple controls:

1. In the Analysis Settings for Relative Standard Curve Example dialog box, select the **Relative Quantitation Settings** tab.
2. In the Select Endogenous Control(s) group, click the **Endogenous Control(s)** drop-down list, then select **Multiple Controls**.

Notes

3. In the Select Multiple Endogenous Controls dialog box, select the targets that you want to use as endogenous controls, then click **OK**.



For More Information

For more information on the analysis settings, open the 7500 Software Help by pressing **F1** when the Analysis Settings dialog box is open.

Notes

View the QC Summary

The QC Summary screen displays a list of the 7500 software flags, and includes the flag frequency and location for the open experiment.

About the Example Experiment

In the standard curve example experiment, you review the QC Summary screen for any flags generated by the experiment data. In the example experiment, the HIGHSD flags has been generated for wells: A7, A8, and A9.

View the QC Summary

1. In the navigation pane, select **Analysis** ▸ **QC Summary**.

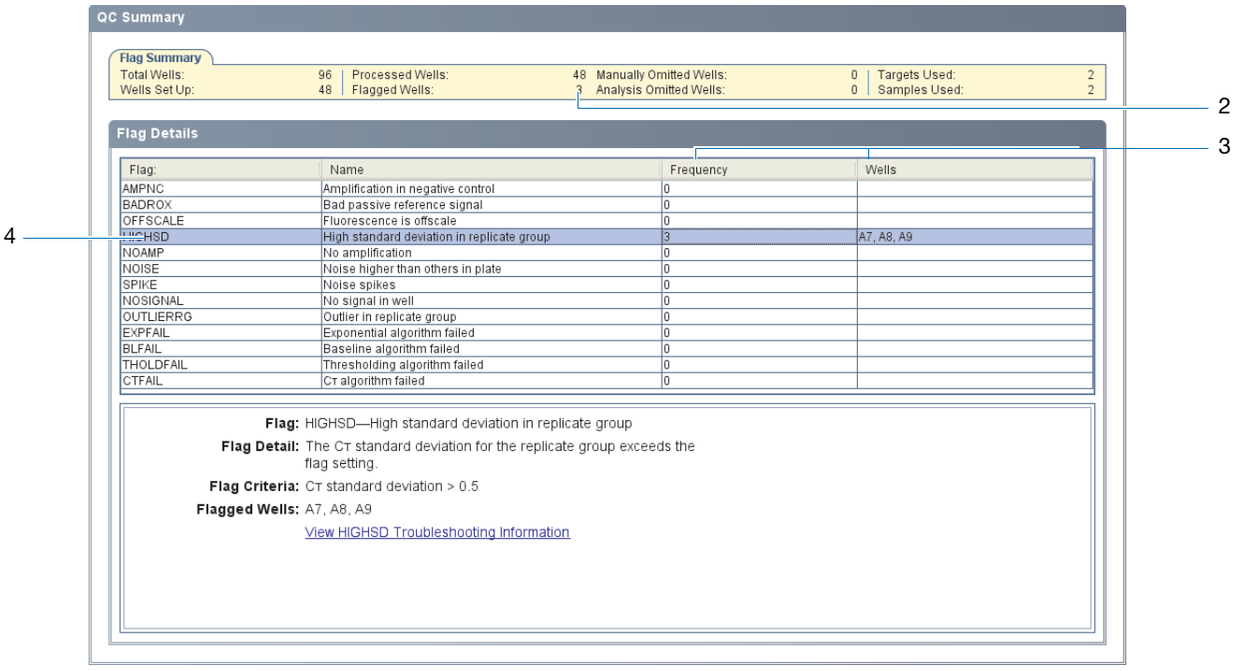
   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary. In the example experiment, three wells are flagged.

3. In the Flag Details table, look in the Frequency and Wells columns to determine which flags appear in the experiment. In the example experiment, the Frequency column displays 3 for the HIGHSD flag.

   Note: The Frequency column displays 0 to indicate that the flag does not appear in the experiment.

4. (Optional) Select the **HIGHSD** flag row to display details about the flag.



Notes

Possible Flags

For standard curve experiments, the flags listed below can be generated by the experiment data.

If a flag does not appear in the experiment, its frequency is 0. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

Flag	Description
AMPNC	Amplification in negative control
BADROX	Bad passive reference signal
BLFAIL	Baseline algorithm failed
CTFAIL	C_T algorithm failed
EXPFAIL	Exponential algorithm failed
HIGHSD	High standard deviation in replicate group
MTP	Multiple Tm peaks **Note:** This flag is displayed only if the experiment includes a melt curve.
NOAMP	No amplification
NOISE	Noise higher than others in plate
NOSIGNAL	No signal in well
OFFSCALE	Fluorescence is offscale
OUTLIERRG	Outlier in replicate group
SPIKE	Noise spikes
THOLDFAIL	Thresholding algorithm failed

Analysis Guidelines

When you analyze your own standard curve experiment:

- In the Flag Details table, click each flag that has a frequency >0 to display details about the flag. If needed, click the troubleshooting link to view information on correcting the flag.
- You can change the flag settings:
  - Adjust the sensitivity so that more wells or fewer wells are flagged.
  - Change the flags that are applied by the 7500 software.

For More Information

For more information on the QC Summary screen or on flag settings, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Omit Wells from the Analysis

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

About the Example Experiment

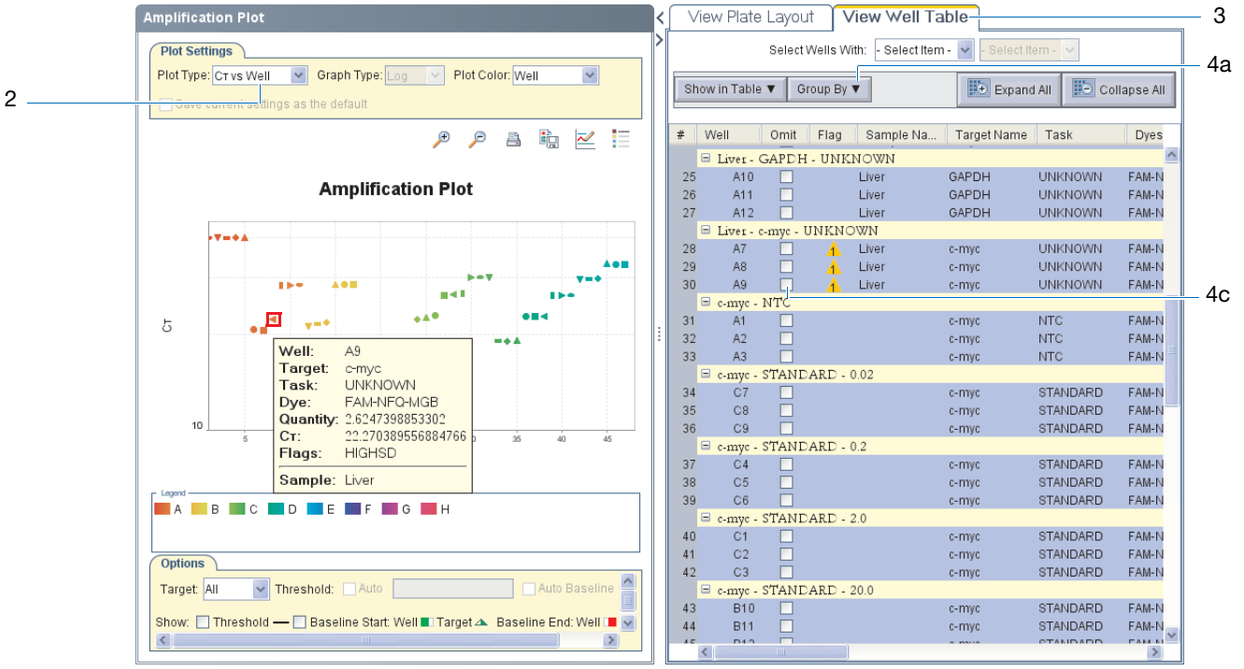
In the example experiment, you use the Well Table to remove well A9 from the analysis. The C_T of well A9 deviates significantly from those of the related technical replicates, which generated the HIGHSD flag due to the degree of variation in their C_T values (see "View the QC Summary" on page 103).

Omit Wells

1. In the navigation pane, select **Analysis** ▸ **Amplification Plot**.

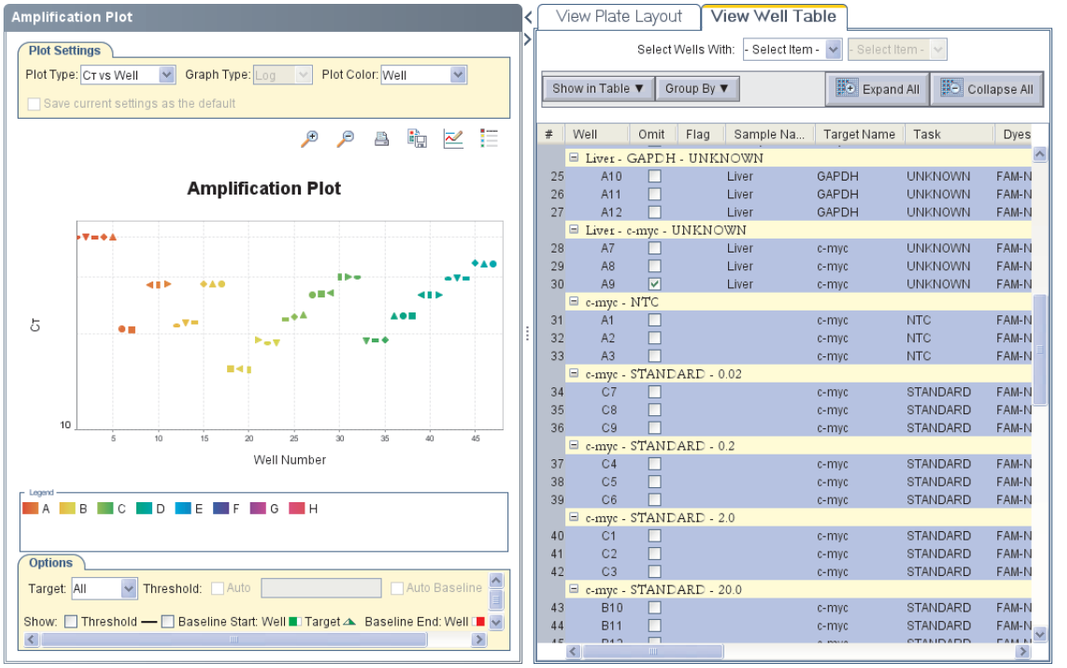
   Note: If no data are displayed, click **Analyze**.

2. In the Plot Type drop-down list, select **C_T vs Well**.
3. Select the **View Well Table** tab.
4. In the Well Table:
   a. In the Group By drop-down list, select **Replicate**.
   b. Look for any outliers in the replicate group (be sure they are flagged). In the example experiment, well A9 is the outlier.
   c. Select the **Omit** check box next to well A9.



Notes

5. Click **Analyze** to reanalyze the experiment data with well A9 removed from the analysis.



Analysis Guidelines

When you analyze your own relative standard curve experiment, carefully view the replicate groups for outliers. If needed, remove outliers manually using the Well Table. See “Omit Wells” on page 105 to remove the outliers in your experiment.

For More Information

For more information on omitting wells from the analysis, open the 7500 Software Help by clicking or pressing **F1**. Within the Help, search for the omit well topics:

1. Select the **Search** tab.
2. Enter **omit well**.
3. Click **List Topics**.
4. Double-click the topics you want to review.

Notes

View the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run.

About the Example Experiment

In the example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

View the Plot

1. In the navigation pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown and standard wells one at a time in the Multicomponent Plot screen:

   a. Select the **View Plate Layout** tab.

   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot screen.

   Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

3. In the Plot Color drop-down list, select **Dye**.

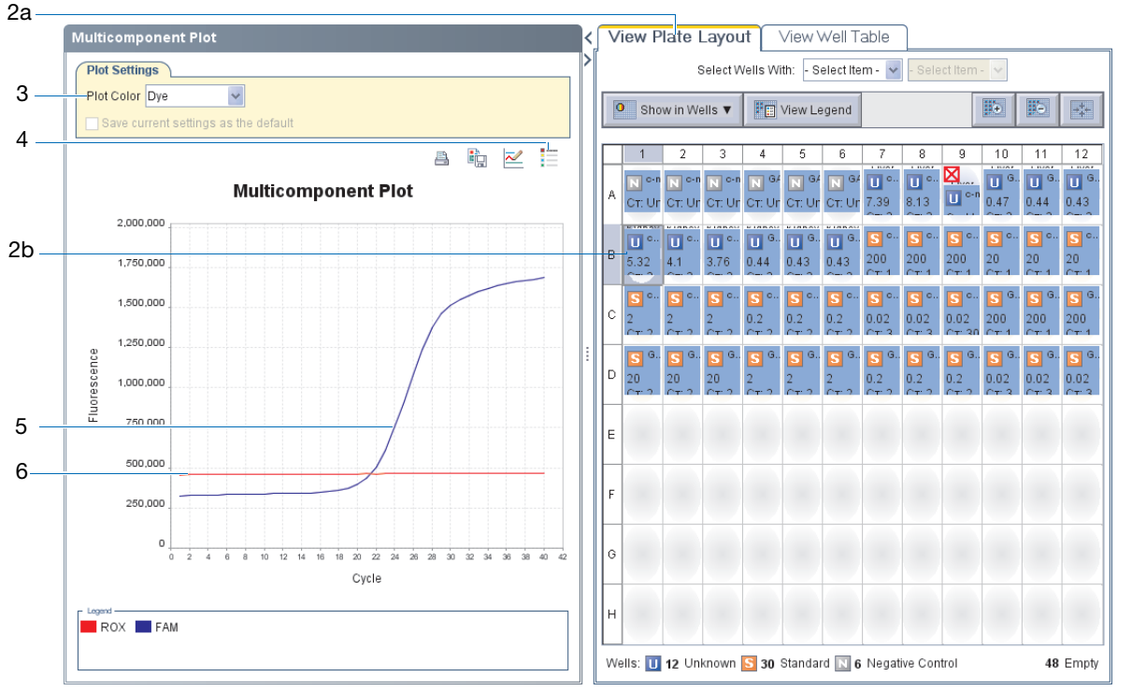
4. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

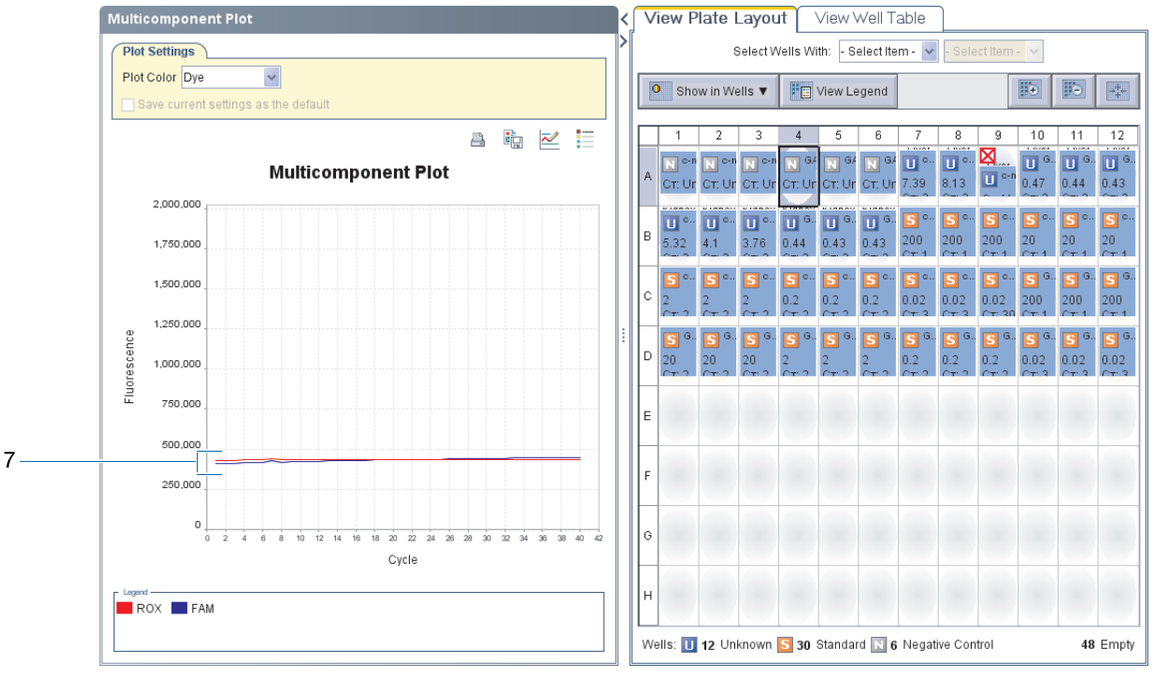
5. View the FAM dye signal. In the example experiment, the FAM dye signal increases throughout the PCR process, which indicates normal amplification.

Notes

6. View the ROX dye signal. In the example experiment, the ROX dye signal is constant throughout the PCR process, which indicates typical data.



7. Select the negative control wells one at a time and check for amplification. In the example experiment, no amplification occurs in the negative control wells.



Notes

Analysis Guidelines

When you analyze your own relative standard curve experiment, look for:

- **Passive reference** – The passive reference dye fluorescence level should be relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Any irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescence.
- **Negative control wells** – There should not be any amplification in the negative control wells.

For More Information

For more information on the Multicomponent Plot screen, open the 7500 Software Help by clicking or pressing **F1**.

View the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

About the Example Experiment

In the relative standard curve example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Plot

1. In the navigation pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display all 96 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the View Plate Layout tab.

3. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

   Note: The legend displays the color code for each row of the reaction plate. In the example shown below, Row A is red, Row B is yellow/green, Row C is green, and so on.

Notes

4. Drag the Show Cycle slider from cycle 1 to cycle 40. In the example experiment, a stable increase occurs in the signal from filter 1, which corresponds to the FAM™ dye.

The filters are:

Filter	1	2	3	4	5
Dye	• FAM™ dye • SYBR® Green dye	• JOE™ dye • VIC® dye	• TAMRA™ dye • NED™ dye • Cy3® dye	• ROX™ dye • Texas Red® dye	Cy5® dye

Analysis Guidelines

When you analyze your own relative standard curve experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

For More Information

For more information on the Raw Data Plot screen, open the 7500 Software Help by clicking or pressing **F1**.

Notes

6 Design the Comparative C_T Experiment

This chapter covers:

- Chapter Overview 112
- Create a New Experiment 113
- Define the Experiment Properties 114
- Define the Methods and Materials 116
- Set Up the Targets 119
- Set Up the Samples 121
- Set Up the Relative Quantitation Settings 124
- Set Up the Run Method 125
- Review the Reaction Setup 127
- Order Materials for the Experiment 131
- Finish the Design Wizard 134

Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

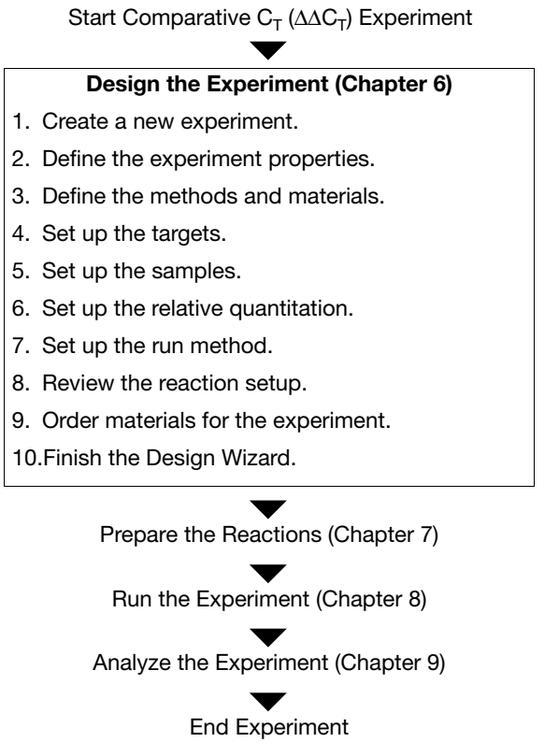
Notes

Chapter Overview

This chapter explains how to use the Design Wizard in the 7500 software to set up the comparative C_T ($\Delta\Delta C_T$) example experiment. The Design Wizard guides you through Applied Biosystems recommended best practices as you enter design parameters for the example experiment.

Note: When you design your own experiments, you can select alternate workflows (see "Using This Guide with Your Own Experiments" on page 13).

Example Experiment Workflow



Notes

Create a New Experiment

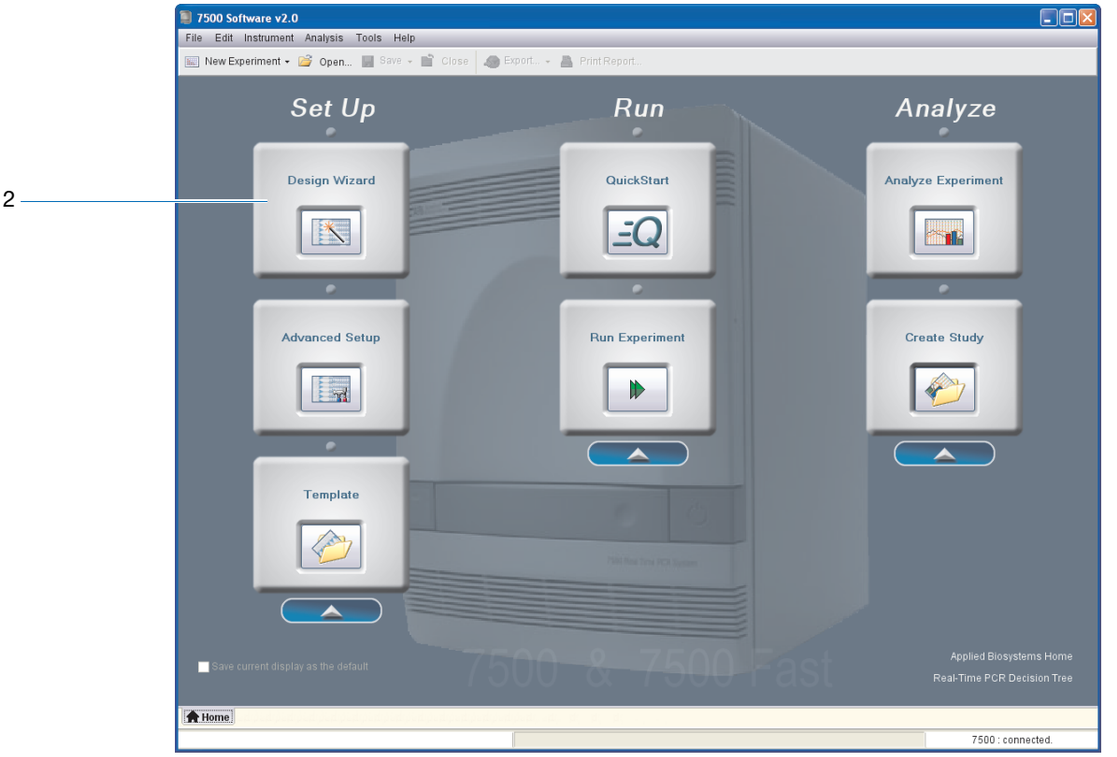
Create a new experiment using the Design Wizard in the 7500 software.

Create an Experiment

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

   where *<software name>* is the current version of the 7500 software.

2. In the Home screen, click **Design Wizard** to open the Design Wizard.



Notes

Define the Experiment Properties

In the Experiment Properties screen, enter identifying information for the experiment, select the instrument type, then select the type of experiment to design.

About the Example Experiment

In the comparative C_T ($\Delta\Delta C_T$) example experiment:

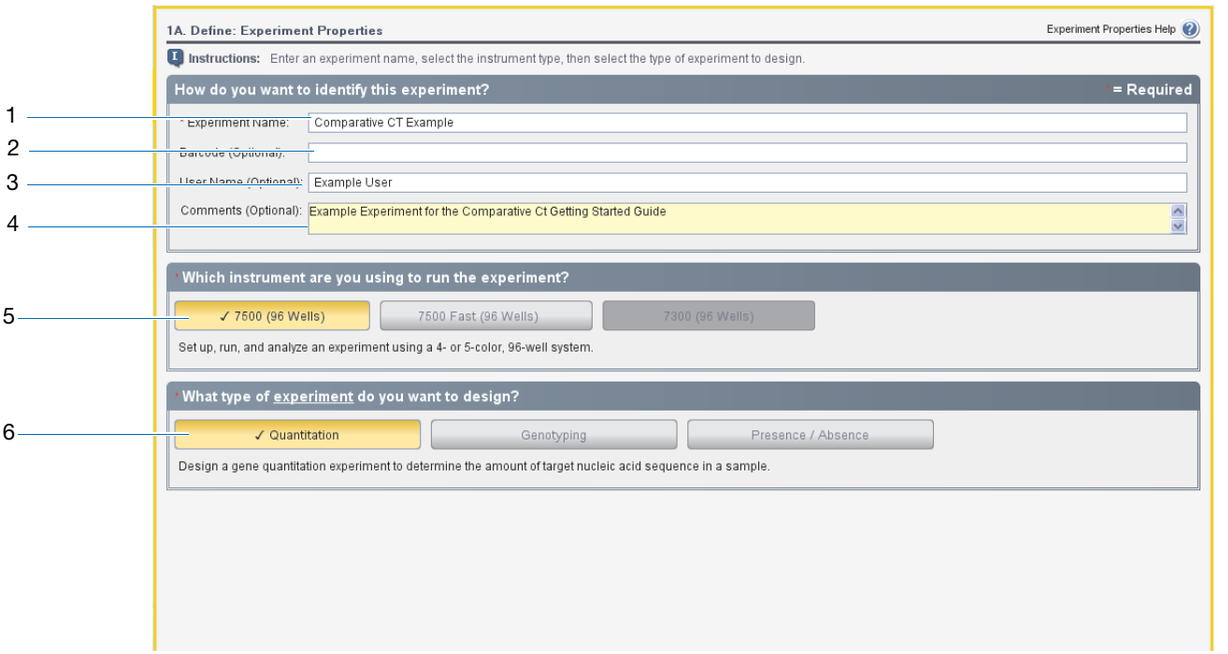
- The experiment is identified as an example.
- The 7500 instrument is selected to run the experiment.
- A MicroAmp® Optical 96-Well Reaction Plate is used.
- The experiment type is quantitation.

Complete the Experiment Properties Screen

1. Click the **Experiment Name** field, then enter **Comparative CT Example**.

   Note: The experiment header is updated with the experiment name you entered.

2. Leave the Barcode field empty.
3. Click the **User Name** field, then enter **Example User**.
4. Click the **Comments** field, then enter **Example Experiment for the Comparative Ct Getting Started Guide**.
5. Select **7500 (96 Wells).**
6. Select **Quantitation** for the experiment type.
7. Click **Next**.



Notes

Design Guidelines

When you design your own comparative C_T experiment:

- Enter an experiment name that is descriptive and easy to remember. You can enter up to 100 characters in the Experiment Name field. You cannot use the following characters in the Experiment Name field: / \ > < * ? " | : ;

  Note: The experiment name is used as the default file name.

- (Optional) Enter a barcode to identify the barcode on the PCR reaction plate. You can enter up to 100 characters in the Barcode field.
- (Optional) Enter a user name to identify the owner of the experiment. You can enter up to 100 characters in the User Name field.
- (Optional) Enter comments to describe the experiment. You can enter up to 1000 characters in the Comments field.
- Select the instrument you are using to run the experiment:
  - **7500 (96 Wells)**
  - **7500 Fast (96 Wells)**

  Note: You can use 7500 software v2.0 to design experiments for the 7500/7500 Fast instrument.

  IMPORTANT! To set the default instrument type, select **Tools ▸ Preferences**, then select the **Defaults** tab (default). In the Instrument Type drop-down list, select the appropriate instrument.

- Select **Quantitation** as the experiment type.

For More Information

For more information on:

- Completing the Experiment Properties screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Consumables – See “Supported Consumables” on page 4.
- Quantitation experiments – Refer to the *Real Time PCR System Reagent Guide*.

Notes

Define the Methods and Materials

In the Methods and Materials screen, select the quantitation method, reagents, ramp speed, and PCR template to use for the experiment.

About the Example Experiment

In the comparative C_T example experiment:

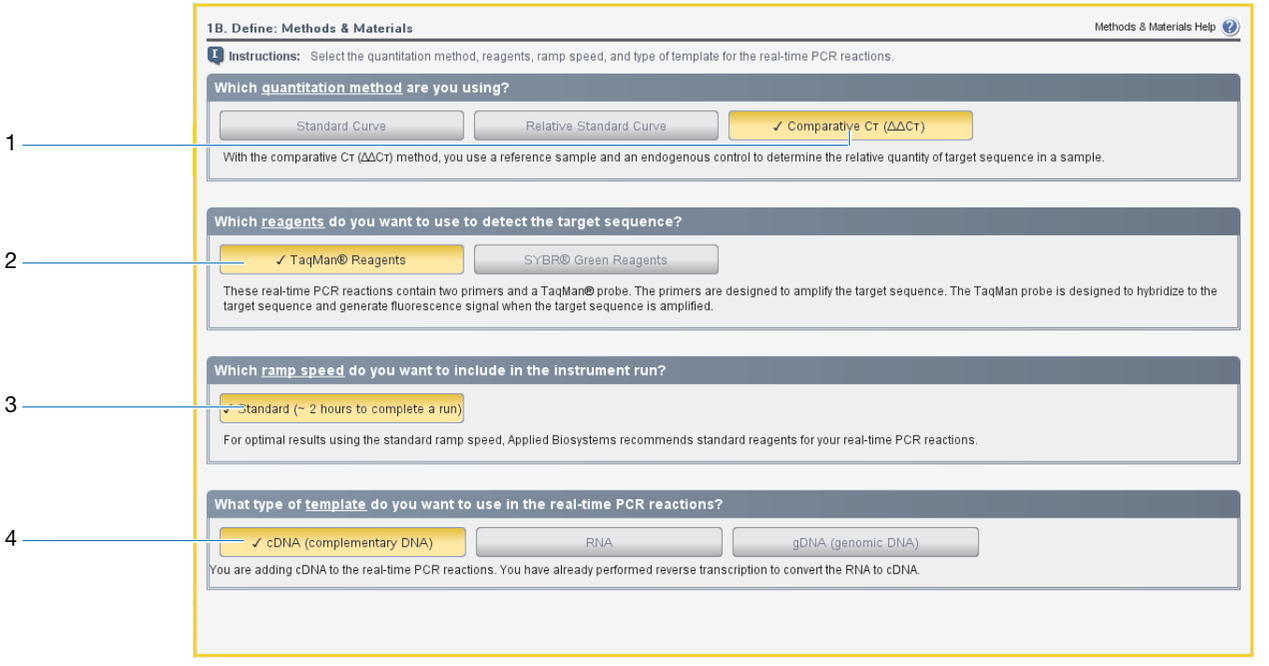
- The comparative C_T quantitation method is used.
- TaqMan® reagents are used.
- The standard ramp speed is used in the instrument run.
- cDNA (prepared from total RNA isolated from liver, kidney, brain, and universal tissues) is the template type. You must first perform reverse transcription to convert the RNA to cDNA (see "Prepare the Template" on page 139).

Complete the Methods and Materials Screen

1. Select **Comparative C_T ($\Delta\Delta C_T$)** as the quantitation method.
2. Select **TaqMan® Reagents** for the reagents. (default)

   IMPORTANT! Fast runs cannot be performed on a 7500 system.

3. Select **Standard (~ 2 hours to complete a run)** for the ramp speed.
4. Select **cDNA (complementary DNA)** for the template type. (default)
5. Click **Next**.



Notes

Design Guidelines

When you design your own comparative C_T experiment:

- Select **Comparative C_T ($\Delta\Delta C_T$)** as the quantitation method. The comparative C_T ($\Delta\Delta C_T$) method is used to determine the relative target quantity in samples. When setting up your reaction plate, the comparative C_T method requires targets, samples, a reference sample, and an endogenous control.

  Note: Before you use the comparative C_T method, Applied Biosystems recommends that you determine that the PCR efficiencies for the target assay and the endogenous control assay are approximately equal. Applied Biosystems TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assays have equivalent amplification efficiencies of 100% (±10%).

- Select the reagents you want to use:
  - Select **TaqMan® Reagents** if you want to use TaqMan reagents to detect amplification and quantify the amount of target in the samples. TaqMan reagents consist of two primers and a TaqMan probe. The primers are designed to amplify the target. The TaqMan probe is designed to hybridize to the target and fluoresce when the target is amplified.
  - Select **SYBR® Green Reagents** if you want to use SYBR Green reagents to detect amplification and quantify the amount of target in the samples. SYBR Green reagents consist of two primers and SYBR Green dye. The primers are designed to amplify the target. The SYBR Green dye fluoresces when it binds to double-stranded DNA. SYBR Green dye is often part of the SYBR Green master mix added to the reaction. If you use SYBR Green dye, select the **Include Melt Curve** check box to perform melt curve analysis of the amplified target.

  IMPORTANT! Although you can use other fluorescence-based reagents on the 7500/7500 Fast system, you must design your experiment using Advanced Setup instead of the Design Wizard.

  Note: Fast runs can be performed only on the 7500 Fast system and an upgraded 7500 system.

- Select the appropriate ramp speed for the instrument run:
  - Select **Fast (~ 40 minutes to complete a run)** if you are using Fast reagents for the PCR reactions.
  - Select **Standard (~ 2 hours to complete a run)** if you are using standard reagents for the PCR reactions.
- Select the appropriate PCR template:
  - Select **cDNA (complementary DNA)** if you are performing 2-step RT-PCR, and you have already performed reverse transcription to convert the RNA to cDNA. You are adding complementary DNA to the PCR reactions.

Notes

– Select **RNA** if you are performing 1-step RT-PCR. You are adding total RNA or mRNA to the PCR reactions.

> **Note:** To use the Fast ramp speed with RNA templates, you must design your experiment using Advanced Setup instead of the Design Wizard.

– Select **gDNA (genomic DNA)** if you have already extracted the gDNA from tissue or sample. You are adding purified genomic DNA to the PCR reactions.

For More Information

For more information on:

- Completing the Methods and Materials screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Determining PCR efficiencies – Open the 7500 Software Help by clicking or pressing **F1**. Within the Help, search as follows:
  a. Select the **Search** tab.
  b. Enter **PCR efficiency**.
  c. Click **List Topics**.
  d. Double-click **Determine Amplification Efficiency**.
- Using Advanced Setup – See "Advanced Setup Workflow" on page 204.
- Using the relative standard curve quantitation method – See Chapters 2 through 5 of this guide.
- Using the standard curve quantitation method – Refer to the *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments*.
- TaqMan and SYBR Green reagents – Refer to the *Real Time PCR System Reagent Guide*.
- PCR, including singleplex vs. multiplex PCR and 1-step vs. 2-step RT PCR – Refer to the *Real Time PCR System Reagent Guide*.

Notes

Set Up the Targets

In the Targets screen, enter the number of targets you want to quantify in the PCR reaction plate, then set up the assay for each target.

About the Example Experiment

In the comparative C_T example experiment:

- Two targets are quantified in the reaction plate.
- The Target 1 assay is set up for the endogenous control. For the example experiment the target is human 18S. 18S serves as the endogenous control because its expression levels tend to be relatively stable.
- The Target 2 through 6 assays are set up for the targets you are studying. For the example experiment, the targets are EGR3, MAOB, OGDH, OSGEP, and SERPING1.

Complete the Targets Screen

1. Click the **How many targets do you want to quantify in the reaction plate?** field, then enter **6**.

   Note: The number of rows in the target assays table is updated with the number you entered.

2. Set up the Target 1 assay:
   a. Click the **Enter Target Name** cell, then enter **18S**.
   b. In the Reporter drop-down list, select **FAM** (default).
   c. In the Quencher drop-down list, select **NFQ-MGB** (default).
   d. Leave the default in the Color field.

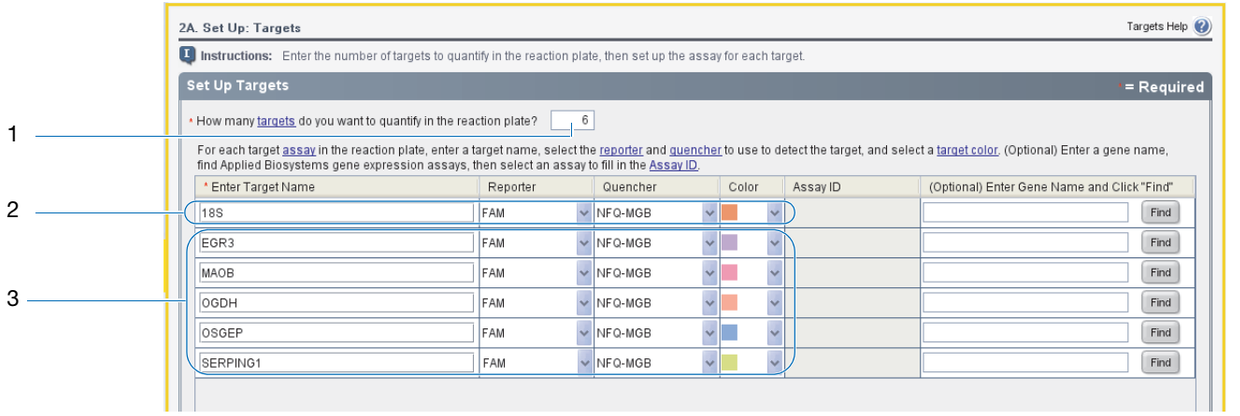
3. Set up the remaining targets as shown below:

Target	Target Name	Reporter	Quencher	Color
2	EGR3	FAM	NFQ-MGB	(Use default color)
3	MAOB	FAM	NFQ-MGB	(Use default color)
4	OGDH	FAM	NFQ-MGB	(Use default color)
5	OSGEP	FAM	NFQ-MGB	(Use default color)
6	SERPING1	FAM	NFQ-MGB	(Use default color)

4. Click **Next**.

Note: For all targets, leave blank the (Optional) Enter Gene Name field. You can search for the gene/assay ID when you order your materials (see "Order Materials for the Experiment" on page 131).

Notes



Design Guidelines

When you design your own comparative C_T experiment:

- Identify each target assay with a unique name and color. You can enter up to 100 characters in the Target Name field.
- Select an endogenous control for each sample. The endogenous control is a target that is present in all samples under investigation. It should be expressed equally in all sample types, regardless of treatment or tissue origin (examples of endogenous controls are β-actin, GAPDH, and 18S ribosomal RNA [18S rRNA]). The endogenous control is used to normalize the PCR results; the endogenous control corrects for variable sample mass, nucleic acid extraction efficiency, reverse transcription efficiency, and pipette calibration errors. Note that:
  - Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.
  - If samples are on multiple plates, each plate must have an endogenous control. Additionally, every reaction plate must include an endogenous control for every sample type on the reaction plate.
  - You can select multiple endogenous controls for a single plate. See "Set Multiple Endogenous Controls" on page 101.
- Select the reporter dye used in the target assay. In the Methods and Materials screen on page 116, if you selected:
  - TaqMan® Reagents – Select the dye attached to the 5′ end of the TaqMan probe.
  - SYBR® Green Reagents – Select **SYBR**.
- Select the quencher used in the target assay. In the Methods and Materials screen on page 116, if you selected:
  - TaqMan® Reagents – Select the quencher attached to the 3′ end of the TaqMan probe.
  - SYBR® Green Reagents – Select **None**.

For More Information

For more information on:

- Completing the Targets screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Selecting an endogenous control – See the Application Note *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies*.

Notes

Set Up the Samples

In the Samples screen, enter the number of samples, replicates, and negative controls to include in the reaction plate, enter the sample names, then select the sample/target reactions to set up.

About the Example Experiment

In the comparative C_T example experiment:

- Four samples are used: cDNA prepared from total RNA isolated from four tissues (brain, kidney, liver, and universal). The samples contain unknown quantities of EGR3, MAOB, OGDH, OSGEP, and SERPING1 (targets), and 18S (endogenous control).
- Four technical replicates are used. The replicates are identical reactions, containing identical reaction components and volumes.
- No negative controls are used. Negative control reactions contain water instead of sample and should not amplify. The software automatically includes three negative controls for each target assay.

IMPORTANT! Although the example experiment does not use negative controls, Applied Biosystems recommends them.

Complete the Samples Screen

1. Click the **How many samples do you want to test in the reaction plate?** field, then enter **4**.

   Note: The number of rows in the samples table is updated with the number you entered.

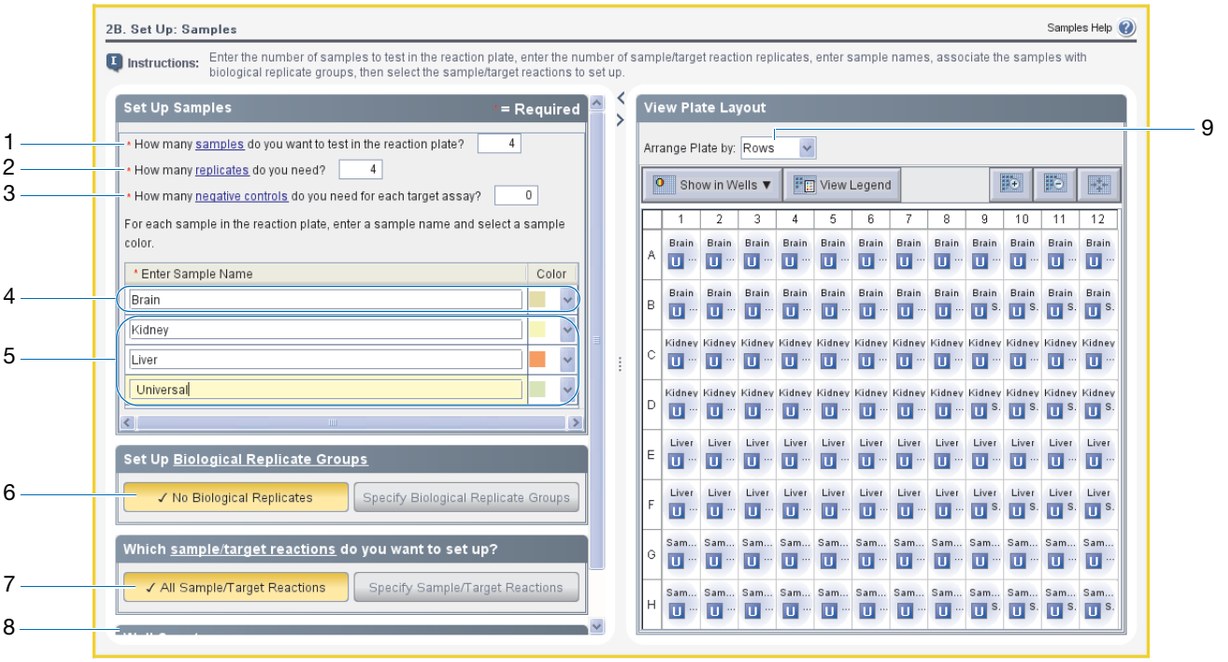
2. Click the **How many replicates do you need?** field, then enter **4**.
3. Click the **How many negative controls do you need for each target assay?** field, then enter **0**.
4. Set up Sample 1:
   a. Click the **Enter Sample Name** field, then enter **Brain**.
   b. Leave the default in the Color field.
5. Set up the remaining samples as shown below:

Sample Name	Color
Kidney	(Use default color)
Liver	(Use default color)
Universal	(Use default color)

6. Select **No Biological Replicates**.
7. Select **All Sample/Target Reactions** to test all targets in all samples.

Notes

8. In the Well Count pane, verify that there are:
   - 96 Unknown wells U
   - 0 Negative Control wells N
   - 0 Empty wells
9. In the Arrange Plate by drop-down list of the View Plate Layout tab, select **Rows** (default).
10. Click **Next**.



Design Guidelines

When you design your own comparative C_T experiment:

- Identify each sample with a unique name and color. You can enter up to 100 characters in the Sample Name field.
- Enter the number of replicates (identical reactions) to set up. Applied Biosystems recommends three replicates for each sample reaction.
- Set up biological replicate groups (see "Set Up Biological Replicate Groups" on page 123).

  Biological replicates allow you to assess the representative nature of your results as they relate to the population being studied. Inclusion of biological replicates can give insight into any natural variation that is present within the population.
- The software automatically includes three negative controls for each target assay.
- Select which targets to test in the samples:
  - Select **All Sample/Target Reactions** to test all targets in all samples.

Notes

– Select **Specify Sample/Target Reactions** to specify the targets to test in each sample.

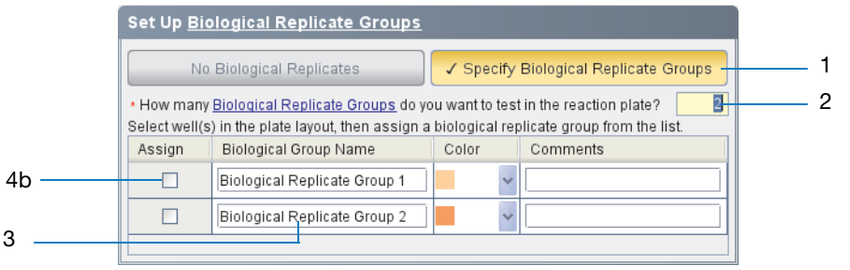
Note: When you use the Design Wizard to set up a comparative C_T experiment, you can set up only singleplex reactions (amplification and detection of one target per well). If you want to set up a comparative C_T experiment with multiplex reactions (amplification and detection of two or more targets per well), design your experiment using Advanced Setup instead of the Design Wizard.

Set Up Biological Replicate Groups

Note: You can also specify biological replicate groups when analyzing comparative C_T experiments as part of a study (see "Define Replicates" on page 192).

1. Select **Specify Biological Replicate Groups**.
2. Enter the number of biological replicate groups that you want to test in the reaction plate.
3. For each biological replicate group, click the cell in the Biological Group Name column, then enter a name for the biological group. For example, **BrainGP**.
4. Assign the biological replicate group(s) to the reaction plate:
   a. Select wells in the plate layout that contain samples associated with the biological replicate group.
   b. In the Assign column, select the check box of the appropriate biological replicate group.

IMPORTANT! A sample cannot belong to more than one biological group.



For More Information

For more information on:

- Completing the Samples screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Using Advanced Setup – See "Advanced Setup Workflow" on page 204.

Notes

Set Up the Relative Quantitation Settings

In the Relative Quantitation Settings screen, select the reference sample and the endogenous control to perform relative quantitation.

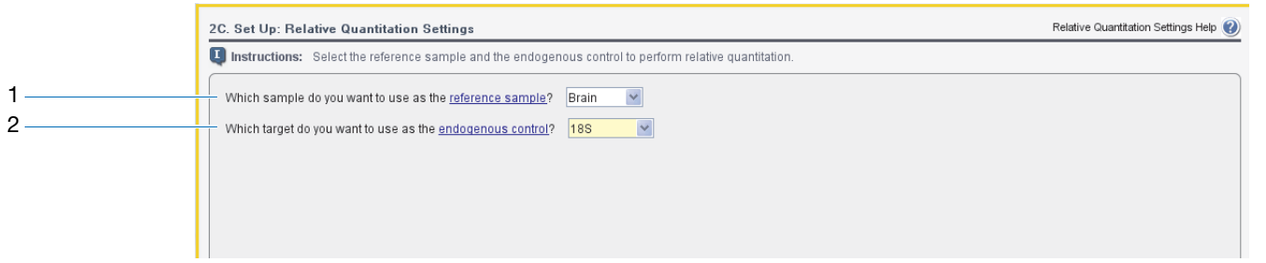
About the Example Experiment

In the comparative C_T example experiment:

- Brain is used as the reference sample.
- 18S is used as the endogenous control.

Complete the Relative Quantitation Settings Screen

1. In the Which sample do you want to use as the reference sample? drop-down list, select **Brain**.
2. In the Which target do you want to use as the endogenous control? drop-down list, select **18S**.
3. Click **Next**.



Design Guidelines

When you design your own comparative C_T experiment:

- Select a reference sample from your previously created samples ("Set Up the Samples" on page 121). Amplification results from the samples are compared to the amplification results from the reference sample to determine relative expression.
- Select an endogenous control from your previously created target assays ("Set Up the Targets" on page 119). Amplification results from the endogenous control are used to normalize the amplification results from the target for differences in the amount of template added to each reaction.

For More Information

For more information on:

- Completing the Relative Quantitation Settings screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Reference samples (also known as calibrators) and endogenous controls – See *User Bulletin #2: Relative Quantitation of Gene Expression*.

Notes

Set Up the Run Method

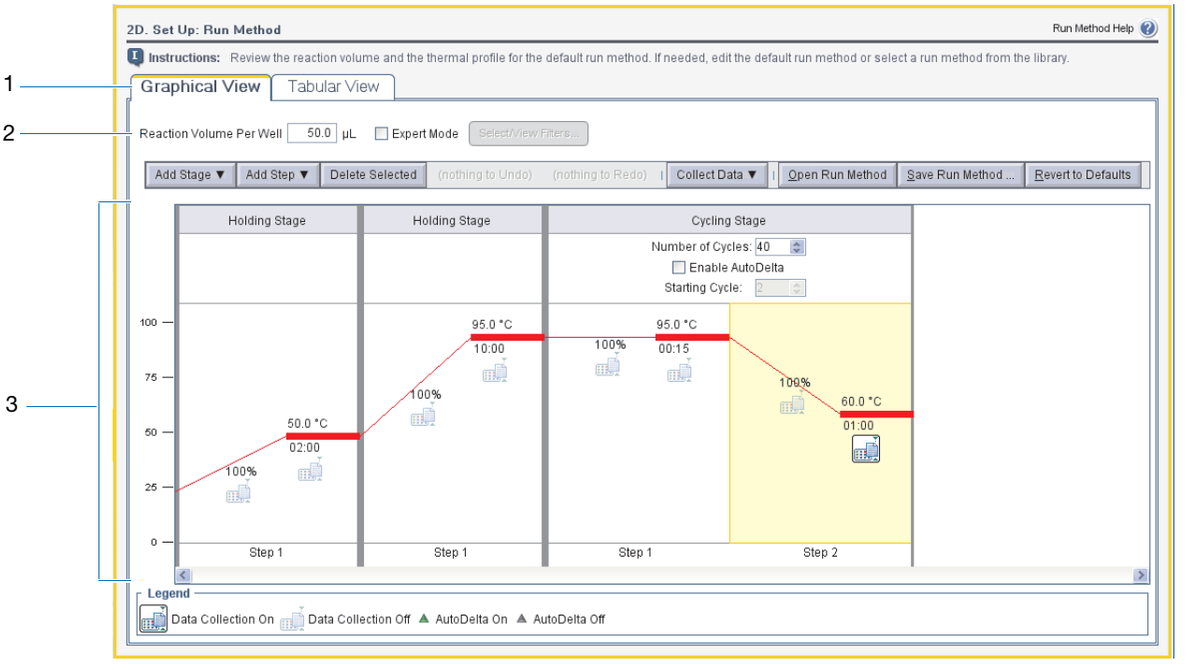
In the Run Method screen, review the reaction volume and the thermal profile for the default run method. If needed, you can edit the default run method or replace it with one from the Run Method library.

About the Example Experiment

In the comparative C_T example experiment, the default run method is used without edits.

Review the Run Method Screen

1. Select either the **Graphical View** tab (default) or **Tabular View** tab.
2. Make sure the Reaction Volume Per Well field displays **50 µL**.
3. Make sure the thermal profile displays the holding and cycling stages shown below. If necessary, add click on a temperature or time setting and change it.
4. Click **Next**.



Notes

Design Guidelines

When you design your own comparative C_T experiment:

- Enter a number from 10 to 100 for the reaction volume/well. The 7500 system supports reaction volumes from 20 to 100 µL. The 7500 Fast system supports reaction volumes from 10 to 30 µL.
- Review the thermal profile:
  - Make sure the thermal profile is appropriate for your reagents.
  - If you are performing 1-step RT-PCR, include a reverse transcription step.

  If your experiment requires a different thermal profile, edit the thermal profile or replace the run method with one from the Run Method library. The Run Method library is included in the 7500 software.

For More Information

For more information on:

- The Run Method library or on completing the Run Method screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Using Advanced Setup – See “Advanced Setup Workflow” on page 204.

Notes

Review the Reaction Setup

In the Reaction Setup screen, select the assay type (if using TaqMan reagents), then review the calculated volumes for preparing the PCR reactions and sample dilutions. If needed, you can edit the reaction volume, excess reaction volume, component concentrations, and/or diluted sample concentration.

About the Example Experiment

In the comparative C_T example experiment:

- Applied Biosystems TaqMan® Gene Expression Assays are used.
- The reaction volume per well is 50 µL.
- The excess reaction volume is 10%.
- The reaction components are:
  - TaqMan® Universal PCR Master Mix (2×)
  - Assay Mix (20×) for each target (18S, EGR3, MAOB, OGDH, OSGEP, and SERPING1)
  - Sample
  - Water
- The diluted sample concentration is 50 ng/µL.
- The sample stock concentration is 100 ng/µL.

Complete the Reaction Setup Screen

IMPORTANT! Perform the following steps for each target assay in the reaction plate.

Complete the Reaction Mix Calculations Tab for the Assays

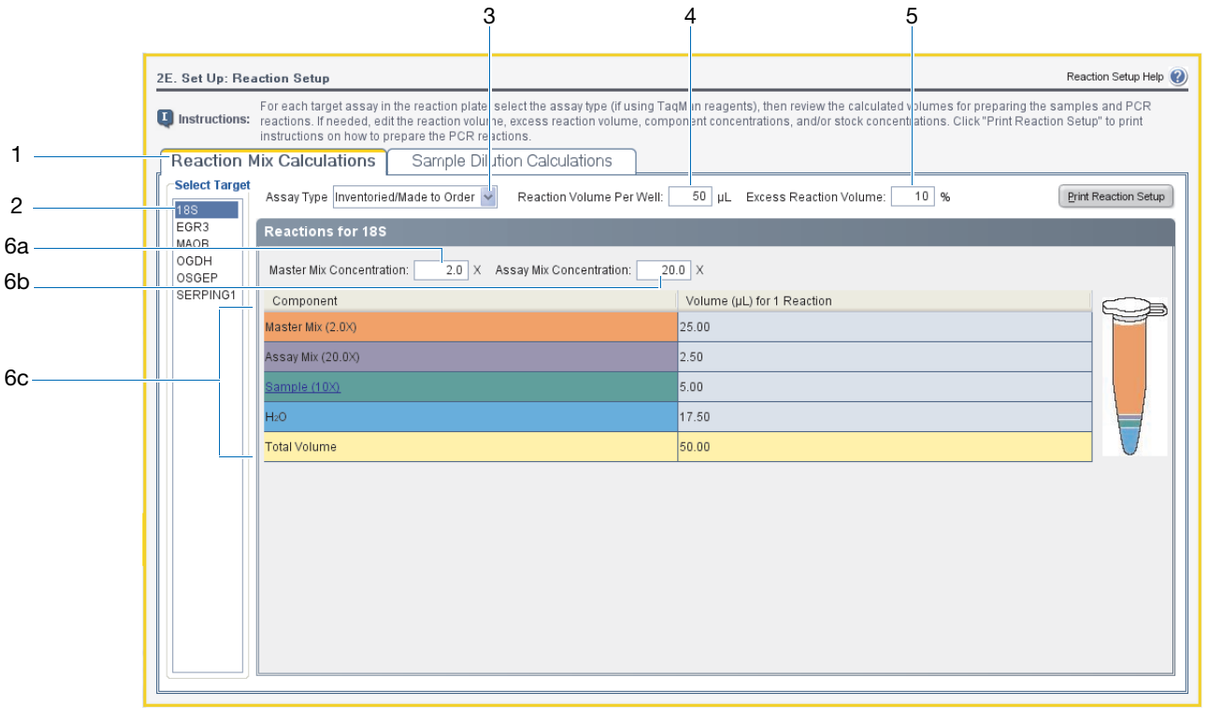
1. Select the **Reaction Mix Calculations** tab (default).
2. In the Select Target pane, select **18S**.
3. In the Assay Type drop-down list, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **50 µL**.
5. Make sure the Excess Reaction Volume field displays **10%**.
6. In the Reactions for 18S pane:
   a. Make sure the Master Mix Concentration field displays **2.0**×.
   b. Make sure the Assay Mix Concentration field displays **20.0**×.

Notes

c. Review the components and calculated volumes for the PCR reactions:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	25.00
Assay Mix (20.0X)	2.50
Sample (10X)	5.00‡
H2O	17.50
Total Volume	50.00

‡ The sample volume is limited to 10% of the total reaction volume.



7. Repeat steps 2 through 6 for the remaining targets listed in the Selected Targets pane (EGR3, MAOB, OGDH, OSGEP, and SERPING1).

Complete the Sample Dilution Calculations Tab

1. Select the **Sample Dilution Calculations** tab.

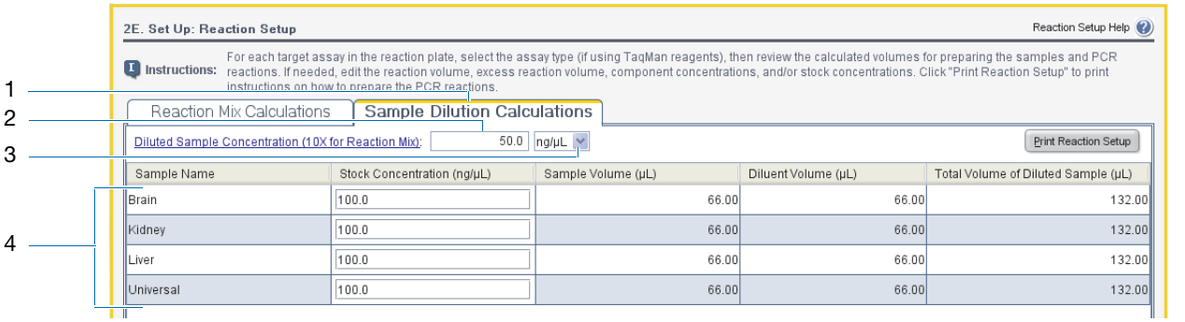
2. Click the **Diluted Sample Concentration (10× for Reaction Mix)** field, then enter **50.0**.

3. In the unit drop-down list, select **ng/µL** (default).

Notes

4. Review the calculated volumes for the sample dilutions:

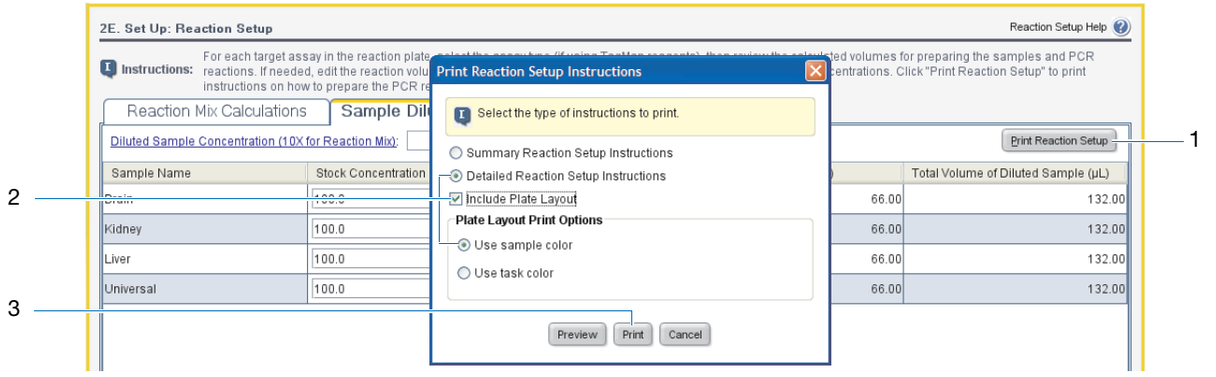
Sample Name	Stock Concentration (ng/µL)	Sample Volume (µL)	Diluent Volume (µL)	Total Volume of Diluted Sample (µL)
Brain	100.0	66.00	66.00	132.00
Kidney	100.0	66.00	66.00	132.00
Liver	100.0	66.00	66.00	132.00
Universal	100.0	66.00	66.00	132.00



Print Reaction Setup Instructions

Print reaction setup instructions for use in Chapter 7, "Prepare the Comparative C_T Reactions."

1. Click **Print Reaction Setup**.
2. In the Print Reaction Setup Instructions dialog box, select:
   - **Detailed Reaction Setup Instructions**
   - **Include Plate Layout**
   - **Use sample color**
3. Click **Print** to print the reaction setup instructions.
4. Click **Next**.



Notes

Design Guidelines

When you design your own comparative C_T experiment:

- If you are using TaqMan reagents, select the type of assay you are using:
  - Select **Inventoried/Made to Order** if you are using Applied Biosystems TaqMan® Gene Expression Assays (Inventoried or Made to Order), or Applied Biosystems Custom TaqMan® Gene Expression Assays.
  - Select **Custom** if you are designing your own assays with Primer Express® software.
- Enter a number from 10 to 100 for the reaction volume/well. The 7500 system supports reaction volumes from 20 to 100 µL. The 7500 Fast system supports reaction volumes from 10 to 30 µL.
- Include excess reaction volume to account for the loss that occurs during pipetting. Applied Biosystems recommends an excess reaction volume of at least 10%.
- Review the reaction mix concentrations for each target: If needed:
  - For TaqMan reagents, edit the master mix and assay mix concentrations.
  - For SYBR Green reagents, edit the master mix, forward primer, and reverse primer concentrations.
  - For 1-step RT-PCR, edit the reverse transcriptase concentration.
- Review the reaction mix components for each target:
  - If you are running Fast PCR reactions, make sure you use Fast master mix in the PCR reactions.
  - If you are running standard PCR reactions, make sure you use standard master mix in the PCR reactions.
  - For 1-step RT-PCR, make sure you include reverse transcriptase in the PCR reactions and use a specific buffer.
- Review the sample dilution calculations for each sample. If needed, edit the diluted sample concentration (including units) and stock concentration.

For More Information

For more information on:

- Completing the Reaction Setup screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Applied Biosystems assays – Refer to the:
  - *TaqMan® Gene Expression Assays Protocol*
  - *Custom TaqMan® Gene Expression Assays Protocol*.

Notes

Order Materials for the Experiment

In the Materials List screen, review the list of materials recommended to prepare the PCR reaction plate. (Optional) Print the materials list, create a shopping list, then order the recommended materials from the Applied Biosystems Store.

Note: You must have an Internet connection to access the Applied Biosystems Store. Product availability and pricing may vary according to your region or country. Online ordering through the Applied Biosystems Store is not available in all countries. Contact your local Applied Biosystems representative for help.

Note: The 7500 software recommends the materials to order based on your experiment design. It is assumed that you will design your experiment, order your materials, then prepare (Chapter 7) and run (Chapter 8) the reaction plate when your materials arrive.

About the Example Experiment

In the comparative C_T example experiment, the recommended materials are:

- MicroAmp® Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- MicroAmp® Splash Free Support Base
- TaqMan® Universal PCR Master Mix (2✕), No AmpErase® UNG
- 18S Assay Mix: Hs99999901_s1
- EGR3 Assay Mix: Hs00231780_m1 (RefSeq NM_004430.2)
- MAOB Assay Mix: Hs00168533_m1 (RefSeq NM_000898.3)
- OGDH Assay Mix: Mm00803121_m1 (RefSeq NM_010956.3)
- OSGEP Assay Mix: Hs00215099_m1 (RefSeq NM_017807.2)
- SERPING1 Assay Mix: Hs00163781_m1 (RefSeq NM_001032295.1/NM_000062.2)

Complete the Ordering Materials Screen

1. Find the target assay in the Applied Biosystems Store:
   a. Click the **Enter Gene Name** field, enter **18S**, then click **Find Assay**.
   b. In the Find Assay Results dialog box, select the **Hs99999901_s1** assay row, then click **Apply Assay Selection**.
2. Repeat step 1 to apply the remaining target assays for the experiment:
   - EGR3 Assay Mix: Hs00231780_m1
   - MAOB Assay Mix: Hs00168533_m1
   - OGDH Assay Mix: Mm00803121_m1
   - OSGEP Assay Mix: Hs00215099_m1
   - SERPING1 Assay Mix: Hs00163781_m1

Notes

3. In the Display drop-down list, select **All Items** (default), then review the recommended materials.

   Note: For more information on a specific item, click the part number link. You are connected to the product information page at the Applied Biosystems Store. To access the Applied Biosystems Store, you need to have an Internet connection.

4. (Optional) Click **Print Materials List** to print the materials list.

5. (Optional) Create a shopping list:

   a. Select the check box next to each of the following items:
      - MicroAmp® Optical 96-Well Reaction Plate
      - MicroAmp™ Optical Adhesive Film
      - MicroAmp® Splash Free Support Base
      - TaqMan® Universal PCR Master Mix (2×), No AmpErase® UNG
      - 18S Assay Mix: Hs99999901_s1
      - EGR3 Assay Mix: Hs00231780_m1
      - MAOB Assay Mix: Hs00168533_m1
      - OGDH Assay Mix: Mm00803121_m1
      - OSGEP Assay Mix: Hs00215099_m1
      - SERPING1 Assay Mix: Hs00163781_m1

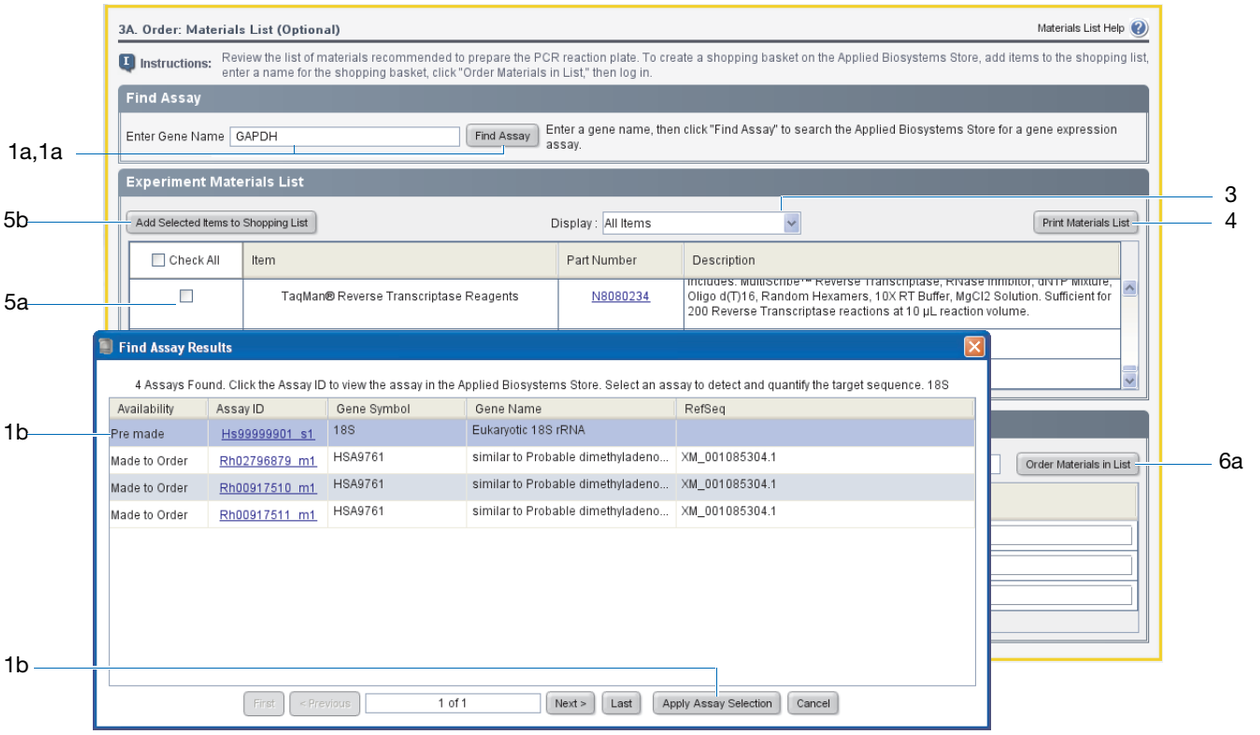
   b. Click **Add Selected Items to Shopping List**.

6. (Optional) Create a shopping basket at the Applied Biosystems Store:

   Note: You must have an Internet connection to access the Applied Biosystems Store. Product availability and pricing may vary according to your region or country. Online ordering through the Applied Biosystems Store is not available in all countries. Contact your local Applied Biosystems representative for help.

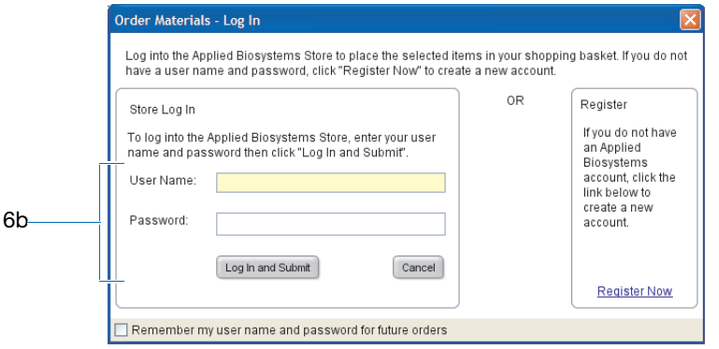
Notes

a. Check that the Experiment Shopping List contains the desired materials and that the quantities are correct, then click **Order Materials in List**.



b. In the Order Materials - Log In dialog box, enter your user name and password for the Applied Biosystems Store, then click **Log In and Submit**.

Note: If you do not have an account with the Applied Biosystems Store, click **Register Now** to create an account.



c. When you are connected to the Applied Biosystems Store, follow the prompts to complete your order.

7. Go to "Finish the Design Wizard" on page 134.

Notes

Design Guidelines

When you design your own comparative C_T experiment:

- Select all the materials you require for your experiment, then add them to your shopping list.
- To access and use the Applied Biosystems Store:
  - Confirm that your computer has an Internet connection.
  - Use the following Applied Biosystems-recommended browsers and versions of Adobe® Acrobat® Reader:

Desktop Operating System	Netscape® Navigator	Microsoft® Internet Explorer	Macintosh® Safari	Adobe® Acrobat® Reader
Windows® 2000/NT/XP/Vista	v6.x or later	v6.x or later	Not applicable	v4.0 or later
Macintosh® OS 9+ or later	Not supported	Not supported	v2.0.4 or later	v4.0 or later

IMPORTANT! Make sure that cookies and JavaScript are turned on.

For More Information

For more information on completing the Materials List screen, open the 7500 Software Help by clicking or pressing **F1**.

Finish the Design Wizard

To finish the Design Wizard, review the plate layout, then select an exit option.

About the Example Experiment

The 7500 software automatically selects locations for the wells in the reaction plate. In the comparative C_T example experiment:

- The wells are arranged as shown below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Brain 18S	Brain MAOB	Brain OSGEP	Kidney 18S	Kidney MAOB	Kidney OSGEP	Liver 18S	Liver MAOB	Liver OSGEP	Universal 18S	Universal MAOB	Universal OSGEP
B	Brain 18S	Brain MAOB	Brain OSGEP	Kidney 18S	Kidney MAOB	Kidney OSGEP	Liver 18S	Liver MAOB	Liver OSGEP	Universal 18S	Universal MAOB	Universal OSGEP
C	Brain 18S	Brain MAOB	Brain OSGEP	Kidney 18S	Kidney MAOB	Kidney OSGEP	Liver 18S	Liver MAOB	Liver OSGEP	Universal 18S	Universal MAOB	Universal OSGEP
D	Brain 18S	Brain MAOB	Brain OSGEP	Kidney 18S	Kidney MAOB	Kidney OSGEP	Liver 18S	Liver MAOB	Liver OSGEP	Universal 18S	Universal MAOB	Universal OSGEP
E	Brain EGR3	Brain OGDH	Brain SERPI...	Kidney EGR3	Kidney OGDH	Kidney SERPI...	Liver EGR3	Liver OGDH	Liver SERPI...	Universal EGR3	Universal OGDH	Universal SERPI...
F	Brain EGR3	Brain OGDH	Brain SERPI...	Kidney EGR3	Kidney OGDH	Kidney SERPI...	Liver EGR3	Liver OGDH	Liver SERPI...	Universal EGR3	Universal OGDH	Universal SERPI...
G	Brain EGR3	Brain OGDH	Brain SERPI...	Kidney EGR3	Kidney OGDH	Kidney SERPI...	Liver EGR3	Liver OGDH	Liver SERPI...	Universal EGR3	Universal OGDH	Universal SERPI...
H	Brain EGR3	Brain OGDH	Brain SERPI...	Kidney EGR3	Kidney OGDH	Kidney SERPI...	Liver EGR3	Liver OGDH	Liver SERPI...	Universal EGR3	Universal OGDH	Universal SERPI...

Notes

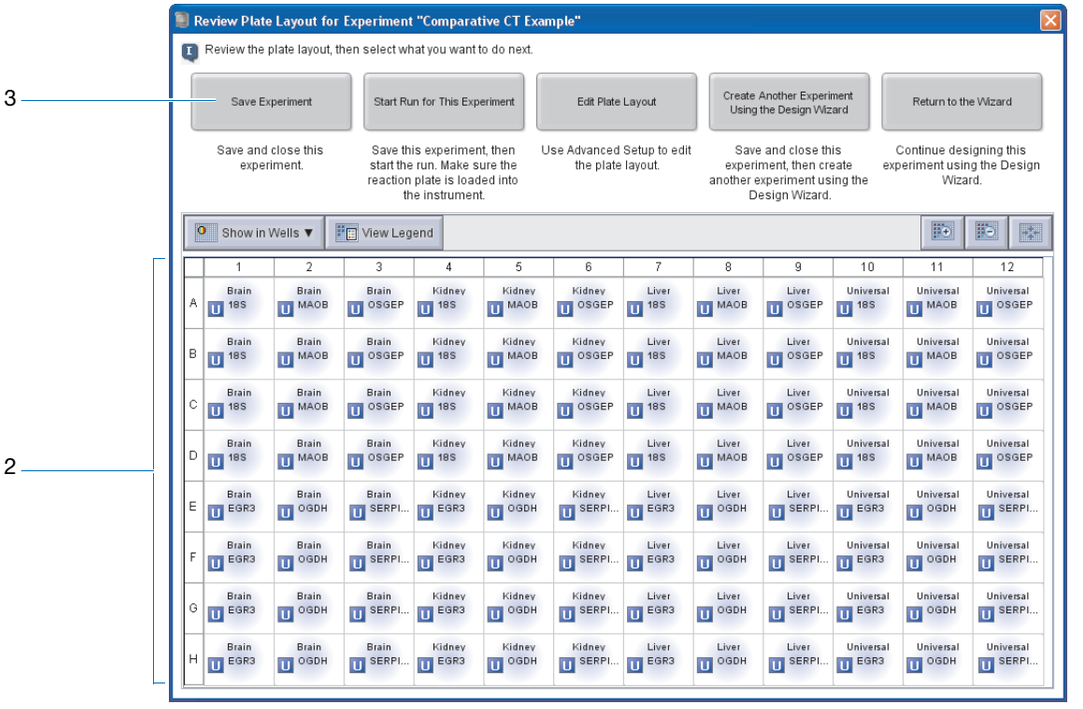
- The experiment is saved as is and closed.

Note: For the example experiment, do not perform the run at this time.

Finish the Design Wizard

1. At the bottom of the 7500 software screen, click **Finish Designing Experiment**.
2. In the Review Plate Layout for Experiment window, review the plate layout. Make sure there are:
   - 96 Unknown wells U
   - 0 Negative Control wells N
   - 0 Empty wells

   Note: If the plate layout is incorrect, click **Return to the Wizard**, then check your entered values.
3. Click **Save Experiment**.



Notes

4. In the Save Experiment dialog box, enter **Comparative Ct Example Setup.eds** in the File name drop-down list, then click **Save**. The example experiment is saved and closed, and you are returned to the Home screen.

IMPORTANT! Do not save the experiment using the default file name. Doing so will overwrite the example experiment already present in the experiments folder.

Note: By default, the example experiment is saved to the <*drive*>:\Applied Biosystems\<*software name*>\experiments folder.

Design Guidelines

When you design your own comparative C_T experiment:

- In the Review Plate Layout for Experiment window, select the appropriate exit option:

Click	To...
Save Experiment	Save and close the experiment without making any further changes or starting the run.
Start Run for This Experiment	Save the experiment and start the run. Make sure the reaction plate is loaded in the instrument.
Edit Plate Layout	Use advanced setup to edit the plate layout.
Create Another Experiment Using the Design Wizard	Save and close the current experiment, then create another experiment using the Design Wizard.
Return to the Wizard	Return to the experiment to make changes using the Design Wizard.

- By default, experiments are saved to:
  <*drive*>:\Applied Biosystems\<*software name*>\experiments
  To change the:
  – Save location for a specific experiment – Navigate to the desired location using the Save Experiment dialog box.
  – Default save location – Select **Tools ▸ Preferences**, then select the **Defaults** tab. In the Data Folder field, browse to then select the desired location.

For More Information

For more information on using Advanced Setup, see “Advanced Setup Workflow” on page 204.

Notes

7 Prepare the Comparative C_T Reactions

This chapter covers:

- Chapter Overview 138
- Prepare the Template. 139
- Prepare the Sample Dilutions 142
- Prepare the Reaction Mix 144
- Prepare the Reaction Plate 146

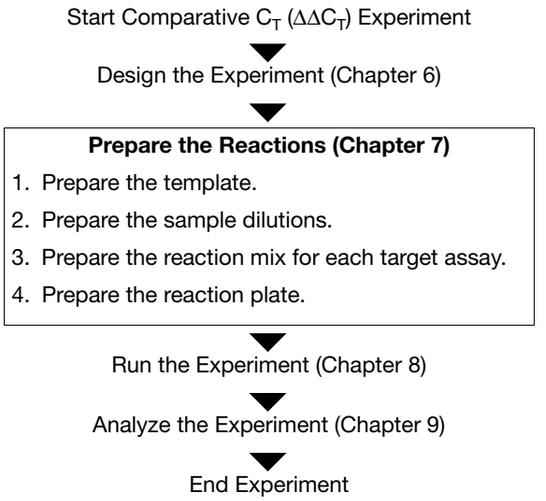
Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

Notes

Chapter Overview

This chapter explains how to prepare the PCR reactions for the comparative C_T ($\Delta\Delta C_T$) example experiment and it provides guidelines for how to prepare the PCR reactions for your own comparative C_T experiment.

Example Experiment Workflow



Notes

Prepare the Template

Prepare the template for the PCR reactions using the High-Capacity cDNA Reverse Transcription Kit.

IMPORTANT! Applied Biosystems recommends that you use the High-Capacity cDNA Reverse Transcription Kit to reverse-transcribe cDNA from total RNA. The TaqMan® Gene Expression Assays are designed to be compatible with the High-Capacity cDNA Reverse Transcription Kit; other protocols have not been tested for use with the TaqMan Gene Expression Assays.

About the Example Experiment

For the comparative C_T example experiment, the template for the PCR reactions is cDNA reverse-transcribed from total RNA samples using the High-Capacity cDNA Reverse Transcription Kit.

Required Materials

- One of the following Ambion® starter packs for RNA isolation:

Kit	Contents	Catalog Number
qRT-PCR Starter Pack	RNA*later*® Tissue Collection: RNA Stabilization Solution	AM7020
	RNase*Zap*® Wipes	AM9786
	RT-PCR Grade Water (nuclease-free)	AM9935
	Silencer® Validated siRNA, Std Purity	AM51331
	Choice of RNA sample preparation products:	
	• RNAqueous®-4PCR Kit **Note:** Recommended if you are isolating RNA from cells or tissues that may be difficult to disrupt or have high RNase activity.	AM1914
	• RiboPure™ Kit **Note:** Recommended if you are: • Isolating RNA from all tissues, including those that may be difficult to disrupt, are rich in lipids, or have high RNase activity, *or* • Labeling and amplifying RNA for use on microarrays.	AM1924
	• TURBO DNA-*free*™ **Note:** Recommended if you are using SYBR® Green reagents. If you are using SYBR Green reagents, use TURBO DNA-free in conjunction with the RiboPure Kit.	AM1907

Notes

Kit	Contents	Catalog Number
PCR Starter Pack	RT-PCR Grade Water (nuclease-free)	AM9935
	DNA*Zap*™	AM9890
	Choice of RNA sample preparation products, as listed above under the qRT-PCR Starter Pack.	See above
High Capacity cDNA Kit	RNA*later*® Tissue Collection: RNA Stabilization Solution	AM7020
	RNase*Zap*® Wipes	AM9786
	Choice of RNA sample preparation products, as listed above under the qRT-PCR Starter Pack.	See above
miRNA Starter Pack	Pre-miR™ miRNA Starter Kit	AM1540

- Total RNA isolated from liver, kidney, brain, and universal tissues
- One of Applied Biosystems High-Capacity cDNA Reverse Transcription Kits:

Kit	Part Number
High-Capacity cDNA Reverse Transcription Kit (200 reactions)	4368814
High-Capacity cDNA Reverse Transcription Kit (1000 reactions)	4368813
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	4374966
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (1000 reactions)	4374967

Note: The High-Capacity cDNA Reverse Transcription Kit was formerly called the High-Capacity cDNA Archive Kit.

Prepare the Template

Use the High-Capacity cDNA Reverse Transcription Kit to reverse-transcribe cDNA from the total RNA samples. Follow the procedures in the *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol* to:

1. Prepare the RT master mix.

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

2. Prepare the cDNA reactions.
3. Perform reverse transcription on a thermal cycler.

Notes

Preparation Guidelines

When you prepare your own comparative C_T ($\Delta\Delta C_T$) experiment Applied Biosystems recommends:

- That you first extract DNA or RNA from the tissue or sample.
- The following templates:
  - **Complementary DNA (cDNA)** – cDNA reverse-transcribed from total RNA samples using a High-Capacity cDNA Reverse Transcription Kit.
  - **RNA** – Purified total RNA or mRNA extracted from tissue or sample.
  - **Genomic DNA (gDNA)** – Purified gDNA already extracted from tissue or sample.

For More Information

For more information on:

- Preparing cDNA templates – Refer to the *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol*. The protocol is not shipped with the High-Capacity cDNA Reverse Transcription Kits. You can download the protocol from:

  http://docs.appliedbiosystems.com/search.taf
- Preparing RNA or gDNA templates – Refer to the protocol for the purification reagents that you select. To locate Applied Biosystems purification reagents, visit:

  http://www.appliedbiosystems.com/

Notes

Prepare the Sample Dilutions

Perform sample dilutions before adding the samples to the final reaction mix. Dilute the samples using the volumes that were calculated by the 7500 software ("Select the Sample Dilution Calculations tab." on page 128).

About the Example Experiment

For the comparative C_T example experiment:

- Sample dilutions are necessary because the sample volume is limited to 10% of the total reaction volume in the 7500 software. Because the total reaction volume is 50 µL/reaction, the sample volume is 5 µL/reaction.
- The stock sample concentration is 100 ng/µL. After diluting the sample according to the Sample Dilutions Calculations table, the sample has a concentration of 50 ng/µL. Adding 5 µL at this concentration to the final reaction mix volume of 50 µL yields a 1× concentration in the final reaction.
- The volumes calculated in the software are:

Sample Name	Stock Concentration (ng/µL)	Sample Volume (µL)	Diluent Volume (µL)	Total Volume of Diluted Sample (µL)
Brain	100.0	66.0	66.0	132.0
Kidney	100.0	66.0	66.0	132.0
Liver	100.0	66.0	66.0	132.0
Universal	100.0	66.0	66.0	132.0

Required Materials

- Water (to dilute the sample)
- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Sample stock
- Vortexer
- Centrifuge

Notes

Prepare the Sample Dilutions

1. Label a separate microcentrifuge tube for each diluted sample:
   - **Brain**
   - **Kidney**
   - **Liver**
   - **Universal**
2. Add the required volume of water (diluent) to each empty tube:

Tube	Sample Name	Diluent Volume (µL)
1	Brain	66.0
2	Kidney	66.0
3	Liver	66.0
4	Universal	66.0

3. Add the required volume of sample stock to each tube:

Tube	Sample Name	Sample Volume (µL)
1	Brain	66.0
2	Kidney	66.0
3	Liver	66.0
4	Universal	66.0

4. Vortex each diluted sample for 3 to 5 seconds, then centrifuge the tubes briefly.
5. Place the diluted samples on ice until you prepare the reaction plate.

Preparation Guidelines

When you prepare your own comparative C_T experiment:

- Sample dilutions may be necessary because the sample volume is limited to 10% of the total reaction volume in the 7500 software. You must perform the sample dilutions before adding the samples to the final reaction mix.
- For optimal performance of TaqMan® Gene Expression Assays or Custom TaqMan® Gene Expression Assays, use 10 to 100 ng of cDNA template per 20-µL reaction.
- Use TE buffer or water to dilute the sample.

For More Information

For more information on Applied Biosystems assays, refer to the:

- *TaqMan® Gene Expression Assays Protocol*
- *Custom TaqMan® Gene Expression Assays Protocol*.

Notes

Prepare the Reaction Mix

Prepare the reaction mix using the components and volumes that were calculated by the 7500 software ("Complete the Reaction Mix Calculations Tab for the Assays" on page 127).

Note: The software calculates the volumes for all components for the PCR reactions. However, when you prepare the reaction mix in this section, include only the master mix, assay mix, and water. Add the sample when you prepare the reaction plate (see "Prepare the Reaction Plate" on page 146).

About the Example Experiment

For the comparative C_T example experiment:

- The reaction mix components are:
  - TaqMan® Universal PCR Master Mix (2X)
  - 18S Assay Mix (20X)
  - EGR3 Assay Mix (20X)
  - MAOB Assay Mix (20X)
  - OGDH Assay Mix (20X)
  - OSGEP Assay Mix (20X)
  - SERPING1 Assay Mix (20X)
  - Water
- The volumes calculated in the software for the target assays are:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	25.0
Assay Mix (20.0X)	2.50
Water	17.50
Total Volume	45.0

Note: The sample is not added at this time.

Required Materials

- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Reaction mix components (listed above)
- Centrifuge

Notes

Prepare the Reaction Mix

IMPORTANT! Prepare the reaction mix for each target assay separately.

1. Label an appropriately sized tube for each reaction mix:
   - **18S Reaction Mix**
   - **EGR3 Reaction Mix**
   - **MAOB Reaction Mix**
   - **OGDH Reaction Mix**
   - **OSGEP Reaction Mix**
   - **SERPING1 Reaction Mix**
2. For the 18S assay, add the required components to the 18S Reaction Mix tube:

Component	Volume (µL) for 1 Reaction	Volume (µL) for 16 Reactions (Plus 10% Excess)
TaqMan® Universal PCR Master Mix (2X)	25.0	440.0
18S Assay Mix (20X)	2.5	44.0
Water	17.5	308.0
Total Reaction Mix Volume	45.0	792.0

3. Repeat step 2 for each remaining assay: EGR3, MAOB, OGDH, OSGEP, and SERPING1.
4. Mix the reaction mix in each tube by gently pipetting up and down, then cap each tube.
5. Centrifuge the tubes briefly to remove air bubbles.
6. Place the reaction mixes on ice until you prepare the reaction plate.

Preparation Guidelines

When you prepare your own comparative C_T experiment:

- If your experiment includes more than one target assay, prepare the reaction mix for each target assay separately.
- Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.
- Include all required components.
- Prepare the reagents according to the manufacturer's instructions.
- Keep the assay mix protected from light (in the freezer) until you are ready to use it. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Mix the master mix thoroughly by swirling the bottle.
  - Resuspend the assay mix by vortexing, then centrifuge the tube briefly.
  - Thaw any frozen samples by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.

Notes

For More Information

For more information on preparing the reaction mix, refer to the protocol appropriate for the reagents you are using in the PCR reactions:

- *TaqMan® Gene Expression Assays Protocol*
- *Custom TaqMan® Gene Expression Assays Protocol*

Prepare the Reaction Plate

Prepare the reactions for each replicate group, then transfer them to the reaction plate. Use the plate layout displayed in the 7500 software.

About the Example Experiment

For the comparative C_T example experiment:

- A MicroAmp® Optical 96-Well Reaction Plate is used.
- The reaction volume is 50 µL/well.
- The reaction plate contains:
  - 96 Unknown wells U
  - 0 Negative Control wells N
  - 0 Empty wells
- The plate layout that is automatically generated by the 7500 software is used:

	1	2	3	4	5	6	7	8	9	10	11	12
A	U Brain 18S	U Brain MAOB	U Brain OSGEP	U Kidney 18S	U Kidney MAOB	U Kidney OSGEP	U Liver 18S	U Liver MAOB	U Liver OSGEP	U Universal 18S	U Universal MAOB	U Universal OSGEP
B	U Brain 18S	U Brain MAOB	U Brain OSGEP	U Kidney 18S	U Kidney MAOB	U Kidney OSGEP	U Liver 18S	U Liver MAOB	U Liver OSGEP	U Universal 18S	U Universal MAOB	U Universal OSGEP
C	U Brain 18S	U Brain MAOB	U Brain OSGEP	U Kidney 18S	U Kidney MAOB	U Kidney OSGEP	U Liver 18S	U Liver MAOB	U Liver OSGEP	U Universal 18S	U Universal MAOB	U Universal OSGEP
D	U Brain 18S	U Brain MAOB	U Brain OSGEP	U Kidney 18S	U Kidney MAOB	U Kidney OSGEP	U Liver 18S	U Liver MAOB	U Liver OSGEP	U Universal 18S	U Universal MAOB	U Universal OSGEP
E	U Brain EGR3	U Brain OGDH	U Brain SERPI...	U Kidney EGR3	U Kidney OGDH	U Kidney SERPI...	U Liver EGR3	U Liver OGDH	U Liver SERPI...	U Universal EGR3	U Universal OGDH	U Universal SERPI...
F	U Brain EGR3	U Brain OGDH	U Brain SERPI...	U Kidney EGR3	U Kidney OGDH	U Kidney SERPI...	U Liver EGR3	U Liver OGDH	U Liver SERPI...	U Universal EGR3	U Universal OGDH	U Universal SERPI...
G	U Brain EGR3	U Brain OGDH	U Brain SERPI...	U Kidney EGR3	U Kidney OGDH	U Kidney SERPI...	U Liver EGR3	U Liver OGDH	U Liver SERPI...	U Universal EGR3	U Universal OGDH	U Universal SERPI...
H	U Brain EGR3	U Brain OGDH	U Brain SERPI...	U Kidney EGR3	U Kidney OGDH	U Kidney SERPI...	U Liver EGR3	U Liver OGDH	U Liver SERPI...	U Universal EGR3	U Universal OGDH	U Universal SERPI...

Required Materials

- Microcentrifuge tubes
- Pipettors
- Pipette tips
- Reaction mixes (from page 145)
- Water
- Samples (from page 143)
- MicroAmp® Optical 96-Well Reaction Plate
- MicroAmp™ Optical Adhesive Film
- Centrifuge

Notes

Prepare the Reaction Plate

1. For each replicate group, prepare the reactions for the unknowns:

   a. To appropriately sized tubes, add the volumes of reaction mix and sample listed below.

Tube	Unknown Reaction	Reaction Mix	Reaction Mix Volume (µL)	Sample	Sample Volume (µL)
1	18S Brain	18S Reaction Mix	198.0	Liver	22.0
2	18S Kidney	18S Reaction Mix	198.0	Kidney	22.0
3	18S Liver	18S Reaction Mix	198.0	Brain	22.0
4	18S Universal	18S Reaction Mix	198.0	Universal	22.0
5	EGR3 Brain	EGR3 Reaction Mix	198.0	Liver	22.0
6	EGR3 Kidney	EGR3 Reaction Mix	198.0	Kidney	22.0
7	EGR3 Liver	EGR3 Reaction Mix	198.0	Brain	22.0
8	EGR3 Universal	EGR3 Reaction Mix	198.0	Universal	22.0
9	MAOB Brain	MAOB Reaction Mix	198.0	Liver	22.0
10	MAOB Kidney	MAOB Reaction Mix	198.0	Kidney	22.0
11	MAOB Liver	MAOB Reaction Mix	198.0	Brain	22.0
12	MAOB Universal	MAOB Reaction Mix	198.0	Universal	22.0
13	OGDH Brain	OGDH Reaction Mix	198.0	Liver	22.0
14	OGDH Kidney	OGDH Reaction Mix	198.0	Kidney	22.0
15	OGDH Liver	OGDH Reaction Mix	198.0	Brain	22.0
16	OGDH Universal	OGDH Reaction Mix	198.0	Universal	22.0
17	OSGEP Brain	OSGEP Reaction Mix	198.0	Liver	22.0
18	OSGEP Kidney	OSGEP Reaction Mix	198.0	Kidney	22.0
19	OSGEP Liver	OSGEP Reaction Mix	198.0	Brain	22.0
20	OSGEP Universal	OSGEP Reaction Mix	198.0	Universal	22.0
21	SERPING1 Brain	SERPING1 Reaction Mix	198.0	Liver	22.0
22	SERPING1 Kidney	SERPING1 Reaction Mix	198.0	Kidney	22.0
23	SERPING1 Liver	SERPING1 Reaction Mix	198.0	Brain	22.0
24	SERPING1 Universal	SERPING1 Reaction Mix	198.0	Universal	22.0

   b. Mix the reactions by gently pipetting up and down, then cap the tubes.

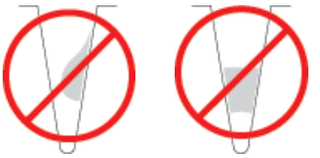
   c. Centrifuge the tubes briefly to remove air bubbles.

   d. Add 50 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

Notes

2. Seal the reaction plate with optical adhesive film.
3. Centrifuge the reaction plate briefly to remove air bubbles.
4. Verify that the liquid is at the bottom of each well of the reaction plate. If not, centrifuge the reaction plate again at a higher speed and for a longer period of time.

IMPORTANT! Do not allow the bottom of the reaction plate to become dirty. Fluids and other contaminants that adhere to the bottom of the reaction plate can contaminate the sample block and cause an abnormally high background signal.

Correct	Incorrect
	
Liquid is at the bottom of the well.	• Not centrifuged with enough force, *or* • Not centrifuged for enough time

5. Place the reaction plate on ice in the dark until you are ready to perform the run.

Preparation Guidelines

When you prepare your own comparative C_T experiment:

- Make sure you use the appropriate consumables.
- Make sure the arrangement of the PCR reactions matches the plate layout displayed in the 7500 software. You can either:
  - Accept the plate layout automatically generated by the software.

    or
  - Use Advanced Setup to change the plate layout in the software.
- If you use optical adhesive film, seal each reaction plate as follows:

  a. Place the reaction plate onto the center of the 96-well base.

  b. Load the reaction plate as desired.

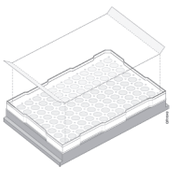
  c. Remove a single optical adhesive film (film) from the box. Fold back one of the end-tabs. Hold the film with its backing side up.

  d. In one continuous movement, peel back the white protective backing from the center sealing surface. Do not touch the center sealing surface.

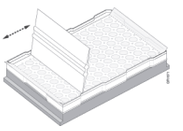
  IMPORTANT! Improper peeling of the optical adhesive film may result in haziness, but does not affect results. Haziness disappears when the film comes into contact with the heated cover in the instrument.

Notes

e. While holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Be sure the film completely covers all wells of the reaction plate.

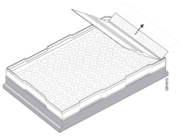


f. While applying firm pressure, move the applicator slowly across the film, horizontally and vertically, to ensure good contact between the film and the entire surface of the reaction plate.



g. While using the applicator to hold the edge of the film in place, grasp one end of the end-tab and pull up and away sharply. Repeat for the other end-tab.

h. Repeat step f to ensure a tight, evaporation-free seal. While applying firm pressure, run the edge of the applicator along all four sides of the outside border of the film.



Note: Optical adhesive films do not adhere on contact. The films require the application of pressure to ensure a tight seal.

i. Inspect the reaction plate to be sure all wells are sealed. You should see an imprint of all wells on the surface of the film.

For More Information

For more information on:

- Preparing the reaction plate – Refer to the protocol appropriate for the reagents you are using in the PCR reactions:
  - *TaqMan® Gene Expression Assays Protocol*
  - *Custom TaqMan® Gene Expression Assays Protocol*
- Consumables – See “Supported Consumables” on page 4.
- Using Advanced Setup to change the plate layout – See “Advanced Setup Workflow” on page 204.

Notes

Notes

8 Run the Comparative C_T Experiment

This chapter covers:

- Chapter Overview 152
- Prepare for the Run 153
- Run the Experiment 154

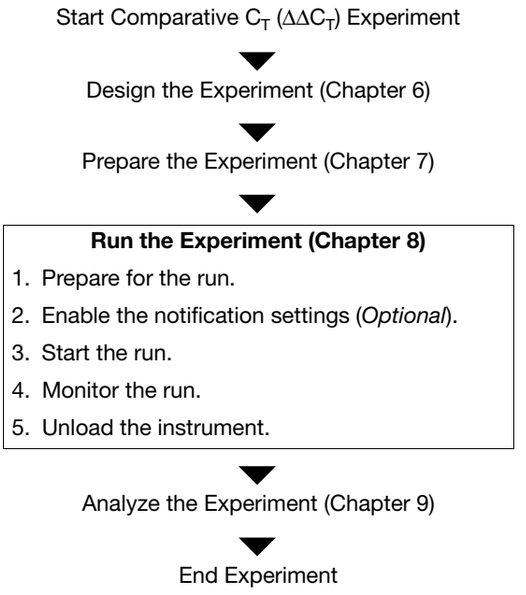
Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

Notes

Chapter Overview

This chapter explains how to perform a run on the Applied Biosystems 7500/7500 Fast Real-Time PCR Systems.

Example Experiment Workflow



Notes

Prepare for the Run

Prepare for the run by opening the example experiment file you created in Chapter 6, then loading the sealed reaction plate into the 7500/7500 Fast instrument.

Open the Example Experiment

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

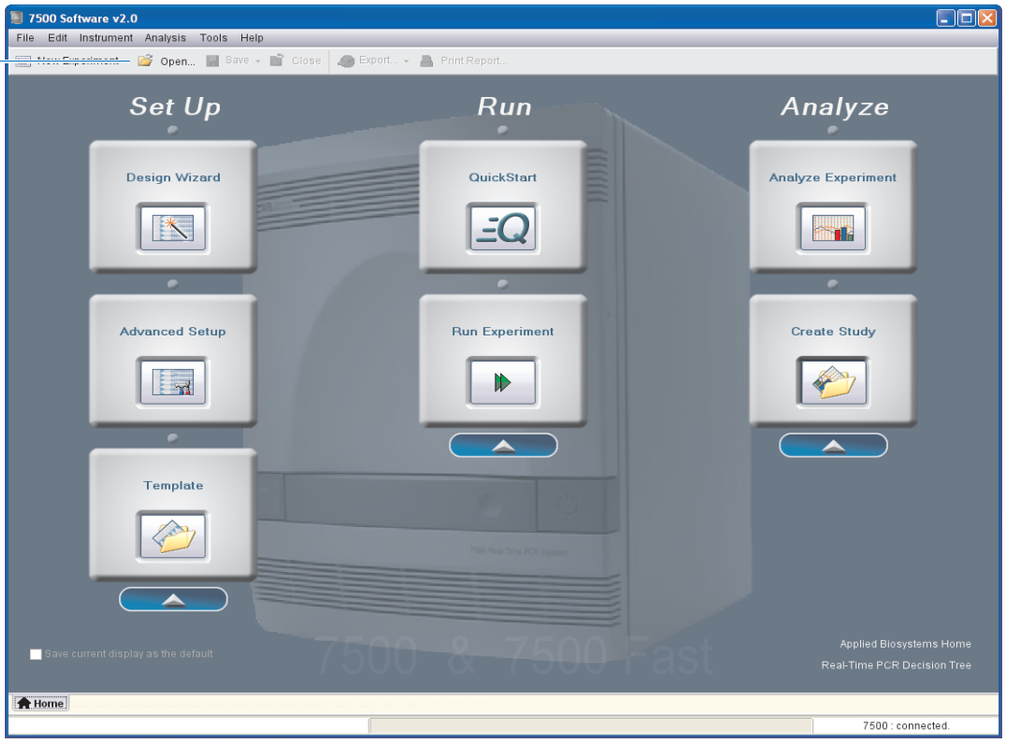
   where *<software name>* is the current version of the 7500 software.

2. In the Home screen, click **Open**.

3. In the Open dialog box, navigate to the **experiments** folder (default) at:

   <drive>:\Applied Biosystems*<software name>*\experiments

4. Double-click the example experiment file that you created in Chapter 6.



Notes

Run the Experiment

Perform the following procedures in Chapter 4 for the comparative C_T experiment:

- “Load the Reaction Plate into the Instrument” on page 68
- “Enable the Notification Settings (Optional)” on page 69
- “Start the Run” on page 71
- “Monitor the Run” on page 72
- “Unload the Instrument” on page 74

Notes

9 Analyze the Comparative C_T Experiment

This chapter covers:

- Chapter Overview 156

Section 9.1 Review Results 157

- Analyze the Experiment 158
- View the Gene Expression Plot and Well Table 162
- View the Amplification Plot 166
- Publish the Data 174

Section 9.2 Troubleshoot (If Needed) 175

- View the Analysis Settings 176
- View the QC Summary 179
- Omit Wells from the Analysis 181
- View the Multicomponent Plot 182
- View the Raw Data Plot 185

Section 9.3 Perform a Study of Multiple Experiments 187

- Section Overview 188
- Design a Study 189
- Analyze the Study 195
- Publish the Data 202

Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

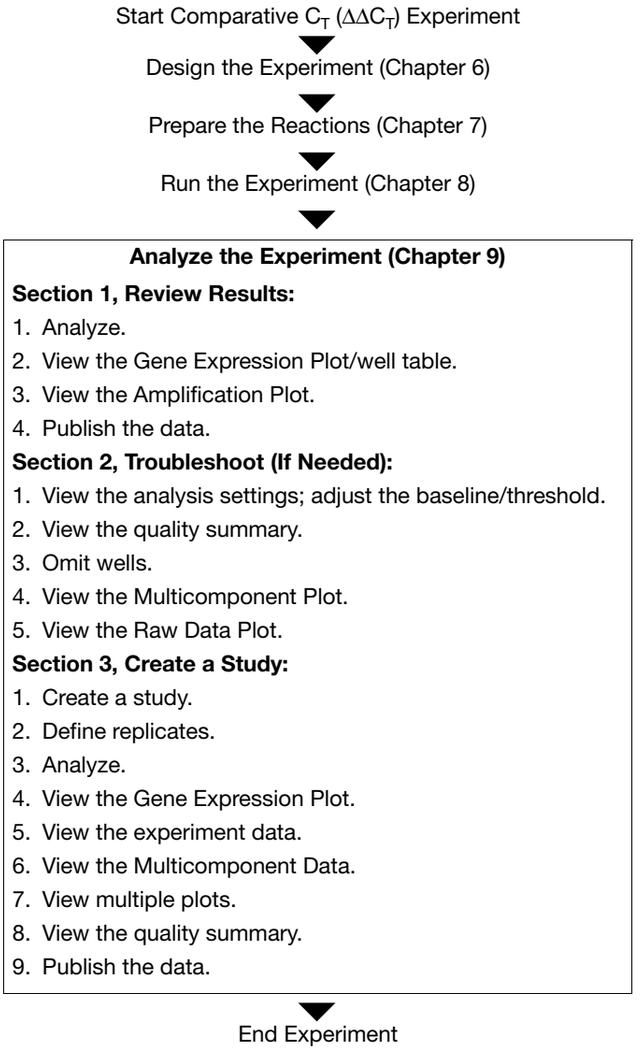
Notes

Chapter Overview

The 7500 software analyzes the data using the comparative C_T ($\Delta\Delta C_T$) quantitation method. This chapter includes the following sections:

- Section 9.1 explains how to review the analyzed data using several of the analysis screens and how to publish the data.
- Section 9.2 explains how to perform some troubleshooting steps if you receive questionable results.
- Section 9.3 explains how to analyze multiple experiments as a study.

Example Experiment Workflow



Notes

Section 9.1 Review Results

This section covers:

- Analyze the Experiment 158
- View the Gene Expression Plot and Well Table 162
- View the Amplification Plot 166
- Publish the Data 174

Notes

Analyze the Experiment

The 7500 software analyzes the experiment and displays results in the analysis screens (for example, the Amplification Plot screen, QC Summary screen, and so on).

About the Example Experiment

For the comparative C_T example experiment, use the data file that is installed with the 7500 software. This data file was created with the same design parameters that are provided in Chapter 6, then run and analyzed on a 7500/7500 Fast instrument.

The data file for the example experiment is on your computer at:

<drive>:\Applied Biosystems*<software name>*\experiments\
Comparative Ct Example.eds

where:

- *<drive>* is the computer hard drive on which the 7500 software is installed.
- *<software name>* is the current version of the 7500 software.

Analyze the Example Experiment

1. Double-click (7500 software) or select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **7500 Software** ▸ ***<software name>***

   where *<software name>* is the current version of the 7500 software.

2. In the Home screen, click **Open**.

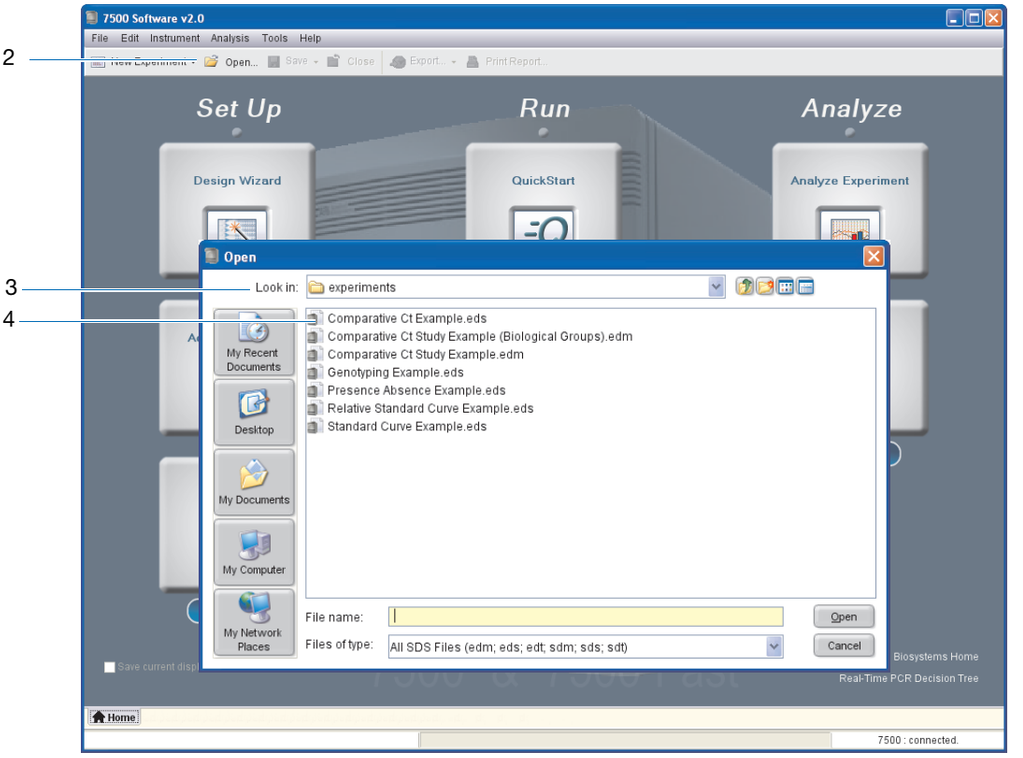
3. In the Open dialog box, navigate to the **experiments** folder at:

   <drive>:\Applied Biosystems*<software name>*\experiments\

4. Double-click **Comparative Ct Example.eds** to open the example experiment data file.

   Note: The examples folder contains several data files; be sure to select **Comparative Ct Example.eds**.

Notes



5. Click **Analyze**. The software analyzes the data using the default analysis settings.
6. See "Navigation Tips" on page 160 for information on navigating within the analysis screens.

Guidelines

When you analyze your own comparative C_T experiment:

- Immediately after a run, the 7500 software automatically analyzes the data using the default analysis settings, then displays the Amplification Plot screen on your computer.
- To reanalyze the data, select all the wells in the plate layout, then click **Analyze**.

Notes

Navigation Tips

How to Select Wells

To display specific wells in the analysis screens, select the wells in the View Plate Layout tab as follows:

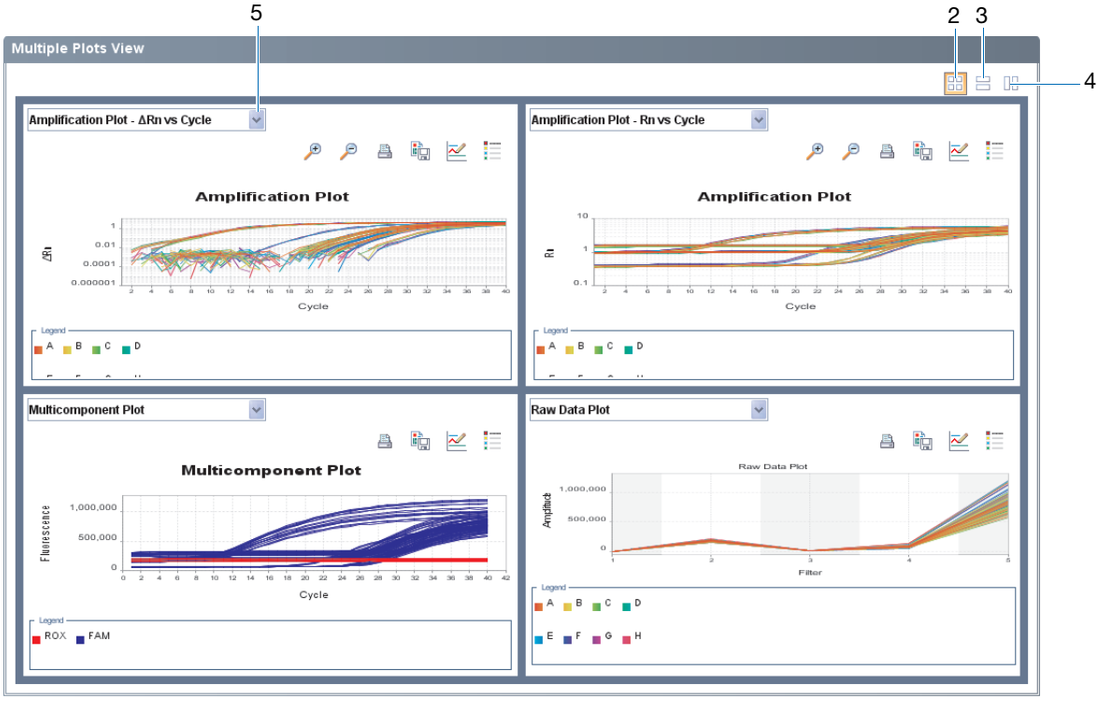
1. To select wells of a specific type, use the Select Wells With drop-down lists, select **Sample**, **Target**, or **Task**, then select the sample, target, or task name.
2. To select a single well, click the well in the plate layout.
3. To select multiple wells, click-drag over the desired wells, or **CTRL+click** or **Shift+click** the desired wells in the plate layout.
4. To select all 96 wells, click the upper left corner of the plate layout.

Notes

How to Display Multiple Plots

Use the Multiple Plots view to display up to four plots simultaneously. To navigate within the Multiple Plots view:

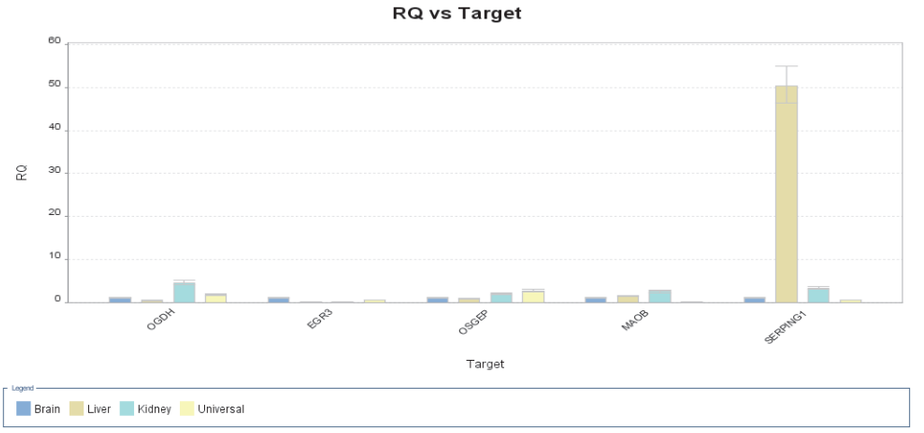
1. In the navigation pane, select **Analysis** ▸ **Multiple Plots View**.
2. To display four plots, click (Show plots in a 2 5 2 matrix).
3. To display two plots in rows, click (Show plots in two rows).
4. To display two plots in columns, click (Show plots in two columns).
5. To display a specific plot, select the plot in the drop-down list above each plot display.



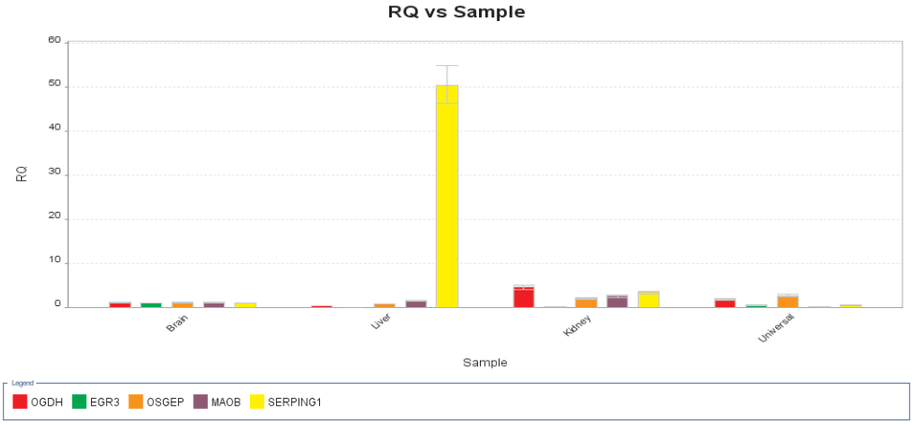
View the Gene Expression Plot and Well Table

Displays the results of relative quantitation calculations in the gene expression profile. Two plots are available:

- **RQ vs Target** – Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. You can display the plot on a linear, log10, Ln, or log2 scale.



- **RQ vs Sample** – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample.You can display the plot on a linear, log10, Ln, or log2 scale.



The Well Table displays data for each well in the reaction plate, including:

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

About the Example Experiment

In the comparative C_T example experiment, you review:

- Each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference sample.
- The Well Table to evaluate the precision of the replicate groups.

Notes

View the Plot and Well Table

1. In the navigation pane, select **Analysis** ▸ **Gene Expression**.

   Note: If no data are displayed, click **Analyze**.

2. In the Gene Expression Plot screen:
   a. In the Plot Type drop-down list, select **RQ vs Sample**.
   b. In the Graph Type drop-down list, select **Log10**.
   c. In the Orientation drop-down list, select **Vertical Bars**.

3. Click (Show a legend for the plot).

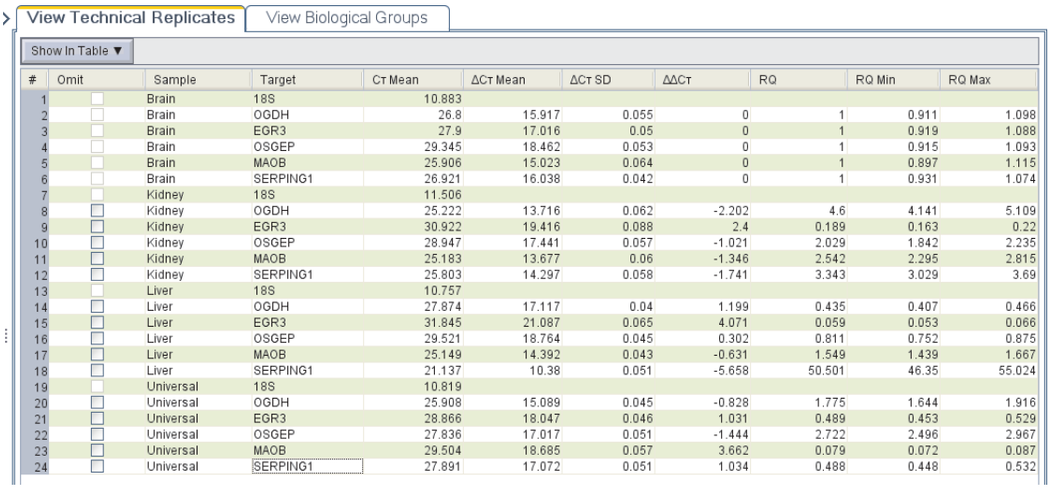
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

   In the example experiment, the expression of EGR3, MAOB, OGDH, OSGEP, and SERPING1 in the liver, kidney, and universal samples are displayed relative to the expression in the reference sample (brain). Because the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).

Notes

4. Click < at the top left of the View Technical Replicates tab.

   The figure below shows the technical replicates table.



View Technical Replicates | View Biological Groups

Show In Table ▼

#	Omit	Sample	Target	CT Mean	ΔCT Mean	ΔCT SD	ΔΔCT	RQ	RQ Min	RQ Max
1		Brain	18S	10.883						
2		Brain	OGDH	26.8	15.917	0.055	0	1	0.911	1.098
3		Brain	EGR3	27.9	17.016	0.05	0	1	0.919	1.088
4		Brain	OSGEP	29.345	18.462	0.053	0	1	0.915	1.093
5		Brain	MAOB	25.906	15.023	0.064	0	1	0.897	1.115
6		Brain	SERPING1	26.921	16.038	0.042	0	1	0.931	1.074
7		Kidney	18S	11.506						
8	☐	Kidney	OGDH	25.222	13.716	0.062	-2.202	4.6	4.141	5.109
9	☐	Kidney	EGR3	30.922	19.416	0.088	2.4	0.189	0.163	0.22
10	☐	Kidney	OSGEP	28.947	17.441	0.057	-1.021	2.029	1.842	2.235
11	☐	Kidney	MAOB	25.183	13.677	0.06	-1.346	2.542	2.295	2.815
12	☐	Kidney	SERPING1	25.803	14.297	0.058	-1.741	3.343	3.029	3.69
13		Liver	18S	10.757						
14	☐	Liver	OGDH	27.874	17.117	0.04	1.199	0.435	0.407	0.466
15	☐	Liver	EGR3	31.845	21.087	0.065	4.071	0.059	0.053	0.066
16	☐	Liver	OSGEP	29.521	18.764	0.045	0.302	0.811	0.752	0.875
17	☐	Liver	MAOB	25.149	14.392	0.043	-0.631	1.549	1.439	1.667
18	☐	Liver	SERPING1	21.137	10.38	0.051	-5.658	50.501	46.35	55.024
19		Universal	18S	10.819						
20	☐	Universal	OGDH	25.908	15.089	0.045	-0.828	1.775	1.644	1.916
21	☐	Universal	EGR3	28.866	18.047	0.046	1.031	0.489	0.453	0.529
22	☐	Universal	OSGEP	27.836	17.017	0.051	-1.444	2.722	2.496	2.967
23	☐	Universal	MAOB	29.504	18.685	0.057	3.662	0.079	0.072	0.087
24	☐	Universal	SERPING1	27.891	17.072	0.051	1.034	0.488	0.448	0.532

Column	Description
Omit	For all technical replicates that evaluate the associated sample/target combination: • A check mark (✓) indicates that all replicates have been removed from the analysis. • A hyphen (–) indicates that one or more replicates have been removed from the analysis.
Sample	The sample associated with the data displayed in the row.
Target	The target assay associated with the data displayed in the row.
C_T Mean	The arithmetic average of the technical replicate C_T values.
ΔC_T Mean	The arithmetic average of the technical replicate C_T values for the sample replicate group.
ΔC_T SD	The sample standard deviation of the sample replicate group level C_T values.
$\Delta\Delta C_T$	The calculated $\Delta\Delta C_T$ value for the replicate group associated with the test sample.
RQ	The calculated relative level of gene expression for the replicate group associated with the test sample.
RQ Min	The minimum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	The maximum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.

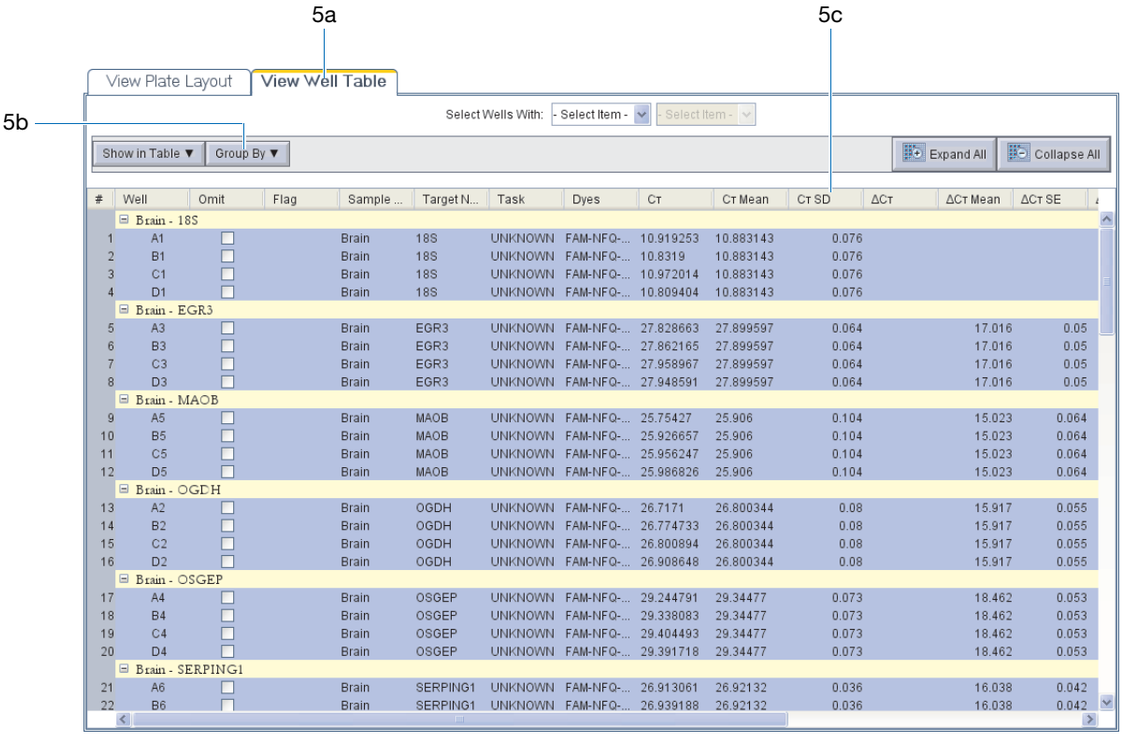
Notes

5. View the Well Table:

   a. In the navigation pane, select **Analysis** ▸ **Amplification Plot**, then select the **View Well Table** tab.

   b. In the Group By drop-down list, select **Replicate**.

   c. Look at the C_T SD column to evaluate the precision of the replicate groups. In the example experiment, there are no outliers.



#	Well	Omit	Flag	Sample ...	Target N...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Mean	ΔCT SE
	Brain - 18S												
1	A1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.919253	10.883143	0.076			
2	B1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.8319	10.883143	0.076			
3	C1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.972014	10.883143	0.076			
4	D1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.809404	10.883143	0.076			
	Brain - EGR3												
5	A3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.828663	27.899597	0.064	17.016		0.05
6	B3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.862165	27.899597	0.064	17.016		0.05
7	C3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.958967	27.899597	0.064	17.016		0.05
8	D3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.948591	27.899597	0.064	17.016		0.05
	Brain - MAOB												
9	A5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.75427	25.906	0.104	15.023		0.064
10	B5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.926657	25.906	0.104	15.023		0.064
11	C5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.956247	25.906	0.104	15.023		0.064
12	D5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.986826	25.906	0.104	15.023		0.064
	Brain - OGDH												
13	A2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.7171	26.800344	0.08	15.917		0.055
14	B2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.774733	26.800344	0.08	15.917		0.055
15	C2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.800894	26.800344	0.08	15.917		0.055
16	D2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.908648	26.800344	0.08	15.917		0.055
	Brain - OSGEP												
17	A4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.244791	29.34477	0.073	18.462		0.053
18	B4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.338083	29.34477	0.073	18.462		0.053
19	C4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.404493	29.34477	0.073	18.462		0.053
20	D4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.391718	29.34477	0.073	18.462		0.053
	Brain - SERPING1												
21	A6			Brain	SERPING1	UNKNOWN	FAM-NFQ-...	26.913061	26.92132	0.036	16.038		0.042
22	B6			Brain	SERPING1	UNKNOWN	FAM-NFQ-...	26.939188	26.92132	0.036	16.038		0.042

Note: To show/hide columns in the Well Table, select/deselect the column name in the Show in Table drop-down list.

Analysis Guidelines

When you analyze your own comparative C_T experiment, look for:

- Differences in gene expression (as a fold change) relative to the reference sample.
- Standard deviation in the replicate groups (C_T SD values). If needed, omit outliers (see "Omit Wells from the Analysis" on page 181).

For More Information

For more information on the Gene Expression Plot screen or Well Table, open the 7500 Software Help by clicking or pressing **F1**.

Notes

View the Amplification Plot

The Amplification Plot screen displays amplification of all samples in the selected wells. Three plots are available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence generated by the reporter at each cycle during the PCR amplification. This plot displays ΔRn as a function of cycle number. You can use this plot to identify and examine irregular amplification and to view threshold and baseline values for the run.
- **Rn vs Cycle** – Rn is the fluorescence from the reporter dye normalized to the fluorescence from the passive reference. This plot displays Rn as a function of cycle number. You can use this plot to identify and examine irregular amplification.
- **C_T vs Well** – C_T is the PCR cycle number at which the fluorescence meets the threshold in the amplification plot. This plot displays C_T as a function of well position. You can use this plot to locate outlying amplification (outliers).

Each plot can be displayed as a linear or log10 graph.

About the Example Experiment

In the comparative C_T example experiment, you review each target in the Amplification Plot for:

- Correct baseline and threshold values
- Outliers

Notes

View the Plot

1. In the navigation pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the 18S wells in the Amplification Plot screen:
   a. Select the **View Plate Layout** tab.
   b. In the Select Wells With drop-down lists, select **Target**, then **18S**.

3. In the Amplification Plot screen, Plot Settings:
   a. In the Plot Type drop-down list, select **ΔRn vs Cycle** (default).
   b. In the Plot Color drop-down list, select **Well** (default).
   c. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

Notes

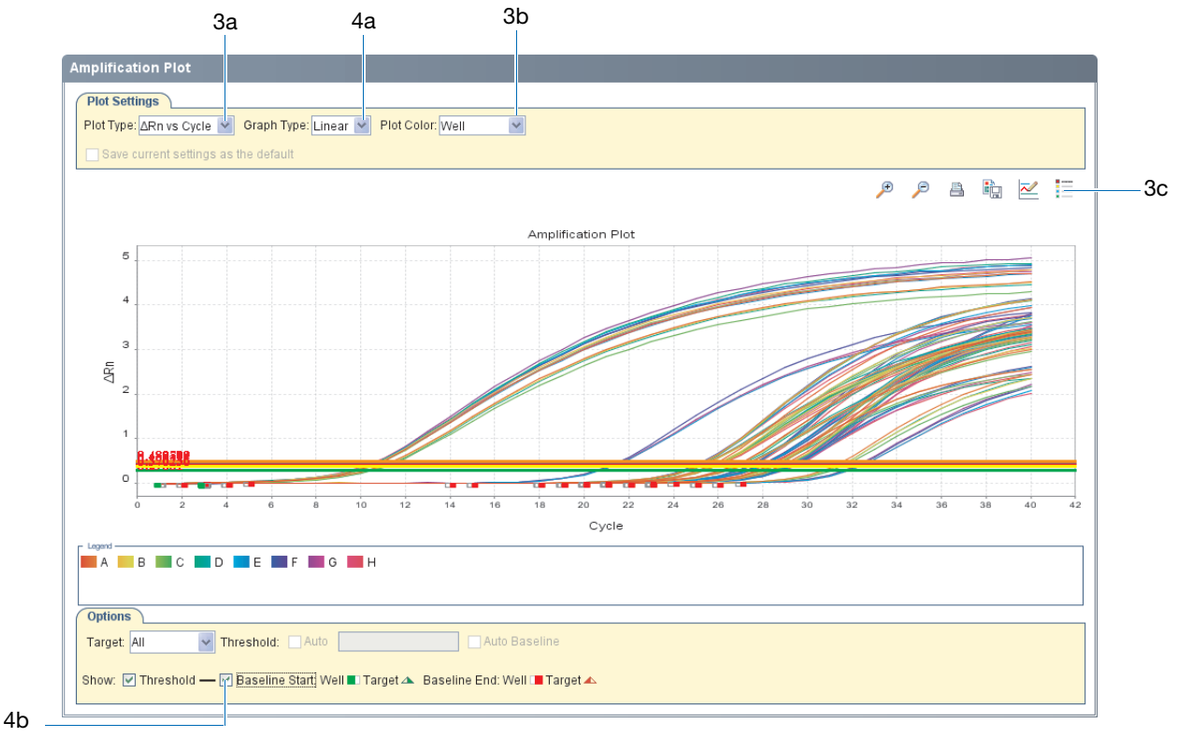
4. View the baseline values (Plot Settings tab):

   a. In the Graph Type drop-down list, select **Linear**.

   b. Select the Baseline Start check box to show the start cycle and end cycle.

   c. Verify that the baseline is set correctly: The end cycle should be set a few cycles before the cycle number where significant florescence is detected. In the example experiment, the baseline is set correctly.

Note: By default, the Amplification Plot displays the data for all targets present in the experiment. Consequently, the plot overlays the baseline and threshold labels for all targets until you select an individual target from the Target drop-down list.



Notes

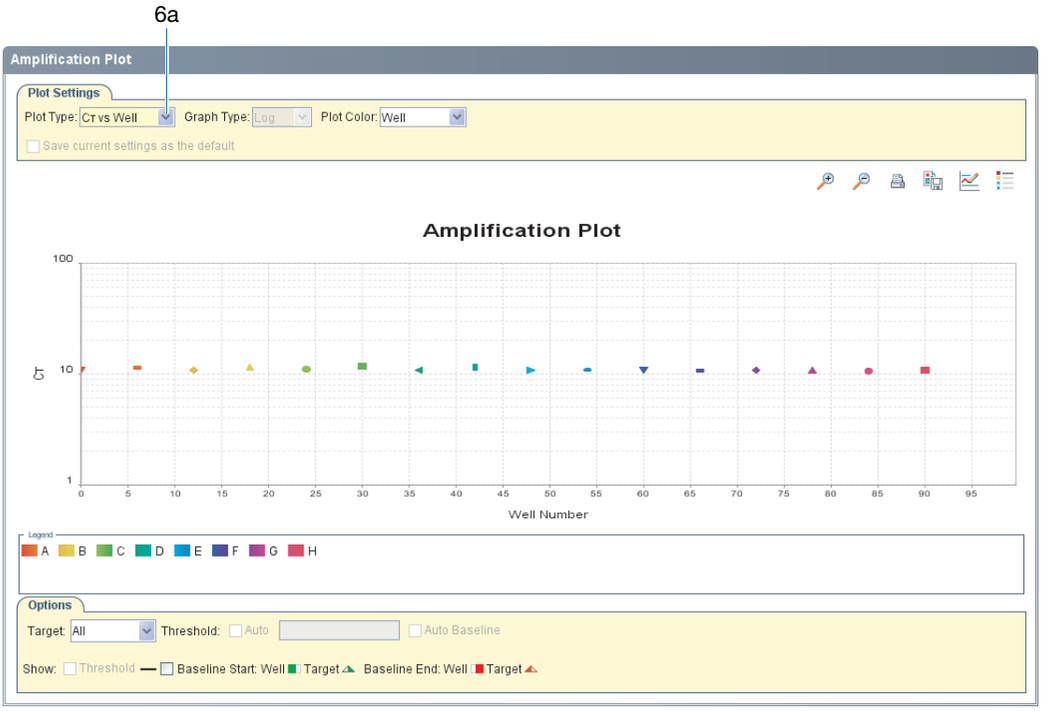
5. View the threshold values:
   a. In Plot Settings tab, select **Log** in the Graph Type drop-down list.
   b. Select **18S** in the Target drop-down list.
   c. In the Options tab, select the **Threshold** check box to show the threshold.
   d. Verify that the threshold is set correctly. The example experiment contains multiple thresholds, one for each target assay. The thresholds for all targets occur in the exponential phase.

Notes

6. Locate any outliers:

   a. In Plot Settings tab, select **C_T vs Well** in the Plot Type drop-down list.

   b. Look for outliers in the amplification plot. In the example experiment, there are no outliers for 18S.



7. Repeat steps 2 through 6 for the EGR3, MAOB, OGDH, OSGEP, and SERPING1 wells. In the example experiment, there are no outliers for any of the targets.

Notes

Analysis Guidelines

When you analyze your own comparative C_T experiment, look for:

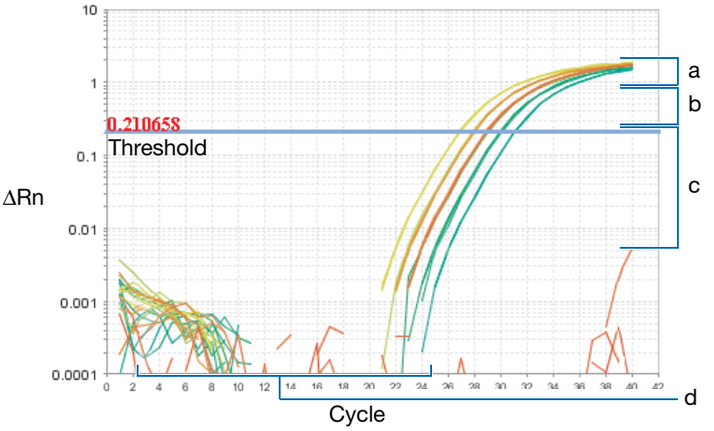
- Outliers
- A typical amplification plot – The 7500 software automatically calculates baseline and threshold values based on the assumption that the data exhibit a *typical* amplification plot. A typical amplification plot has four distinct sections:

  a. Plateau phase

  b. Linear phase

  c. Exponential (geometric phase)

  d. Baseline



IMPORTANT! Experimental error (such as contamination or pipetting errors) can produce atypical amplification curves that can result in incorrect baseline and threshold value calculations by the 7500 software. Therefore, Applied Biosystems recommends that you examine the Amplification Plot screen and review the assigned baseline and threshold values for each well after analysis is complete.

- Correct baseline and threshold values – See the "Threshold Examples" on page 172 and "Baseline Examples" on page 173.

Notes

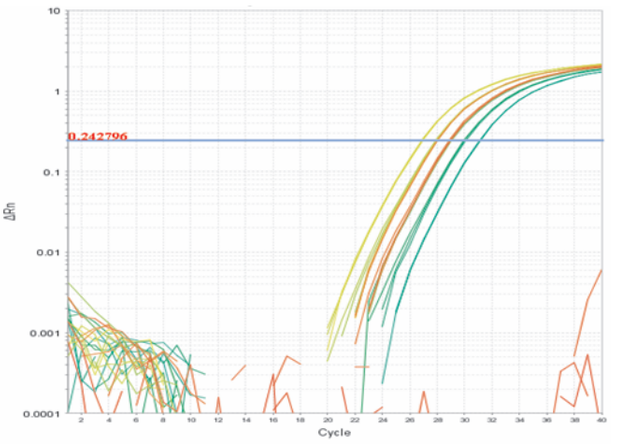
Threshold Examples

Threshold Set Correctly

The threshold is set in the exponential phase of the amplification curve.

Threshold settings above or below the optimum increase the standard deviation of the replicate groups.

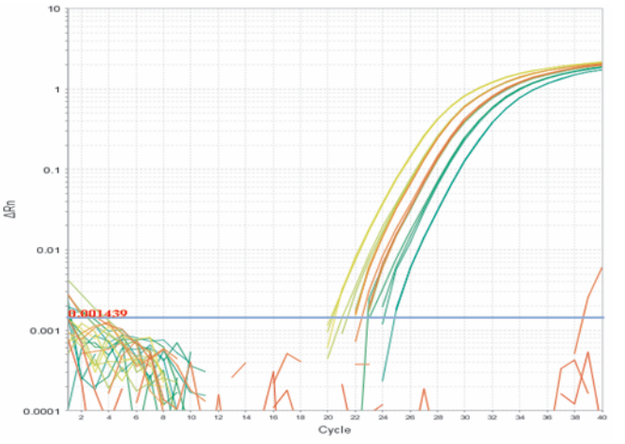
Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...	Comm
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
UNKNOWN	FAM-NFQ-...	28.96287	28.923796	0.074	2,484.31	2,551.476	126.2	
UNKNOWN	FAM-NFQ-...	28.838797	28.923796	0.074	2,697.054	2,551.476	126.2	
UNKNOWN	FAM-NFQ-...	28.96972	28.923796	0.074	2,473.064	2,551.476	126.2	



Threshold Set Too Low

The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the exponential phase of the curve.

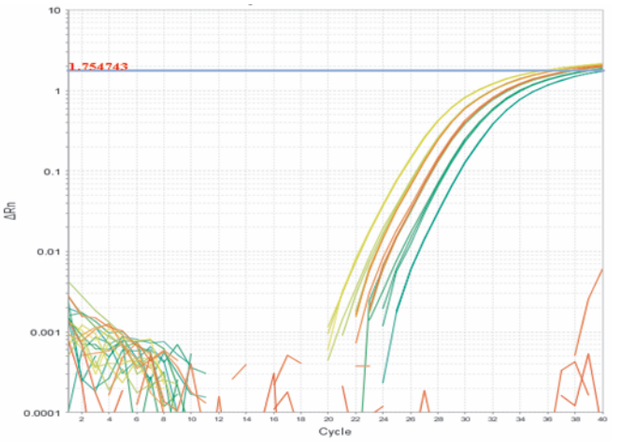
Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	38.453182					
UNKNOWN	FAM-NFQ-...	22.85404	22.761744	0.252	2,314.852	2,472.463	400.435
UNKNOWN	FAM-NFQ-...	22.476973	22.761744	0.252	2,927.722	2,472.463	400.435
UNKNOWN	FAM-NFQ-...	22.954218	22.761744	0.252	2,174.816	2,472.463	400.435



Threshold Set Too High

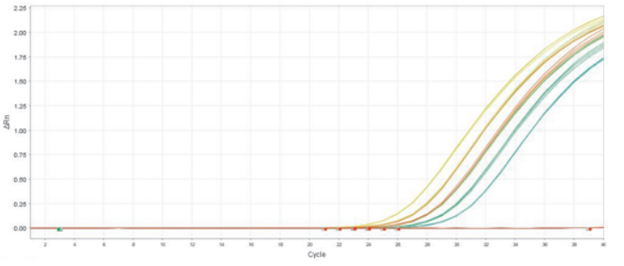
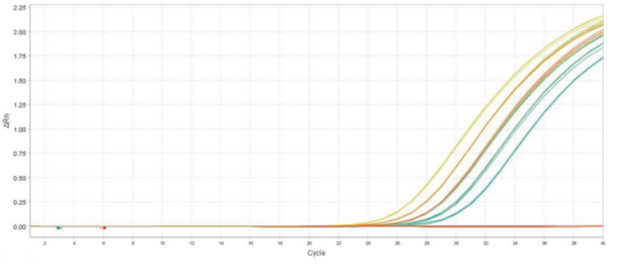
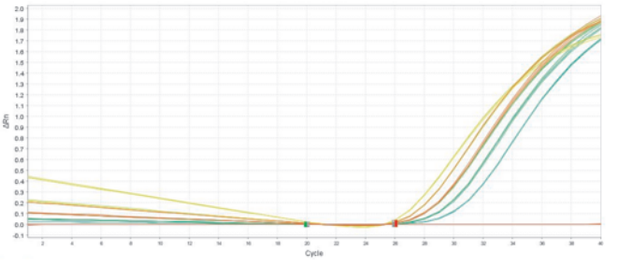
The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.

Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...	Comme
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
UNKNOWN	FAM-NFQ-...	37.681107	37.49507	0.202	2,571.177	2,888.429	353.775	
UNKNOWN	FAM-NFQ-...	37.27971	37.49507	0.202	3,269.923	2,888.429	353.775	
UNKNOWN	FAM-NFQ-...	37.524395	37.49507	0.202	2,824.187	2,888.429	353.775	



Notes

Baseline Examples

Baseline Set Correctly The amplification curve begins after the maximum baseline.	
Baseline Set Too Low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline Set Too High The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

If your experiment does not meet the guidelines above, troubleshoot as follows:

- Omit wells (see "Omit Wells from the Analysis" on page 181).

  or

- Manually adjust the baseline and/or threshold (see "View the Analysis Settings" on page 176).

For More Information

For more information on the Amplification Plot screen, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Publish the Data

You can publish the experiment data in several ways:

- Save the plot as an image file
- Print the plot
- Print the reaction plate layout
- Create slides
- Print a report
- Export data to applications such as Microsoft® Excel® and Microsoft® PowerPoint®.

For information on performing these procedures, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Section 9.2 Troubleshoot (If Needed)

This section covers:

- View the Analysis Settings 176
- View the QC Summary 179
- Omit Wells from the Analysis 181
- View the Multicomponent Plot 182
- View the Raw Data Plot 185

Notes

View the Analysis Settings

The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, relative quantitation, and advanced options. If the default analysis settings in the 7500 software are not suitable for your experiment, you can change the settings in the Analysis Settings dialog box, then reanalyze your experiment.

About the Example Experiment

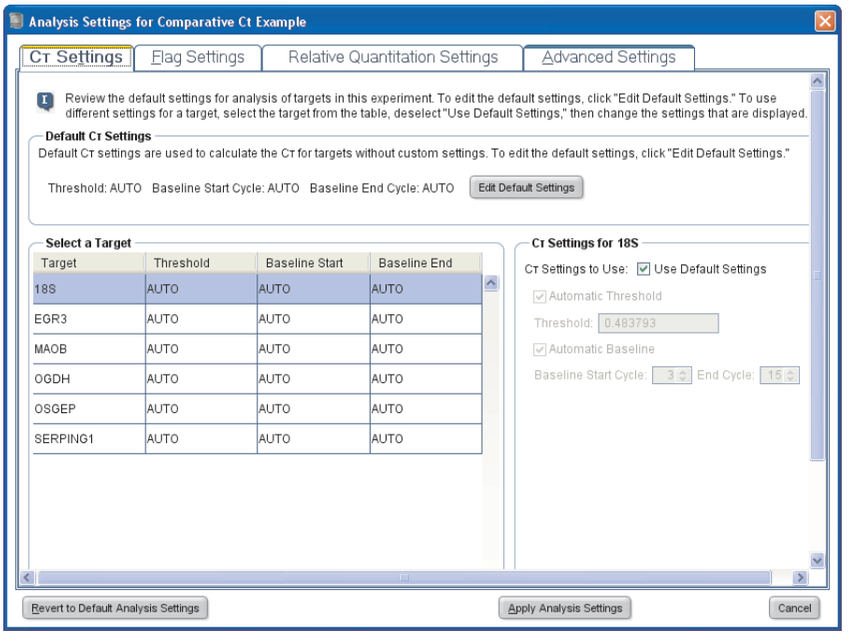
In the comparative C_T example experiment, the default analysis settings are used without changes.

View the Settings

1. In the navigation pane, select **Analysis**.
2. Click **Analysis Settings** to open the Analysis Settings dialog box.

   In the example experiment, the default analysis settings are used from each of the following tabs:

   - C_T Settings
   - Flag Settings
   - Relative Quantitation Settings
   - Advanced Settings



3. Click **Apply Analysis Settings**.

Notes

Analysis Guidelines

Unless you have already determined the optimal settings for your experiment, use the default analysis settings in the 7500 software. If the default settings are not suitable for your experiment, you can change the:

- **C_T Settings** – Use this tab to manually set the threshold and baseline. When manually setting the threshold and baseline, Applied Biosystems recommends the following:

Setting	Recommendation
Threshold	Enter a value for the threshold so that the threshold is: • Above the background. • Below the plateau and linear regions of the amplification curve. • Within the exponential phase of the amplification curve.
Baseline	Select the Start Cycle and End Cycle values so that the baseline ends before significant fluorescent signal is detected.

- **Flag Settings** – Use this tab to:
  - Adjust the sensitivity so that more wells or fewer wells are flagged.
  - Change the flags that are applied by the 7500 software.
- **Relative Quantitation Settings** – Use this tab to:
  - Select the type of analysis to perform.
  - Select the reference sample(s).
  - Select the endogenous control. You can set more than one endogenous control. See "Set Multiple Endogenous Controls" below.
  - Set the efficiency. See "Set Efficiency" on page 178.
  - Reject outliers.
  - Select the algorithm to use to determine RQ Min/Max values (confidence level or standard deviations).
- **Advanced Settings** – Use this tab to change baseline settings well by well.

Set Multiple Endogenous Controls

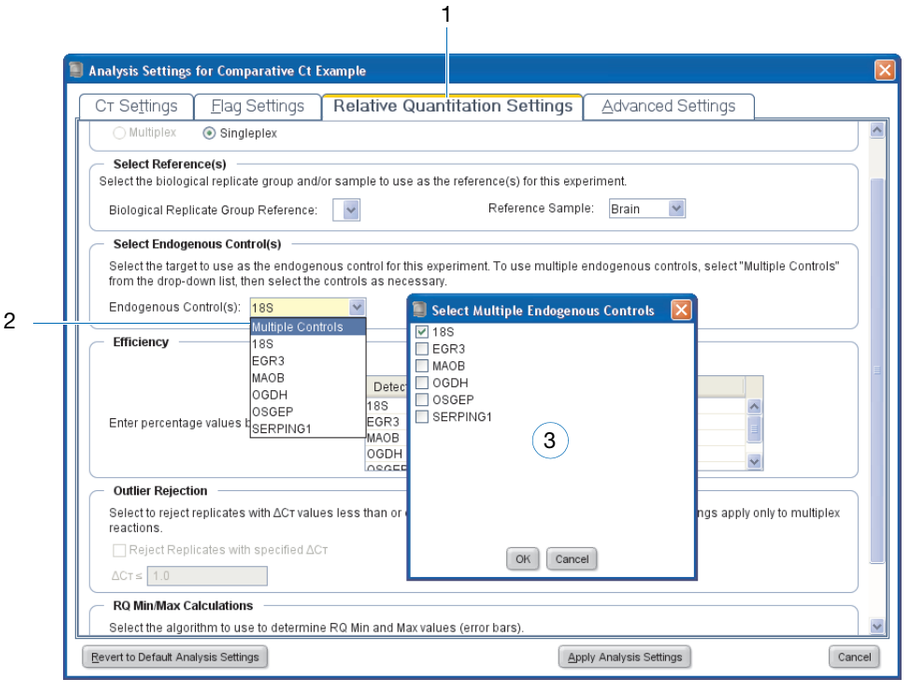
You can select multiple endogenous controls for a comparative C_T experiment.

To select multiple controls:

1. In the Analysis Settings for Comparative Ct Example dialog box, select the **Relative Quantitation Settings** tab.
2. In the Select Endogenous Control(s) group, click the **Endogenous Control(s)** drop-down list, then select **Multiple Controls**.

Notes

3. In the Select Multiple Endogenous Controls dialog box, select the targets that you want to use as endogenous controls, then click **OK**.

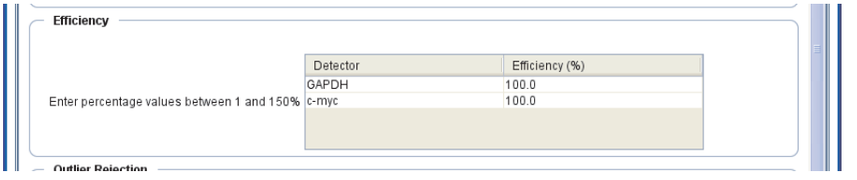


Set Efficiency

You can specify efficiency on a per-assay basis. When you set an assay to have less than 100% efficiency, the 7500 software uses the relative standard curve algorithm.

By setting the correct assay efficiencies for your experiment, you enable the 7500 software to produce more precise quantitation results for reactions that do not amplify with 100% efficiency. By default, the 7500 software calculates sample quantities based on assays that have calculated amplification efficiencies of 100%. Applied Biosystems TaqMan® Gene Expression Assays (Inventoried and Made to Order) have an amplification efficiency of 100% +/- 10% when measured over a broad dynamic range (6 logs) in samples that are free of PCR inhibitors. To determine efficiency of custom designed assays, use at least 5-6 logs dilution range of template.

To set assay efficiency, click **100.0** in the Efficiency (%) column of an assay, then type a percentage value between 1% and 100%.



For More Information

For more information on the analysis settings, open the 7500 Software Help by pressing **F1** when the Analysis Settings dialog box is open.

Notes

View the QC Summary

The QC Summary screen displays a list of the 7500 software flags, and includes the flag frequency and location for the open experiment.

About the Example Experiment

In the comparative C_T example experiment, you review the QC Summary screen for any flags generated by the experiment data; however, no flags are generated.

View the QC Summary

1. In the navigation pane, select **Analysis** ▸ **QC Summary**.

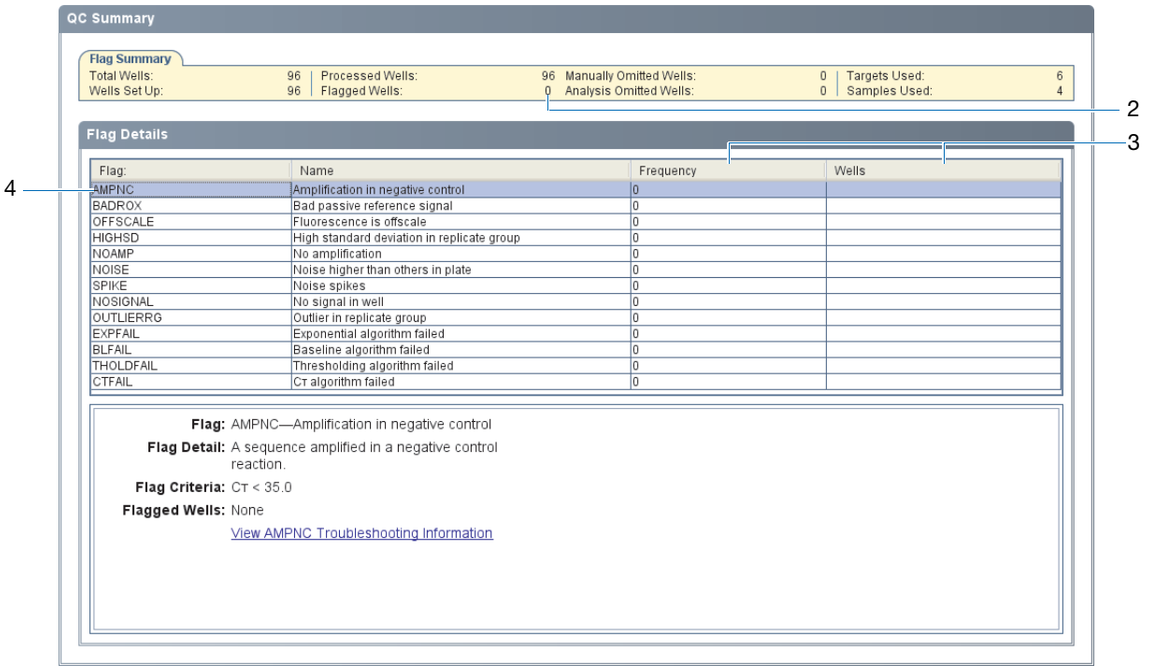
   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary. In the example experiment, there are no flagged wells.

3. In the Flag Details table, look in the Frequency and Wells columns to determine the flags that appear in the experiment. In the example experiment, no wells were flagged by the 7500 software.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment.

4. (Optional) Click each flag row to display details about the flag.



Notes

Possible Flags

For comparative C_T experiments, the flags listed below may be generated by the experiment data.

If a flag does not appear in the experiment, its frequency is 0. If the frequency is >0, the flag appears somewhere in the experiment; the well position is listed in the Wells column.

Flag	Description
AMPNC	Amplification in negative control
BADROX	Bad passive reference signal
BLFAIL	Baseline algorithm failed
CTFAIL	C_T algorithm failed
EXPFAIL	Exponential algorithm failed
HIGHSD	High standard deviation in replicate group
MTP	Multiple Tm peaks **Note:** This flag is displayed only if the experiment includes a melt curve.
NOAMP	No amplification
NOISE	Noise higher than others in reaction plate
NOSIGNAL	No signal in well
OFFSCALE	Fluorescence is offscale
OUTLIERRG	Outlier in replicate group
SPIKE	Noise spikes
THOLDFAIL	Thresholding algorithm failed

Analysis Guidelines

When you analyze your own comparative C_T experiment:

- In the Flag Details table, click each flag that has a frequency >0 to display details about the flag. If needed, click the troubleshooting link to view information on correcting the flag.
- You can change the flag settings:
  - Adjust the sensitivity so that more wells or fewer wells are flagged.
  - Change the flags that are applied by the 7500 software.

For More Information

For more information on the QC Summary screen or on flag settings, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Omit Wells from the Analysis

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

About the Example Experiment

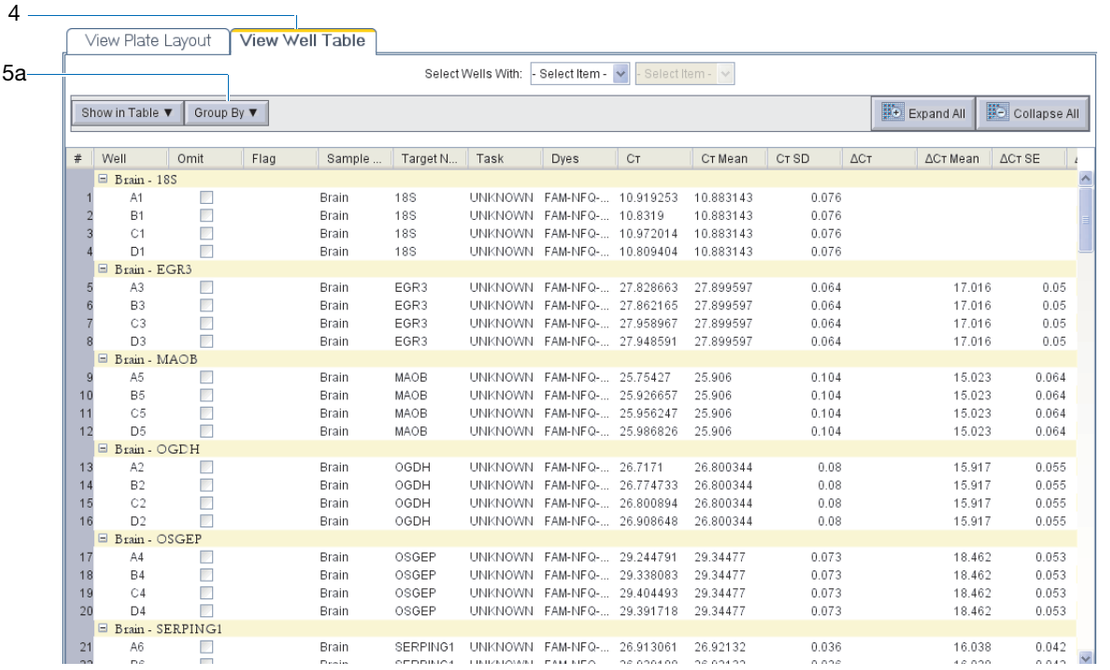
In the comparative C_T example experiment, there are no outliers. No wells need to be removed from analysis.

Omit Wells

1. In the navigation pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the Amplification Plot screen, select **C_T vs Well** in the Plot Type drop-down list.
3. Click the upper left corner of the plate layout in the View Plate Layout tab to display all wells.
4. Select the **View Well Table** tab.
5. In the Well Table:
   a. In the Group By drop-down list, select **Replicate**.
   b. Look for any outliers in the replicate group (be sure they are flagged). In the example experiment, there are no outliers.



#	Well	Omit	Flag	Sample ...	Target N...	Task	Dyes	CT	CT Mean	CT SD	ΔCT	ΔCT Mean	ΔCT SE
	Brain - 18S												
1	A1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.919253	10.883143	0.076			
2	B1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.8319	10.883143	0.076			
3	C1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.972014	10.883143	0.076			
4	D1			Brain	18S	UNKNOWN	FAM-NFQ-...	10.809404	10.883143	0.076			
	Brain - EGR3												
5	A3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.828663	27.899597	0.064	17.016	0.05	
6	B3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.862165	27.899597	0.064	17.016	0.05	
7	C3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.958967	27.899597	0.064	17.016	0.05	
8	D3			Brain	EGR3	UNKNOWN	FAM-NFQ-...	27.948591	27.899597	0.064	17.016	0.05	
	Brain - MAOB												
9	A5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.75427	25.906	0.104	15.023	0.064	
10	B5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.926657	25.906	0.104	15.023	0.064	
11	C5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.956247	25.906	0.104	15.023	0.064	
12	D5			Brain	MAOB	UNKNOWN	FAM-NFQ-...	25.986826	25.906	0.104	15.023	0.064	
	Brain - OGDH												
13	A2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.7171	26.800344	0.08	15.917	0.055	
14	B2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.774733	26.800344	0.08	15.917	0.055	
15	C2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.800894	26.800344	0.08	15.917	0.055	
16	D2			Brain	OGDH	UNKNOWN	FAM-NFQ-...	26.908648	26.800344	0.08	15.917	0.055	
	Brain - OSGEP												
17	A4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.244791	29.34477	0.073	18.462	0.053	
18	B4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.338083	29.34477	0.073	18.462	0.053	
19	C4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.404493	29.34477	0.073	18.462	0.053	
20	D4			Brain	OSGEP	UNKNOWN	FAM-NFQ-...	29.391718	29.34477	0.073	18.462	0.053	
	Brain - SERPING1												
21	A6			Brain	SERPING1	UNKNOWN	FAM-NFQ-...	26.913061	26.92132	0.036	16.038	0.042	

Notes

Analysis Guidelines

When you analyze your own comparative C_T experiment, carefully view the replicate groups for outliers. If needed, remove outliers manually using the Well Table:

1. In the navigation pane, select **Analysis** ▸ **Amplification Plot**.

   Note: If no data are displayed, click **Analyze**.

2. In the Plot Type drop-down list of the Amplification Plot screen, select **C_T vs Well**.
3. Select the **View Well Table** tab.
4. In the Well Table:
   a. In the Group By drop-down list, select **Replicate**.
   b. Look for any outliers in the replicate group (be sure they are flagged).
   c. Select the **Omit** check box next to the outlying well(s).
5. Click **Analyze** to reanalyze the experiment data with the outlying well(s) removed from the analysis.

For More Information

For more information on omitting wells from the analysis, open the 7500 Software Help by clicking or pressing **F1**. Within the Help, search for the omit well topics:

1. Select the **Search** tab.
2. Enter **omit well**.
3. Click **List Topics**.
4. Double-click the topics you want to review.

View the Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye in a selected well over the duration of the PCR.

About the Example Experiment

In the comparative C_T example experiment, you review the Multicomponent Plot screen for:

- ROX™ dye (passive reference)
- FAM™ dye (reporter)
- Spikes, dips, and/or sudden changes
- Amplification in the negative control wells

Notes

View the Multicomponent Plot

1. In the navigation pane, select **Analysis** ▸ **Multicomponent Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display the unknown wells one at a time in the Multicomponent Plot screen:
   a. Click the **View Plate Layout** tab.
   b. Select one well in the plate layout; the Multicomponent Plot shows the well data.

   Note: If you select multiple wells, the Multicomponent Plot screen displays the data for all selected wells simultaneously.

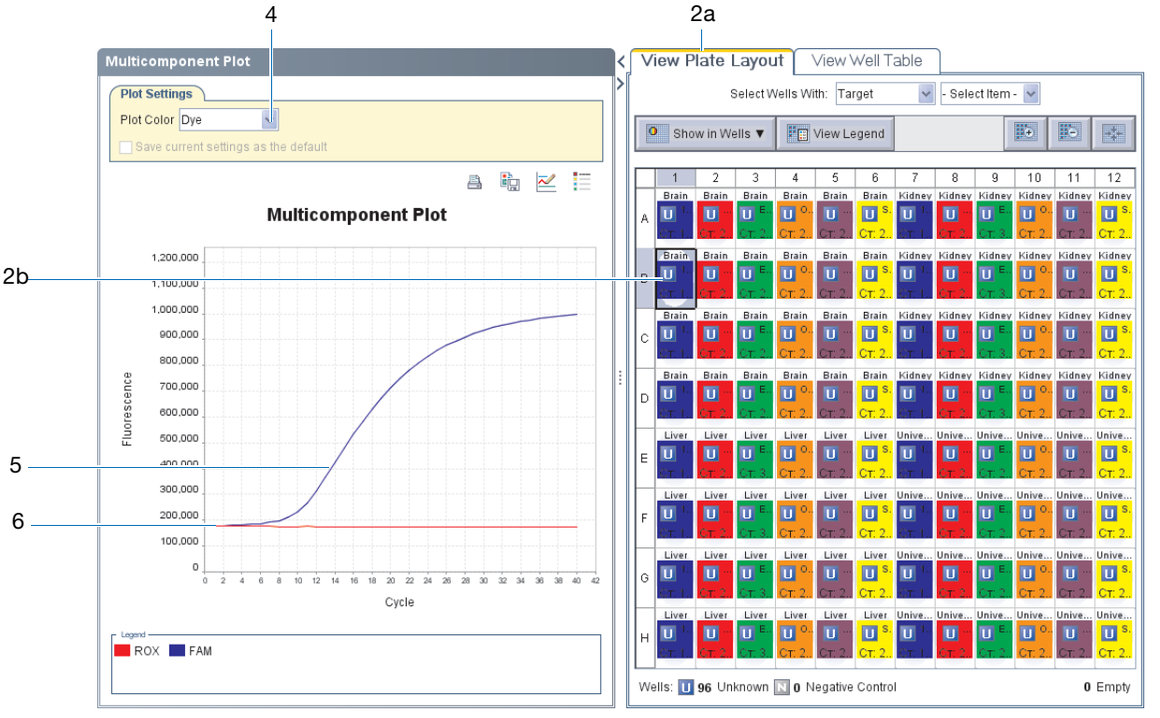
3. In the Plot Color drop-down list, select **Dye**.

4. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

5. View the FAM dye signal. In the example experiment, the FAM dye signal increases throughout the PCR process, which indicates normal amplification.

6. View the ROX dye signal. In the example experiment, the ROX dye signal is constant throughout the PCR process, which indicates typical data.



Notes

Analysis Guidelines

When you analyze your own comparative C_T experiment, look for:

- **Passive reference** – The passive reference dye fluorescence level should remain relatively constant throughout the PCR process.
- **Reporter dye** – The reporter dye fluorescence level should display a flat region corresponding to the baseline, followed by a rapid rise in fluorescence as the amplification proceeds.
- **Any irregularities in the signal** – There should not be any spikes, dips, and/or sudden changes in the fluorescence.
- **Negative control wells** – There should not be any amplification in the negative control wells.

For More Information

For more information on the Multicomponent Plot screen, open the 7500 Software Help by clicking or pressing **F1**.

Notes

View the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence (not normalized) for each optical filter for the selected wells during each cycle of the real-time PCR.

About the Example Experiment

In the comparative C_T example experiment, you review the Raw Data Plot screen for a stable increase in signal (no abrupt changes or dips) from the appropriate filter.

View the Plot

1. In the navigation pane, select **Analysis** ▸ **Raw Data Plot**.

   Note: If no data are displayed, click **Analyze**.

2. Display all 96 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the View Plate Layout tab.

3. Click (Show a legend for the plot).

   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

   Note: The legend displays the color code for each row of the reaction plate. In the example shown below, Row A is red, Row B is yellow/green, Row C is green, and so on.

Notes

4. Drag the Show Cycle slider from cycle 1 to cycle 40. In the example experiment, a stable increase occurs in signal from filter 1, which corresponds to the FAM™ dye filter.

The filters are:

Filter	1	2	3	4	5
Dye	• FAM™ dye • SYBR® Green dye	• JOE™ dye • VIC® dye	• TAMRA™ dye • NED™ dye • Cy3® dye	• ROX™ dye • Texas Red® dye	Cy5® dye

Analysis Guidelines

When you analyze your own comparative C_T experiment, look for the following in each filter:

- Characteristic signal growth
- No abrupt changes or dips

For More Information

For more information on the Raw Data Plot screen, open the 7500 Software Help by clicking or pressing **F1**.

Notes

Section 9.3 Perform a Study of Multiple Experiments

This section covers:

- Section Overview 188
- Design a Study 189
- Analyze the Study 195
- Publish the Data 202

Notes

Section Overview

The 7500 software can combine the analysis of experiments that use the comparative C_T method ($\Delta\Delta C_T$) of relative quantitation.

In a comparative C_T study, you can...	You cannot...
• Specify the endogenous control(s) and reference sample for the study. • Select the control type when applicable. • Set baseline and threshold values and confidence levels, or set the number of standard deviations for Comparative C_T Min/Max. • Omit wells individually or together through their association with replicate groups (technical or biological).	• Create, add, or modify samples. • Create, add, or modify targets. • Change assay tasks. **Note:** You can perform these operations in comparative C_T experiments.

For more information about methods of calculating relative quantitation, refer to the *Real-Time PCR Systems Reagent Guide*.

About the Example Study

In this section, you use two files:

- In the first half, you create a comparative C_T example study file that contains setup data, then save it to the experiments folder on your computer.
- In the second half, you view results in a example study file that is installed with the 7500 software. You can find the data file for the example study on your computer at:

  <*drive*>:\Applied Biosystems\<*software name*>\experiments\
  Comparative Ct Study Example.edm

  where:

  – <*drive*> is the computer hard drive on which the 7500 software is installed.

  – <*software name*> is the current version of the 7500 software.

Note: The Comparative Ct Study Example.edm file is not designed to demonstrate the use of biological replicate groups; an additional example study provided with the 7500 software provides a better example. You can find the alternate study file on your computer at: <*drive*>:\Applied Biosystems\<*software name*>\experiments\ Comparative Ct Study Example (Biological Groups).edm.

Notes

Design a Study

This section explains how to use the 7500 software to set up a comparative C_T example study. It also describes Applied Biosystems recommended best practices as you create the study

Create a New Study

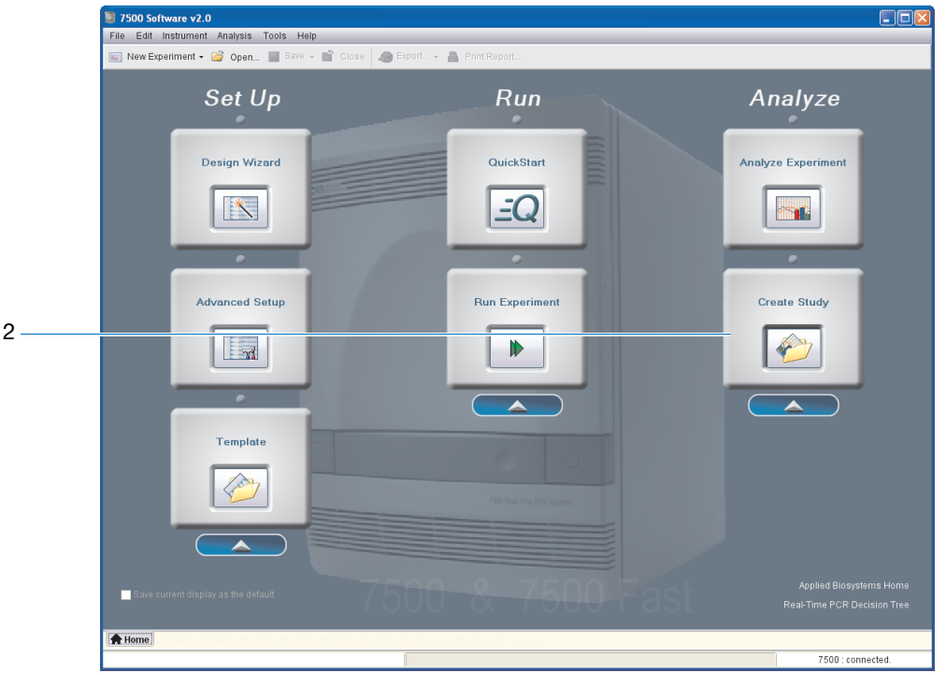
To create a new experiment using the Design Wizard in the 7500 software:

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

   where *<software name>* is the current version of the 7500 software.

2. In the Home screen, click **Create Study**.

Note: If you do not see the Create Study icon, click the arrow beneath the Analyze Experiment icon to expand the Analyze menu.



Notes

Define Study Properties

In the Experiment Properties screen, enter identifying information for the study, then add experiments to the study.

About the Example Study

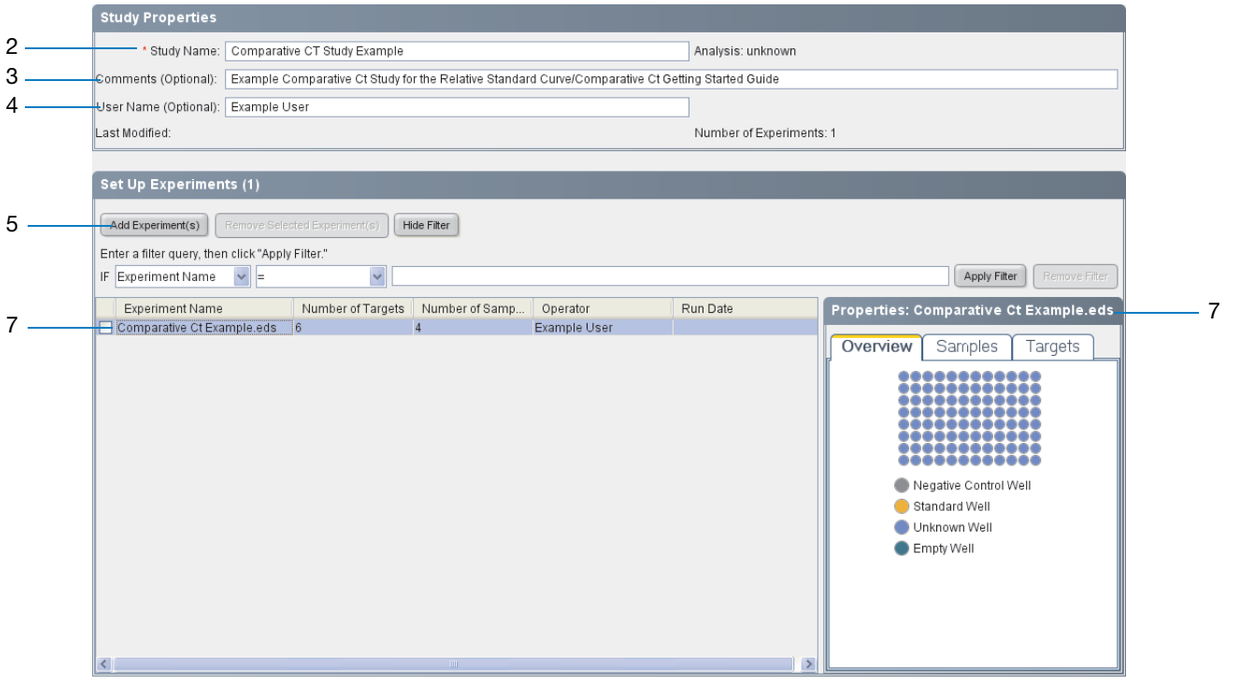
In the example study, the:

- Study is identified as an example.
- Experiment that you add to the study is the same example experiment analyzed in Section 9.1 and Section 9.2 of this chapter.

Complete the Study Properties Screen

1. In the navigation pane, select **Setup** ▸ **Study Properties**.
2. In the Study Properties pane, click the **Study Name** field, then enter **Comparative Ct Study Example**.
3. Click the **Comments** field, then enter **Example Comparative Ct Study for the Relative Standard Curve/Comparative Ct Getting Started Guide**.
4. Click the **User Name** field, then enter **Example User**.
5. In the Set Up Experiments pane, click **Add Experiment(s)**.
6. In the Open dialog box, open the **Comparative Ct Example.eds** file at:

   <drive>:\Applied Biosystems\<software name>\experiments\
7. In the Set Up Experiments table, select the **Comparative Ct Example** experiment. The 7500 software displays the details of the experiment in the Properties pane.



Notes

Design Guidelines

When you design your own comparative C_T study:

- Enter an study name that is descriptive and easy to remember. You can enter up to 100 characters in the Study Name field. You cannot use the following characters in the Study Name field: / \ > < * ? " | : ;

  Note: The study name is used as the default file name.

- (Optional) Enter a user name to identify the owner of the study. You can enter up to 100 characters in the User Name field.
- (Optional) Enter comments to describe the study. You can enter up to 1000 characters in the Comments field.
- Add up to 100 comparative C_T experiments to the study.

  To add experiments to a study they must have:

  – One or more common endogenous control(s).

  – Identical thermal cycling parameters (the same number of steps, cycles, sample volume, and emulation mode).

  IMPORTANT! The 7500 software cannot combine in the same study experiments that use Fast and standard thermal cycling conditions.

  IMPORTANT! The 7500 software automatically analyzes a study after more than one experiment is added to it. Consequently, to ensure that the software uses the correct settings, Applied Biosystems recommends that you review the analysis settings of your study after adding multiple experiments.

  Note: The 7500 software automatically assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment added to it.

  Note: If experiments that contain biological replicate groups are added to a study, the 7500 software automatically merges the matching biological groups.

- When adding experiments to the study, **Ctrl+click** multiple experiments in the Open dialog box to add them to the study.
- Select an experiment that has been added to the study to view its properties in the Properties pane.
- Filter the experiments added to the study to simplify the list for easier review. See “How to Simplify Data Lists Using the Filter Query” on page 192.

Notes

How to Simplify Data Lists Using the Filter Query

To narrow your search for an experiment, define and apply a filter as follows:

1. In the left-most drop-down list, select an experiment attribute to query.
2. In the center drop-down list, select an operator for the query.
3. In the right-most field, enter the condition to for which to test, then click **Apply Filter**.

Note: After applying a filter, click **Hide Filter/Show Filter** to hide or show the filter tool, or click **Remove Filter** to remove the filter.

For More Information

For more information on:

- Completing the Experiment Properties screen – Open the 7500 Software Help by clicking or pressing **F1**.
- Quantitation experiments – Refer to the *Real Time PCR System Reagent Guide*.

Define Replicates

In the Define Replicates screen, create biological replicate groups and use them to associate samples for the analysis. Biological replicates allow you to assess the representative nature of your results as they relate to the population being studied. Inclusion of biological replicates can give insight into any natural variation that is present within the population.

About the Example Study

The comparative C_T example does not contain biological replicate groups. You will create and apply the biological group "LKBGP" as an exercise.

Complete the Define Replicates Screen

1. In the navigation pane, select **Setup** ▸ **Define Replicates**.
2. Click **Add Biological Group**.
3. Set up the example LKBGP biological replicate group:
   a. In the Add Biological Replicate Group dialog box, click the **Biological Group Name** field, then enter **LKBGP**.

      Note: In the example study, the LKBGP (liver, kidney, brain group) biological group is not used for the analysis.

   b. In the Color field, leave the default.
   c. Click the **Comments** field, then enter **Example Biological Replicate Group for the Example Comparative Ct Study**.
   d. In the Select Plate drop-down list, select **Comparative Ct Experiment.eds**.

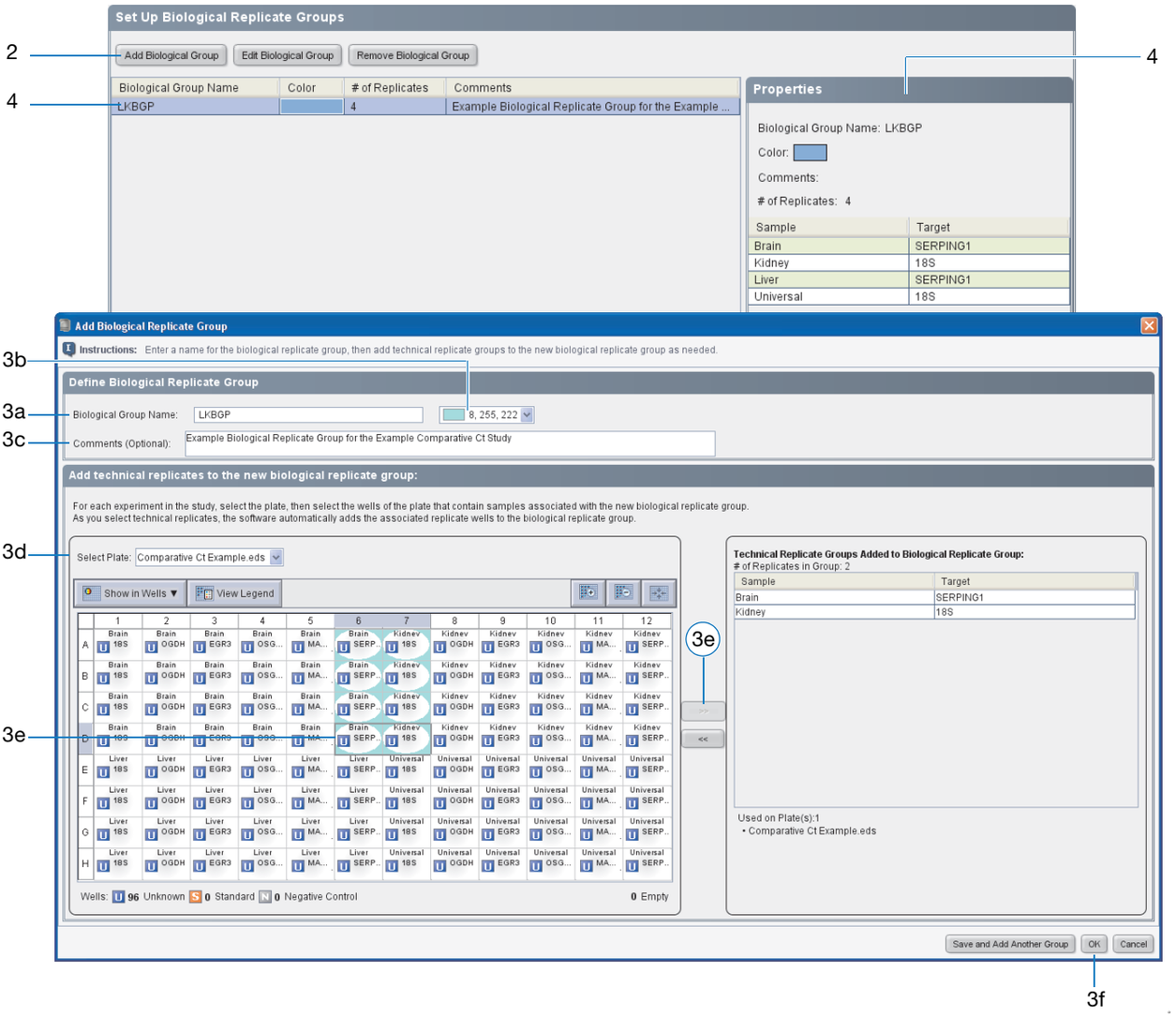
Notes

e. In the plate layout, select wells **D6** and **D7**, then click >> to add the technical replicate wells associated with the selected wells to the biological group.

   The 7500 software automatically adds technical replicates of the selected wells to the biological group.

f. Click **OK**.

4. Select the LKBGP group that you just added to the study. The 7500 software displays the details of the biological group in the Properties pane.



Notes

Design Guidelines

When you add a biological replicate group:

- Enter a biological replicate group name that is descriptive and easy to remember. You can enter up to 100 characters in the Biological Group field. You cannot use the following characters in the Biological Group field: / \ > < * ? " | : ;
- (Optional) Enter comments to describe the biological replicate group. You can enter up to 1000 characters in the Comments field.
- Add an unlimited number of technical replicates to a biological group.

  IMPORTANT! A sample cannot belong to more than one biological group.

- Click-drag over the desired wells, or **CTRL+click** or **Shift+click** in the plate layout to select multiple wells.
- Click the upper left corner of the plate layout to select all 96 wells.
- You can use the Define Replicates screen to change the name of a biological replicate group, change its color identification and description, and add or remove technical replicates. See "Edit a Biological Replicate Group" below.
- Delete an existing biological replicate group by selecting the desired biological group, then clicking **Remove Biological Group**.

  IMPORTANT! After you remove a biological replicate group from a study, you cannot restore it.

Edit a Biological Replicate Group

1. Click **Edit Biological Group**.
2. In the Add Biological Replicate Group window, click the **Biological Group Name** drop-down list, then select a biological group to edit. Add or remove technical replicates to or from the biological group as needed.

   Note: To change the name of the group, click the **Edit** button after the Biological Group Name field.

3. To save your changes, click **OK**. To leave the group unchanged, click **Cancel**.

Notes

Analyze the Study

This section explains how to use the 7500 software to analyze the comparative C_T example study. It also describes Applied Biosystems recommended best practices as you perform the analysis.

Note: The Comparative Ct Study Example.edm file is not designed to demonstrate the use of biological replicate groups; an additional example study provided with the 7500 software provides a better example. You can find the alternate study file on your computer at: *<drive>*:\Applied Biosystems*<software name>*\experiments\Comparative Ct Study Example (Biological Groups).edm.

Open the Example Study

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

   where *<software name>* is the current version of the 7500 software.

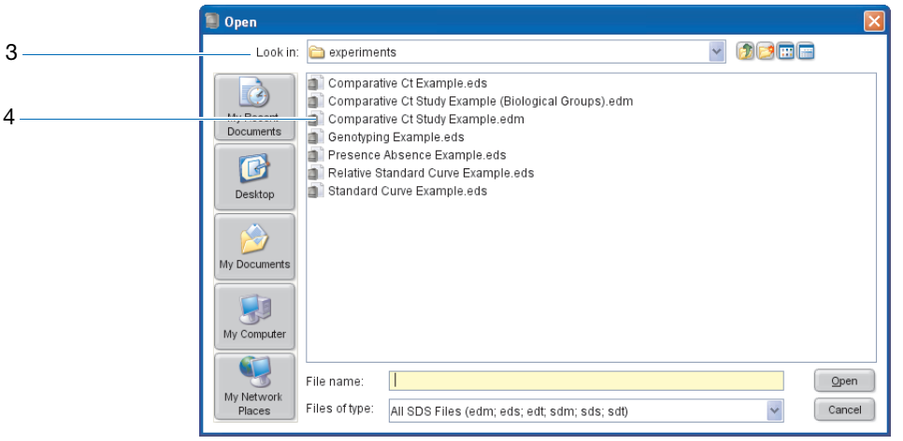
2. In the Home screen, click **Open**.

3. In the Open dialog box, navigate to the **experiments** folder at:

   <drive>:\Applied Biosystems*<software name>*\experiments\

4. Double-click **Comparative Ct Study Example.edm** to open the example study data file.

   Note: The examples folder contains several data files; be sure to select **Comparative Ct Study Example.edm**.



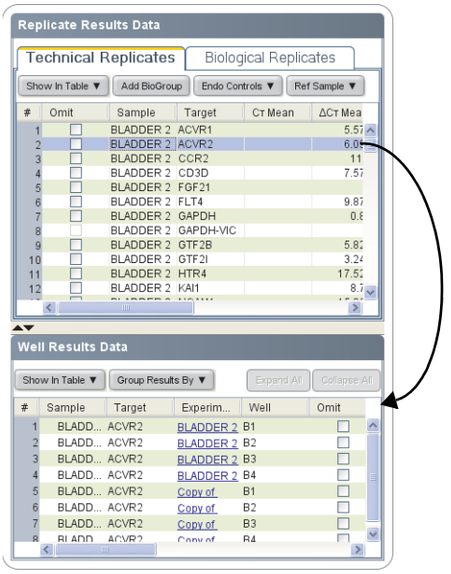
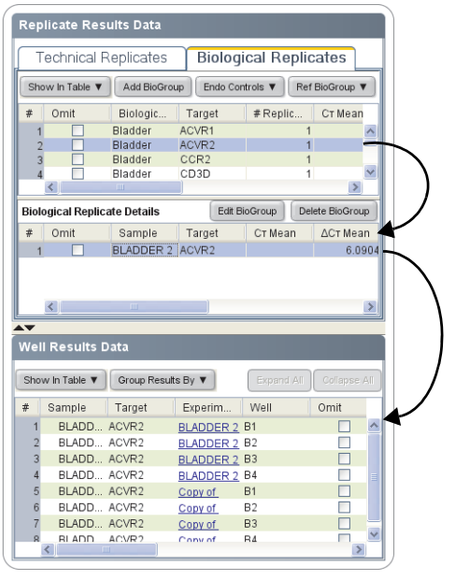
Notes

View the Gene Expression Plot, Replicate Results Table, and Well Table

The Gene Expression Plot screen displays the results of the relative quantitation calculations in the gene expression profile. Two plots are available:

- **RQ vs Target** – Groups the relative quantitation (RQ) values by target. Each sample is plotted for each target. You can view the plot as a linear, log10, Ln, or log2 graph.
- **RQ vs BioGroup** – Groups the relative quantitation (RQ) values by biological replicate group (if present). Each target is plotted for each biological group.You can view the plot as a linear, log10, Ln, or log2 graph.

The Replicate Results Data group organizes the results of the gene expression study by biological or technical replicate association. The data are organized into two tabs:

Technical Replicates Tab	Biological Replicates Tab
This tab groups the results of the relative quantitation analysis by technical replicate group. The 7500 software displays the results for each sample/target combination as a row in the table. You can view the members of a technical replicate group by selecting the appropriate row in the table. When a row is selected, the Well Result Data table displays the wells that make up the group.	This tab groups the results of the relative quantitation analysis by biological replicate group. The 7500 software displays the results for each biological group as a row in the table. You can view the members of a group by selecting the appropriate row from the table. When a row is selected, the Biological Replicate Details table displays the technical replicates that make up the biological group. You can then display the members of a technical replicate group by selecting a row in the Biological Replicate Details table.
	

Note: The Omit column of the Replicate Results Data group tabs indicates the omission status of the members of the associated replicate groups. For example, a check mark (✔) indicates that all members of a group have been omitted from the analysis. A hyphen (–) indicates that one or more members of the group have been omitted.

Notes

The Well Results Data group displays data for each well in the reaction plates that are added to the study, including:

- The sample name, target name, task, and dyes
- The calculated threshold cycle (C_T), normalized fluorescence (Rn), and quantity values
- Flags

About the Example Study

In the comparative C_T example study, you review:

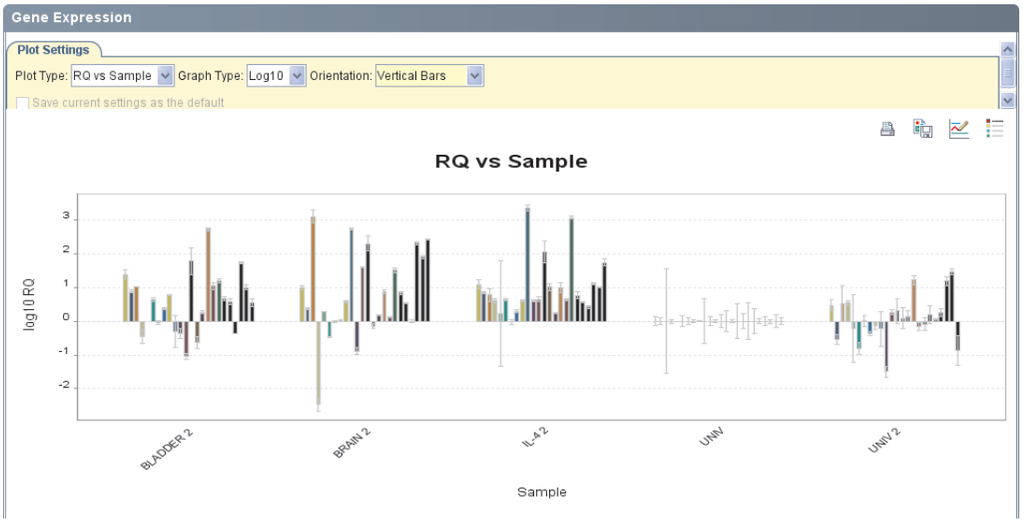
- Each target in the Gene Expression Plot screen for the expression level (or fold change) of the target sample relative to the reference sample.
- The Replicate Results Data group to evaluate the precision of the replicate groups.

View the Gene Expression Plot

1. In the navigation pane, select **Analysis** ▸ **Gene Expression**.
2. In the Gene Expression Plot screen:
   a. In the Plot Type drop-down list, select **RQ vs Sample**.
   b. In the Graph Type drop-down list, select **Log10**.
   c. In the Orientation drop-down list, select **Vertical Bars**.
3. Click (Show a legend for the plot).

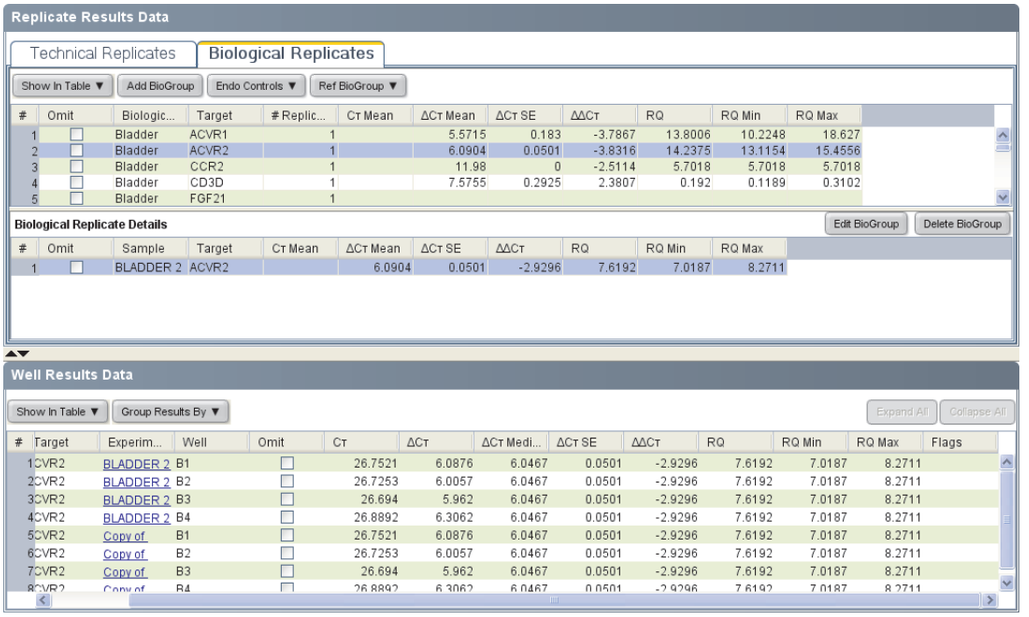
Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

In the example experiment, the expression level of multiple targets in samples are displayed relative to the expression level of the same targets in the reference sample (universal). Because the reference sample is compared to itself, the relative expression level is 1. When the result is displayed in the Log10 graph type, the expression level of the reference sample appears as 0 in the graph (log10 of 1 = 0).



Notes

4. Click < at the top left of the Replicate Results Data group. The figure below shows the replicate results and well results tables.



Column	Description
Omit	For all technical replicates that evaluate the associated sample/target combination: • A check mark (✓) indicates that all replicates have been removed from the analysis. • A hyphen (–) indicates that one or more replicates have been removed from the analysis.
Sample	The sample associated with the data displayed in the row.
Target	The target assay associated with the data displayed in the row.
C_T Mean	The arithmetic average of the technical replicate C_T values.
ΔC_T Mean	The arithmetic average of the technical replicate C_T values for the sample replicate group.
ΔC_T SE	The sample standard deviation of the sample replicate group level C_T values.
$\Delta\Delta C_T$	The calculated $\Delta\Delta C_T$ value for the replicate group associated with the test sample.
RQ	The calculated relative level of gene expression for the replicate group associated with the test sample.
RQ Min	The minimum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. **Note:** The minimum includes the variability associated with the endogenous control and targets in only the test samples.
RQ Max	The maximum relative level of gene expression in the test samples calculated using the confidence level set in the Analysis Settings dialog box. **Note:** The maximum includes the variability associated with the endogenous control and targets in only the test samples.
Flag	The number of QC flags the well generated as listed in the ▲ symbol.

Notes

Analysis Guidelines

When you review the Gene Expression Plot of a comparative C_T study:

- Select the Technical Replicates tab or the Biological Replicates tab to organize and view the sample data according to the associated technical replicate group or biological replicate group.
- Change the endogenous control by clicking **Endo Control**, then selecting a new target.
- Change the reference sample by clicking **Ref Sample**, then selecting a new sample.
- Omit biological or technical replicates from the analysis. See "Omit Wells using the Well Table" on page 200
- Display a subset of the data in the study by selecting one or more rows in the Technical Replicates tab or the Biological Replicates tab, then by selecting **Hide unselected data from plot** in bottom of the Gene Expression Plot.

Note: Comparative Ct Example Study.edm does not contain biological replicate groups. However, another example study that installed with the 7500 software better demonstrates the use of biological groups. You can find the data file for the example study on your computer at: <*drive*>:\Applied Biosystems\<*software name*>\experiments\Comparative Ct Study Example (Biological Groups).edm

Omit Replicates from the Analysis

You can use the Technical Replicates and Biological Replicates tabs of the Gene Expression screen to omit technical replicates and biological replicates from the analysis.

To omit a technical or biological replicate:

1. In the navigation pane, select **Analysis** ▸ **Gene Expression**.
2. In the Gene Expression screen, select the **Technical Replicates** or **Biological Replicates** tab according to the type of replicate that you want to omit.
3. In the replicate table, scroll to the biological or technical replicate of interest, then select the check box in the Omit column.
4. Click **Analyze** when you are finished omitting wells.

IMPORTANT! You cannot omit *all* technical replicates that belong to a reference sample, belong to a reference biological group, or serve as the endogenous control for a study.

Note: You can also omit the technical replicates in the Biological Replicate Details table at the bottom of the Biological Replicates tab.

For More Information

For more information on:

- Viewing the Gene Expression Plot – See "View the Gene Expression Plot and Web Table" on page 162, or open the 7500 Software Help by clicking or pressing **F1**.
- Calculating Relative Quantitation Values – Refer to the *User Bulletin #2: Relative Quantitation of Gene Expression*.

Notes

View the Amplification Plot

The Amplification Plot displays post-run amplification of the samples of the experiments added to the study. The plot is identical to the plot of the same name described in the experiment-level analysis with the exception of the Experiment Data table, which allows you to select the experiment data displayed by the plot. For more information, see "View the Amplification Plot" on page 166.

Note: To display a subset of the study data in the Amplification Plot, select one or more rows in the Experiment Data tab or the Well Table tab, then select **Hide unselected data from plot** to display data only from the selected rows.

Omit Wells using the Well Table

You can use the Well Table to omit individual wells from the analysis. To omit a well:

1. In the Experiment Data group, select the experiment that contains the well of interest.
2. In the View Well Table tab, select the check box in the Omit column for the well of interest.

IMPORTANT! You cannot omit all technical replicates that belong to a reference sample, belong to a reference biological group, or serve as the endogenous control.

View the Multicomponent Plot

The Multicomponent Plot displays complete spectral contribution of each dye over the duration of the PCR run in a selected well of any experiment added to the study. The plot is identical to the plot of the same name described in the experiment-level analysis with the exception of the Experiment Data table, which allows you to select the experiment data displayed by the plot. For more information, see "View the Multicomponent Plot" on page 182.

Note: To display a subset of the study data in the Multicomponent Plot, select one or more rows in the Experiment Data tab or the Well Table tab, then select **Hide unselected data from plot** to display data only from the selected rows.

Note: You can use the View Well Table tab of the Multicomponent Plot to omit individual wells from the analysis. The procedure for omitting wells is identical to that for the Amplification Plot. See "Omit Wells using the Well Table" above.

Notes

View Multiple Plots

In the Study Menu, select **Multiple Plots View**. The operation of the plot is identical to the plot of the same name described in the experiment-level analysis with the exception of the Experiment Data table, which allows you to select the experiment data displayed by the plot. For more information, see "How to Display Multiple Plots" on page 161.

Note: To display a subset of the study data in the Multiple Plots View, select one or more rows in the Experiment Data tab or the Well Table tab, then select **Hide unselected data from plot** to display data only from the selected rows.

Note: You can reduce the data displayed in the plots by applying a filter using the filter function at the top of the screen. See "How to Simplify Data Lists Using the Filter Query" on page 192 for more information on using filters.

Note: You can use the View Well Table tab of the Multiple Plots View to omit individual wells from the analysis. The procedure for omitting wells is identical to that for the Amplification Plot. See "Omit Wells using the Well Table" on page 200.

View the QC Summary

The QC Summary screen displays a list of the 7500 software flags, and it includes the flag frequency and location for the experiments added to the open study. The summary is identical to the QC Summary that appears in the experiment-level analysis. For more information, see "View the QC Summary" on page 179.

Notes

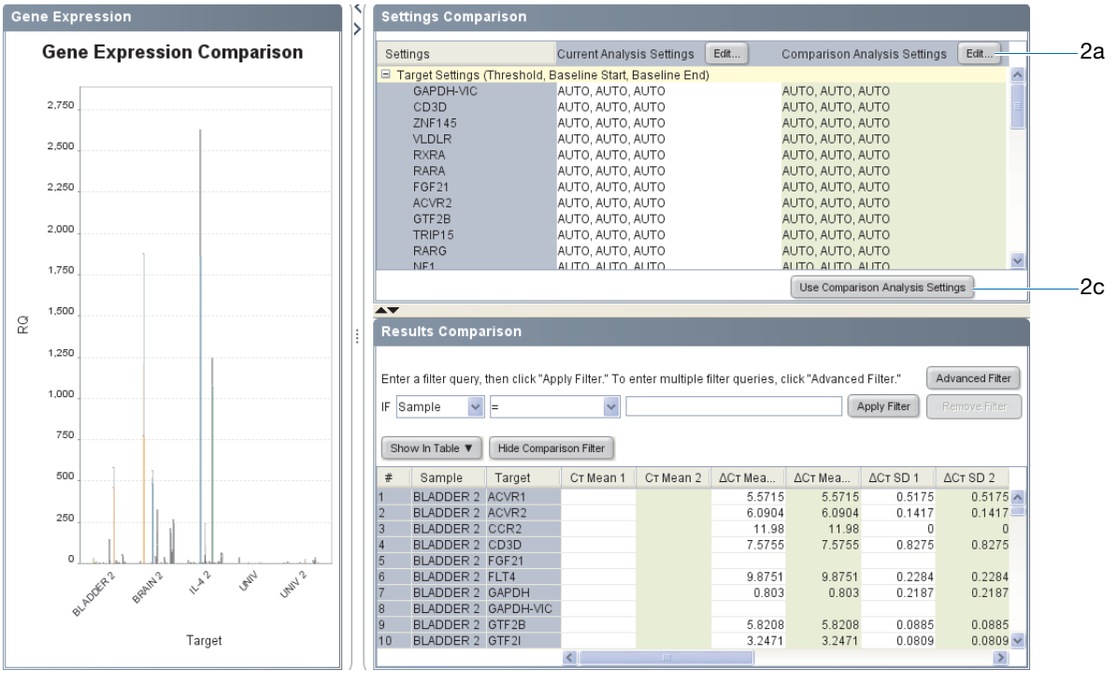
Compare Analysis Settings

The Compare Settings screen allows you to perform a side-by-side comparison of analysis settings for a comparative C_T study.

Complete the Compare Settings Screen

1. In the navigation pane, select **Analysis** ▸ **Compare Settings**.
2. In the Comparison Settings group, modify the comparison analysis settings:
   a. Click **Edit** to the right of the Comparison Analysis Settings heading.
   b. Edit the comparison analysis settings as desired, then click **Analyze**.
   c. Click **Use Comparison Analysis Settings** to analyze the study data using the modified settings.

   Note: Click **Use Comparison Analysis Settings** to use the previous analysis settings.
3. Compare the results in the Results Comparison pane. The table uses shaded cells to distinguish the results from the comparison settings.



Publish the Data

You can publish the study data in the same way you publish experiment data. See "Publish the Data" on page 97 for more information.

Notes

A

Alternate Experiment Workflows

This appendix covers:

- Advanced Setup Workflow 204
- QuickStart Workflow 206
- Template Workflow 208
- Export/Import Workflow 210

Note: For more information about any of the topics discussed in this guide, open the Help from within Applied Biosystems 7500/7500 Fast Real-Time PCR Software v2.0 by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ 7500 Software Help**.

Notes

Advanced Setup Workflow

When you create an experiment using Advanced Setup in the 7500 software, you can set up the experiment according to your own design.

1. Double-click (7500 software) or select **Start ▸ All Programs ▸ Applied Biosystems ▸ 7500 Software ▸ *<software name>***

   where *<software name>* is the current version of the 7500 software.

2. In the Home screen, click **Advanced Setup**.

   Note: If you do not see the Advanced Setup icon, click the arrow beneath the Design Wizard icon to expand the Set Up menu.

3. Complete the setup screens to set up a new experiment:

   a. Click **Experiment Properties** (default), enter the experiment name, then select the experiment properties.

   b. Click **Plate Setup:**

Experiment Type	Action
Genotyping	Define the SNP assays, then assign them to wells in the reaction plate.
All other experiments	Define the targets, then assign them to wells in the reaction plate.

   c. Click **Add Biological Group** to create biological replicates and assign replicates to samples for each biological group in the reaction plate.

   d. Click **Run Method**, review the reaction volume and thermal profile, then edit as needed.

   e. Click **Reaction Setup**, review the components and calculated volumes for the PCR reactions, then edit as needed.

   f. (Optional) Click **Materials List**, review the list of materials, then order the materials you need to prepare the reaction plate.

Notes

4. Prepare the PCR reactions:

Experiment Type	Prepare the…
Relative standard curve	a. Template. b. Sample dilutions. c. Standard dilution series. d. Reaction mix. e. Reaction plate.
Standard curve	
Comparative C_T	a. Template. b. Sample dilutions. c. Reaction mix. d. Reaction plate.
Genotyping	
Presence/absence	

5. Run the experiment:

IMPORTANT! While the 7500/7500 Fast instrument is performing a run, do not create experiments, perform maintenance, or allow the computer to run antivirus software or to enter hibernation. Performing such activities while the instrument is running an experiment will cause gaps in data collection.

   a. Load the reaction plate into the instrument.

   b. Start the run.

   c. (Optional) Monitor the run.

   d. Unload the reaction plate from the instrument.

6. Analyze the data:

   a. Open the experiment in the 7500 software.

   b. In the navigation pane, click **Analysis**.

   c. If the data are not analyzed, click **Analyze**.

   d. In the navigation pane, select an analysis screen to view the data (for example, select **QC Summary** to view a quality summary of the data).

Notes

QuickStart Workflow

When you create an experiment using QuickStart, you can run the reactions on the instrument with no reaction plate setup information.

1. Prepare the PCR reactions:

Experiment Type	Prepare the…
Relative standard curve	a. Template. b. Sample dilutions. c. Standard dilution series. d. Reaction mix. e. Reaction plate.
Standard curve	
Comparative C_T	a. Template. b. Sample dilutions. c. Reaction mix. d. Reaction plate.
Genotyping	
Presence/absence	

2. QuickStart the experiment:
   a. Double-click (7500 software) or select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **7500 Software** ▸ ***<software name>***
      where *<software name>* is the current version of the 7500 software.
   b. In the Home screen, click **QuickStart**.
   c. Select the **Experiment Properties** tab (default), enter the experiment name, then select the experiment properties.
   d. Select the **Run Method** tab, review the reaction volume and thermal profile, then edit as needed.
3. Run the experiment:

   IMPORTANT! While the 7500/7500 Fast instrument is performing a run, do not create experiments, perform maintenance, or allow the computer to run antivirus software or to enter hibernation. Performing such activities while the instrument is running an experiment will cause gaps in data collection.

   a. Load the reaction plate into the instrument.
   b. Start the run.
   c. (Optional) Monitor the run.
   d. Unload the reaction plate from the instrument.

Notes

4. In the 7500 software, complete the reaction plate setup:

Experiment Type	Select, then complete the…
Genotyping	a. **Define SNP Assays and Samples** tab. b. **Assign SNP Assays and Samples** tab.
All other experiments	a. **Define Targets and Samples** tab. b. **Assign Targets and Samples** tab.

5. Analyze the data:
   a. Open the experiment in the 7500 software.
   b. In the navigation pane, click **Analysis**.
   c. If the data are not analyzed, click **Analyze**.
   d. In the navigation pane, select an analysis screen to view the data (for example, select **QC Summary** to view a quality summary of the data).

Notes

Template Workflow

You can use a template to create a new experiment. Templates allow you to create many experiments with the same setup information.

Create a Template

1. Double-click (7500 software) or select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **7500 Software** ▸ ***<software name>***

   where *<software name>* is the current version of the 7500 software.

2. Open an existing experiment, or create a new experiment.

   Note: You can create a new experiment using the Design Wizard (see Chapter 2 and Chapter 6) or Advanced Setup (see page 204).

3. Select **File** ▸ **Save As Template**.

4. Enter a file name, select a location for the template, then click **Save**.

5. Click **Close**.

Create an Experiment Using a Template

1. In the Home screen, click **Template**.

   Note: If you do not see the Template icon, click the arrow below the Design Wizard icon to expand the Set Up menu.

2. Locate, then select the template you created above, then click **Open**. A new experiment is created using the following setup information from the template:
   - Experiment properties
   - Plate setup
   - Run method
   - Reaction setup

3. (Optional) If you want to modify the experiment, use Advanced Setup (see page 204).

4. Click **Save**, enter a file name, then click **Save** to save the experiment.

Notes

5. Prepare the PCR reactions:

Experiment Type	Prepare the…
Relative standard curve	a. Template. b. Sample dilutions. c. Standard dilution series. d. Reaction mix. e. Reaction plate.
Standard curve	
Comparative C_T	a. Template. b. Sample dilutions. c. Reaction mix. d. Reaction plate.
Genotyping	
Presence/absence	

6. Run the experiment:

IMPORTANT! While the 7500/7500 Fast instrument is performing a run, do not create experiments, perform maintenance, or allow the computer to run antivirus software or to enter hibernation. Performing such activities while the instrument is running an experiment will cause gaps in data collection.

   a. Load the reaction plate into the instrument.

   b. Start the run.

   c. (Optional) Monitor the run.

   d. Unload the reaction plate from the instrument.

7. Analyze the data:

   a. Open the experiment in the 7500 software.

   b. In the navigation pane, click **Analysis**.

   c. If the data are not analyzed, click **Analyze**.

   d. In the navigation pane, select an analysis screen to view the data (for example, select **QC Summary** to view a quality summary of the data).

Notes

Export/Import Workflow

Use the Export/Import workflow to set up a new experiment using setup data exported from another experiment. Only reaction plate setup data are exported and imported.

Export Setup Data

1. Double-click (7500 software) or select **Start** ▸ **All Programs** ▸ **Applied Biosystems** ▸ **7500 Software** ▸ ***<software name>***

   where *<software name>* is the current version of the 7500 software.

2. Open an existing experiment, or create a new experiment.

   Note: You can create a new experiment using the Design Wizard (see Chapter 2 and Chapter 6) or Advanced Setup (see page 204).

3. Select **File** ▸ **Export**.

4. Select the **Export Properties** tab (default), then:

   a. Select **Sample Setup**.

   b. Select **One File** in the drop-down list.

   c. Enter a name, then select a location for the export file.

   d. Select **(*.txt)** in the File Type drop-down list.

   IMPORTANT! You cannot export *.xml files.

5. (Optional) Select the **Customize Export** tab, then select the appropriate options.

6. Click **Start Export**.

7. When prompted, click **Close Export Tool**.

Create an Experiment with an Exported Text File

You can import reaction plate setup data from an exported text file (*.txt) to complete the reaction plate setup data for your experiment.

IMPORTANT! Be sure the exported text file that you select contains only reaction plate setup data and that the experiment types match.

1. Import the reaction plate setup data from the exported text file:

   a. Using a spreadsheet application (such as Microsoft® Excel software), open an exported text file.

   b. Replace the parameters of the text file as needed. When you finish, save the file as a tab-delimited text file.

Notes

c. In the Home screen, click **Advanced Setup**.

Note: If you do not see the Advanced Setup icon, click the arrow below the Design Wizard icon to expand the Set Up menu.

d. Create a new experiment or open an existing experiment.

e. Select **File ▸ Import**.

f. Click **Browse**, locate and select the text file (*.txt), then click **Select**.

g. Click **Start Import**. The setup data from the exported text file is imported into the open experiment.

Note: If your experiment already contains reaction plate setup information, the software prompts you to replace the reaction plate setup with the data from the text file. Click **Yes** to replace the reaction plate setup.

2. Use Advanced Setup to finish setting up your experiment (see page 204).

3. Prepare the PCR reactions:

Experiment Type	Prepare the…
Relative standard curve	a. Template. b. Sample dilutions. c. Standard dilution series. d. Reaction mix. e. Reaction plate.
Standard curve	
Comparative C_T	a. Template. b. Sample dilutions. c. Reaction mix. d. Reaction plate.
Genotyping	
Presence/absence	

4. Run the experiment:

IMPORTANT! While the 7500/7500 Fast instrument is performing a run, do not create experiments, perform maintenance, or allow the computer to run antivirus software or to enter hibernation. Performing such activities while the instrument is running an experiment will cause gaps in data collection.

a. Load the reaction plate into the instrument.

b. Start the run.

c. (Optional) Monitor the run.

d. Unload the reaction plate from the instrument.

Notes

5. Analyze the data:
   a. Open the experiment in the 7500 software.
   b. In the navigation pane, click **Analysis**.
   c. If the data are not analyzed, click **Analyze**.
   d. In the navigation pane, select an analysis screen to view the data (for example, select **QC Summary** to view a quality summary of the data).

Notes

Bibliography

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

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.

Glossary

Advanced Setup — In the 7500 software, a feature that allows you to set up your experiment according to your experiment design. Advanced Setup provides you with maximum flexibility in the design and setup of your experiment.

AIF — See assay information file (AIF).

allele — For a given target, any of the different sequences that occurs in the population.

allelic discrimination plot — Display of data collected during the post-PCR read. The allelic discrimination plot is a graph of the normalized reporter signal from the allele 1 probe plotted against the normalized reporter signal from the allele 2 probe.

amplicon — A segment of DNA amplified during PCR.

amplification — Part of the instrument run in which PCR produces amplification of the target. For quantitation experiments, fluorescence data collected during amplification are displayed in an amplification plot, and the data are used to calculate results. For genotyping or presence/absence experiments, fluorescence data collected during amplification are displayed in an amplification plot, and the data can be used for troubleshooting.

amplification efficiency (EFF%) — Calculation of efficiency of the PCR amplification. The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to −3.32 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:

- **Range of standard quantities** – To increase the accuracy and precision of the efficiency measurement, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
- **Number of standard replicates** – To increase the precision of the standard quantities and decrease the effects of pipetting inaccuracies, include replicates.
- **PCR inhibitors** – PCR inhibitors in the reaction can reduce amplification and alter measurements of the efficiency.

amplification plot — Display of data collected during the cycling stage of PCR amplification. Can be viewed as:

- Baseline-corrected normalized reporter (ΔRn) vs. cycle
- Normalized reporter (Rn) vs. cycle
- Threshold cycle (C_T) vs. well

amplification stage Part of the instrument run in which PCR produces amplification of the target. The amplification stage is called a cycling stage in the thermal profile and consists of denaturing, primer annealing, and polymerization steps that are repeated.

For quantitation experiments, fluorescence data collected during the amplification stage are displayed in an amplification plot, and the data are used to calculate results. For genotyping or presence/absence experiments, fluorescence data collected during the amplification stage are displayed in an amplification plot, and the data can be used for troubleshooting. See also cycling stage.

assay In the 7500/7500 Fast system, a PCR reaction mix that contains primers to amplify a target and a reagent to detect the amplified target.

Assay ID Identifier assigned by Applied Biosystems to TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays.

assay information file (AIF) Data file on a CD shipped with each assay order. The file name includes the number from the barcode on the plate. The information in the AIF is provided in a tab-delimited format.

assay mix PCR reaction component in Applied Biosystems TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays. The assay mix contains primers designed to amplify a target and a TaqMan® probe designed to detect amplification of the target.

AutoDelta In the run method, a setting to increase or decrease the temperature and/or time for a step with each subsequent cycle in a cycling stage. When AutoDelta is enabled for a cycling stage, the settings are indicated by an icon in the thermal profile:

- AutoDelta on: ▲
- AutoDelta off: ▲

automatic baseline An analysis setting in which the software calculates the baseline start and end values for the amplification plot. You can apply the automatic baseline setting to specific wells in the reaction plate. See also baseline.

automatic C_T An analysis setting in which the software calculates the baseline start and end values and the threshold in the amplification plot. The software uses the baseline and threshold to calculate the threshold cycle (C_T). See also threshold cycle (C_T).

baseline In the amplification plot, a line fit to the fluorescence levels during the initial stages of PCR, when there is little change in fluorescence.

baseline-corrected normalized reporter (ΔRn)

The magnitude of normalized fluorescence generated by the reporter:

1. In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs. Cycle amplification plot, ΔRn is calculated at each cycle as:

   ΔRn (cycle) = Rn (cycle) − Rn (baseline), where Rn = normalized reporter

2. In genotyping experiments and presence/absence experiments, the difference in normalized fluorescence generated by the reporter between the pre-PCR read and the post-PCR read. In the allelic discrimination plot (genotyping experiments) and the presence/absence plot (presence/absence experiments), ΔRn is calculated as:

   ΔRn = Rn (post-PCR read) − Rn (pre-PCR read), where Rn = normalized reporter

See also normalized reporter (Rn).

biological replicates

Reactions that contain identical components and volumes, but evaluate separate samples of the same biological source (for example, samples from three different mice of the same strain, or separate extractions of the same cell line or tissue sample). See also technical replicates.

blocked IPC

In presence/absence experiments, a reaction that contains IPC blocking agent, which blocks amplification of the internal positive control (IPC). In the 7500 software, the task for the IPC target in wells that contain IPC blocking agent. See also negative control-blocked IPC wells.

C_T

See threshold cycle (C_T).

calibrator

See reference sample.

chemistry

See reagents.

comparative C_T ($\Delta\Delta C_T$) method

Method for determining relative target quantity in samples. With the comparative C_T ($\Delta\Delta C_T$) method, the 7500 software measures amplification of the target and of the endogenous control in samples and in a reference sample. Measurements are normalized using the endogenous control. The software determines the relative quantity of target in each sample by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

custom dye

Dye that is not supplied by Applied Biosystems. Custom dyes may be adapted for use in experiments on the 7500/7500 Fast system. When using custom dyes, the custom dye should be added to the Dye Library and a custom dye calibration performed.

cycle threshold

See threshold cycle (C_T).

cycling stage

In the thermal profile, a stage that is repeated. A cycling stage is also called an amplification stage. For cycling stages, you can enable AutoDelta settings. See also amplification stage.

data collection A process during the instrument run in which an instrument component detects fluorescence data from each well of the reaction plate. The instrument transforms the signal to electronic data, and the data are saved in the experiment file. In the 7500 software, a data collection point is indicated by an icon in the thermal profile:

- Data collection on:
- Data collection off:

delta Rn (ΔRn) See baseline-corrected normalized reporter (ΔRn).

derivative reporter (–Rn′) The negative first-derivative of the normalized fluorescence generated by the reporter during PCR amplification. In the derivative reporter (–Rn′) vs. temperature melt curve, the derivative reporter signal is displayed in the y-axis.

Design Wizard A feature in the 7500 software that helps you set up your experiment by guiding you through best practices as you enter your experiment design.

diluent A reagent used to dilute a sample or standard before adding it to the PCR reaction. The diluent can be water or buffer.

Diluted Sample Concentration (10× for Reaction Mix) In the 7500 software, a field displayed on the Sample Dilution Calculations tab of the Reaction Setup screen. For this field, enter the sample concentration you want to use to add to the reaction mix for all samples in the experiment. "10× for Reaction Mix" indicates that the software assumes the sample or standard component of the reaction mix is at a 10× concentration. For example, if the diluted sample concentration is 50.0 ng/µL (10×), the final sample concentration in the reaction is 5 ng/µL (1×).

dilution factor See serial factor.

dissociation curve See melt curve.

EFF% See amplification efficiency (EFF%).

endogenous control A target or gene that should be expressed at similar levels in all samples you are testing. Endogenous controls are used in relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments to normalize fluorescence for the target you are quantifying. Housekeeping genes can be used as endogenous controls. See also housekeeping gene.

endpoint read See post-PCR read.

experiment Refers to the entire process of performing a run using the 7500/7500 Fast system, including setup, run, and analysis. The types of experiments you can perform using the 7500/7500 Fast systems:

- Quantitation - standard curve
- Quantitation - relative standard curve
- Quantitation - comparative C_T ($\Delta\Delta C_T$)
- Melt curve
- Genotyping
- Presence/absence

experiment name Entered during experiment setup, the name that is used to identify the experiment. Experiment names cannot exceed 100 characters and cannot include any of the following characters: forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), or semicolon (;).

experiment type The type of experiment you are performing using the 7500/7500 Fast system:

- Standard curve
- Comparative C_T ($\Delta\Delta C_T$)
- Relative standard curve
- Melt curve (not available in the Design Wizard)
- Genotyping
- Presence/absence

The experiment type you select affects the setup, run, and analysis.

forward primer Oligonucleotide that flanks the 5′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

holding stage In the thermal profile, a stage that includes one or more steps. You can add a holding stage to the thermal profile to activate enzymes, to inactivate enzymes, or to incubate a reaction.

housekeeping gene A gene that is involved in basic cellular functions and is constitutively expressed. Housekeeping genes can be used as endogenous controls. See also endogenous control.

internal positive control (IPC) In presence/absence experiments, a short synthetic DNA template that is added to PCR reactions. You can use the IPC to distinguish between true negative results (that is, the target is absent in the samples) and negative results caused by PCR inhibitors, incorrect assay setup, or reagent or instrument failure.

inventoried assays TaqMan® Gene Expression Assays and TaqMan® SNP Genotyping Assays that have been previously manufactured, passed quality control specifications, and stored in inventory.

IPC In presence/absence experiments, abbreviation for internal positive control (IPC). In the 7500 software, the task for the IPC target in wells that contain the IPC and do not contain IPC blocking agent. See also internal positive control (IPC).

IPC blocking agent Reagent added to PCR reactions to block amplification of the internal positive control (IPC).

IPC+ See negative control-IPC wells.

made-to-order assays TaqMan® Gene Expression Assays or TaqMan® SNP Genotyping Assays that are manufactured at the time of order. Only assays that pass manufacturing quality control specifications are shipped.

manual baseline An analysis setting in which you enter the baseline start and end values for the amplification plot. You can apply the manual baseline setting to specific wells in the reaction plate.

manual C_T An analysis setting in which you enter the threshold value and select whether to use automatic baseline or manual baseline values. The software uses the baseline and the threshold values to calculate the threshold cycle (C_T).

melt curve A plot of data collected during the melt curve stage. Peaks in the melt curve can indicate the melting temperature (Tm) of the target or can identify nonspecific PCR amplification. In the 7500 software, you can view the melt curve as normalized reporter (Rn) vs. temperature or as derivative reporter ($-Rn'$) vs. temperature. Also called dissociation curve.

melt curve stage In the thermal profile, a stage with a temperature increment to generate a melt curve.

melting temperature (Tm) In melt curve experiments, the temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA. The Tm is displayed in the melt curve.

multicomponent plot A plot of the complete spectral contribution of each dye for the selected well(s) over the duration of the PCR run.

negative control (NC) In the 7500 software, the task for targets or SNP assays in wells that contain water or buffer instead of sample. No amplification of the target should occur in negative control wells. Previously called no template control (NTC).

negative control-blocked IPC wells In presence/absence experiments, wells that contain IPC blocking agent instead of sample in the PCR reaction. No amplification should occur in negative control-blocked IPC wells because the reaction contains no sample and amplification of the IPC is blocked. Previously called no amplification control (NAC).

negative control-IPC wells In presence/absence experiments, wells that contain IPC template and buffer or water instead of sample. Only the IPC template should amplify in negative control-IPC wells because the reaction contains no sample. Previously called IPC+.

no amplification control (NAC) See negative control-blocked IPC wells.

no template control (NTC) See negative control (NC).

nonfluorescent quencher-minor groove binder (NFQ-MGB) Molecules that are attached to the 3′ end of TaqMan® probes. When the probe is intact, the nonfluorescent quencher (NFQ) prevents the reporter dye from emitting fluorescence. Because the NFQ does not fluoresce, it produces lower background signals, resulting in improved precision in quantitation. The minor groove binder (MGB) increases the melting temperature (Tm) without increasing probe length. It also allows the design of shorter probes.

normalized quantity Quantity of target divided by the quantity of endogenous control.

normalized reporter (Rn) Fluorescence from the reporter dye normalized to the fluorescence of the passive reference.

omit well An action that you perform before reanalysis to omit one or more wells from analysis. Because no algorithms are applied to omitted wells, omitted wells contain no results.

outlier For a set of data, a datapoint that is significantly smaller or larger than the others.

passive reference A dye that produces fluorescence. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in concentrations or volume. Normalization to the passive reference signal allows for high data precision.

plate layout An illustration of the grid of wells and assigned content in the reaction plate. In the 7500/7500 Fast system, the grid contains 8 rows and 12 columns.

In the 7500 software, you can use the plate layout as a selection tool to assign well contents, to view well assignments, and to view results. The plate layout can be printed, included in a report, exported, and saved as a slide for a presentation.

point One standard in a standard curve. The standard quantity for each point in the standard curve is calculated based on the starting quantity and serial factor.

positive control In genotyping experiments, a DNA sample with a known genotype, homozygous or heterozygous. In the 7500 software, the task for the SNP assay in wells that contain a sample with a known genotype.

post-PCR read Used in genotyping and presence/absence experiments, the part of the instrument run that occurs after amplification. In genotyping experiments, fluorescence data collected during the post-PCR read are displayed in the allelic discrimination plot and used to make allele calls. In presence/absence experiments, fluorescence data collected during the post-PCR read are displayed in the presence/absence plot and used to make detection calls. Also called endpoint read.

pre-PCR read Used in genotyping and presence/absence experiments, the part of the instrument run that occurs before amplification. The pre-PCR read is optional but recommended. Fluorescence data collected during the pre-PCR read can be used to normalize fluorescence data collected during the post-PCR read.

primer mix PCR reaction component that contains the forward primer and reverse primer designed to amplify the target.

primer/probe mix PCR reaction component that contains the primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.

pure dye See custom dye and system dye.

quantitation method In quantitation experiments, the method used to determine the quantity of target in the samples. In 7500/7500 Fast systems, there are three types of quantitation methods: standard curve, relative standard curve, and comparative C_T ($\Delta\Delta C_T$).

quantity In quantitation experiments, the amount of target in the samples. Absolute quantity can refer to copy number, mass, molarity, or viral load. Relative quantity refers to the fold-difference between normalized quantity of target in the sample and normalized quantity of target in the reference sample.

quencher A molecule attached to the 3′ end of TaqMan® probes to prevent the reporter from emitting fluorescence while the probe is intact. With TaqMan® reagents, a nonfluorescent quencher-minor groove binder (NFQ-MGB) can be used as the quencher. With SYBR® Green reagents, no quencher is used.

QuickStart A feature in 7500/7500 Fast systems that allows you to run an experiment without entering plate setup information.

R^2 value Regression coefficient calculated from the regression line in the standard curve. The R^2 value indicates the closeness of fit between the standard curve regression line and the individual C_T data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.

ramp The rate at which the temperature changes during the instrument run. Except for the melt curve step, the ramp is defined as a percentage. For the melt curve step, the ramp is defined as a temperature increment. In the graphical view of the thermal profile, the ramp is indicated by a diagonal line.

ramp speed Speed at which the temperature ramp occurs during the instrument run. Available ramp speeds include fast and standard.

- For optimal results using the fast ramp speed, Applied Biosystems recommends using TaqMan® Fast reagents in your PCR reactions.
- For optimal results using the standard ramp speed, Applied Biosystems recommends using standard reagents in your PCR reactions.

IMPORTANT! TaqMan Fast reagents are not supported for genotyping or presence/absence experiments.

raw data plot A plot of raw fluorescence (not normalized) for each optical filter.

reaction mix A solution that contains all components to run the PCR reaction, except for the template (sample, standard, or control).

reagents The PCR reaction components you are using to amplify the target and to detect amplification. Types of reagents used on the 7500/7500 Fast systems:

- TaqMan® reagents
- SYBR® Green reagents
- Other reagents

real-time PCR Process of collecting fluorescence data during PCR. Data from the real-time PCR are used to calculate results for quantitation experiments or to troubleshoot results for genotyping or presence/absence experiments.

reference sample In relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments, the sample used as the basis for relative quantitation results. Also called the calibrator.

refSNP ID Identifies the reference SNP (refSNP) cluster ID. Generated by the Single Nucleotide Polymorphism Database of Nucleotide Sequence Variation (dbSNP) at the National Center for Biotechnology Information (NCBI). The refSNP ID can be used to search the Applied Biosystems Store for an Applied Biosystems SNP Genotyping Assay. Also called an rs number.

regression coefficients Values calculated from the regression line in standard curves, including the R^2 value, slope, and y-intercept. You can use the regression coefficients to evaluate the quality of results from the standards. See also standard curve.

regression line In standard curve and relative standard curve experiments, the best-fit line from the standard curve. Regression line formula:

$C_T = m [\log (Qty)] + b$

where m is the slope, b is the y-intercept, and Qty is the standard quantity.

See also regression coefficients.

reject well An action that the software performs during analysis to remove one or more wells from further analysis if a specific flag is applied to the well. Rejected wells contain results calculated up to the point of rejection.

relative standard curve method Method for determining relative target quantity in samples. With the relative standard curve method, the 7500 software measures amplification of the target and of the endogenous control in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates target quantity in the samples and in the reference sample. The software determines the relative quantity of target in each sample by comparing target quantity in each sample to target quantity in the reference sample.

replicate group A set of identical reactions in an experiment.

replicates See technical replicates or biological replicates.

reporter Fluorescent dye used to detect amplification. If you are using TaqMan® reagents, the reporter dye is attached to the 5′ end. If you are using SYBR® Green reagents, the reporter dye is SYBR® Green dye.

reverse primer An oligonucleotide that flanks the 3′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

reverse transcriptase An enzyme that converts RNA to cDNA. Reverse transcriptase is added to the PCR reaction to perform 1-step RT-PCR.

Rn See normalized reporter (Rn).

ROX™ dye A dye supplied by Applied Biosystems and precalibrated on the 7500/7500 Fast systems. ROX dye is used as the passive reference.

rs number See refSNP ID.

run method Definition of the reaction volume and the thermal profile for the 7500/7500 Fast instrument run.

sample The template that you are testing.

Sample DNA (10×) In the 7500 software, a reaction component displayed on the Reaction Mix Calculations tab of the Reaction Setup screen. The software assumes the sample DNA is added to the reaction mix at a 10× concentration. For example, if the reaction volume is 20 µL, the calculated volume of sample for 1 reaction is 2 µL.

Sample Library In the 7500 software, a collection of samples. The Sample Library contains the sample name and the sample color.

Sample or Standard (10×) In the 7500 software, a reaction component displayed on the Reaction Mix Calculations tab of the Reaction Setup screen. The software assumes the sample or standard is added to the reaction mix at a 10× concentration. For example, if the reaction volume is 20 µL, the calculated volume of sample or standard for 1 reaction is 2 µL.

sample/SNP assay reaction In genotyping experiments, the combination of which sample to test and which SNP assay to perform in one PCR reaction. Each PCR reaction can contain only one sample and one SNP assay.

sample/target reaction In quantitation experiments, the combination of which sample to test and which target to detect and quantify in one PCR reaction. In the Design Wizard, you can detect and quantify only one target in one PCR reaction. Use Advanced Setup to detect and quantify more than one target in one PCR reaction.

serial factor In the 7500 software, a numerical value that defines the sequence of quantities in the standard curve. The serial factor and the starting quantity are used to calculate the standard quantity for each point in the standard curve. For example, if the standard curve is defined with a serial factor of 1:10 or 10×, the difference between any 2 adjacent points in the curve is 10-fold.

series See standard dilution series.

slope Regression coefficient calculated from the regression line in the standard curve. The slope indicates the PCR amplification efficiency for the assay. A slope of −3.32 indicates 100% amplification efficiency. See also amplification efficiency (EFF%) and regression line.

SNP Abbreviation for single nucleotide polymorphism. The SNP can consist of a base difference or an insertion or deletion of one base.

SNP assay Used in genotyping experiments, a PCR reaction that contains primers to amplify the SNP and two probes to detect different alleles.

SNP Assay Library In the 7500 software, a collection of SNP assays to add to genotyping experiments. The SNP assays in the library contain the SNP assay name, SNP assay color, and for each allele, the allele name or base(s), reporter, quencher, and allele colors. The SNP assays in the library may also contain the assay ID and comments about the SNP assay.

region of interest (ROI) calibration Type of 7500/7500 Fast system calibration in which the system maps the positions of the wells in the sample block. ROI calibration data are used so that the software can associate increases in fluorescence during a run with specific wells in the reaction plate.

stage In the thermal profile, a group of one or more steps. There are three types of stages: holding stage (including pre-PCR read and post-PCR read), cycling stage (also called amplification stage), and melt curve stage.

standard Sample that contains known standard quantities. Standard reactions are used in quantitation experiments to generate standard curves. See also standard curve and standard dilution series.

standard curve In standard curve and relative standard curve experiments:

- The best-fit line in a plot of the C_T values from the standard reactions plotted against standard quantities. See also regression line.
- A set of standards containing a range of known quantities. Results from the standard curve reactions are used to generate the standard curve. The standard curve is defined by the number of points in the dilution series, the number of standard replicates, the starting quantity, and the serial factor. See also standard dilution series.

standard curve method Method for determining absolute target quantity in samples. With the standard curve method, the 7500 software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples. See also standard and standard curve.

standard dilution series In standard curve and relative standard curve experiments, a set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards. For example, the standard stock is used to prepare the first dilution point, the first dilution point is used to prepare the second dilution point, and so on. In the 7500 software, the volumes needed to prepare a standard dilution series are calculated by the number of dilution points, the number of standard replicates, the starting quantity, the serial factor, and the standard concentration in the stock. See also standard curve.

standard quantity A known quantity in the PCR reaction.

- In standard curve experiments, the quantity of target in the standard. In the 7500 software, the units for standard quantity can be for mass, copy number, viral load, or other units for measuring the quantity of target.
- In relative standard curve experiments, a known quantity in the standard. Standard quantity can refer to the quantity of cDNA or the quantity of standard stock in the PCR reaction. The units are not relevant for relative standard curve experiments because they cancel out in the calculations.

starting quantity When defining a standard curve in the 7500 software, corresponds to the highest or lowest quantity.

step A component of the thermal profile. For each step in the thermal profile, you can set the ramp rate (ramp increment for melt curve steps), hold temperature, hold time (duration), and you can turn data collection on or off for the ramp or the hold parts of the step. For cycling stages, a step is also defined by the AutoDelta status.

SYBR® Green reagents PCR reaction components that consist of two primers designed to amplify the target and SYBR® Green dye to detect double-stranded DNA.

system dye Dye supplied by Applied Biosystems and precalibrated on the 7500/7500 Fast system. Before you use system dyes in your experiments, make sure the system dye calibration is current in the Instrument Maintenance Manager.

System dyes of the 7500/7500 Fast system include:

- FAM™ dye
- JOE™ dye
- NED™ dye
- ROX™ dye
- SYBR® Green dye
- TAMRA™ dye
- VIC® dye
- CY3 dye
- CY5 dye
- TEXAS RED® dye

TaqMan® reagents PCR reaction components that consist of primers designed to amplify the target and a TaqMan® probe designed to detect amplification of the target.

target The nucleic acid sequence that you want to amplify and detect.

target color In the 7500 software, a color assigned to a target to identify the target in the plate layout and analysis plots.

Target Library In the 7500 software, a collection of targets to add to experiments. The targets in the library contain the target name, reporter, quencher, and target color. The target in the library may also contain comments about the target.

task In the 7500 software, the type of reaction performed in the well for the target or SNP assay. Available tasks:

- Unknown
- Negative Control
- Standard (standard curve and relative standard curve experiments)
- Positive control (genotyping experiments)
- IPC (presence/absence experiments)
- Blocked IPC (presence/absence experiments)

technical replicates Identical reactions that contain identical components and volumes and evaluate the same sample.

See also biological replicates.

template In the Design Wizard of the 7500 software (and in QuickStart for quantitation experiments), the type of nucleic acid to add to the PCR reaction. The recommended template varies according to experiment type:

- Quantitation experiments (standard curve, relative standard curve, and comparative C_T) – cDNA (complementary cDNA), RNA, or gDNA (genomic DNA)

  For quantitation experiments, the template type selection affects the run method, reaction setup, and materials list.
- Genotyping experiments – Wet DNA (gDNA or cDNA) or dry DNA (gDNA or cDNA)

  For genotyping experiments, the template type selection affects the reaction setup.
- Presence/absence experiments - DNA

  For presence/absence experiments, Applied Biosystems recommends adding DNA templates to the PCR reactions.

thermal profile Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the 7500/7500 Fast instrument run.

threshold

1. In amplification plots, the level of fluorescence above the baseline and within the exponential growth region The threshold can be determined automatically (see automatic C_T) or can be set manually (see manual C_T).
2. In presence/absence experiments, the level of fluorescence above which the 7500 software assigns a presence call.

threshold cycle (C_T) The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.

Tm See melting temperature (Tm).

unknown In the 7500 software, the task for the target or SNP assay in wells that contain the sample you are testing:

- In quantitation experiments, the task for the target in wells that contain a sample with unknown target quantities.
- In genotyping experiments, the task for the SNP assay in wells that contain a sample with an unknown genotype.
- In presence/absence experiments, the task for the target in wells that contain a sample in which the presence of the target is not known.

unknown-IPC wells In presence/absence experiments, wells that contain a sample and internal positive control (IPC).

y-intercept In the standard curve, the value of y where the regression line crosses the y-axis. The y-intercept indicates the expected threshold cycle (C_T) for a sample with quantity equal to 1.

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Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

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