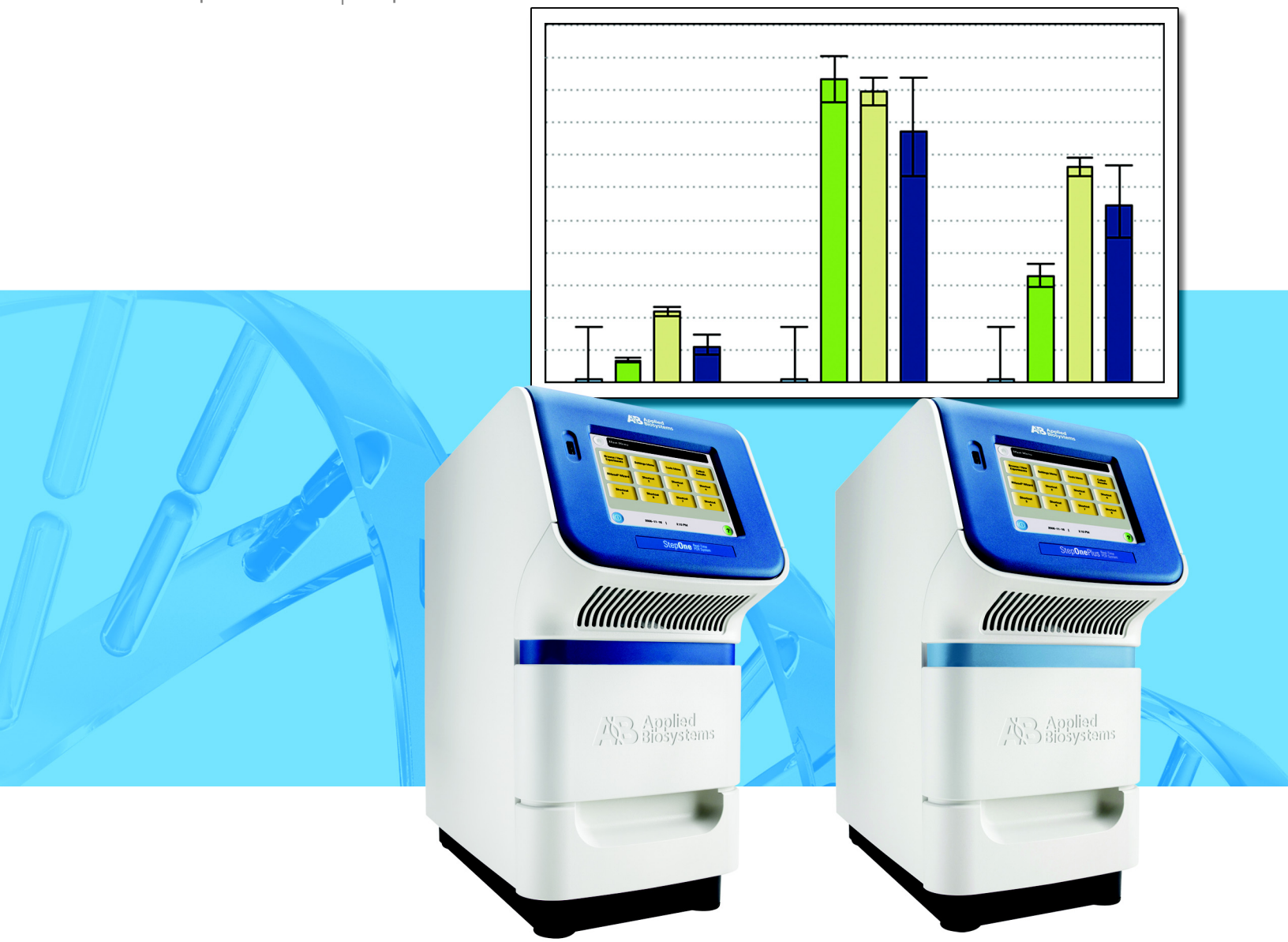
Applied Biosystems StepOne™ and StepOnePlus™

Real-Time PCR Systems

Relative Standard Curve and Comparative C_T Experiments



Applied Biosystems

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Relative Standard Curve and Comparative C_T Experiments

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Contents

Preface

How to Use This Guide

About the System Documentation

The guides listed below are shipped with the Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems (StepOne™ and StepOnePlus™ systems).

Guide	Purpose and Audience	PN
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Genotyping Experiments	Explains how to perform experiments on the StepOne and StepOnePlus systems. Each Getting Started Guide functions as both: • A tutorial, using example experiment data provided with the Applied Biosystems StepOne™ Real-Time PCR Software (StepOne™ software). • A guide for your own experiments. Intended for laboratory staff and principal investigators who perform experiments using the StepOne or StepOnePlus system.	4376786
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Presence/Absence Experiments		4376787
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments		4376785
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments		4376784
Applied Biosystems 7500/7500Fast, StepOne™, and StepOnePlus™ Real-Time PCR Systems Quick Reference Card for Comparative C_T Experiments and Studies		4411937
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking, and Maintenance Guide	Explains how to install and maintain the StepOne and StepOnePlus systems. Intended for laboratory staff responsible for the installation and maintenance of the StepOne or StepOnePlus system	4376782
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation Quick Reference Card		4376783

Guide	Purpose and Audience	PN
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Reagent Guide	Provides information about the reagents you can use on the StepOne and StepOnePlus systems, 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 StepOne or StepOnePlus system.	4379704
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Site Preparation Guide	Explains how to prepare your site to receive and install the StepOne and StepOnePlus systems. Intended for personnel who schedule, manage, and perform the tasks required to prepare your site for installation of the StepOne or StepOnePlus system.	4376768
Applied Biosystems StepOne™ Real-Time PCR Software Help	Explains how to use the StepOne software to: • Set up, run, and analyze experiments using the StepOne and StepOnePlus systems. • Monitor networked StepOne and StepOnePlus instruments. • Calibrate StepOne and StepOnePlus instruments. • Verify the performance of StepOne and StepOnePlus instruments with an RNase P run. Intended for: • Laboratory staff and principal investigators who perform experiments using the StepOne or StepOnePlus system. • Laboratory staff responsible for the installation and maintenance of the StepOne or StepOnePlus 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 StepOne or StepOnePlus system into your existing laboratory data flow.

Text Conventions

This guide uses the following conventions:

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

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

  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▸) separates successive commands you select from a drop-down 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 xiv.

How to Obtain More Information

Related Documentation

Other StepOne and StepOnePlus System Documents

The documents listed in the table below are not shipped with the StepOne or StepOnePlus instrument.

Document	PN
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation Performance Verification Protocol	4376791
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation Qualification-Operation Qualification Protocol	4376790
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Planned Maintenance Protocol	4376788

Documents Related to Genotyping Experiments

Document	PN
Allelic Discrimination Pre-Developed TaqMan® Assay Reagents Quick Reference Card	4312212
Custom TaqMan® Genomic Assays Protocol	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
PrepMan® Ultra Sample Preparation Reagent Protocol	4318925

Documents Related to Relative Standard Curve and Comparative C_T Experiments

Document	PN
Amplification Efficiency of TaqMan® Gene Expression Assays Application Note	127AP05
High-Capacity cDNA Reverse Transcription Kits Protocol	4375575
High Capacity RNA-to-cDNA Kit Protocol	4387951
High Capacity RNA-to-cDNA Master Mix Protocol	4377474
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 (2×) 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 xiii.

Obtaining Information from the Software Help

The StepOne Software Help describes how to use each feature of the user interface. Access the Help from within the software by doing one of the following:

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

To find topics of interest in the Help:

- Review the table of contents.
- Search for a specific topic.
- Search an 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 xiii).

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 StepOne™ or StepOnePlus™ 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 reagent kit use, or safe use of a chemical.

– 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.

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

– Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is 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 symbols that are affixed to Applied Biosystems instruments (see "Safety Symbols" on page xvi).

Examples

IMPORTANT! You must create a separate sample entry spreadsheet for each 96-well plate.

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.

WARNING **PHYSICAL INJURY HAZARD.** During instrument operation, the the heated cover and sample block can reach temperatures in excess of 100 °C.

DANGER **ELECTRICAL HAZARD.** Grounding circuit continuity is vital for the safe operation. Never operate the system with the grounding conductor disconnected.

Symbols on Instruments

Electrical Symbols

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

Symbol	Description
	Indicates the **On** position of the main power switch.
	Indicates the **Off** position of the main power switch.
	Indicates 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 the **On/Off** position of a push-push main power switch.

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

Safety Symbols

The following table describes the safety symbols that may be displayed on Applied Biosystems instruments. Each symbol may appear by itself or with text that explains the relevant hazard (see "Safety Labels on Instruments" on page xvii). 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 a hot surface or other high-temperature hazard and to proceed with appropriate caution.

Symbol	Description
	Indicates the presence of moving parts 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 laser inside the instrument and to proceed with appropriate caution.

Environmental Symbols

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.

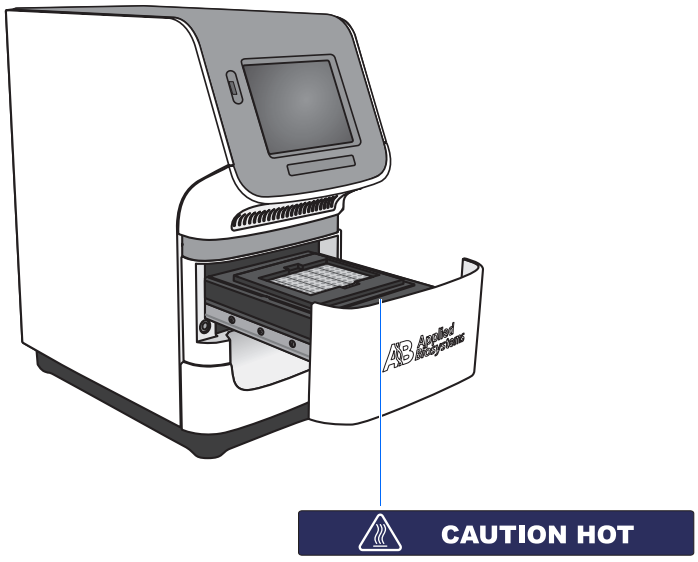
Safety Labels on Instruments

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	Francais
CAUTION Hazardous chemicals. Read the Material Safety Data Sheets (MSDSs) before handling.	**ATTENTION** Produits chimiques dangereux. Lire les fiches techniques de sûreté de matériels avant la manipulation des produits.
CAUTION 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.
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.
DANGER Class 3B (III) visible and/or invisible LED radiation present when open and interlocks defeated. Avoid exposure to beam.	**DANGER** Rayonnement visible ou invisible d'un faisceau LED de Classe 3B (III) en cas d'ouverture et de neutralisation des dispositifs de sécurité. Evitez toute exposition au faisceau.

Locations of Warnings

The StepOne and StepOnePlus instruments contain a warning at the location shown below:



General Instrument Safety

WARNING **PHYSICAL INJURY HAZARD.** Using the 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 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 xix.

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 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 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 xix.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For more 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 more 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 in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

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 **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
   a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.
   b. Select the language of your choice.

c. Click **Search**.

3. To view, download, or print the document of interest:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the document
      - **Save Target As** – To download a PDF version of the document to a destination that you choose
      - **Print Target** – To print the document
4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:
   a. Select **Fax** or **Email** below the document title.
   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
   c. Enter the required information.
   d. Click **View/Deliver Selected Documents Now**.

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

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 more 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 more safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying a 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

DANGER **ELECTRICAL SHOCK HAZARD.** Severe electrical shock can result from operating the StepOne or StepOnePlus instrument 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

WARNING **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.

WARNING **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

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

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

DANGER **ELECTRICAL HAZARD.** Plug the instrument into a properly grounded receptacle with adequate current capacity.

Overvoltage Rating

The StepOne and StepOnePlus instruments have an installation (overvoltage) category of II, and they are classified as portable equipment.

LED Safety

To ensure safe LED operation:

- The system must be maintained by an Applied Biosystems Technical Representative.
- All instrument panels must be in place on the instrument while the instrument is operating. When all panels are installed, there is no detectable radiation present. If any panel is removed when the LED is operating (during service with safety interlocks disabled), you may be exposed to LED emissions in excess of the Class **3B** rating.
- Do not remove safety labels or disable safety interlocks.

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 performed 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; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).

- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

More information about biohazard guidelines is available at:

http://www.cdc.gov

Workstation Safety

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

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

To minimize musculoskeletal and repetitive motion risks:

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

Safety and Electromagnetic Compatibility (EMC) Standards

U.S. and Canadian Safety Standards

The StepOne and StepOnePlus instruments have been tested to and comply with standard:

UL 61010A-1/CAN/CSA C22.2 No. 1010.1-92, "Safety Requirements for Electrical Equipment for Measurement, Control, and Laboratory Use, Part 1: General Requirements."

UL 61010A-2-010/CAN/CSA 1010.2.010, "Particular Requirements for Laboratory Equipment for the Heating of Materials."

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."

EN 61010-2-010, "Particular Requirements for Laboratory Equipment for the Heating of Materials."

EN 61010-2-081, "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:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

About the StepOne™ and StepOnePlus™ Systems

There are two models available for this Real-Time PCR System:

System	Features
Applied Biosystems StepOne™ Real-Time PCR System (StepOne™ system)	• 48-well platform • Three-color system
Applied Biosystems StepOnePlus™ Real-Time PCR System (StepOnePlus™ system)	• 96-well platform • Four-color system • VeriFlex™ sample blocks

The StepOne and StepOnePlus systems use fluorescent-based polymerase chain reaction (PCR) reagents to provide:

- Quantitative detection of target nucleic acid sequences (targets) using real-time analysis.
- Qualitative detection of targets using post-PCR (endpoint) analysis.
- Qualitative analysis of the PCR product (achieved by melt curve analysis that occurs post-PCR).

About Data Collection

The StepOne and StepOnePlus systems collect raw fluorescence data at different points during a PCR, depending on the type of run that the instruments perform:

Run Type		Data Collection Point
Real-time runs	Standard curve	The instrument collects data following 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 StepOne™ or StepOnePlus™ 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.

Notes

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 StepOne™ software stores the raw fluorescent image for analysis.

After a run, the StepOne software uses calibration data (spatial, dye, and background) to determine the location and intensity of the fluorescent signals in each read, the dye associated with each fluorescent signal, and the significance of the signal.

About the Filters

The StepOne and StepOnePlus systems use the following filters:

StepOne system		StepOnePlus system	
Filter	**Dye**	**Filter**	**Dye**
1	FAM™ dye	1	FAM™ dye
	SYBR® Green dye		SYBR® Green dye
2	JOE™ dye	2	JOE™ dye
	VIC® dye		VIC® dye
3	ROX™ dye	3	TAMRA™ dye
			NED™ dye
		4	ROX™ dye

About the VeriFlex™ Technology

The StepOnePlus instrument contains six independently thermally regulated VeriFlex™ blocks to help you optimize your thermal cycling conditions. You can set a different temperature for one or more of the VeriFlex blocks, creating up to six different zones for samples, or you can set the same temperature for each of the VeriFlex blocks.

For More Information

For information on:

- The StepOne and StepOnePlus systems, refer to *Applied Biosystems StepOne™ Real-Time PCR Software Help*.

  Note: To access the Help, select **Help ▸ StepOne Software Help** from within the StepOne software.

- Genotyping experiments, refer to *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Genotyping Experiments*.
- Presence/absence experiments, refer to *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Presence/Absence Experiments*.
- Standard curve experiments, refer to *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve and Experiments*.

Notes

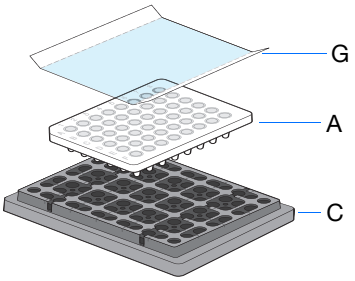
Supported Consumables

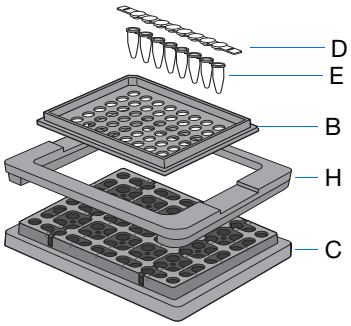
StepOne System

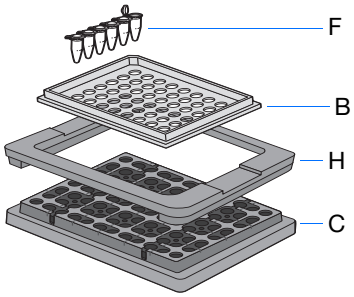
The StepOne system supports the consumables listed below. These consumables are for use with both standard and Fast reagents/protocols.

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

Consumable	Part Number
• MicroAmp® Fast Optical 48-Well Reaction Plate • MicroAmp® 48-Well Optical Adhesive Film	• 4375816 • 4375323 and 4375928
• MicroAmp® Fast 8-Tube Strip • MicroAmp® Optical 8-Cap Strip	• 4358293 • 4323032
• MicroAmp® Fast Reaction Tube with Cap	• 4358297
• MicroAmp® Fast 48-Well Tray • MicroAmp® 48-Well Base Adaptor • MicroAmp® 96-Well Support Base	• 4375282 • 4375284 • 4379590







#	Consumable
A	MicroAmp® Fast Optical 48-Well Reaction Plate
B	MicroAmp® Fast 48-Well Tray
C	MicroAmp® 96-Well Support Base
D	MicroAmp® Optical 8-Cap Strip
E	MicroAmp® Fast 8-Tube Strip
F	MicroAmp® Fast Reaction Tube with Cap
G	MicroAmp® 48-Well Optical Adhesive Film
H	MicroAmp® 48-Well Base Adaptor

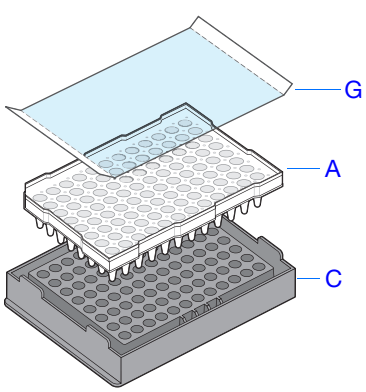
Notes

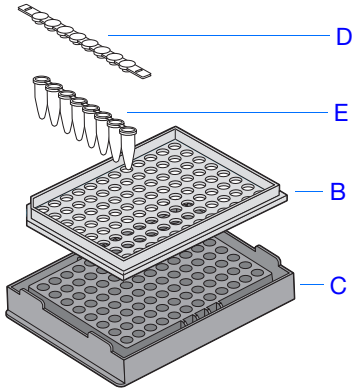
StepOnePlus System

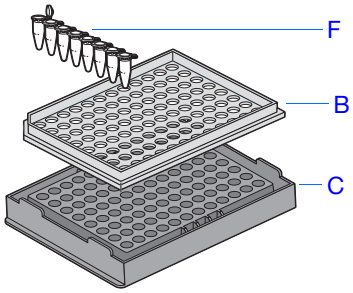
The StepOnePlus system supports the consumables listed below. These consumables are for use with both standard and Fast reagents/protocols.

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

Consumable	Part Number
• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode • MicroAmp® Optical Adhesive Film	• 4346906 and 4366932 • 4360954 and 4311971
• MicroAmp® Fast 8-Tube Strip • MicroAmp® Optical 8-Cap Strip	• 4358293 • 4323032
• MicroAmp® Fast Reaction Tube with Cap	• 4358297
• MicroAmp® 96-Well Tray for VeriFlex™ Blocks • MicroAmp® 96-Well Support Base	• 4379983 • 4379590
• MicroAmp® Adhesive Film Applicator • MicroAmp® Cap Installing Tool (Handle)	• 4333183 • 4330015







#	Consumable
A	MicroAmp® Fast Optical 96-Well Reaction Plate
B	MicroAmp® 96-Well Tray for VeriFlex™ Blocks
C	MicroAmp® 96-Well Support Base
D	MicroAmp® Optical 8-Cap Strip
E	MicroAmp® Fast 8-Tube Strip
F	MicroAmp® Fast Reaction Tube with Cap
G	MicroAmp® Optical Adhesive Film

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 (Kwok and Higuchi, 1989).
- 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 (Saiki *et al.*, 1985).

Note: In this guide, the term *experiment* refers to the entire process of performing a run using the StepOne or StepOnePlus 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 StepOne 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 vs. 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.

Notes

- **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 signals 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.

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 StepOne 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 vs. 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 signals 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:

Experiment Type	Description	Advantage	Limitation
Relative standard curve	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.	Requires the least amount of validation because the PCR efficiencies of the target and endogenous control do not need to be equivalent.	A standard curve must be constructed for each target, which requires more reagents and more space in the reaction plate.
Comparative C_T ($\Delta\Delta C_T$)	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.	• Relative levels of target in samples can be determined without the use of a standard curve, provided that the PCR efficiencies of the target and endogenous control are relatively equivalent. • Reduced reagent usage. • More space available in the reaction plate.	• 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.

PCR Options

When performing real-time PCR, choose between:

- Singleplex and multiplex PCR (below)

  and

- 1-step and 2-step RT-PCR (page 9)

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.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA™ dye as a reporter or quencher with the StepOne system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus system.

Notes

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.

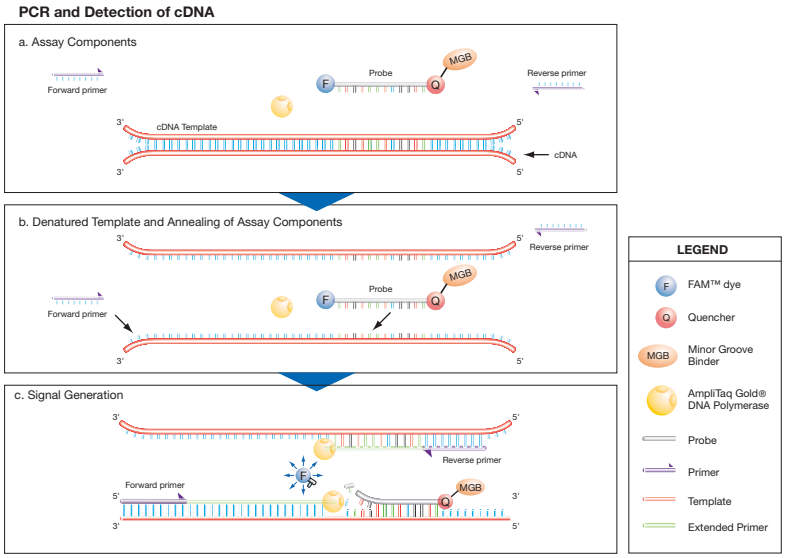
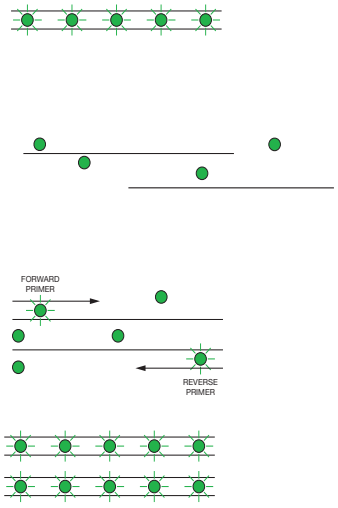
Note: For more information on AmpErase® UNG, refer to the *Real-Time PCR Systems Reagent Guide*.

Notes

Supported Reagents

TaqMan® and SYBR® Green Reagents

Applied Biosystems offers TaqMan® and SYBR® Green reagents for use on the StepOne and StepOnePlus systems. Both reagent types are briefly described in the table below.

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.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA dye as a reporter or quencher with the StepOne system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus system.

Notes

Other Reagents

You can use other fluorescent-based reagents on the StepOne and StepOnePlus systems, but note the following:

- You must design your experiment using Advanced Setup instead of the Design Wizard. (See "Advanced Setup Workflow" on page 260.)
- For Applied Biosystems TaqMan and SYBR Green reagents, the StepOne software automatically calculates reaction volumes in the Reaction Setup screen.

For More Information

For more information on real-time PCR experiments, PCR options, and reagents, refer to the *Real-Time PCR Systems 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

By using the example experiment data provided with the StepOne software, you can use this guide as a tutorial for performing a relative standard curve or comparative C_T experiment on a StepOne or StepOnePlus system. Follow the procedures in the appropriate chapters:

Chapter		Procedure
Relative Standard Curve	**Comparative C_T**	
2	6	Design the experiment using the Design Wizard in the StepOne 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 StepOne or StepOnePlus instrument (standalone or colocated layout).
5	9	Analyze the results.

For more information, see "About the Example Experiments" on page 12.

Using This Guide With Your Own Experiments

After completing the tutorial exercises in Chapters 2 to 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 to 9 includes a set of guidelines that you can use to perform your own experiments.

Notes

Additionally, you can use one of the other workflows provided in the StepOne software to perform your experiments. The table below provides a summary of all the workflows available in the StepOne software.

Workflow	Description	See...
Design Wizard	Set up a new experiment with guidance from the software. The Design Wizard walks 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 260
QuickStart	Run a new experiment with no plate setup information. If desired, you can add all design parameters after the run.	page 261
Template	Set up a new experiment using setup information from a template.	page 263
Export/Import	Import experiment designs from ASCII text files that contain experiment setup information.	page 265

About the Example Experiments

To illustrate how to perform relative standard curve and comparative C_T experiments, this guide leads you through the process of designing, preparing, running, and analyzing an example experiment. The example experiment represents a typical setup that you can use to quickly familiarize yourself with the StepOne or StepOnePlus system.

Description of the Relative Standard Curve Example Experiment

The objective of the relative standard curve example experiment is to compare the expression 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:

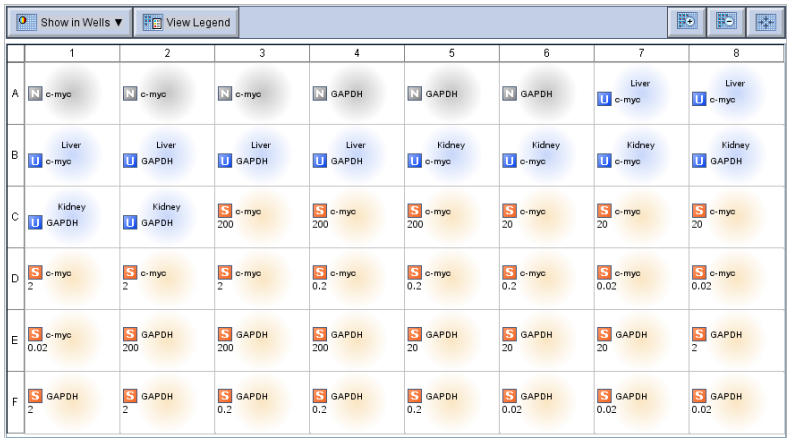
- The samples are cDNA prepared from total RNA isolated from liver and kidney tissues.
- The target is human c-myc.
- The endogenous control is human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
- The reference sample is RNA isolated from kidney tissue.
- One standard curve is set up for c-myc (target). The standard used for the standard dilution series is a cDNA sample of known quantity prepared from RNA isolated from lung tissue.

Notes

- 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® Fast 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_002046.2).

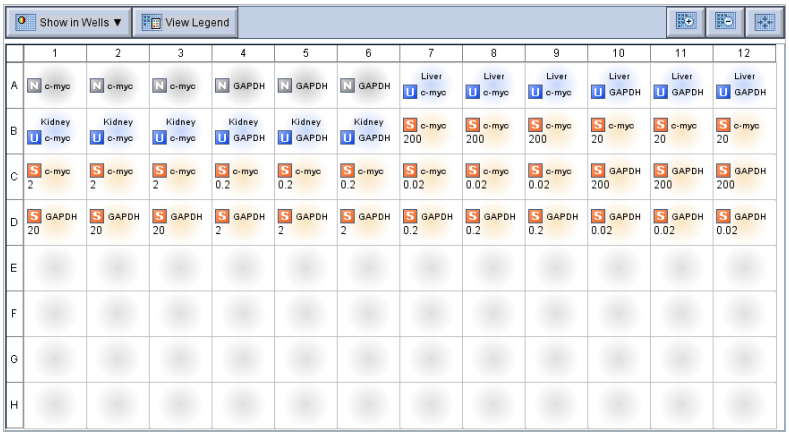
Reaction Plate Layout

The relative standard curve example experiment was created for a StepOne instrument. For the StepOne instrument, the software displays a 48-well reaction plate layout:



You can create the example experiment for a StepOnePlus instrument; however, your reaction plate layout will differ from the 48-well reaction plate layout shown throughout this guide. For the StepOnePlus instrument, the software displays a 96-well reaction plate layout:

Notes



Description of the Comparative C_T Example Experiment

The objective of the comparative C_T example experiment is to compare the expression of TP53 (a transcription factor that regulates other genes) in liver, kidney, and brain tissues.

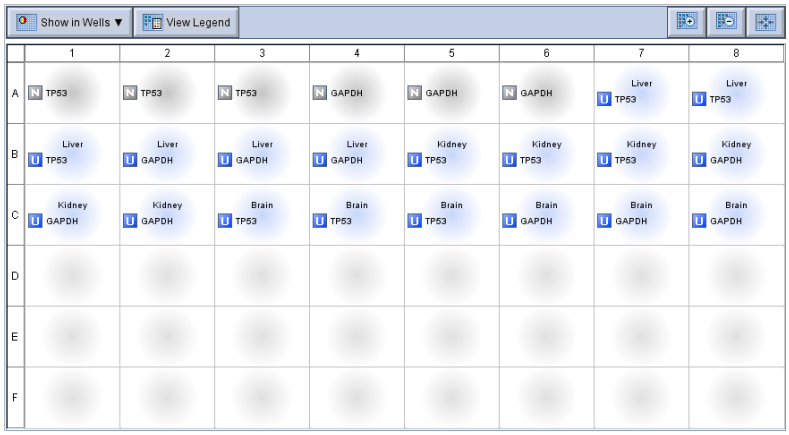
In the comparative C_T example experiment:

- The samples are cDNA prepared from total RNA isolated from liver, kidney, and brain tissues.
- The target is TP53.
- The reference sample is brain.
- The endogenous control is human GAPDH.
- The experiment is designed for singleplex PCR, where the target (TP53) 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® Fast 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 (TP53), the assay ID is Hs00153340_m1 (RefSeq NM_000546.2).
  - For the endogenous control assay (GAPDH), the Human GAPD (GAPDH) Endogenous Control kit (PN 4333764T) is used.

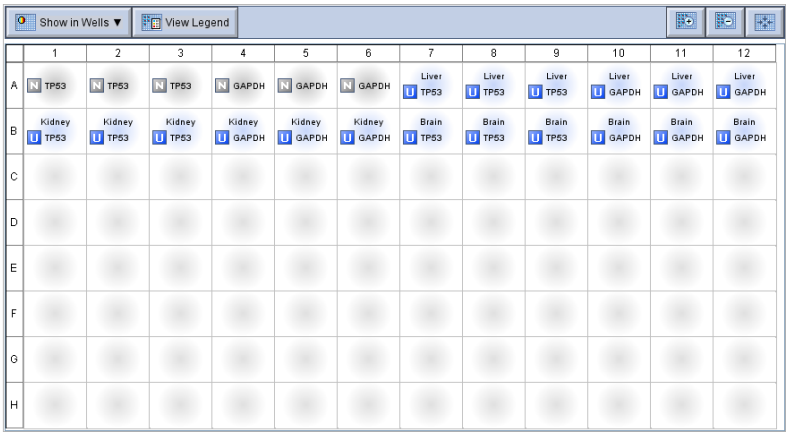
Reaction Plate Layout

The comparative CT example experiment was created for a StepOne instrument. For the StepOne instrument, the software displays a 48-well reaction plate layout:

Notes



You can create the example experiment for a StepOnePlus instrument; however, your reaction plate layout will differ from the 48-well reaction plate layout shown throughout this guide. For the StepOnePlus instrument, the software displays a 96-well reaction plate layout:



Notes

Description of the Comparative C_T Example Study

In the comparative C_T example study:

- 11 reaction plates (experiments) are used.
- The cDNA was prepared from total RNA that was isolated from the following 11 samples:

Sample Name		Sample Name
Placenta		Spleen
Liver		Stomach
Brain		Heart
Lung		Colon
Testes		Ovary
Bladder		

- 16 targets (assays) are used:

Target	Assay ID No.		Target	Assay ID No.
18S‡	Hs99999901_s1		CENPA	Hs00156455_m1
HPRT1‡	Hs99999909_m1		DCT	Hs00157244_m1
UBC‡	Hs00824723_m1		GABRD	Hs00181309_m1
POLR2A‡	Hs00172187_m1		PLA2G2A	Hs00179898_m1
ACTN2	Hs00153809_m1		SFPQ	Hs00192574_m1
ATP1A2	Hs00265131_m1		SHH	Hs00179843_m1
C1orf61	Hs00197247_m1		VCAM1	Hs00174239_m1
CD58	Hs00156385_m1		WNT2	Hs00608224_m1

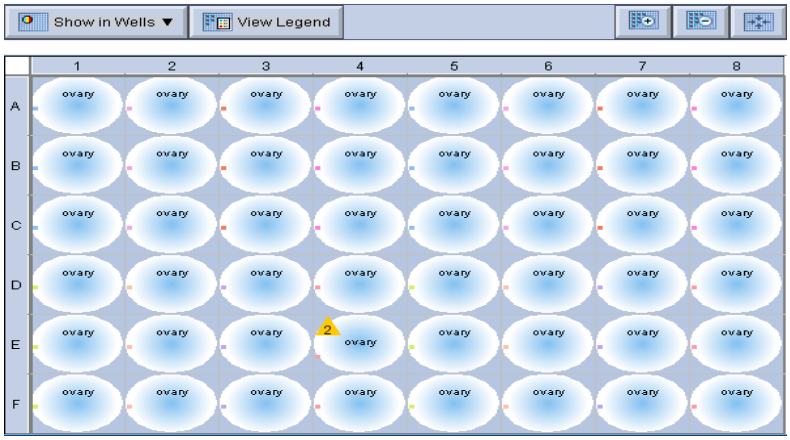
‡ Can be used as an endogenous control.

- The reference sample is bladder.
- The endogenous control is 18S.
- Each experiment in the study was designed for singleplex PCR, where the target and endogenous control assays are performed in separate wells.
- For each experiment in the study, reactions were set up for 2-step RT-PCR:
  - The cDNA was reverse-transcribed from total RNA samples using High Capacity RNA-to-cDNA Master Mix.
  - The reactions were prepared using TaqMan® Fast Universal PCR Master Mix (2✕).

Notes

Reaction Plate Layout

The comparative C_T example study was created for a StepOne instrument. For the StepOne instrument, the software displays a 48-well reaction plate layout:



You can create the example study for a StepOnePlus instrument; however, your reaction plate layout will differ from the 48-well reaction plate layout shown throughout this guide. For the StepOnePlus instrument, the software displays a 96-well reaction plate layout.

About the Example Experiment and Example Study Data

In this getting started guide you will use six files:

- In Chapters 2 and 6, you will create example experiment files that contain setup data (the Relative Standard Curve Example.eds file in Chapter 2 and the Comparative CT Example.eds file in Chapter 6). Save these files to the experiments folder on your computer at:

  <drive>:\Applied Biosystems*<software name>*\experiments\
- In Chapters 5 and 9, you will view results in example experiment files that contain run data (the Relative Standard Curve Example.eds file in Chapter 5 and the Comparative CT Example.eds file in Chapter 9). The data files for the example experiments are installed with the StepOne software. You can find the data files for the example experiments on your computer at:

  <drive>:\Applied Biosystems*<software name>*\experiments\examples
- In Chapter 10, you will:
  - Create an example study file that contains setup data (the Comparative CT Study Example.edm file). Save the file to the experiments folder on your computer at:

    <drive>:\Applied Biosystems*<software name>*\experiments
  - View results in an example study file that contains run data (the Comparative CT Study Example.edm). The data file for the example study is installed with the StepOne software. You can find the data file for the example study on your computer at:

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

Notes

where:

- *<drive>* is the computer hard drive on which the StepOne software is installed. The default installation drive for the software is the D drive.
- *<software name>* is the current version of the StepOne software.

Data Files in the Examples Folder

The examples folder contains several data files that you can refer to when you analyze your own data, as listed in the table below. The data files are installed with the StepOne software.

Note: Be sure to use the Relative Standard Curve Example.eds, Comparative CT Example.eds, and Comparative CT Study Example.edm files when you perform the tutorial procedures in this guide. The 96-Well Relative Standard Curve Example.eds and 96-Well Comparative CT Example.eds files are different examples of relative standard curve and comparative C_T methods.

StepOne Instrument	StepOnePlus Instrument
Comparative CT Example.eds	96-Well Comparative CT Example.eds
Comparative CT Study Bio Replicates Example.edm	—
Comparative CT Study Example.edm	—
Genotyping Example.eds	96-Well Genotyping Example.eds
Multiplex Example.eds	96-Well Multiplex Example.eds
Presence Absence Example.eds	96-Well Presence Absence Example.eds
Relative Standard Curve Example.eds	96-Well Relative Standard Curve Example.eds
RNase P Experiment.eds	96-Well RNase P Experiment.eds
Standard Curve Example.eds	96-Well Standard Curve Example.eds
SYBR Example.eds	96-Well SYBR Example.eds

Example Experiment Workflow

The figure on page 19 shows the workflow for the relative standard curve and comparative C_T example experiments.

The figure on page 20 shows the workflow for the comparative C_T example study.

Notes

Relative Standard Curve Experiment
Start 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. (Optional) Enable the notification settings.
3. Start the run.
4. Monitor the run.
5. Unload the instrument and transfer the data.

Analyze the Experiment (Chapter 5)

Section 1, Review Results:

1. Analyze.
2. View the standard curve.
3. View the amplification plot.
4. View the gene expression plot/results 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
Start 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. (Optional) Enable the notification settings.
3. Start the run.
4. Monitor the run.
5. Unload the instrument and transfer the data.

Analyze the Experiment (Chapter 9)

Section 1, Review Results:

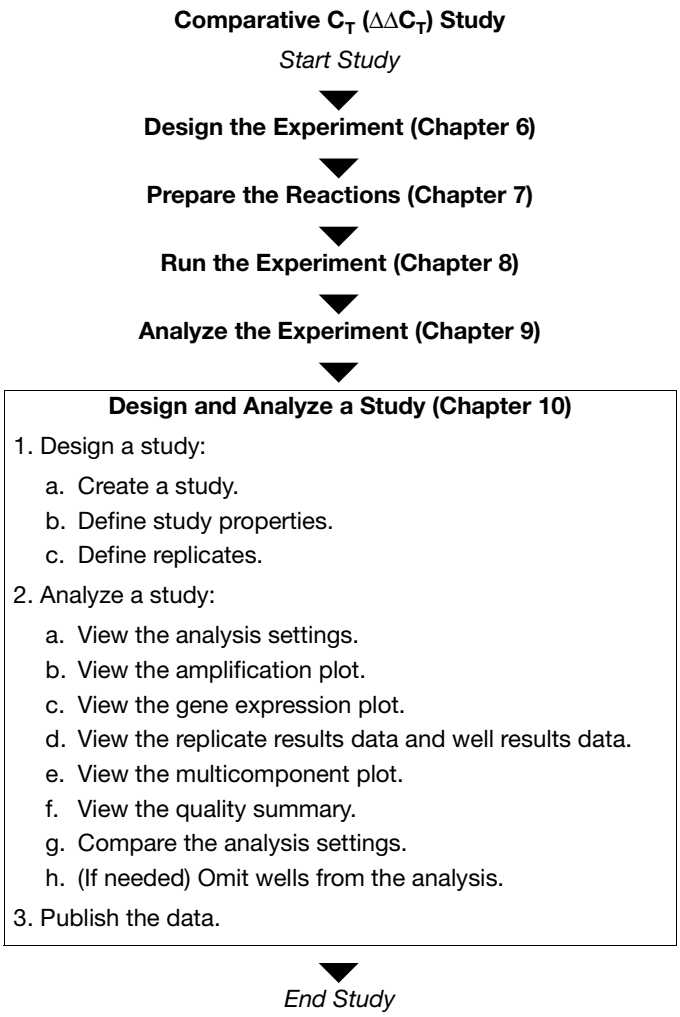
1. Analyze.
2. View the gene expression plot/results table.
3. View the amplification plot.
4. Publish the data.

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.

End Experiment

Notes


Comparative C_T ($\Delta\Delta C_T$) Study
Start Study
Design the Experiment (Chapter 6)
Prepare the Reactions (Chapter 7)
Run the Experiment (Chapter 8)
Analyze the Experiment (Chapter 9)
Design and Analyze a Study (Chapter 10)
1. Design a study:
a. Create a study.
b. Define study properties.
c. Define replicates.
2. Analyze a study:
a. View the analysis settings.
b. View the amplification plot.
c. View the gene expression plot.
d. View the replicate results data and well results data.
e. View the multicomponent plot.
f. View the quality summary.
g. Compare the analysis settings.
h. (If needed) Omit wells from the analysis.
3. Publish the data.
End Study

Notes

2 Design the Relative Standard Curve Experiment

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

Notes

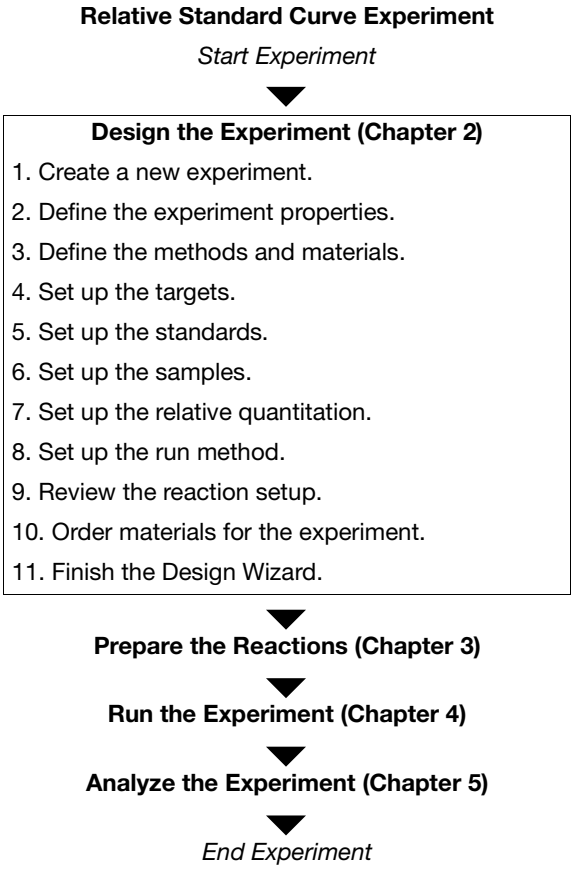
Chapter Overview

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

Example Experiment Workflow

The workflow for designing the example experiment provided with this getting started guide is shown below.

Note: Design the example experiment using the Design Wizard in the StepOne software. When you design your own experiments, you can select alternate workflows (see "Using This Guide With Your Own Experiments" on page 11).



Notes

Create a New Experiment

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

Log In to the Software and Create an Experiment

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

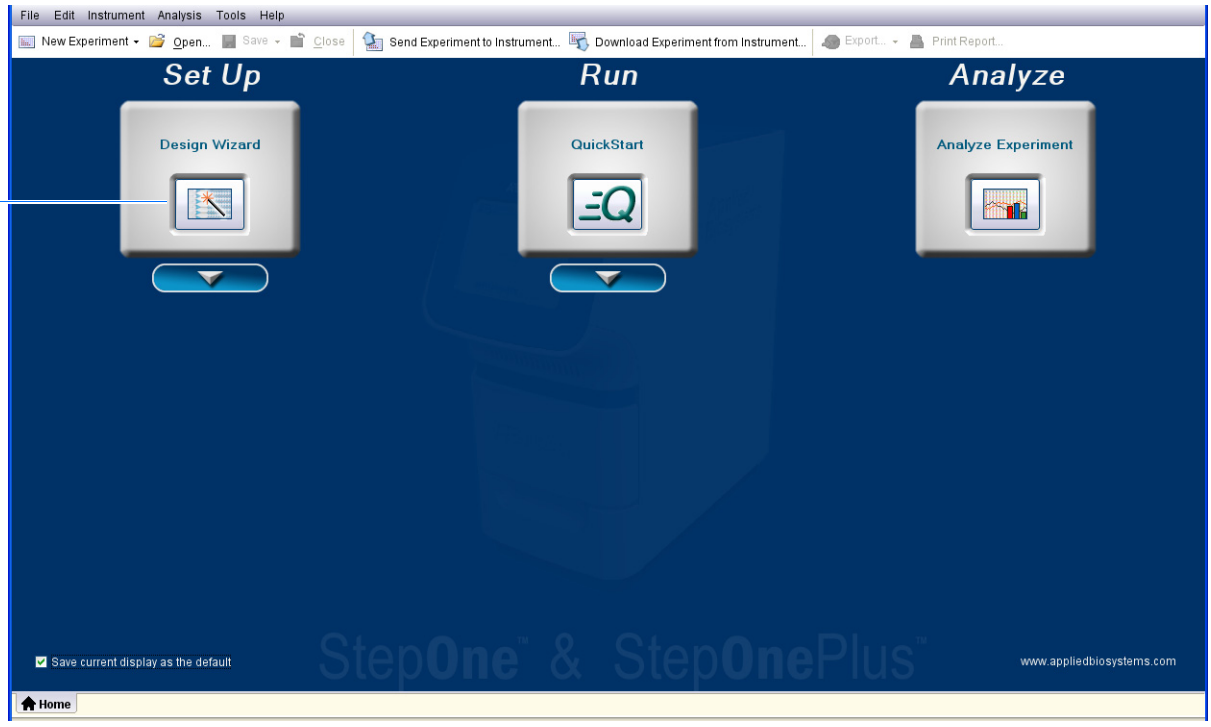
   where *<software name>* is the current version of the StepOne software.

2. In the Login dialog box, create a user name:

   a. In the User Name field, enter **EXAMPLEUSER**. You cannot enter spaces in the User Name field.

   b. Click **OK**.

3. From the Home screen, click (Design Wizard) to open the Design Wizard.



Notes

4. See "Software Elements" on page 25 for information on navigating within the Design Wizard.

Design Guidelines

When you design your own experiment, you can do one of the following in the Login dialog box:

- Log in as a new user – In the User Name field, enter a user name, then click **OK**.

  Note: You cannot use the following characters in the User Name field: space, forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), or semicolon (;).

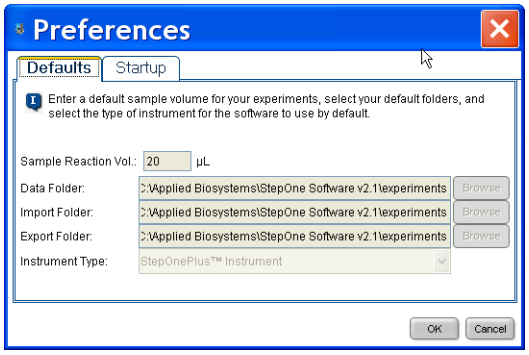
- Log in as an existing user – From the User Name dropdown menu, select an existing user, then click **OK**.
- Log in anonymously – Click **Log in as Guest**.

Applied Biosystems recommends that you log in with a user name. If you log in with a user name, you can set preferences in the software. The next time you log in to the software with the same user name, the software uses the preferences you set as the defaults.

IMPORTANT! If you log in to the software as a Guest, you cannot set preferences.

You can set preferences as follows:

- **Save as the default checkboxes** – Select the **Save as the default** checkboxes as needed. The Save as the default checkboxes appear on the Home screen, on the Export Data dialog box, and on several Analysis screens.
- **Preferences dialog box** – Select **Tools** ▸ **Preferences** to open the Preferences dialog box. In the Defaults and/or Startup tabs, change the preferences as desired.



For More Information

For more information, access the StepOne Software Help by clicking ? or pressing **F1**.

Notes

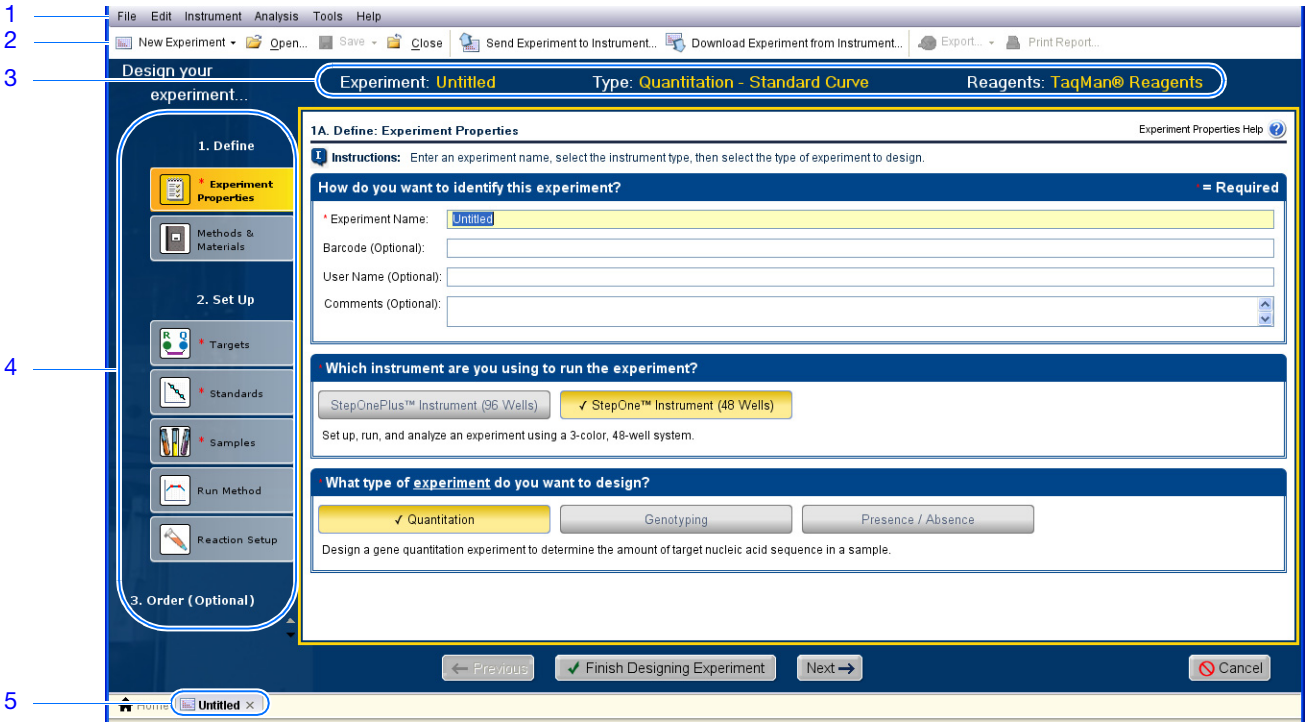
Software Elements

The StepOne software elements for the Design Wizard are illustrated below.

1. Menu bar – Displays the menus available in the software:
   - File
   - Edit
   - Instrument
   - Analysis
   - Tools
   - Help
2. Toolbar – Displays the tools available in the software:
   - New Experiment
   - Open
   - Close
   - Send Experiment to Instrument
   - Download Experiment from Instrument
3. Experiment header – Displays the experiment name, experiment type, and reagents for the open experiment.
4. Navigation pane – Provides links to all screens in the Design Wizard:
   - Experiment Properties
   - Methods & Materials
   - Targets
   - Relative Quantitation Settings
   - Standards
   - Samples
   - Run Method
   - Reaction Setup
   - Materials List

   Note: The Design Wizard initially displays the Quantitation - Standard Curve experiment type. The available Design Wizard screens may change when you select a different experiment type. For example, the Relative Quantitation Settings screen is not displayed until you select the relative standard curve or comparative C_T ($\Delta\Delta C_T$) experiment type.
5. Experiment tab(s) – Displays a tab for each open experiment.

Notes


1
2
3
4
5
File Edit Instrument Analysis Tools Help
New Experiment
Open...
Save
Close
Send Experiment to Instrument...
Download Experiment from Instrument...
Export...
Print Report...
Design your experiment...
Experiment: Untitled
Type: Quantitation - Standard Curve
Reagents: TaqMan® Reagents
1. Define
* Experiment Properties
Methods & Materials
2. Set Up
* Targets
* Standards
* Samples
Run Method
Reaction Setup
3. Order (Optional)
1A. Define: Experiment Properties
Experiment Properties Help
Instructions: Enter an experiment name, select the instrument type, then select the type of experiment to design.
How do you want to identify this experiment?
* = Required
* Experiment Name:
Untitled
Barcode (Optional):
User Name (Optional):
Comments (Optional):
* Which instrument are you using to run the experiment?
StepOnePlus™ Instrument (96 Wells)
✓ StepOne™ Instrument (48 Wells)
Set up, run, and analyze an experiment using a 3-color, 48-well system.
* What type of experiment do you want to design?
✓ Quantitation
Genotyping
Presence / Absence
Design a gene quantitation experiment to determine the amount of target nucleic acid sequence in a sample.
Previous
✓ Finish Designing Experiment
Next
Cancel
Home
Untitled

Notes

Define the Experiment Properties

On 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 selected to run the experiment is the StepOne instrument.
- A MicroAmp® Fast Optical 48-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 updates with the experiment name you entered.

2. Leave the Barcode field empty.

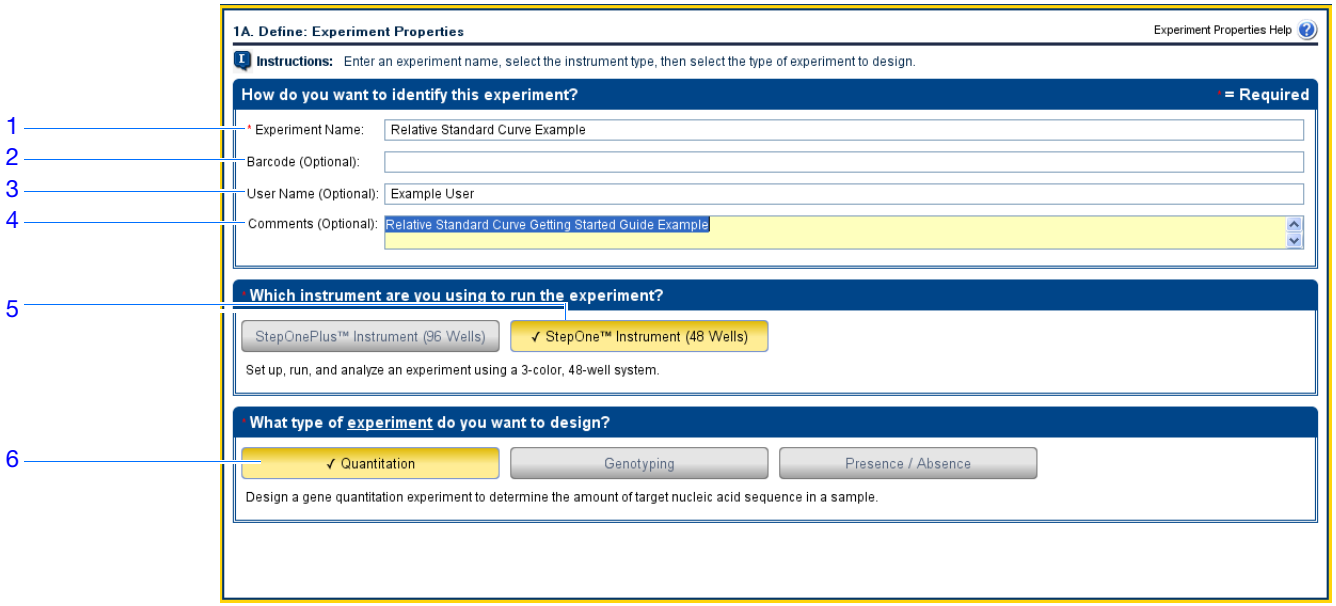
   Note: The MicroAmp Fast Optical 48-Well Reaction Plate does not have a barcode.

3. Click the **User Name** field, then enter **Example User**.
4. Click the **Comments** field, then enter **Relative Standard Curve Getting Started Guide Example**.
5. Select **StepOne™ Instrument (48 Wells)**.

   Note: The example experiment was created for a StepOne instrument. You can create the example experiment for a StepOnePlus instrument; however, your reaction plate layout will differ from the layout shown in this guide. The software displays a 48-well reaction plate layout for the StepOne instrument and a 96-well reaction plate layout for the StepOnePlus instrument. To create the example experiment for a StepOnePlus instrument, select **StepOnePlus™ Instrument (96 Wells)**.

6. Select **Quantitation** for the experiment type.
7. Click **Next >**.

Notes



Design Guidelines

When you design your own relative standard curve experiment:

- Enter an experiment name:
  - The experiment name is used as the default file name.
  - Enter a name that is descriptive and easy to remember. You can enter up to 100 characters in the Experiment Name field.

  Note: You cannot use the following characters in the Experiment Name field: forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), semicolon (;), and sign (&), percent sign (%), dollar sign ($), at sign (@), circumflex (^), left parenthesis (( ), right parenthesis ( )), or exclamation point (!).

  IMPORTANT! If you run the instrument in standalone mode from the instrument touchscreen, you cannot enter more than 32 characters in the Experiment Name field and you cannot include spaces in the name.

- (Optional) If you use a MicroAmp® Fast Optical 96-Well Reaction Plate, enter a barcode to identify the barcode on the PCR reaction plate. You can enter up to 100 characters in the Barcode field.

  Note: The MicroAmp Fast Optical 48-Well Reaction Plate does not have a barcode.

- (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.

Notes

- Select the instrument you are using to run the experiment:
  - **StepOne™ Instrument (48 Wells)**
  - **StepOnePlus™ Instrument (96 Wells)**

Note: You can use StepOne Software v2.1 or later to design experiments for both the StepOne and StepOnePlus instruments. The instrument you select in the Experiment Properties screen affects the reaction plate layout and materials list.

Note: To set the default instrument type, select **Tools** ▸ **Preferences**, then select the **General** tab (default). From the Default Instrument Type dropdown menu, select the appropriate instrument. To change the default instrument type, you must be logged into the software with a user name, not as a Guest. For more information, see the "Design Guidelines" for logging in on page 24.

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

For More Information

For more information on:

- Completing the Experiment Properties screen, access the StepOne Software Help by clicking or pressing **F1**.
- Consumables, see "Supported Consumables" on page 4.
- Quantitation experiments, refer to the *Real-Time PCR Systems Reagent Guide*.

Notes

Define the Methods and Materials

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

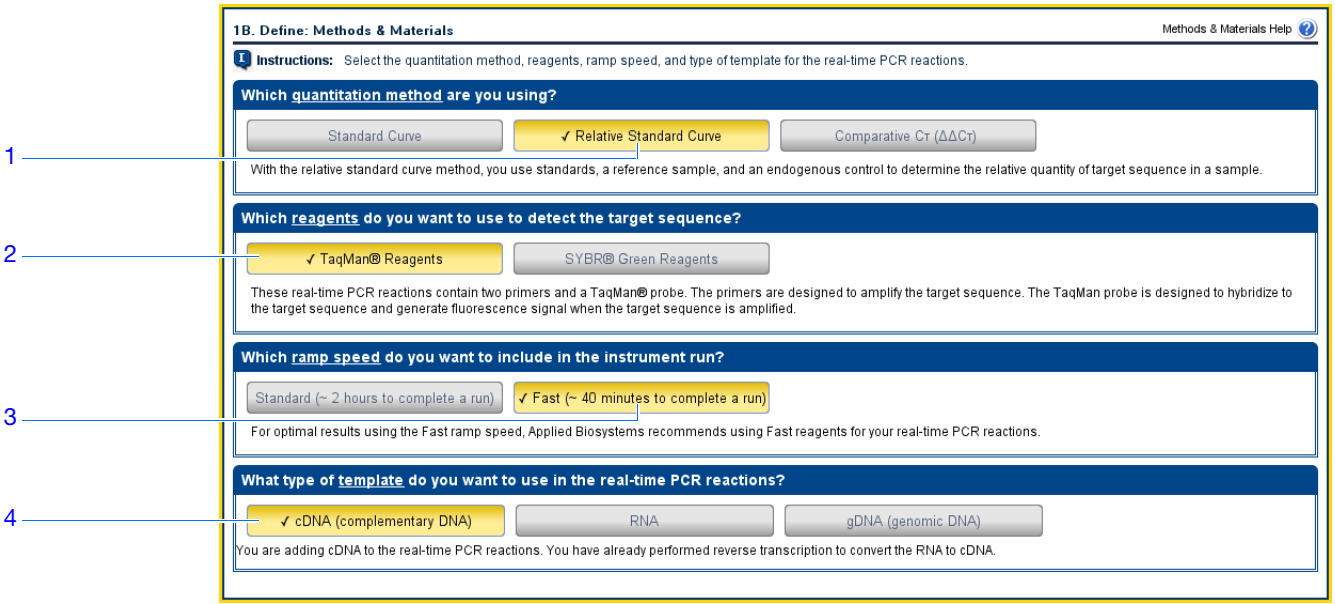
About the Example Experiment

In the relative standard curve example experiment:

- The relative standard curve quantitation method is used.
- TaqMan® reagents are used.
- The Fast ramp speed is used in the instrument run.
- cDNA (prepared from total RNA isolated from liver and kidney tissues) is the template type. Before using cDNA template, you must first perform reverse transcription to convert the RNA to cDNA (see "Prepare the Template" on page 59).

Complete the Methods & Materials Screen

1. Select **Relative Standard Curve** as the quantitation method.
2. Select **TaqMan® Reagents** for the reagents.
3. Select **Fast (~ 40 minutes to complete a run)** for the ramp speed.
4. Select **cDNA (complementary DNA)** for the template type.
5. Click **Next >**.



Design Guidelines

When you design your own relative standard curve experiment:

- 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.

Notes

- 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 generate fluorescence when the target is amplified.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA™ dye as a reporter or quencher with the StepOne™ system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus™ system.

  - 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 generates fluorescence when it binds to double-stranded DNA. SYBR Green dye is often part of the SYBR Green master mix that is added to the reaction. If you use SYBR Green dye, select the **Include Melt Curve** checkbox to perform melt curve analysis of the amplified target.

Note: You can use other fluorescence-based reagents on the StepOne and StepOnePlus systems, but you must design your experiment using Advanced Setup instead of the Design Wizard.

- Select the appropriate ramp speed for the instrument run:
  - Select **Fast (~ 40 minutes to complete a run)** if you use fast reagents for the PCR reactions.
  - Select **Standard (~ 2 hours to complete a run)** if you use 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.

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 & Materials screen, access the StepOne Software Help by clicking or pressing **F1**.
- Using Advanced Setup, see "Advanced Setup Workflow" on page 260.
- Using the comparative C_T quantitation method, see Chapters 6 to 9 of this guide.

Notes

- Using the standard curve quantitation method, refer to the *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*.
- TaqMan and SYBR Green reagents, refer to the *Real-Time PCR Systems Reagent Guide*.
- PCR, including singleplex vs. multiplex PCR and 1-step vs. 2-step RT PCR, refer to the *Real-Time PCR Systems Reagent Guide*.

Set Up the Targets

On 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 relative standard curve example experiment:

- Two targets are quantified in the reaction plate.
- The Set Up Standards checkbox is selected. When this checkbox 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 35).
- The Target 1 assay is set up for the target you are studying. For the example experiment, this is human c-myc (an oncoprotein that activates the transcription of growth-associated genes).
- The Target 2 assay is set up for the endogenous control. For the example experiment this is human glyceraldehyde-3-phosphate (GAPDH). GAPDH serves as the endogenous control because its expression levels tend to be relatively stable.

Complete the Targets Screen

1. 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 updates with the number you entered.

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

   Note: The Set Up Standards checkbox is selected by default.

3. Set up the Target 1 assay:
   a. Click the **Enter Target Name** cell, then enter **c-myc**.
   b. From the Reporter dropdown menu, select **FAM** (default).
   c. From the Quencher dropdown menu, select **NFQ-MGB** (default).
   d. Leave the default in the Color field.
4. Set up the Target 2 assay:

Notes

a. Click the **Enter Target Name** cell, then enter **GAPDH**.

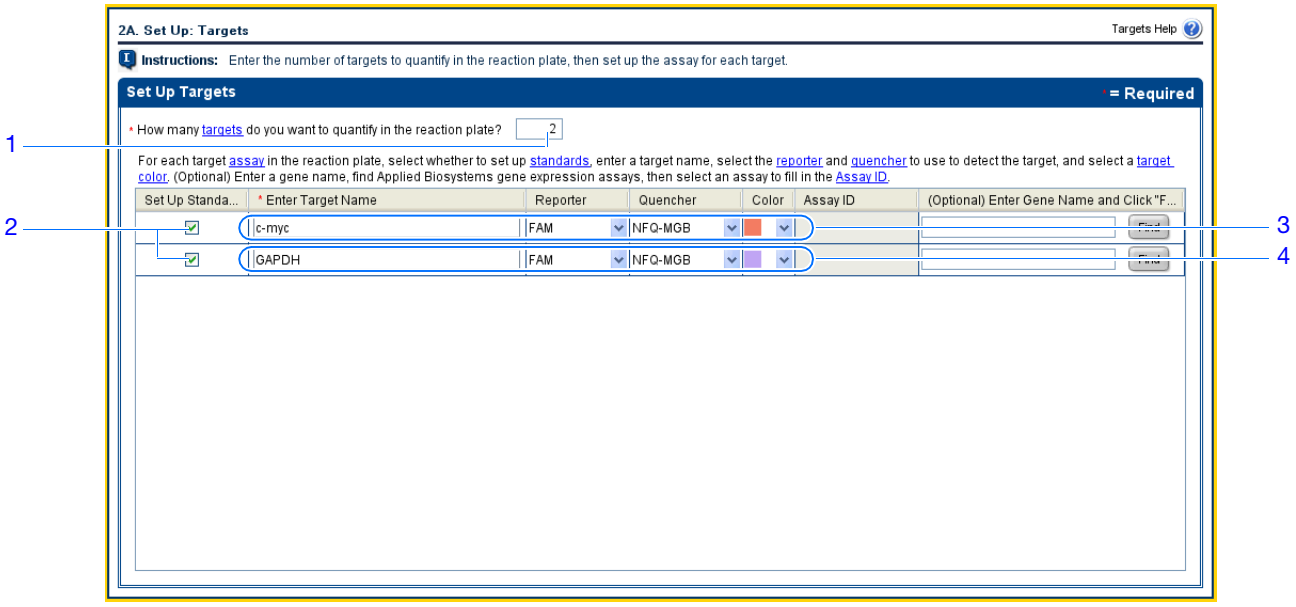
b. From the Reporter dropdown menu, select **FAM** (default).

c. From the Quencher dropdown menu, select **NFQ-MGB** (default).

d. Leave the default in the Color field.

5. Click **Next >**.

Note: For all targets, leave the (Optional) Enter Gene Name field blank. You can search for the gene/assay ID when you order your materials (see "Order Materials for the Experiment" on page 50).



Design Guidelines

When you design your own relative standard curve experiment:

- Select the **Set Up Standards** checkbox. 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 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.

Notes

  - If samples are spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.
- Select the reporter dye used in the target assay. In the Methods & Materials screen on page 30, 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 & Materials screen on page 30, if you selected:
  - TaqMan® Reagents, select the quencher attached to the 3′ end of the TaqMan probe.
  - SYBR® Green Reagents, select **None**.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA dye as a reporter or quencher with the StepOne system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus system.

For More Information

For more information on:

- Completing the Targets screen, access the StepOne 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

On 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

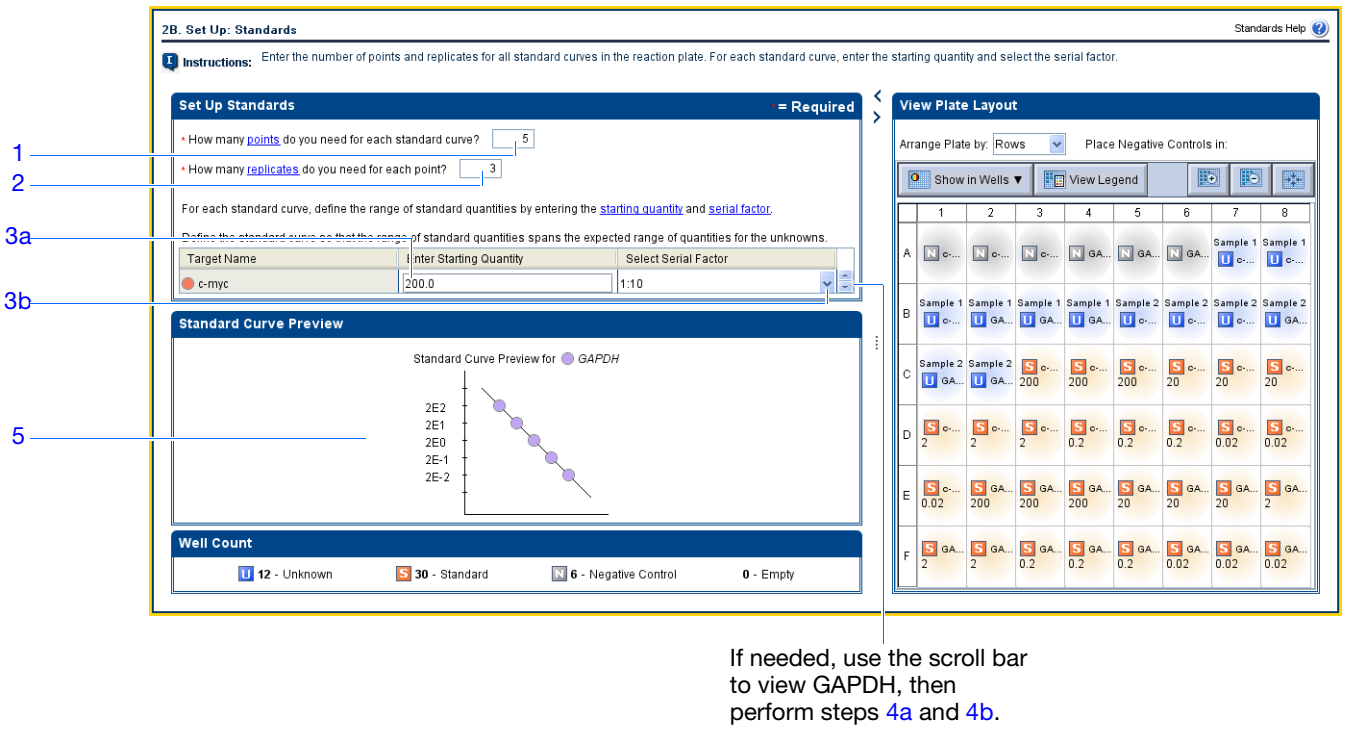
In the relative standard curve example experiment:

- 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. 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**.
   b. From the Select Serial Factor dropdown menu, select **1:10**.
4. Define the range of standard quantities for the GAPDH assay:
   a. Click the **Enter Starting Quantity** field, then enter **200**.
   b. From the Select Serial Factor dropdown menu, 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 on the Targets screen ("Set Up the Targets" on page 32).
- 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, spanning between 5 and 6 logs. 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 fall within a given 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 dilution 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, access the StepOne 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

On 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. 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 updates with the number you entered.

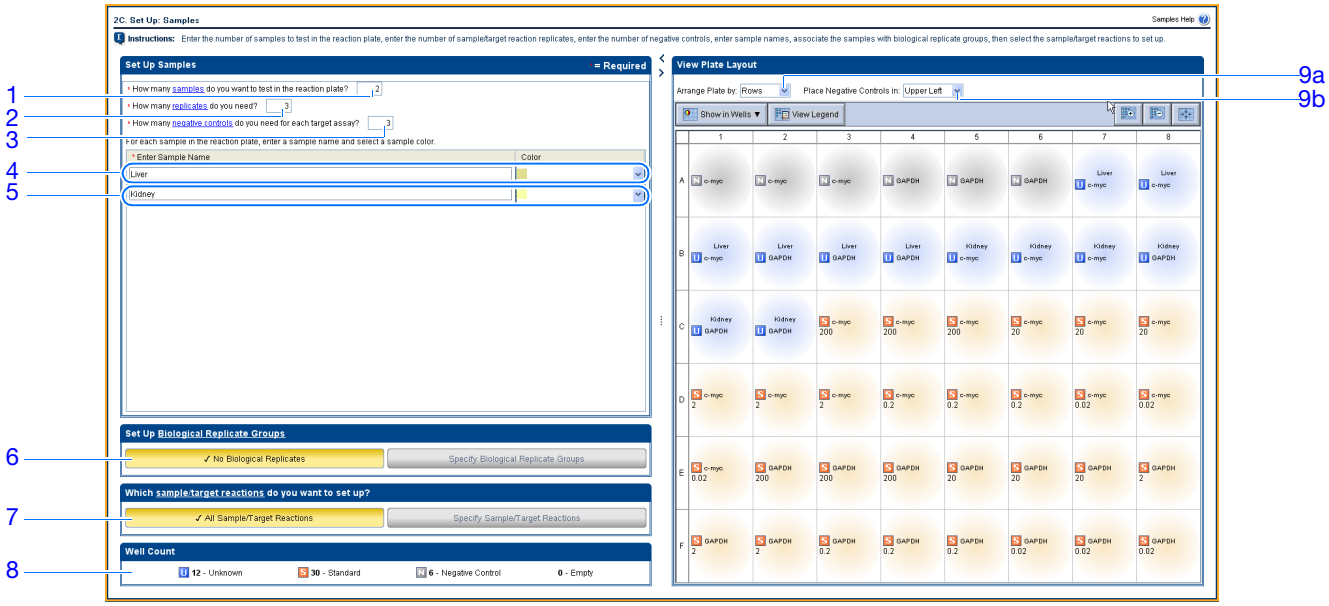
2. Click the **How many replicates do you need?** field, then enter **3**.
3. Click the **How many negative controls do you need for each target assay?** field, then enter **3**.
4. Set up Sample 1:
   a. Click the **Enter Sample Name** field, then enter **Liver**.
   b. Leave the default in the Color field.
5. Set up Sample 2:
   a. Click the **Enter Sample Name** field, then enter **Kidney**.
   b. Leave the default in the Color field.
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, confirm that there are:
   - 12 Unknown wells
   - 30 Standard wells
   - 6 Negative control wells
   - 0 Empty wells

9. In the View Plate Layout tab:
   a. From the Arrange Plate by dropdown menu, select **Rows** (default).
   b. From the Place Negative Controls in dropdown menu, select **Upper Left** (default).

10. Click **Next >**.



Design Guidelines

When you design your own relative standard curve experiment:

- Identify each sample using 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 the biological replicates groups:
  – Select **No Biological Replicates** if you do not want to include biological replicate groups in the experiment.
  – Select **Specify Biological Replicate Groups** to include biological replicate groups in the experiment. For more information on biological replicate groups, see Chapter 10.

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.

Note: 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.

- If you are running the experiment on a StepOnePlus instrument and plan to edit the Run Method (page 41) to set a different temperature for one or more of the VeriFlex blocks, you need to:

  a. Design your experiment using Advanced Setup instead of the Design Wizard.

  b. In the Plate Setup screen, select the **Assign Targets and Samples** tab, select the **View Plate Layout** tab, then select the **Enable VeriFlex™ Block** checkbox.

IMPORTANT! If you do not select the **Enable VeriFlex™ Block** checkbox in the Plate Setup screen, you will not be able to set a different temperature for one or more of the VeriFlex blocks in the Run Method screen (page 41).

For More Information

For more information on:

- Completing the Samples screen, access the StepOne Software Help by clicking or pressing **F1**.
- Using Advanced Setup, see "Advanced Setup Workflow" on page 260.

Set Up the Relative Quantitation Settings

On 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. From the Which sample do you want to use as the reference sample? dropdown menu, select **Kidney**.
2. From the Which target do you want to use as the endogenous control? dropdown menu, select **GAPDH**.
3. Click **Next >**.

Notes

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 37). 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 32). 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, access the StepOne 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*.

Notes

Set Up the Run Method

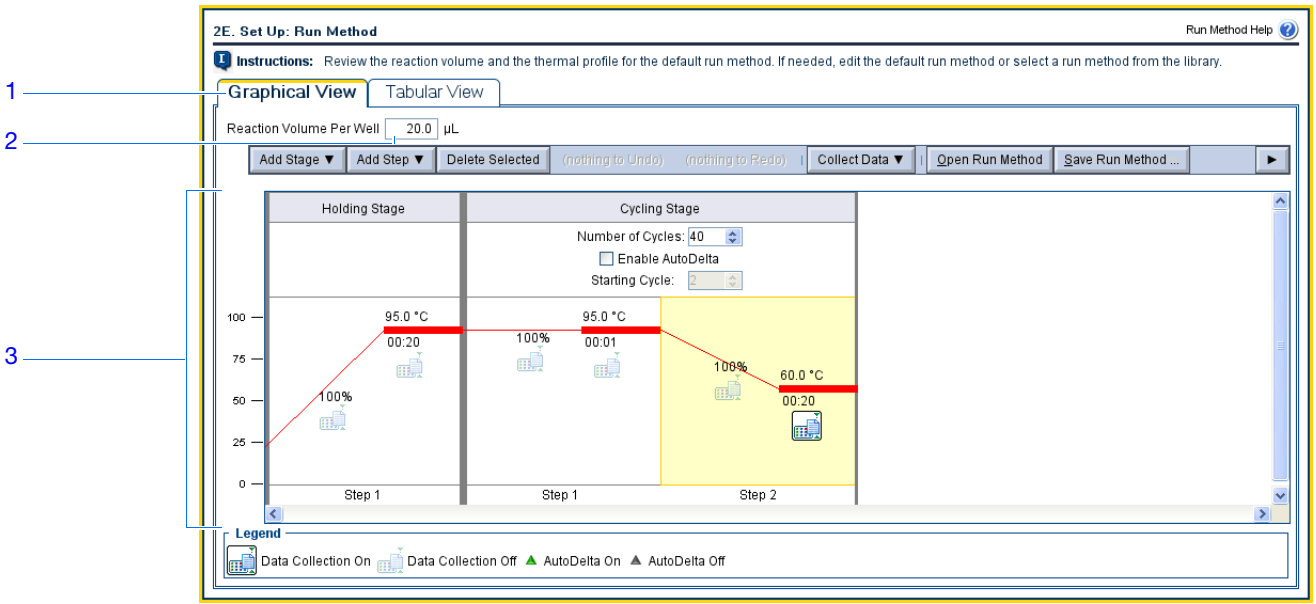
On the Run Method screen, review the reaction volume and the thermal profile for the default run method. If needed, you can edit the default run method or replace it with one from the Run Method library.

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. Click either the **Graphical View** tab (default) or **Tabular View** tab.
2. Make sure the Reaction Volume Per Well field displays **20 µL**.
3. Make sure the thermal profile displays the holding and cycling stages shown below.
4. Click **Next >**.



Design Guidelines

When you design your own relative standard curve experiment:

- Enter a number from 10 to 30 for the reaction volume/well. The StepOne and StepOnePlus systems support 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 StepOne software.

Notes

- If you are running the experiment on a StepOnePlus instrument and you want to set a different temperature for one or more of the VeriFlex blocks, you need to:

  a. Design your experiment using Advanced Setup instead of the Design Wizard.

  b. In the Plate Setup screen (page 39), select the **Assign Targets and Samples** tab, select the **View Plate Layout** tab, then select the **Enable VeriFlex™ Block** checkbox.

  IMPORTANT! If you do not select the **Enable VeriFlex™ Block** checkbox in the Plate Setup screen, you will not be able to set a different temperature for one or more of the VeriFlex blocks in the Run Method screen.

  c. In the Run Method screen, select the Graphical View tab.

  d. For each VeriFlex™ block you want to change, click the temperature, then enter the desired value.

Note: You can set a different temperature for one or more of the VeriFlex blocks, or set each of the VeriFlex blocks to the same temperature. If neighboring VeriFlex blocks are not set to the same temperature, the temperature difference must be between 0.1 and 5.0 °C. The maximum temperature is 99.9 °C.

For More Information

For more information on:

- The Run Method library or on completing the Run Method screen, access the StepOne Software Help by clicking or pressing **F1**.
- Setting temperatures for the VeriFlex blocks, access the StepOne Software Help by clicking or pressing FI.
- Using Advanced Setup, see "Advanced Setup Workflow" on page 260.

Notes

Review the Reaction Setup

On 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.

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

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 20 µL.
- The excess reaction volume is 10%.
- The reaction components are:
  - TaqMan® Fast Universal PCR Master Mix (2×)
  - c-myc Assay Mix (20×)
  - GAPDH Assay Mix (20×)
  - Sample or standard
  - Water
- The standard concentration in stock is 200 ng/µL.
- The diluted sample concentration is 5.0 ng/µL.
- The sample stock concentration is 100 ng/µL.

Complete the Reaction Setup Screen

Complete the Reaction Mix Calculations Tab for the c-myc Assay

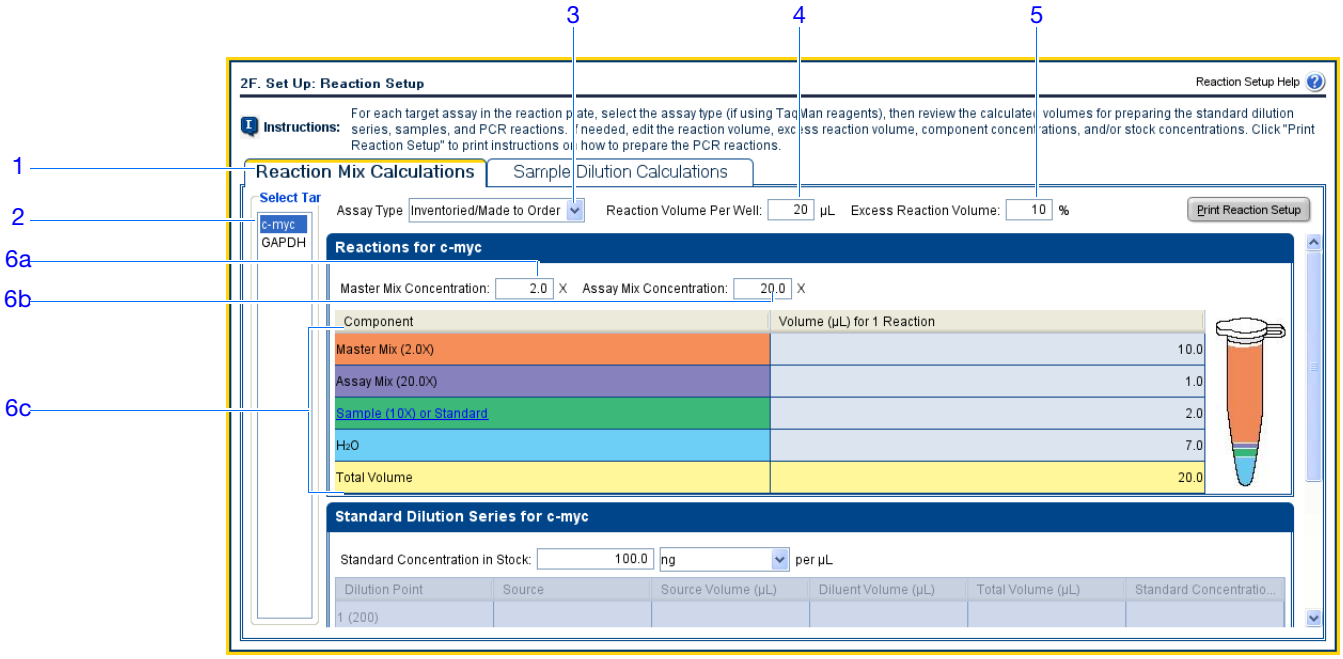
1. Select the **Reaction Mix Calculations** tab (default).
2. From the Select Target pane, select **c-myc**.
3. From the Assay Type dropdown menu, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **20 µL**.
5. Make sure the Excess Reaction Volume field displays **10%**.
6. In the Reactions for c-myc pane:
   a. Make sure the Master Mix Concentration field displays **2.0×**.
   b. Make sure the Assay Mix Concentration field displays **20.0×**.
   c. Review the components and calculated volumes for the PCR reactions:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0×)	10.0

Notes

Component	Volume (µL) for 1 Reaction
Assay Mix (20.0X)	1.0
Sample (10X) or Standard	2.0‡
H_2O	7.0
Total Volume	20.0

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



7. In the Standard Dilution Series for c-myc pane:

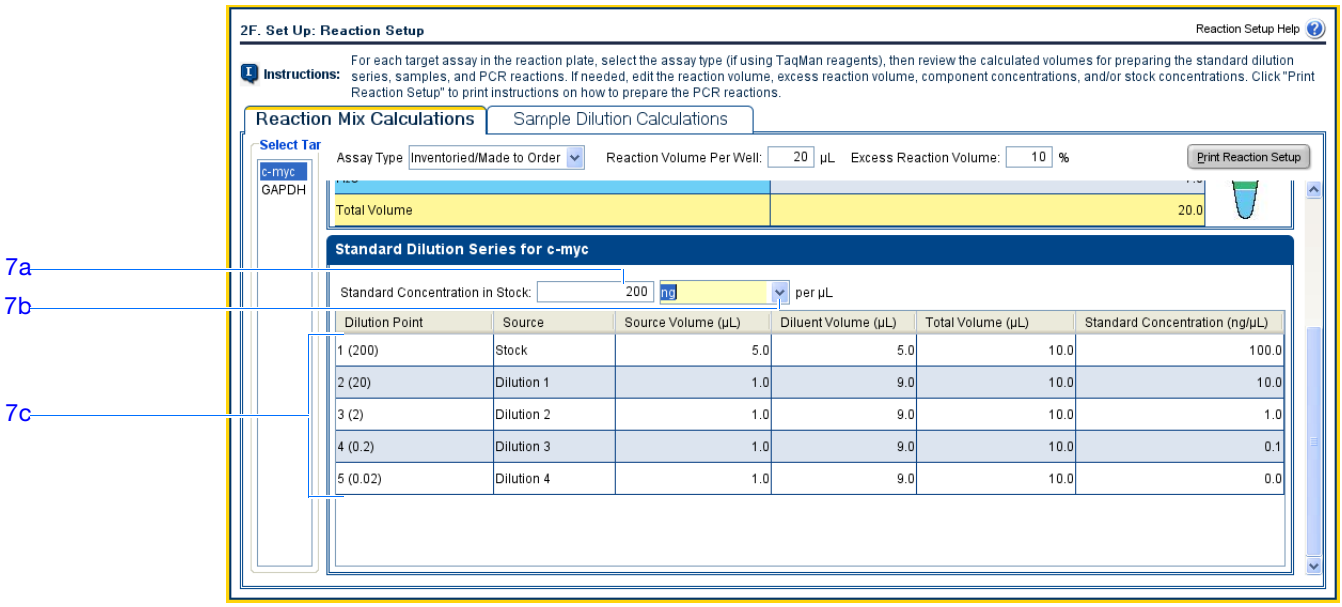
   a. Click the **Standard Concentration in Stock** field, then enter **200**.

   b. In the units field, select **ng** per µL (default) from the dropdown menu.

   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	5.0	5.0	10.0	100.0
2 (20)	Dilution 1	1.0	9.0	10.0	10.0
3 (2)	Dilution 2	1.0	9.0	10.0	1.0
4 (0.2)	Dilution 3	1.0	9.0	10.0	0.1
5 (0.02)	Dilution 4	1.0	9.0	10.0	0.01

Notes



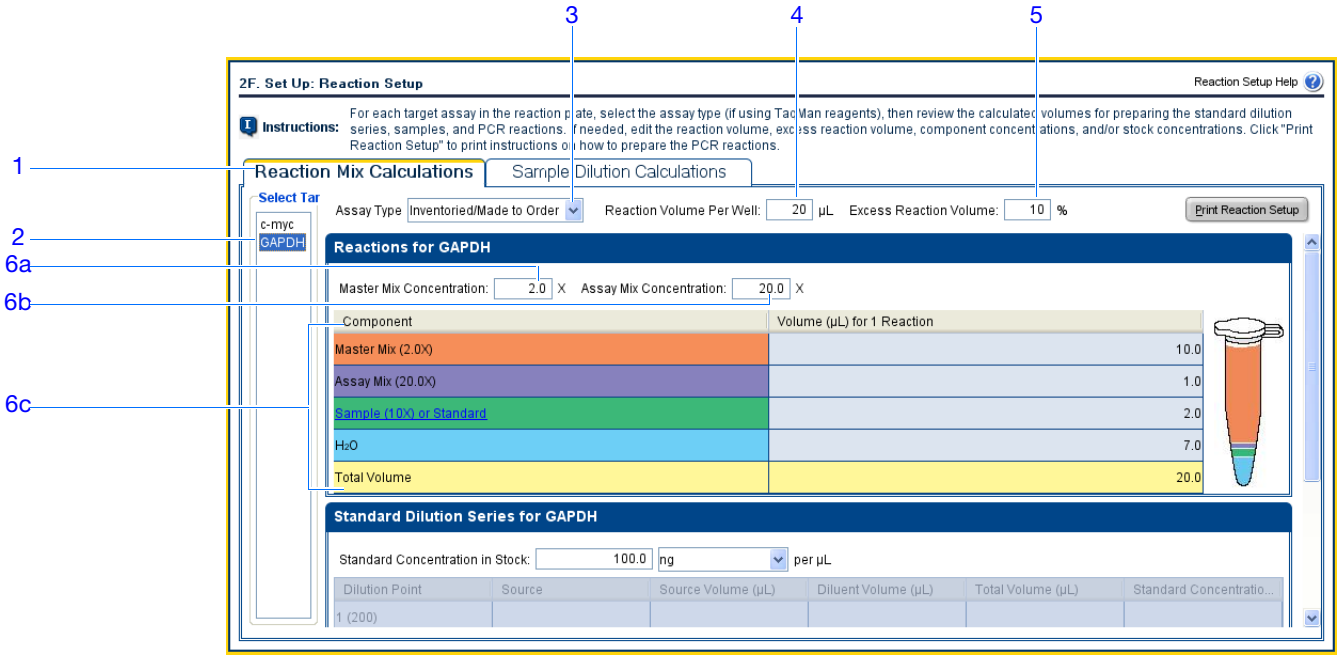
Complete the Reaction Mix Calculations Tab for the GAPDH Assay

1. Select the **Reaction Mix Calculations** tab (default).
2. From the Select Target pane, select **GAPDH**.
3. From the Assay Type dropdown menu, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **20 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×**.
   c. Review the components and calculated volumes for the PCR reactions:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0×)	10.0
Assay Mix (20.0×)	1.0
Sample (10×) or Standard	2.0‡
H_2O	7.0
Total Volume	20.0

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

Notes



7. In the Standard Dilution Series for GAPDH pane:

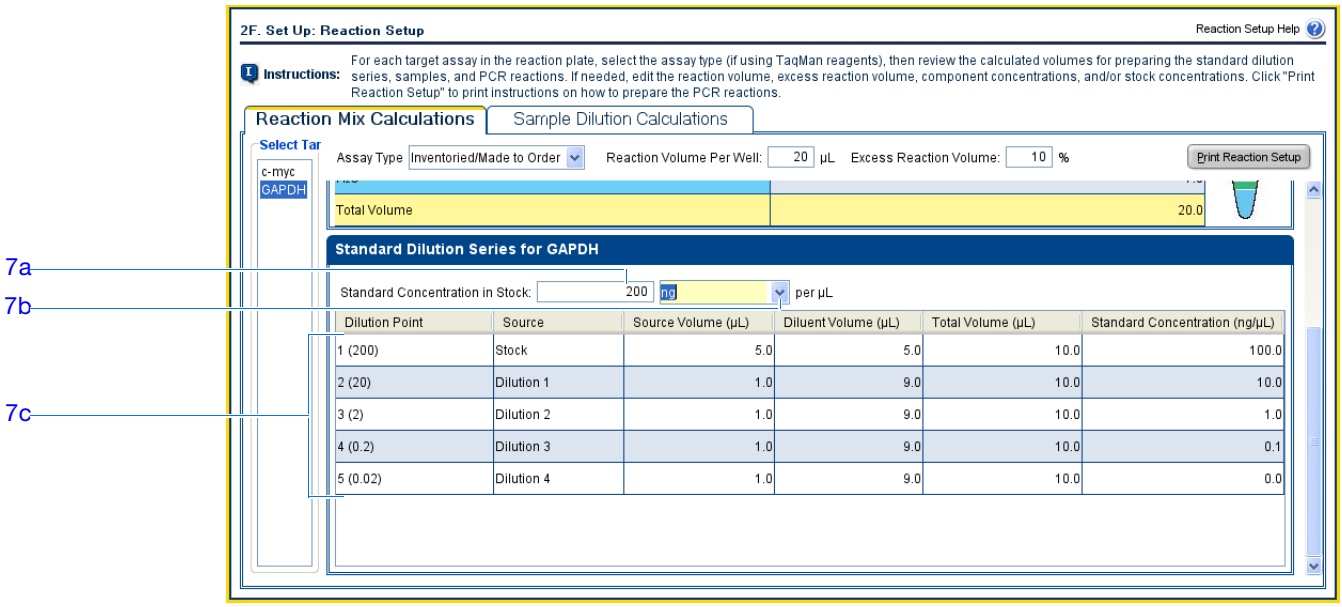
   a. Click the **Standard Concentration in Stock** field, then enter **200**.

   b. In the units field, select **ng** per µL (default) from the dropdown menu.

   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	5.0	5.0	10.0	100.0
2 (20)	Dilution 1	1.0	9.0	10.0	10.0
3 (2)	Dilution 2	1.0	9.0	10.0	1.0
4 (0.2)	Dilution 3	1.0	9.0	10.0	0.1
5 (0.02)	Dilution 4	1.0	9.0	10.0	0.01

Notes

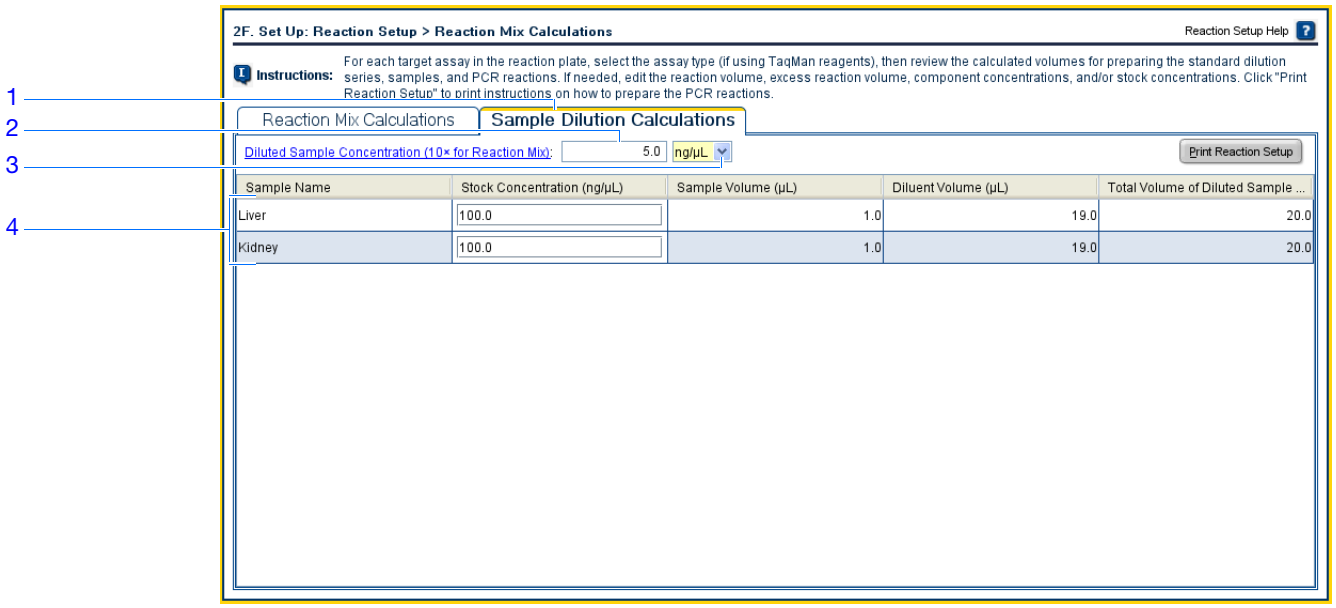


Complete the Sample Dilution Calculations Tab

1. Select the **Sample Dilution Calculations** tab.
2. Click the **Diluted Sample Concentration (10× for Reaction Mix)** field, then enter **5.0**.
3. From the unit dropdown menu, 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	1.0	19.0	20.0
Kidney	100.0	1.0	19.0	20.0

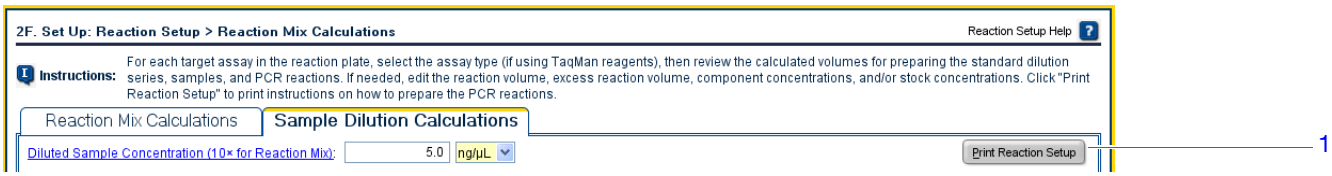
Notes



Print Reaction Setup Instructions

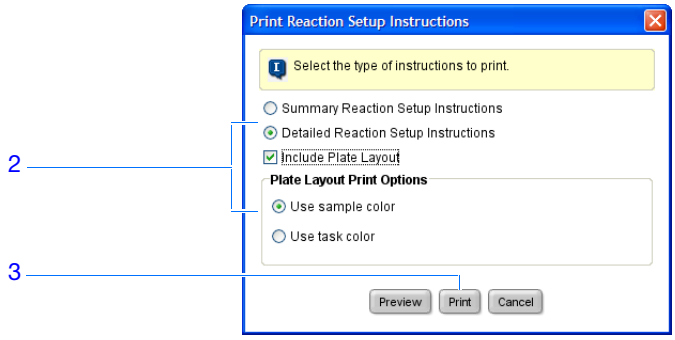
Print detailed reaction setup instructions, then save the instructions for Chapter 3, "Prepare the Relative Standard Curve Reactions."

1. Click **Print Reaction Setup**.



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

3. Click **Print** to send the reaction setup instructions to your printer.



Notes

4. Click **Next >**.

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 30 for the reaction volume/well. The StepOne and StepOnePlus systems support 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 (including units).

  Note: For the Standard Concentration in Stock units field, you can select **ng** or **µg** from the dropdown menu or you can enter another unit in the field (for example, **copies**, **IU**, [International Units], **nmol**, **pg**, and so on). The table updates 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, access the StepOne 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

On 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: To access the Applied Biosystems Store, you need to have an Internet connection. 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 StepOne 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® Fast Optical 48-Well Reaction Plate
- MicroAmp® 48-Well Optical Adhesive Film
- MicroAmp® 96-Well Support Base
- TaqMan® Fast Universal PCR Master Mix (2✕), No AmpErase® UNG
- c-myc Assay Mix: Hs00153408_m1 (RefSeq NM_002467.3)
- GAPDH Assay Mix: Hs99999905_m1 (RefSeq NM_002046.2)

Note: The example experiment was created for a StepOne instrument. If you selected the StepOnePlus instrument in the Experiment Properties screen (page 27), the 96-well consumables (for example, the MicroAmp® Fast Optical 96-Well Reaction Plate) are listed in place of the 48-well consumables.

Complete the Ordering Materials Screen

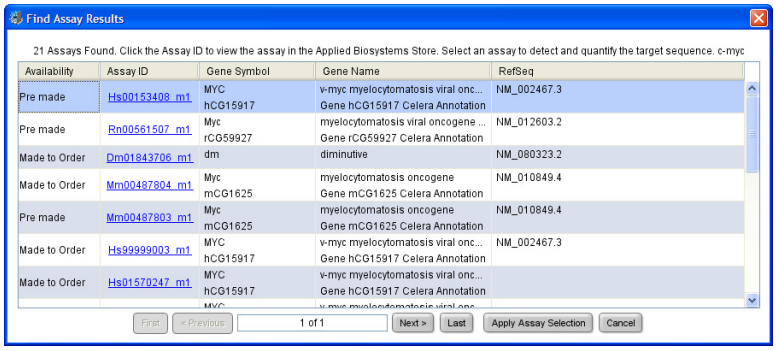
1. Find the target assay on the Applied Biosystems Store:

   Note: To access the Applied Biosystems Store, you need to have an Internet connection.

   a. Click the **Enter Gene Name** field, enter **c-myc**, then click **Find Assay**.

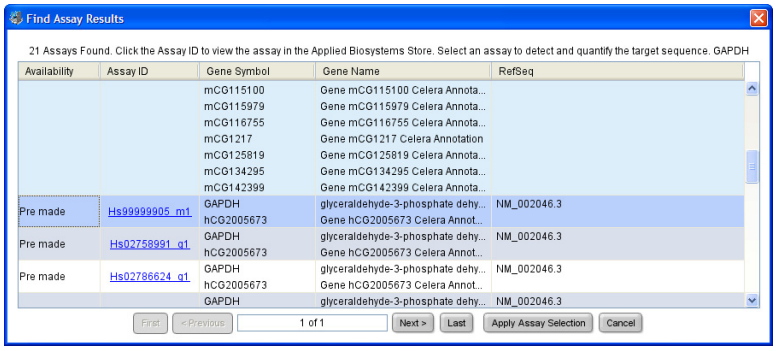
   b. In the Find Assay Results dialog box, select the **Hs00153408_m1** row, then click **Apply Assay Selection**.

Notes



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

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



2. From the Display dropdown menu, select **All Items** (default), then review the recommended materials. If needed, use the scroll bar at right to see all items.

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

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

4. (Optional) Create a shopping list:

   a. Select the checkbox next to each of the following items:
      - MicroAmp® Fast Optical 48-Well Reaction Plate
      - MicroAmp® 48-Well Optical Adhesive Film
      - MicroAmp® 96-Well Support Base
      - TaqMan® Fast Universal PCR Master Mix (2✕), No AmpErase® UNG
      - Hs00153408_m1 (c-myc Assay Mix)
      - Hs99999905_m1 (GAPDH Assay Mix)

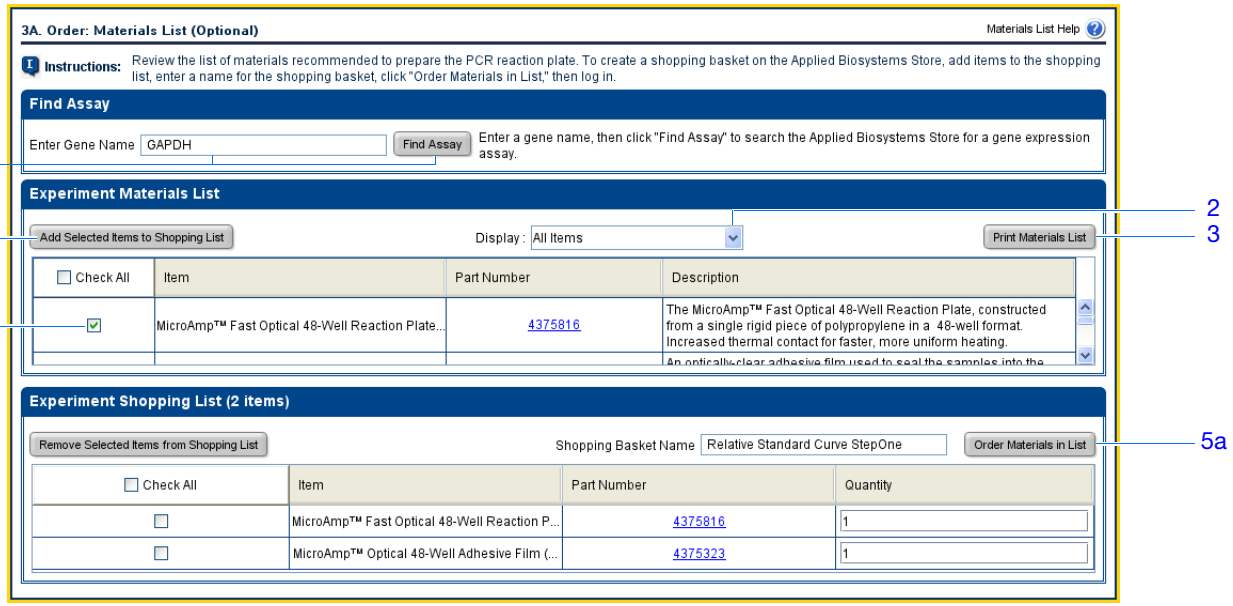
   b. Click **Add Selected Items to Shopping List**.

Notes

5. (Optional) Create a shopping basket on the Applied Biosystems Store:

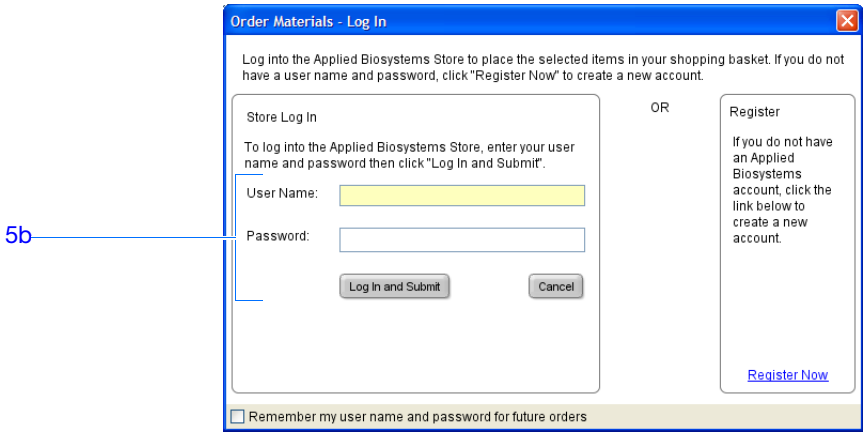
Note: To access the Applied Biosystems Store, you need to have an Internet connection. Product availability and pricing may vary according to your region or country. Online ordering through the Applied Biosystems Store is not available in all countries. Contact your local Applied Biosystems representative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

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 **Login and Submit**.

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



Notes

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 54.

Design Guidelines

When you design your own relative standard curve experiment:

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

Desktop Operating System	Microsoft® Internet Explorer	Adobe® Acrobat® Reader
Windows® XP (Service Pack 2 or Service Pack 3)	v6.x	v4.0 or later
Windows® Vista	v7.x or later	v4.0 or later

Note: Make sure that cookies and Java Script are turned on for the web site to function correctly.

For More Information

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

Notes

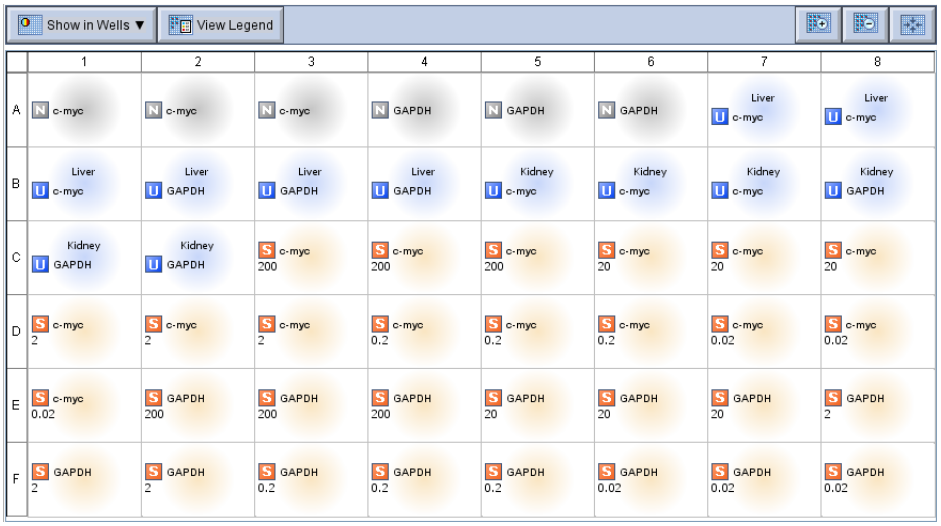
Finish the Design Wizard

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

About the Example Experiment

The StepOne 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.



Note: The example experiment was created for a StepOne instrument. If you selected the StepOnePlus instrument in the Experiment Properties screen (page 27), your reaction plate layout will differ from the layout shown above. The software displays a 96-well reaction plate layout for the StepOnePlus instrument. For an example of the 96-well reaction plate layout, see page 13.

- The experiment is saved as is and closed.

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

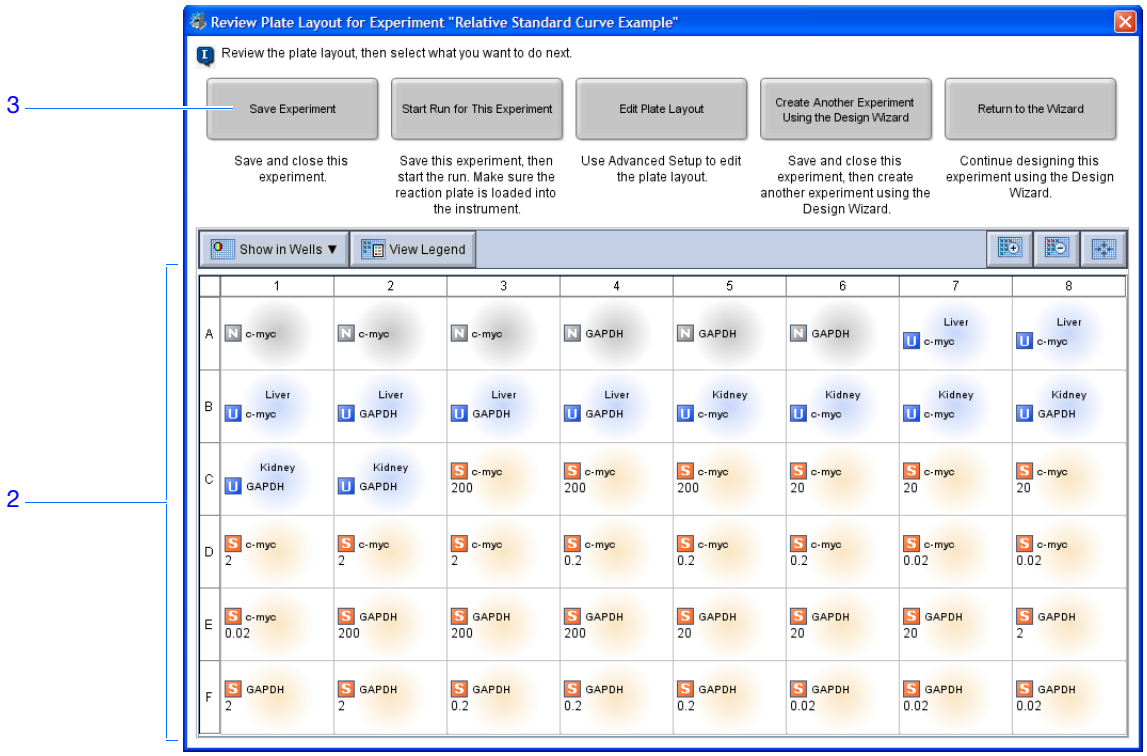
Finish the Design Wizard

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

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

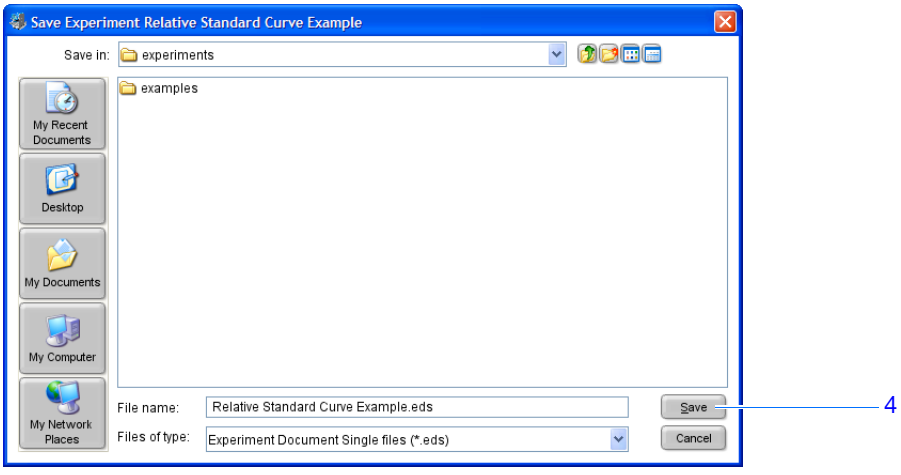
Notes

3. Click **Save Experiment**.



4. In the Save Experiment dialog box, click **Save** to accept the default file name and location. The example experiment is saved and closed, and you are returned to the Home screen.

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 for Experiment window, select the appropriate exit option:

Click	If you want 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. • (*StepOnePlus instrument only*) Set a different temperature for one or more of the VeriFlex blocks using Advanced Setup.
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 the <*drive*>:\Applied Biosystems\<*software name*>\experiments folder. 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 **General** tab (default). In the Default Data Folder field, browse to the desired location.

Note: To change the default save location, you must be logged in to the software with a user name, not as a Guest. For more information, see the "Design Guidelines" for logging in on page 24.

For More Information

For more information on using Advanced Setup, see "Advanced Setup Workflow" on page 260.

Notes

3 Prepare the Relative Standard Curve Reactions

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

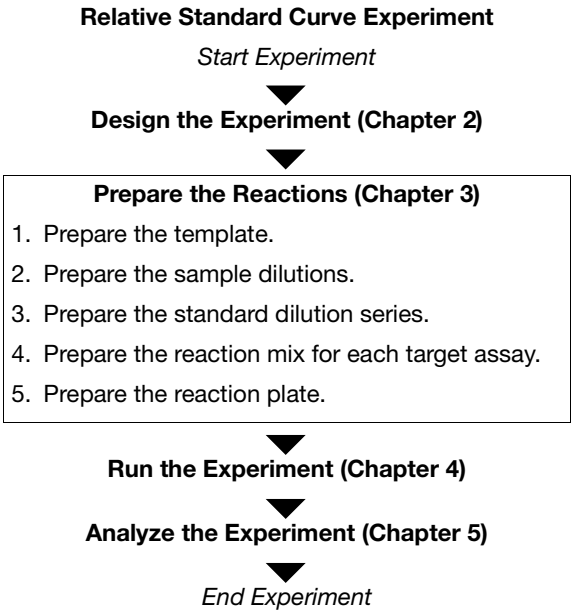
Notes

Chapter Overview

This chapter explains how to prepare the PCR reactions for the relative standard curve example experiment and provides guidelines for how to prepare the PCR reactions for your own relative standard curve experiment.

Example Experiment Workflow

The workflow for preparing the PCR reactions for the example experiment provided with this getting started guide is shown below.



Notes

Prepare the Template

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

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 Ambion® starter packs listed below 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 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
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

Notes

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

IMPORTANT! Be sure to prepare template for both your samples and your standards.

- 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 is used to perform the example experiment. For a list of other kits you can use to perform your own experiment, see "Preparation Guidelines" on page 61.

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 one of the following kits:

Kit	Part Number
High Capacity RNA-to-cDNA Kit (50 reactions)	4387406
High Capacity RNA-to-cDNA Master Mix (200 reactions)	4390778
High-Capacity cDNA Reverse Transcription Kit (200 reactions)	4368814
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	4374966

  - **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 protocol for the kit you select:
  - *High Capacity RNA-to-cDNA Kit Protocol*
  - *High Capacity RNA-to-cDNA Master Mix Protocol*
  - *High-Capacity cDNA Reverse Transcription Kits Protocol*

  Note: The *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits 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 StepOne™ software ("Complete the Sample Dilution Calculations Tab" on page 47).

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 StepOne software. The total reaction volume is 20 µL/reaction, so the sample volume is 2 µL/reaction.
- The stock concentration is 100 ng/µL. After diluting the sample according to the Sample Dilutions Calculations table, the sample will have a concentration of 5.0 ng/µL. This is a 10× concentration when adding 2 µL to the final reaction mix volume of 20 µL. You will have 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	1.0	19.0	20.0
Kidney	100.0	1.0	19.0	20.0

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	19.0
2	Kidney	19.0

Notes

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

Tube	Sample Name	Sample Volume (µL)
1	Liver	1.0
2	Kidney	1.0

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 StepOne 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. For Fast reagents, Applied Biosystems recommends 10 ng.
- 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*.

Prepare the Standard Dilution Series

Prepare the standard dilution series using the volumes that were calculated by the StepOne software ("Complete the Reaction Mix Calculations Tab for the c-myc Assay" on page 43 and "Complete the Reaction Mix Calculations Tab for the GAPDH Assay" on page 45):

About the Example Experiment

For the relative standard curve example experiment:

- The standard concentration in stock is 200 ng/µL.
- The volumes calculated in the software for the both the c-myc and GAPDH assays are:

Dilution Point	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
1 (200)	Stock	5.0	5.0	10.0	100.0
2 (20)	Dilution 1	1.0	9.0	10.0	10.0
3 (2)	Dilution 2	1.0	9.0	10.0	1.0

Notes

Dilution Point	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
4 (0.2)	Dilution 3	1.0	9.0	10.0	0.1
5 (0.02)	Dilution 4	1.0	9.0	10.0	0.01

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**
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	5.0
2	c-myc Std. 2	9.0
3	c-myc Std. 3	9.0
4	c-myc Std. 4	9.0
5	c-myc Std. 5	9.0

3. Prepare dilution 1 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 5.0 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. Prepare dilution 2 in the c-myc Std. 2 tube:
   a. Using a new pipette tip, add 1.0 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.

Notes

5. Prepare dilution 3 in the c-myc Std. 3 tube:
   a. Using a new pipette tip, add 1.0 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. Prepare dilution 4 in the c-myc Std. 4 tube:
   a. Using a new pipette tip, add 1.0 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. Prepare dilution 5 in the c-myc Std. 5 tube:
   a. Using a new pipette tip, add 1.0 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**
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	5.0
2	GAPDH Std. 2	9.0
3	GAPDH Std. 3	9.0
4	GAPDH Std. 4	9.0
5	GAPDH Std. 5	9.0

3. Prepare dilution 1 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 5.0 µL of stock to the GAPDH Std. 1 tube.
   c. Vortex Std. 1 for 3 to 5 seconds, then centrifuge the tube briefly.
4. Prepare dilution 2 in the GAPDH Std. 2 tube:
   a. Using a new pipette tip, add 1.0 µL of dilution 1 to GAPDH Std. 2 tube.
   b. Vortex Std. 2 for 3 to 5 seconds, then centrifuge the tube briefly.

Notes

5. Prepare dilution 3 in the GAPDH Std. 3 tube:
   a. Using a new pipette tip, add 1.0 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. Prepare dilution 4 in the GAPDH Std. 4 tube:
   a. Using a new pipette tip, add 1.0 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. Prepare dilution 5 in the GAPDH Std. 5 tube:
   a. Using a new pipette tip, add 1.0 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.

Prepare the Reaction Mix

Prepare the reaction mix using the components and volumes that were calculated by the StepOne software ("Complete the Reaction Mix Calculations Tab for the c-myc Assay" on page 43 and "Complete the Reaction Mix Calculations Tab for the GAPDH Assay" on page 45).

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 69).

About the Example Experiment

For the relative standard curve example experiment:

- The reaction mix components are:
  - TaqMan® Fast Universal PCR Master Mix (2×)
  - c-myc Assay Mix (20×)
  - GAPDH Assay Mix (20×)
  - Water

Notes

- The volumes calculated in the software for both target assays are:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	10.0
Assay Mix (20.0X)	1.0
H_2O	7.0
Total Volume	18.0

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

Required Materials

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

Prepare the Reaction Mix

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

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® Fast Universal PCR Master Mix (2X)	10.0	264.0
c-myc Assay Mix (20X)	1.0	26.4
Water	7.0	184.8
Total Reaction Mix Volume	18.0	475.2

Notes

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® Fast Universal PCR Master Mix (2X)	10.0	264.0
GAPDH Assay Mix (20X)	1.0	26.4
Water	7.0	184.8
Total Reaction Mix Volume	18.0	475.2

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 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 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*

Notes

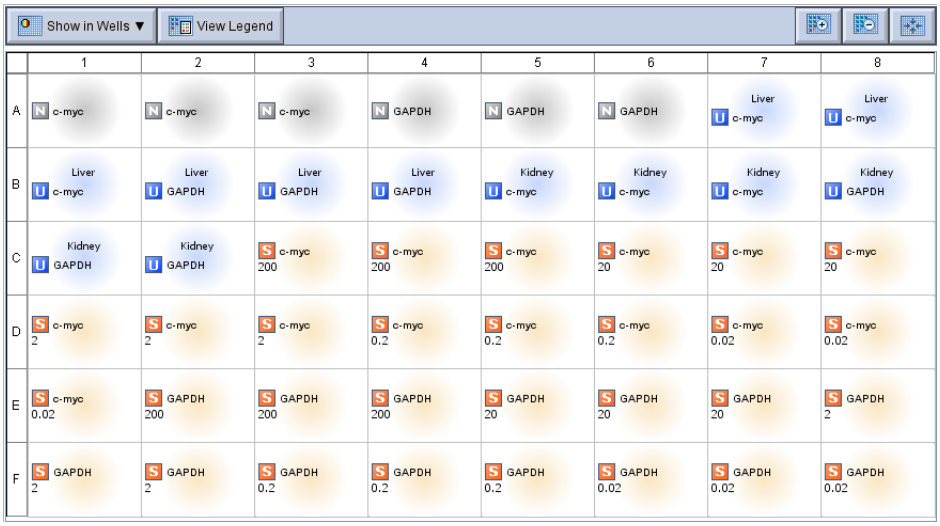
Prepare the Reaction Plate

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

About the Example Experiment

For the relative standard curve example experiment:

- A MicroAmp® Fast Optical 48-Well Reaction Plate is used.
- The reaction volume is 20 µL/well.
- The reaction plate contains:
  - 12 Unknown wells U
  - 30 Standard wells S
  - 6 Negative control wells N
  - 0 Empty wells
- The plate layout automatically generated by the StepOne software is used:



Note: The example experiment was created for a StepOne instrument. If you selected the StepOnePlus instrument in the Experiment Properties screen (page 27), your reaction plate layout will differ from the layout shown above. The software displays a 96-well reaction plate layout for the StepOnePlus instrument. For an example of the 96-well reaction plate layout, see page 13.

Required Materials

- Microcentrifuge tubes
- Pipettors
- Pipette tips
- c-myc reaction mix (from page 67)
- GAPDH reaction mix (from page 67)
- Water

Notes

- c-myc standards (from page 64)
- GAPDH standards (from page 65)
- Samples (from page 62)
- MicroAmp® Fast Optical 48-Well Reaction Plate
- MicroAmp® Optical 48-Well 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 20 µL of the negative control reaction to the appropriate wells in the reaction plate.

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	59.4	c-myc Std 1	6.6
2	c-myc Std 2	c-myc reaction mix	59.4	c-myc Std 2	6.6
3	c-myc Std 3	c-myc reaction mix	59.4	c-myc Std 3	6.6
4	c-myc Std 4	c-myc reaction mix	59.4	c-myc Std 4	6.6
5	c-myc Std 5	c-myc reaction mix	59.4	c-myc Std 5	6.6
6	GAPDH Std 1	GAPDH reaction mix	59.4	GAPDH Std 1	6.6
7	GAPDH Std 2	GAPDH reaction mix	59.4	GAPDH Std 2	6.6

Notes

Tube	Standard Reaction	Reaction Mix	Reaction Mix Volume (µL)	Standard	Standard Volume (µL)
8	GAPDH Std 3	GAPDH reaction mix	59.4	GAPDH Std 3	6.6
9	GAPDH Std 4	GAPDH reaction mix	59.4	GAPDH Std 4	6.6
10	GAPDH Std 5	GAPDH reaction mix	59.4	GAPDH Std 5	6.6

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 20 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	59.4	Liver	6.6
2	c-myc Kidney	c-myc reaction mix	59.4	Kidney	6.6
3	GAPDH Liver	GAPDH reaction mix	59.4	Liver	6.6
4	GAPDH Kidney	GAPDH reaction mix	59.4	Kidney	6.6

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 20 µL of the unknown (sample) reaction to the appropriate wells in the reaction plate.

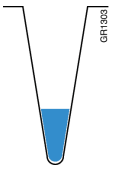
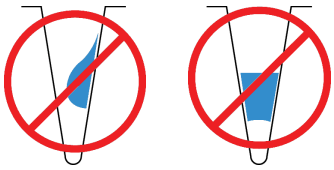
4. Seal the reaction plate with optical adhesive film.

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

Notes

6. Confirm 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(s) 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

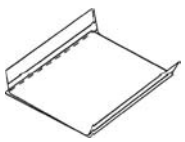
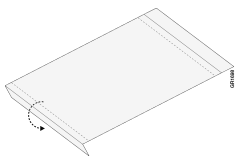
7. Until you are ready to perform the run, place the reaction plate on ice in the dark.

Preparation Guidelines

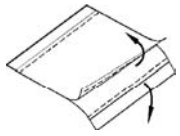
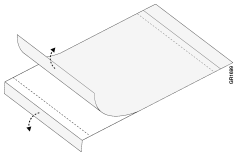
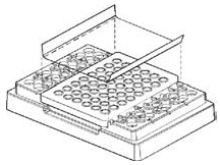
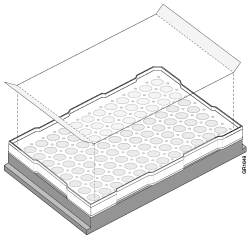
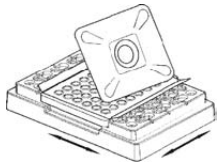
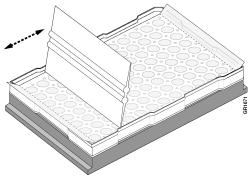
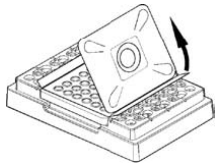
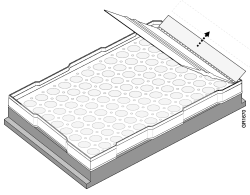
When you prepare your own relative standard curve experiment:

- Make sure you use the appropriate consumables.
- Make sure the arrangement of the PCR reactions matches the plate layout displayed in the StepOne 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 to seal your reaction plates, seal each reaction plate as follows:

Action	Example	
	StepOne™ System	StepOnePlus™ System
1. Place the reaction plate onto the center of the 96-well base. Be sure the reaction plate is flush with the top surface of the 96-well base.		
2. Load the reaction plate as desired.		
3. Remove a single optical adhesive film (film) from the box. • *For the StepOne system reaction plate,* bend both end-tabs upward. Hold the film backing side up. • *For the StepOnePlus system reaction plate*, fold back one of the end-tabs. Hold the film backing side up.		

Notes

Action	Example	
	StepOne™ System	**StepOnePlus™ System**
4. In one swift 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 will not affect results. Haziness will disappear when the film comes into contact with the heated cover in the instrument.		
5. Holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Be sure the film is completely covering all wells of the reaction plate.		
6. 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.		
7. 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.		
8. To ensure a tight, evaporation-free seal: a. Repeat step 6. b. 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, evaporation-free seal.		
9. 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 260.

Notes

Notes

4 Run the Relative Standard Curve Experiment

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

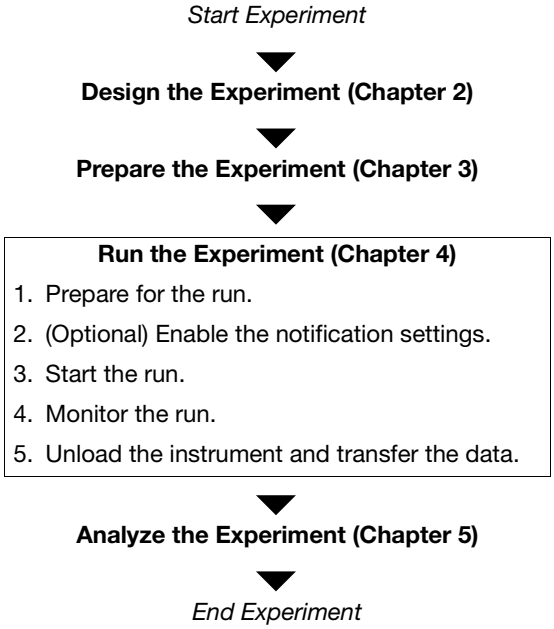
Notes

Chapter Overview

This chapter explains how to perform a run on the Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems.

Example Experiment Workflow

The workflow for running the example experiment provided with this getting started guide is shown below.



Notes

Prepare for the Run

Prepare for the run by opening the example experiment file you created in Chapter 2, then loading the sealed reaction plate into the StepOne™ or StepOnePlus™ instrument.

Open the Example Experiment

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

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

2. In the Login dialog box, select **EXAMPLEUSER** from the User Name dropdown menu, then click **OK**.

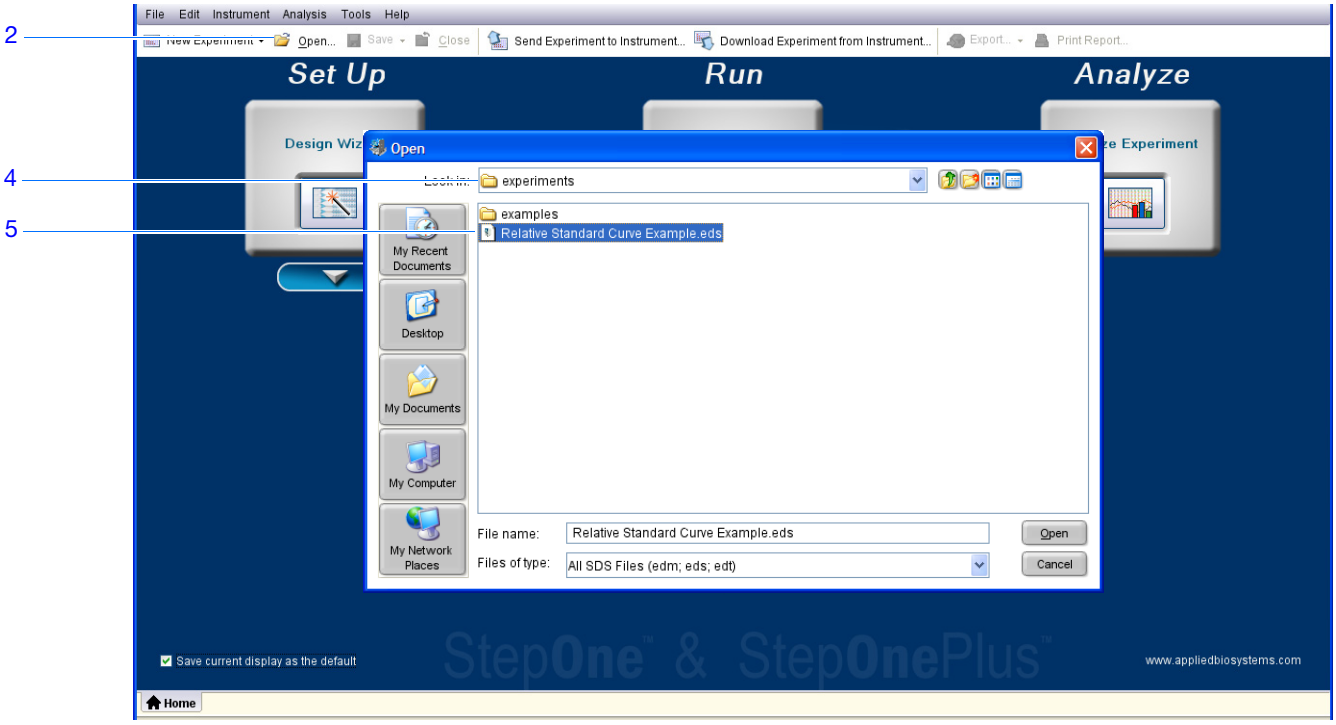
   Note: EXAMPLEUSER is the user name you created when designing the relative standard curve experiment (page 23).

3. From the Home screen, click **Open**.

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

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

5. Double-click **Relative Standard Curve Example** to open the example experiment file you created in Chapter 2.



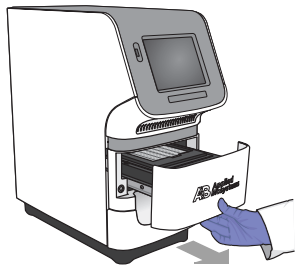
Notes

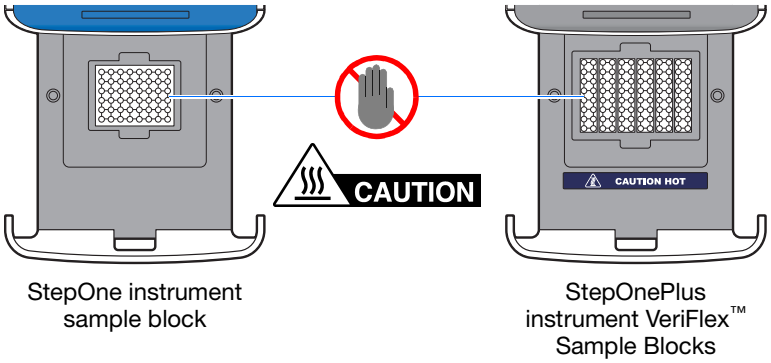
Load the Reaction Plate Into the Instrument

CAUTION **PHYSICAL INJURY HAZARD.** During instrument operation, the temperature of the sample block(s) can exceed 100 °C. If the instrument has been used recently, keep your hands away until the sample block(s) reach room temperature.

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

1. Open the instrument drawer.





2. Place the reactions in the sample block(s):
   - If using a reaction plate: Place the reaction plate in the sample block(s) with well A1 at the back-left corner.
   - If using reaction tube strips: Place the tray containing the tube strips in the sample block(s).
   - If using reaction tubes: Place the tray containing the tubes in the sample block(s).

Notes

IMPORTANT! For optimal performance with partial loads:

StepOnePlus instruments – Load at least 16 tubes and arrange them in:

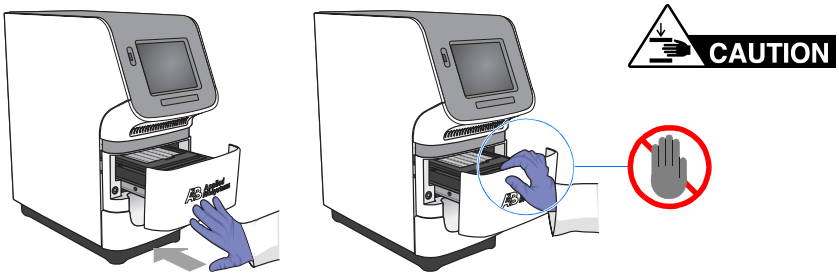
- Adjacent columns of 8 tubes, using rows A through H. For example, fill wells in column 1 (rows A through H) and column 2 (rows A through H).

  or

- Adjacent rows of 8 tubes, using columns 3 through 10. For example, fill wells in row A (columns 3 through 10) and row B (columns 3 through 10).

StepOne instruments – Load at least 4 tubes in the sample block.

3. Close the instrument drawer carefully.



(Optional) Enable the Notification Settings

Enable the notification settings so that the StepOne software alerts you by e-mail when the StepOne or StepOnePlus instrument begins and completes the run, or if an error occurs during the run. You can also set up the software to attach a completed run file to the Run Completed e-mail notification. Enabling the notifications settings feature is optional and does not affect the performance of the StepOne™ and StepOnePlus™ systems or the duration of the run.

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

Note: The notification system is also available to computers that are monitoring a StepOne or StepOnePlus instrument remotely. For more information, see "Remote Monitor" on page 90.

About the Example Experiment

In the example experiment:

- The StepOne software is set up to send notifications to three users (scientist, supervisor, and technician at mycompany.com) when the StepOne or StepOnePlus system ends the run and if it encounters any errors during operation.
- The example SMTP server (www.mycompany.com) is set up for Secure Sockets Layer (SSL) encryption and requires authentication for use.

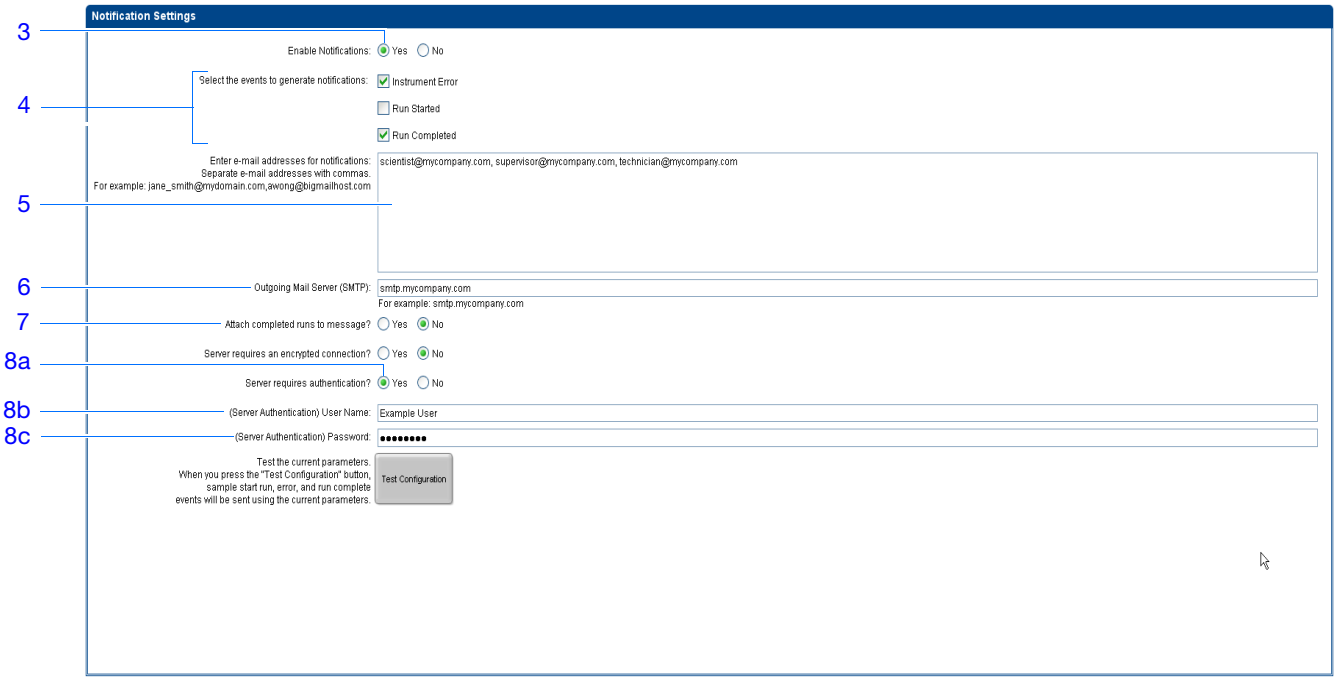
Notes

Set Up the Notification Settings

1. In the StepOne software, click **Run** in the navigation pane.
2. Click **Notification Settings**.
3. Select **Yes** for Enable Notifications.
4. Select the events that 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. Select **No** next to "Attach completed runs to message."

   Note: This option applies only to e-mail notifications that are generated when the instrument completes a run (that is, **Run Completed** is selected in step 4).

8. 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 StepOne or StepOnePlus system for automatic notification:

- Your system must be set up for network use. Refer to the *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking, and Maintenance Guide*.
- Select the events for which you want to receive e-mail notifications:
  - **Instrument Error** – When selected, recipients are e-mailed all errors encountered by the instrument during each run.
  - **Run Started** – When selected, recipients are e-mailed every time the instrument starts a run.
  - **Run Completed** – When selected, recipients are e-mailed every time the instrument completes a run.
- Obtain e-mail addresses to receive notifications.

  IMPORTANT! Separate addresses with a comma (,).

- Contact your systems administrator or information technology department if you need:
  - E-mail addresses for users who will receive notifications
  - A network address for a simple mail transfer protocol (SMTP) server on the 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)
- Click **Test Configuration** to test your notification settings. If the notification settings are set up correctly, sample e-mails are sent to the addresses you entered.

Notes

Start the Run

Start the run according to the layout of your StepOne or StepOnePlus system:

Layout	Description	See...
Colocated	The yellow cable connects the computer to the instrument	"Colocated Startup" below
Standalone	• The computer and the instrument are not connected, or • The computer and the instrument are connected to the same network.	"Standalone Startup" on page 83

Colocated Startup

Perform this procedure if your computer is directly connected to your StepOne or StepOnePlus instrument by the yellow cable.

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

Notes

Standalone Startup

Perform these procedures if your computer and StepOne or StepOnePlus instrument *are not* directly connected by the yellow cable. Start with:

- "Send the Experiment to the Instrument Over the Network" on page 83 if your computer and instrument are connected to the same network.

  or

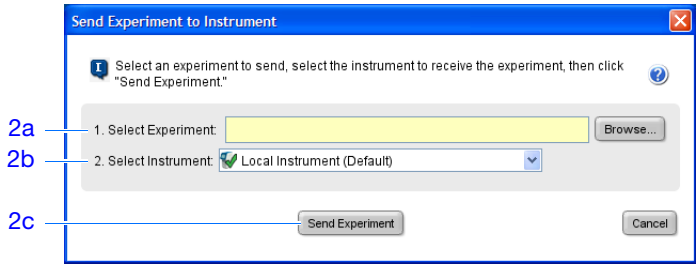
- "Transfer the Experiment to the Instrument Using a USB Drive" on page 83 if your computer and instrument are not connected to the same network.

Send the Experiment to the Instrument Over the Network

1. In the StepOne software, click **Send Experiment to Instrument**.
2. In the Send Experiment to Instrument dialog box:
   a. Click **Browse**, navigate to the example experiment file, then click **Open**.
   b. Select the instrument to receive the experiment file.

   Note: If your instrument is not listed, set up the instrument for monitoring as explained in the *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking, and Maintenance Guide*.

   c. Click **Send Experiment** to send the experiment to the your instrument over the network.



3. When prompted, click **OK** to close the confirmation.
4. Go to "Start the Instrument Run Using the Touchscreen" on page 84.

Transfer the Experiment to the Instrument Using a USB Drive

1. Connect the USB drive to one of the USB ports on the computer.
2. In the StepOne software, select **Save** ▸ **Save As**.
3. In the Save dialog box, navigate to the USB drive, then click **Save**.

Notes

4. Remove the USB drive from your computer, then connect it to the USB port of your StepOne or StepOnePlus instrument.

5. Go to "Start the Instrument Run Using the Touchscreen" below.

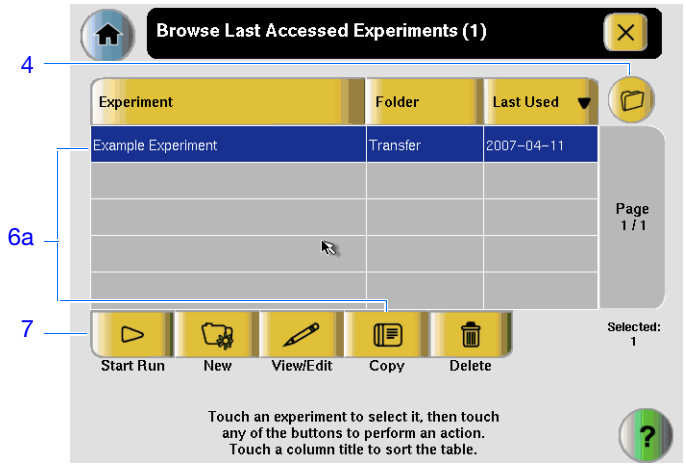
Start the Instrument Run Using the Touchscreen

1. Touch the StepOne or StepOnePlus instrument touchscreen to awaken it.

   Note: If the touchscreen is not at the Main Menu screen, touch .

2. Wait for the USB sign to appear on the touchscreen.
3. In the Main Menu screen, touch **Browse/New Experiments**.
4. In the Browse screen, touch **Folders**.
5. In the Choose an Experiment Folder screen:
   - Touch **USB** if you transferred the experiment on a USB drive.
   - Touch **Default** if you sent the experiment over a network connection.
6. Before starting the run, save the example experiment to your instrument:
   a. In the Browse screen, touch the example experiment name, then touch **Copy**.
   b. In the Save Experiment screen, navigate to a destination folder, then click **Save & Exit**.
7. In the Browse screen, touch the example experiment name, then touch **Start Run**.

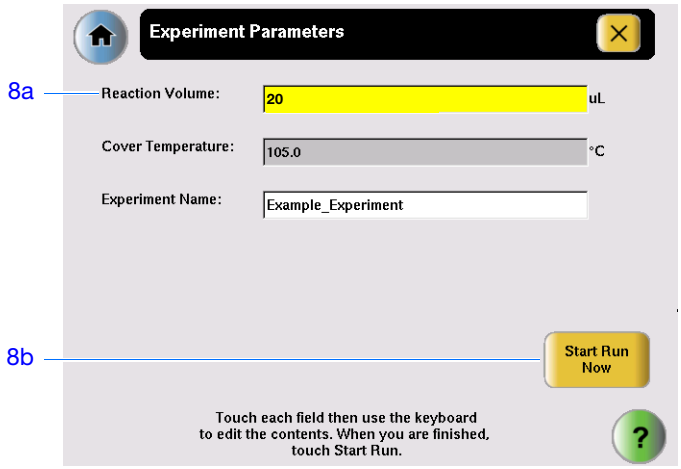
Notes



8. In the Run Parameters screen:

   a. Touch the **Reaction Volume** field, use the keypad to enter the reaction volume for the example experiment, then touch **Done**.

   b. Touch **Start Run Now**.



Notes

Monitor the Run

Monitor the run according to the layout of your StepOne or StepOnePlus system:

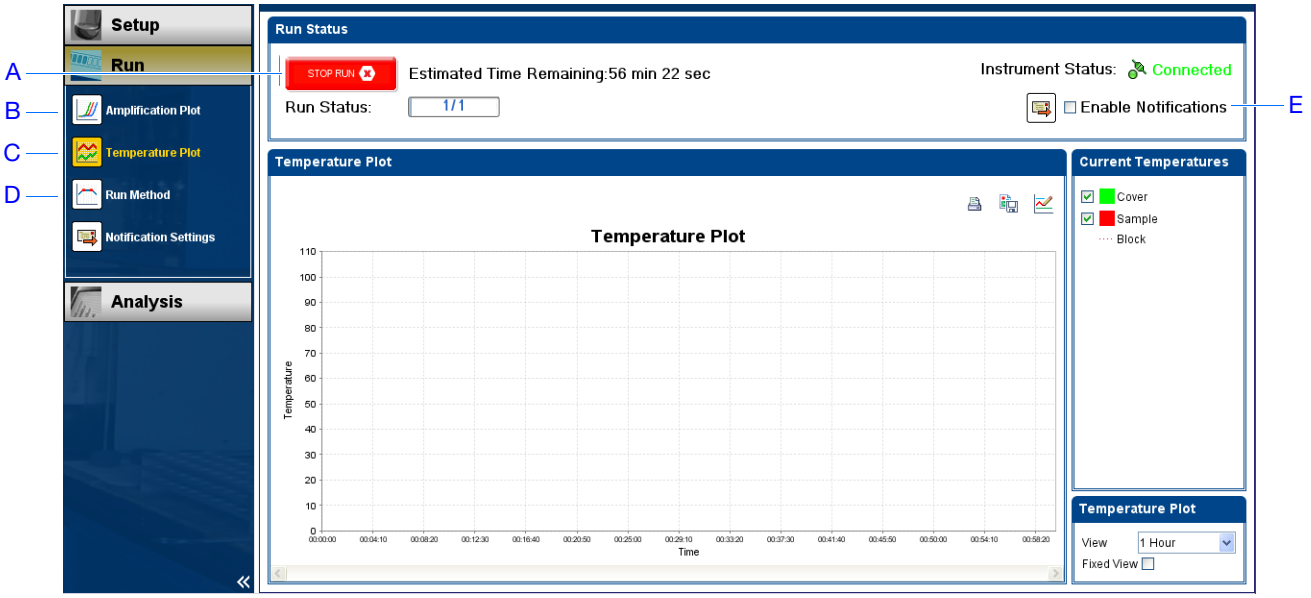
Layout	Description	See...
Colocated	The yellow cable connects the computer to the instrument.	"Colocated Monitoring" below
Standalone (Networked)	The computer and the instrument are connected to the same network.	"Remote Monitor" on page 90
Standalone (Basic)	The computer and the instrument are not connected.	"Standalone Monitoring" on page 92

Colocated Monitoring

If your computer is directly connected to your StepOne or StepOnePlus instrument by the yellow cable, you can view the progress of the run in realtime as described below. During the run, periodically view all three plots available from the StepOne software for potential problems.

#	To...	Action
A	Stop the run	1. In the StepOne software, click **STOP RUN**. 2. In the Stop Run dialog, 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.
B	View amplification data in realtime	Select **Amplification Plot**. See "About the Amplification Plot Screen" on page 87.
C	View temperature data for the run in realtime	Select **Temperature Plot**. See "About the Temperature Plot Screen" on page 88.
D	View progress of the run in the Run Method screen	Select **Run Method**. See "About the Run Method Screen" on page 89.
E	Enable/disable the Notification Settings	Select or deselect **Enable Notifications**. See "(Optional) Enable the Notification Settings" on page 79.

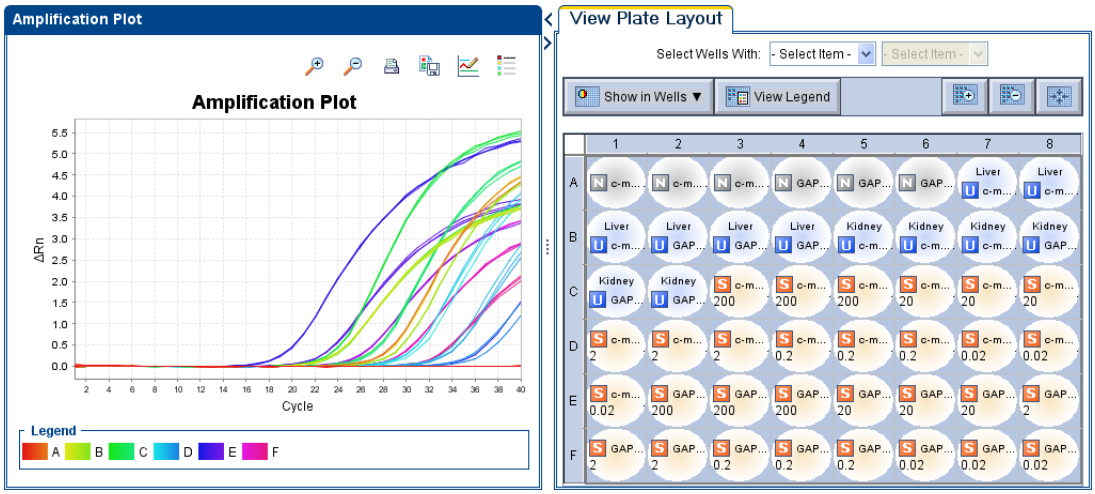
Notes



About the Amplification Plot Screen

The Amplification Plot screen allows you to view 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 contrasts normalized dye fluorescence (ΔRn) and 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.



The Amplification Plot screen is useful for identifying and examining abnormal amplification. Abnormal amplification can include the following:

- Increased fluorescence in negative control wells.

Notes

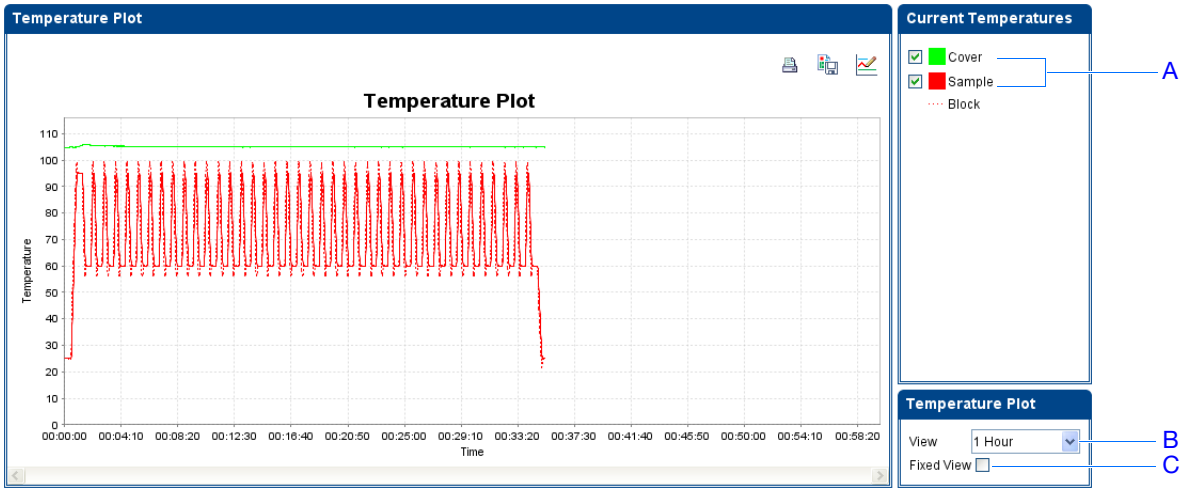
- Absence of detectable fluorescence at an expected cycle (determined from previous similar experiments run using the same reagents under the same conditions).

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

About the Temperature Plot Screen

During a run, the Temperature Plot screen displays the temperatures of the sample block(s), the heated cover, and samples (calculated) in realtime. The figure below shows the Temperature Plot screen as it appears during the example experiment.

	To...	Action
A	Add/remove temperature plots	Select **Cover** or **Sample Block** to toggle the presence of the associated data in the plot.
B	Change the time displayed by plot	From the **View** dropdown menu, select the amount of time to display in the plot.
C	Display a fixed time window during the instrument run If the entire plot does not fit in the screen, the screen is not updated as the run progresses. For example, if you select 10 minutes from the View dropdown menu, the plot will show data for 10 minutes. If the run lasts more than 10 minutes: • The plot updates as the run progresses with Fixed View deselected. • The plot does not update as the run progresses with Fixed View selected.	Select **Fixed View**.



The Temperature Plot screen can be useful for identifying hardware failures. When monitoring the Temperature Plot screen, observe the Sample and Block plots for abnormal behavior.

Notes

- In general, the Sample and Block plots should mirror each other approximately. A significant deviation of the plots may indicate a problem.
- The Cover plot should maintain the constant temperature specified in the method. A departure from the **constant** temperature may indicate a problem.

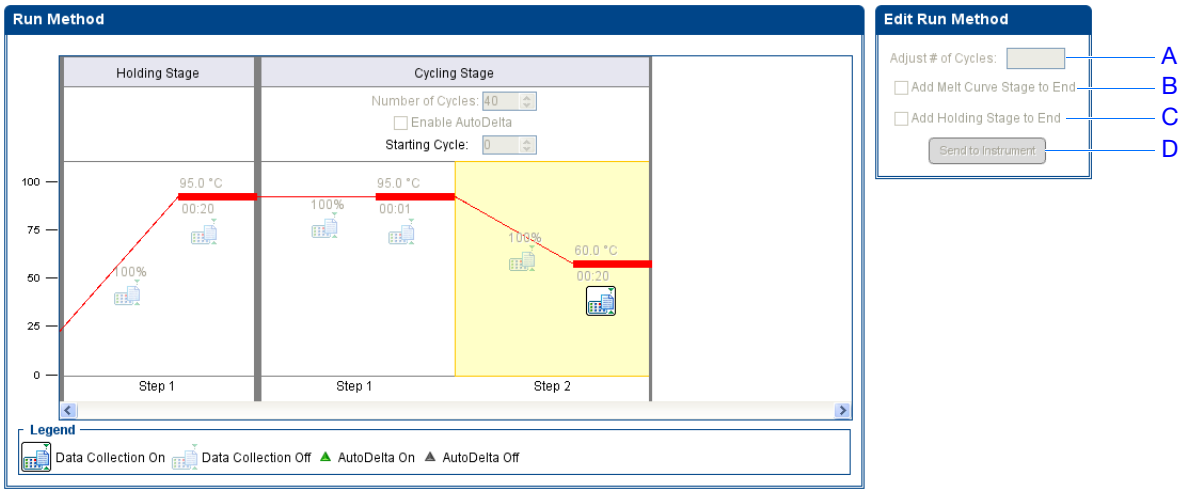
If you notice an abnormal temperature plot, troubleshoot the error as explained in the StepOne Software Help (click or press **F1**).

Note: The Sample temperature displayed in the Current Temperatures group is an estimated value.

About the Run Method Screen

The Run Method screen displays the run method selected 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 Adjust # of Cycles field, enter the number of cycles to apply to the Cycling Stage.
B	Add a melt curve stage to the end of the run	Select **Add Melt Curve Stage to End**.
C	Add a Hold stage to the end of the run	Select **Add Holding Stage to End**.
D	Apply your changes	Click **Send to Instrument**.



If an alert appears, click the error for more information and troubleshoot the problem as explained in the StepOne Software Help (click or press **F1**).

Notes

Remote Monitor

If your StepOne or StepOnePlus instrument is connected to a network, you can use the Remote Monitor in the StepOne software to view the progress of the run in realtime from any computer on the network.

IMPORTANT! Networked computers cannot control the StepOne or StepOnePlus instrument, only monitor it.

To monitor your instrument remotely:

1. In the StepOne software, select **Instrument ▸ Remote Monitor**.
2. In the navigation pane, select your instrument.

   If the navigation pane does not list your instrument:

   a. Click **Add Instrument**.

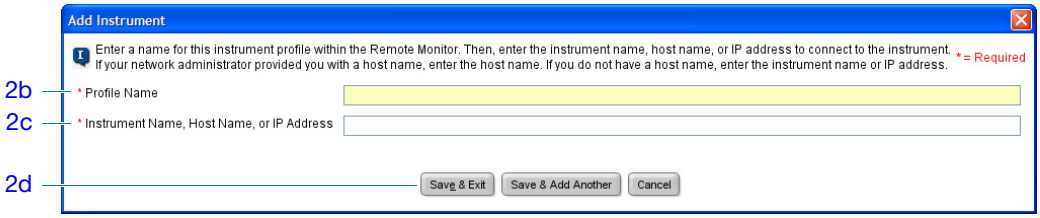
   b. Enter a name for the instrument profile within the Remote Monitor.

   Note: Enter any name that helps you identify the instrument. The profile name you enter will be displayed in the Remote Monitor and in the instrument dropdown menus when you send experiments, download experiments, or monitor instruments.

   c. In the Instrument Name, Host Name, or IP Address field:
      - If you know the host name, enter the host name.
      - If you do not know the host name, enter the instrument name or IP address.

   Note: The instrument name and IP address are displayed on the instrument touchscreen. Go to **Settings Menu ▸ Admin Menu ▸ Set Instrument Name** or **Set IP Address**. Contact your systems administrator or information technology department for the host name.

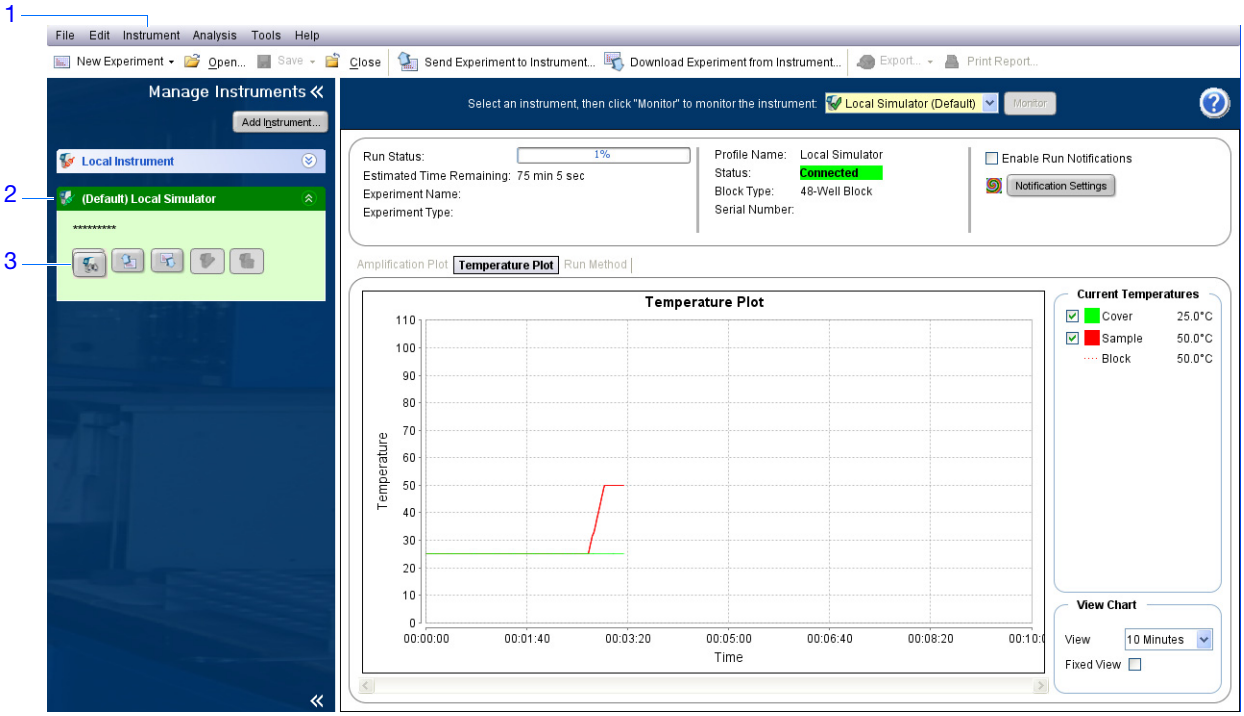
   d. Click **Save & Exit**.



Note: For more information on configuring the StepOne or StepOnePlus instrument for network use or for the Remote Monitor feature, refer to the *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking, and Maintenance Guide*.

Notes

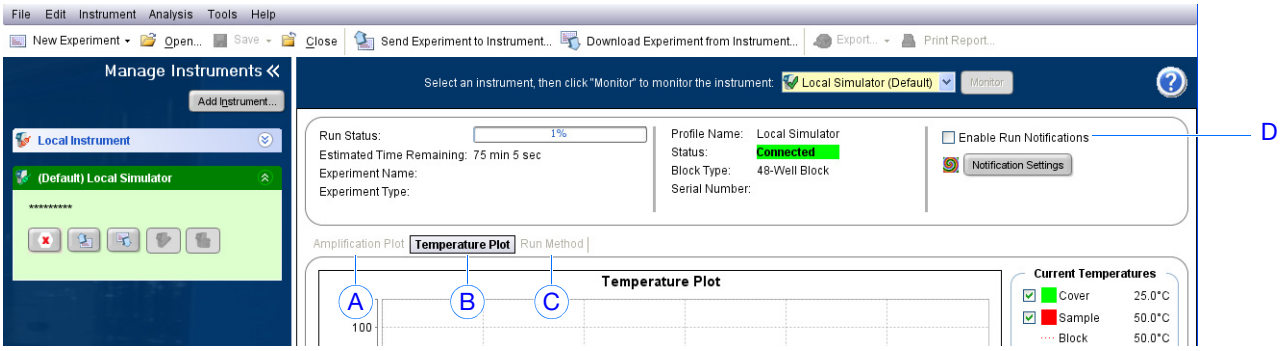
3. Click **Start monitoring the instrument** for your instrument. It may take several minutes for the instrument to send the information to your computer.



4. View data as described below.

#	To...	Action
A	View amplification data in realtime	Click **Amplification Plot**. See "About the Amplification Plot Screen" on page 87.
B	View temperature data for the run in realtime	Click **Temperature Plot**. See "About the Temperature Plot Screen" on page 88.
C	View progress of the run in the Run Method screen	Click **Run Method**. See "About the Run Method Screen" on page 89.
D	Enable/disable the Notification Settings	Select or deselect **Enable Notifications**. See "(Optional) Enable the Notification Settings" on page 79.

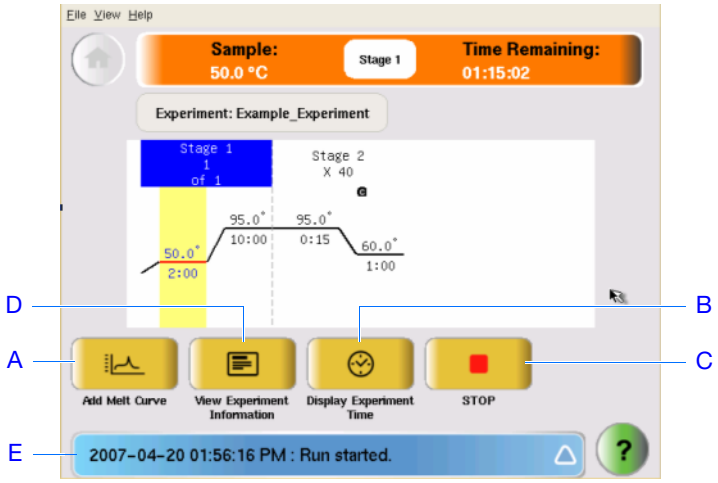
Notes



Standalone Monitoring

If you started the run from your StepOne or StepOnePlus instrument, you can view the progress of the run from the touchscreen. The Run Method screen displays the method for the experiment and highlights the thermal profile steps as the instrument performs them.

#	To...	Action
A	Add a melt curve stage to the run	Touch **Add Melt Curve**, then touch **OK**.
B	Display the time remaining in the run	Touch **Display Experiment Time**, then touch ✕ to return to the Run Method screen.
C	Stop the run	Touch **STOP**, then touch: • **Stop** to stop the run after the instrument completes the current cycle or hold. • **Abort** to stop the run immediately. • ✕ to continue the run with no changes.
D	View experiment information	Touch **View Experiment Information**, then touch ✕ to return to the Run Method screen.
E	View the Error Log	Touch the status bar to display the error log.



Notes

Unload the Instrument and Transfer the Data

When your StepOne or StepOnePlus instrument displays the Main Menu screen, unload the reaction plate from the instrument and transfer the experiment data to the computer for analysis.

Unload the Reaction Plate

CAUTION **PHYSICAL INJURY HAZARD.** During instrument operation, the temperature of the sample block(s) can exceed 100 °C. Keep your hands away until the sample block(s) reach room temperature.

Note: When the StepOne or StepOnePlus instrument completes a run, the system saves the details of the run to the run history, which remains present in the system until the instrument completes another run.

1. When the Run Report screen appears in the StepOne or StepOnePlus instrument touchscreen, touch  .
2. Open the instrument drawer.
3. Remove the reaction plate from the sample block(s).
4. Carefully close the instrument drawer.

Select a Data Transfer Method

Transfer the experiment to your computer for analysis according to the layout of your StepOne or StepOnePlus system:

Layout	Description	See...
Colocated	The yellow cable connects the computer and the instrument.	"Colocated Data Transfer" below
Standalone (Networked)	The computer and the instrument are connected to the same network.	"Remote Data Transfer" on page 94
Standalone (Basic)	The computer and the instrument are not connected.	"Standalone Data Transfer" on page 95

Notes

Colocated Data Transfer

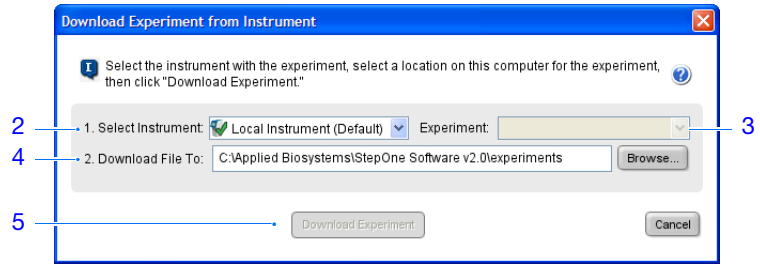
If your computer is directly connected to your StepOne or StepOnePlus instrument by the yellow cable, no action is necessary. The StepOne software automatically transfers the experiment data from the instrument to the computer after the run.

Note: In a colocated layout, you can start the run from the computer or from the instrument touchscreen. However, the StepOne software only transfers the experiment data automatically when a run is started from the computer (see "Colocated Startup" on page 82).

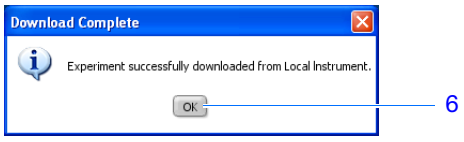
Remote Data Transfer

If your computer and StepOne or StepOnePlus instrument are connected to the same Ethernet network, download the experiment from the instrument over the network:

1. In the StepOne software, click **Download Experiment from Instrument** to open the Download Experiment from Instrument dialog box.
2. From the Select Instrument dropdown menu, select your instrument.
3. From the Experiment dropdown menu, select the example experiment file.
4. In the Download File To field:
   a. Click **Browse**.
   b. Navigate to:
   <*drive*>:\Applied Biosystems\<*software name*>\experiments\
   where:
   <*drive*> is the computer hard drive on which the StepOne software is installed. The default installation drive for the software is the D drive.
   <*software name*> is the current version of the StepOne software.
   c. Click **Select**.
5. Click **Download Experiment** to download the example experiment file from your instrument to your computer over the network.



6. When prompted, click **OK** to close the confirmation.



Notes

Standalone Data Transfer

If your computer is not connected to your StepOne or StepOnePlus instrument, use the USB drive to transfer the experiment from the instrument to the computer:

1. If not already connected to the instrument, connect a USB drive to the USB port.

2. Touch the StepOne or StepOnePlus instrument touchscreen to awaken it.

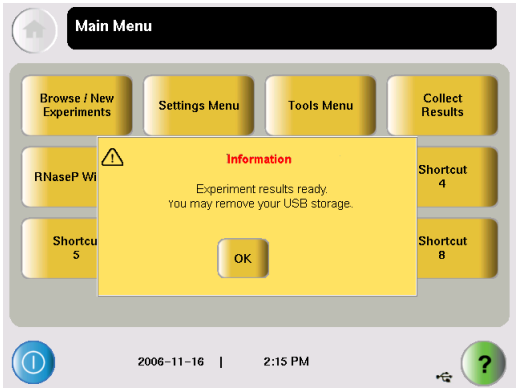
Note: If the touchscreen is not at the Main Menu screen, touch .

3. Wait for the USB sign to appear on the touchscreen.

4. In the Main Menu, touch **Collect Results** to save the data to the USB drive.

Note: If your instrument cannot find the USB drive, remove the USB drive, then try again. If the instrument still does not recognize the USB drive, try another USB drive.

5. When prompted that the data has been transferred successfully, touch **OK**.



6. Remove the USB drive from your instrument, then connect it to one of the USB ports on your computer.

7. In the computer desktop, use the Windows explorer open the USB drive.

Notes

8. Copy the example experiment file to:

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

   where:

   - <*drive*> is the computer hard drive on which the StepOne software is installed. The default installation drive for the software is the D drive.
   - <*software name*> is the current version of the StepOne software.

Notes

5 Analyze the Relative Standard Curve Experiment

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

Notes

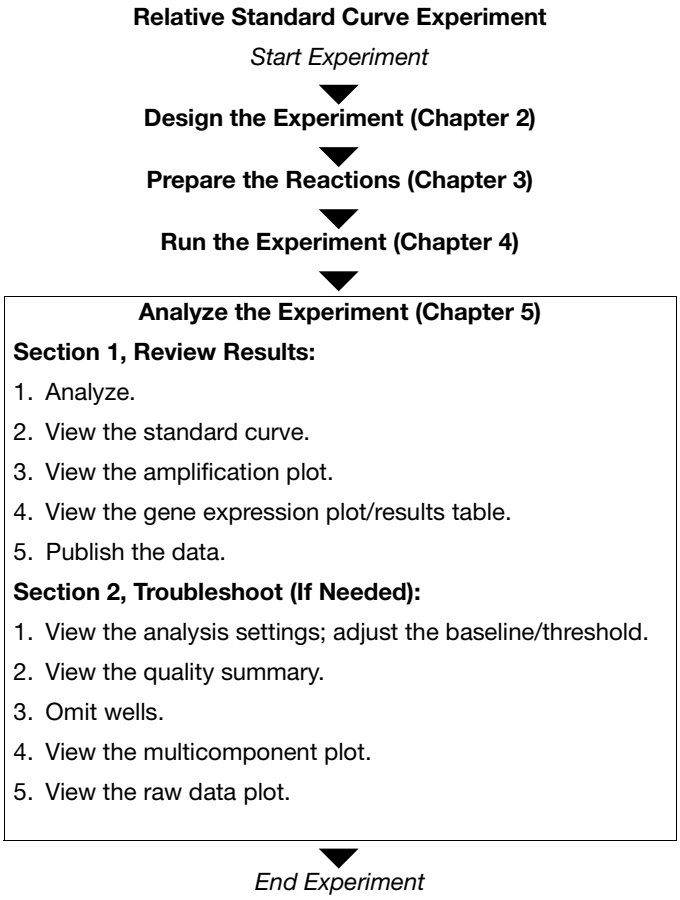
Chapter Overview

The StepOne™ 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. If you receive questionable results, Section 2 of this chapter explains how to perform some troubleshooting steps.

Note: The relative standard curve example experiment includes four flagged wells. The flags reflect 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

The workflow for analyzing the example experiment provided with this getting started guide is shown below.



Notes

Section 5.1 Review Results

This section covers:

Notes

Analyze the Experiment

The StepOne 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 installs with the StepOne software. The data file was created with the same design parameters provided in Chapter 2, then run and analyzed on a StepOne™ instrument.

You can find the data file for the example experiment on your computer:

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

where:

- <*drive*> is the computer hard drive on which the StepOne software is installed. The default installation drive for the software is the D drive.
- <*software name*> is the current version of the StepOne software.

Analyze the Example Experiment

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

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

2. In the Login dialog box, select **EXAMPLEUSER** from the User Name dropdown menu, then click **OK**.

   Note: EXAMPLEUSER is the user name you created when you designed the relative standard curve experiment (page 23).

3. From the Home screen, click **Open**.

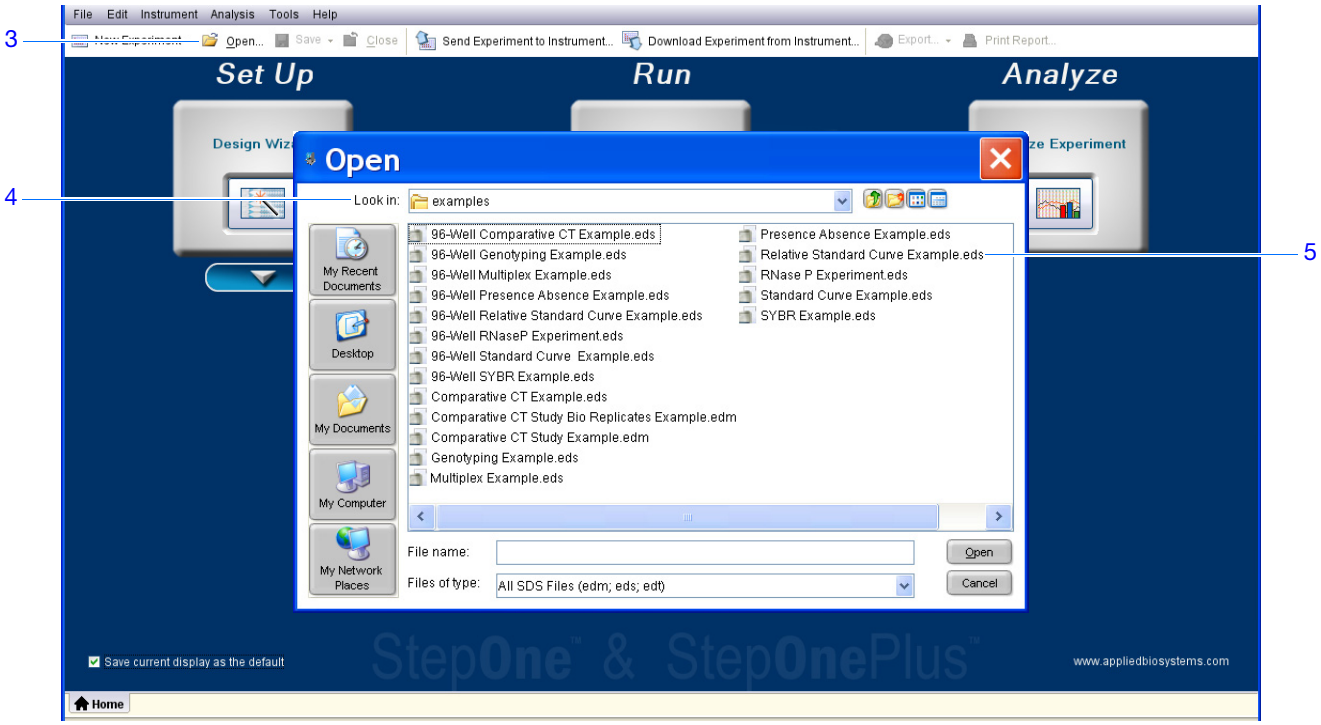
4. In the Open dialog box, navigate to the **examples** folder:

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

5. Double-click **Relative Standard Curve Example** to open the example experiment data file.

   Note: The examples folder contains several data files; be sure to select **Relative Standard Curve Example**. For information on the other data files, see "Data Files in the Examples Folder" on page 18.

Notes



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

7. See "Software Elements" below and "Navigation Tips" on page 104 for information on navigating within the analysis screens.

Guidelines

When you analyze your own relative standard curve experiment:

- Immediately after a run, the StepOne 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**.

Software Elements

The StepOne software elements for the analysis screens are illustrated below.

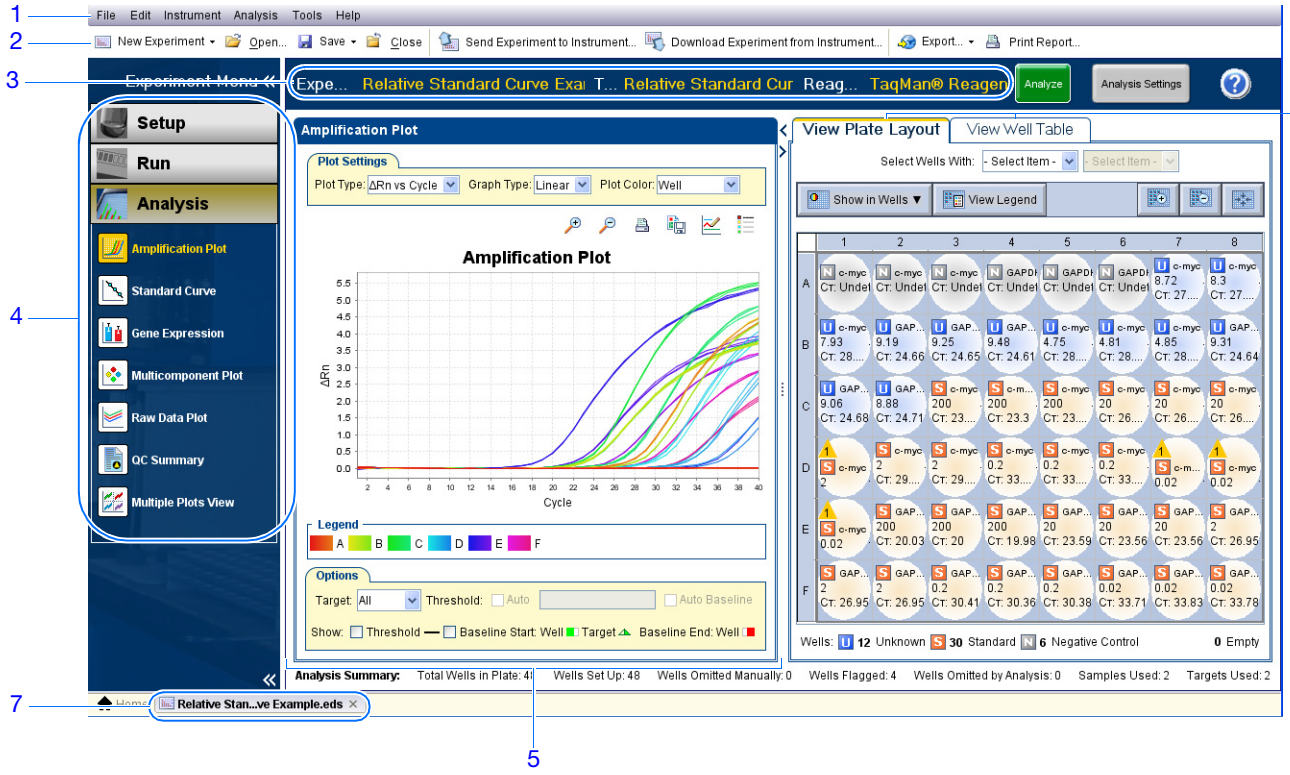
1. Menu bar – Displays the menus available in the software:
   - File
   - Edit
   - Instrument
   - Analysis
   - Tools

Notes

- Help

2. Toolbar – Displays the tools available in the software:
   - New Experiment
   - Open
   - Save
   - Close
   - Send Experiment to Instrument
   - Download Experiment from Instrument
   - Export
   - Print Report
3. Experiment header - Displays the experiment name, experiment type, and reagents for the open experiment.
4. Experiment Menu pane – Provides links to the following software screens:
   - Setup screens
   - Run screens
   - Analysis screens:
     - Amplification Plot
     - Standard Curve
     - Gene Expression
     - Multicomponent Plot
     - Raw Data Plot
     - QC Summary
     - Multiple Plots View
5. Plot pane – Displays the selected analysis screen for the open experiment.
6. View tabs – Displays the plate layout or Well Table for the open experiment
7. Experiment tab(s) – Displays a tab for each open experiment.

Notes


1
2
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File Edit Instrument Analysis Tools Help
New Experiment Open... Save Close Send Experiment to Instrument... Download Experiment from Instrument... Export... Print Report...
Experiment Menu
Setup
Run
Analysis
Amplification Plot
Standard Curve
Gene Expression
Multicomponent Plot
Raw Data Plot
QC Summary
Multiple Plots View
Relative Standard Curve Exa... Relative Standard Cur... TaqMan® Reagen...
Analyze
Analysis Settings
Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle Graph Type: Linear Plot Color: Well
Amplification Plot
ΔRn
Cycle
Legend
A B C D E F
Options
Target: All Threshold: Auto Auto Baseline
Show: Threshold Baseline Start: Well Target Baseline End: Well
View Plate Layout
View Well Table
Select Wells With: - Select Item -
Show in Wells
View Legend
Wells: 12 Unknown 30 Standard 6 Negative Control 0 Empty
Analysis Summary: Total Wells in Plate: 4 Wells Set Up: 48 Wells Omitted Manually: 0 Wells Flagged: 4 Wells Omitted by Analysis: 0 Samples Used: 2 Targets Used: 2
Relative Stan...ve Example.eds

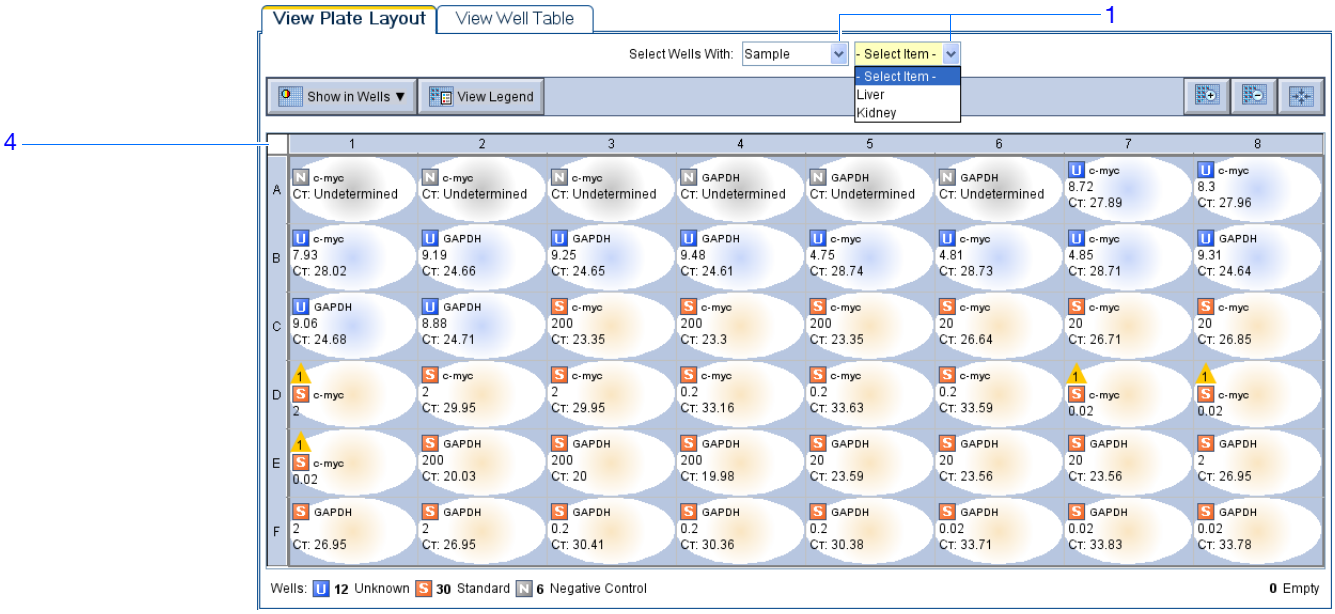
Notes

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 dropdown menus: 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 and drag over the desired wells, press **CTRL+click**, or press **Shift+click** in the plate layout.
4. To select all 48 wells, click the upper left corner of the plate layout.

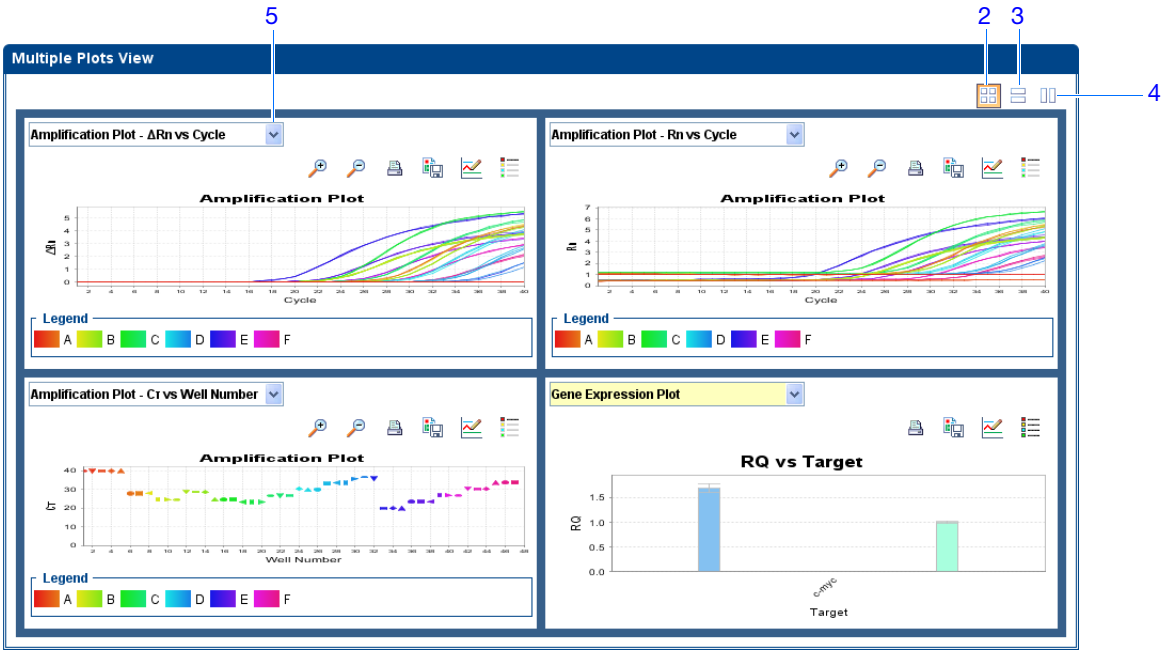


Notes

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. From the Experiment Menu 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 from the dropdown menu above each plot display.



Notes

View the Standard Curve

The Standard Curve screen displays the standard curve for samples designated as standards. The StepOne 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. From the Experiment Menu pane, select **Analysis** ▸  **Standard Curve**.

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

2. Display all 48 wells in the Standard Curve screen by clicking the upper left corner of the plate layout in the View Plate Layout tab.
3. From the Target dropdown menu, select **All** (default).
4. From the Plot Color dropdown menu, select **Default**.
5. Click  **Show a legend for the plot** (default).

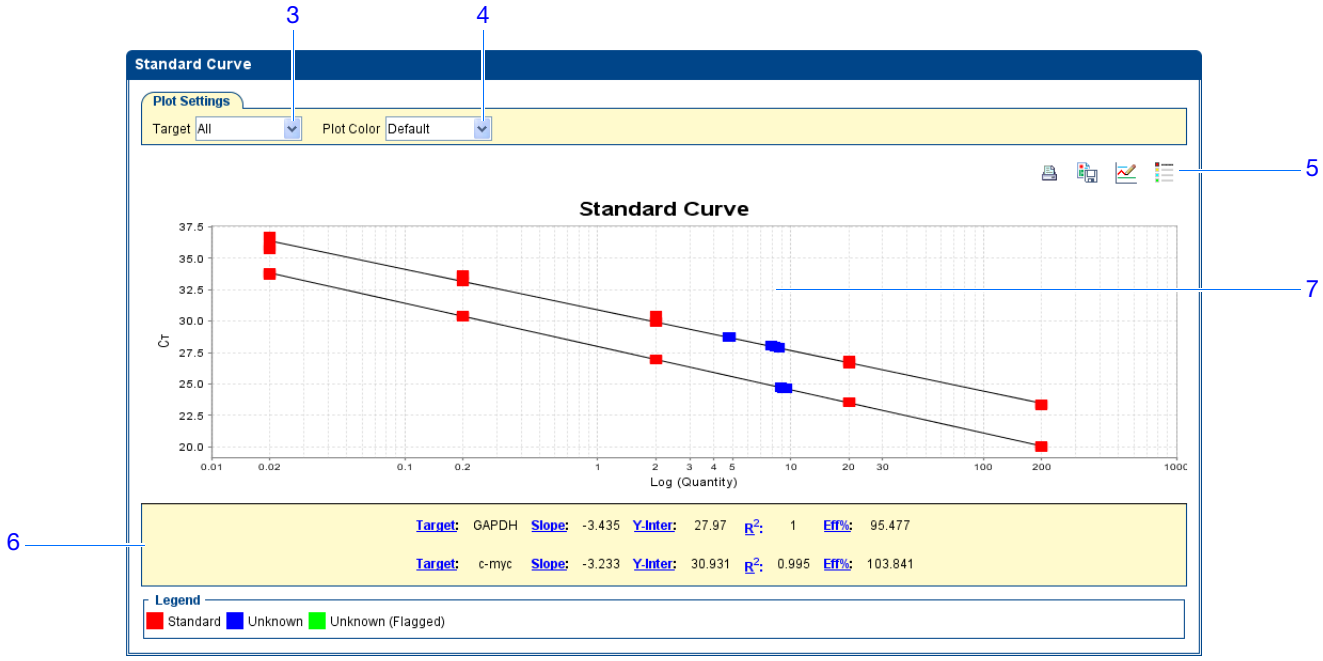
   Note: This is a toggle button. When the legend is displayed, the button changes to Hide the plot legend.

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

Target	Slope	R^2 Value	Amplification Efficiency (Eff%)
GAPDH	–3.435	1	95.477
c-myc	–3.233	0.995	103.841

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).

Notes



8. Check the C_T values:

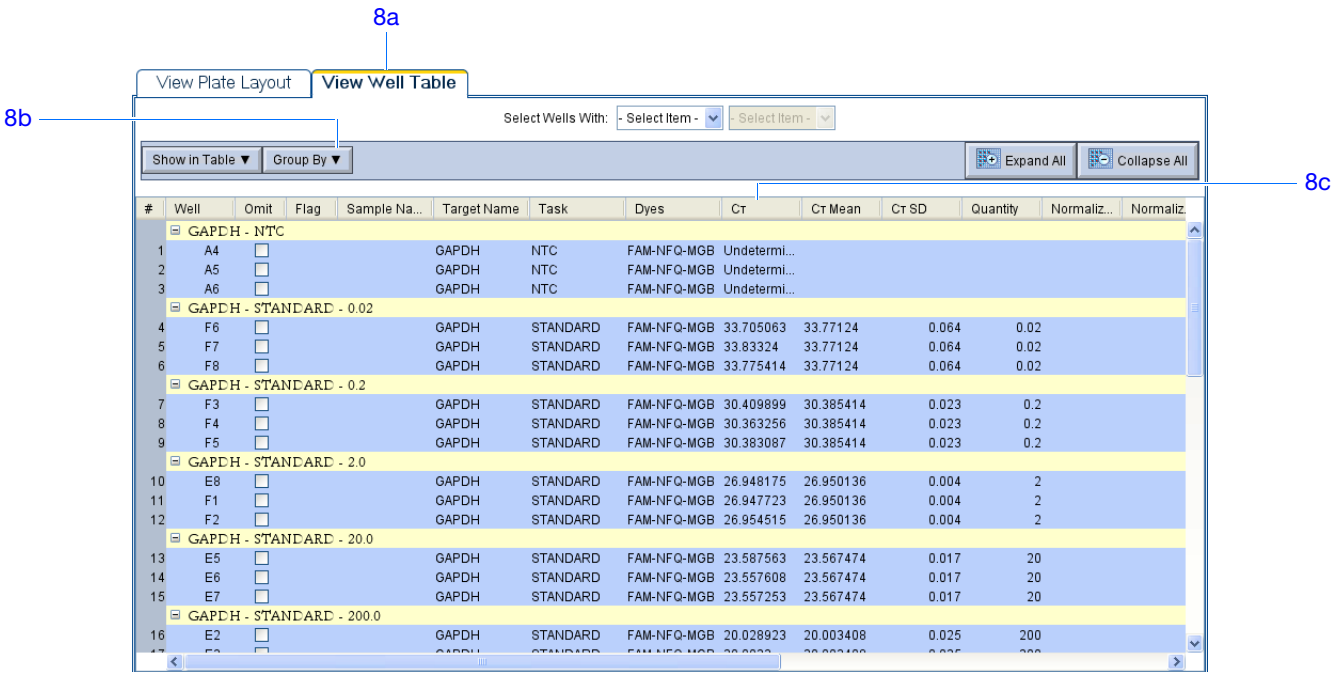
   a. Click the **View Well Table** tab.

   b. From the Group By dropdown menu, select **Replicate**.

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

   Note: The C_T values for wells D7, D8, and E1 are >35 due to a low amount of target. The wells do not need to be removed from the analysis.

Notes



Analysis Guidelines

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

- **Slope/amplification efficiency values** – The amplification efficiency is 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:
  - Range of standard quantities – For more accurate and precise efficiency measurements, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
  - 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)** – The R^2 value is 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 threshold cycle (C_T) is the PCR cycle number at which the fluorescence level meets 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 126).

  Or
- Rerun the experiment.

Notes

For More Information

For more information on:

- The Standard Curve screen, access the StepOne Software Help by clicking or pressing **F1**.
- Amplification efficiency, refer to the *Amplification Efficiency of TaqMan® Gene Expression Assays Application Note*.

View the Amplification Plot

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

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal 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 signal from the reporter dye normalized to the fluorescence signal 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 viewed as the following graph types: linear or log10.

About the Example Experiment

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

- Correct baseline and threshold values
- Outliers

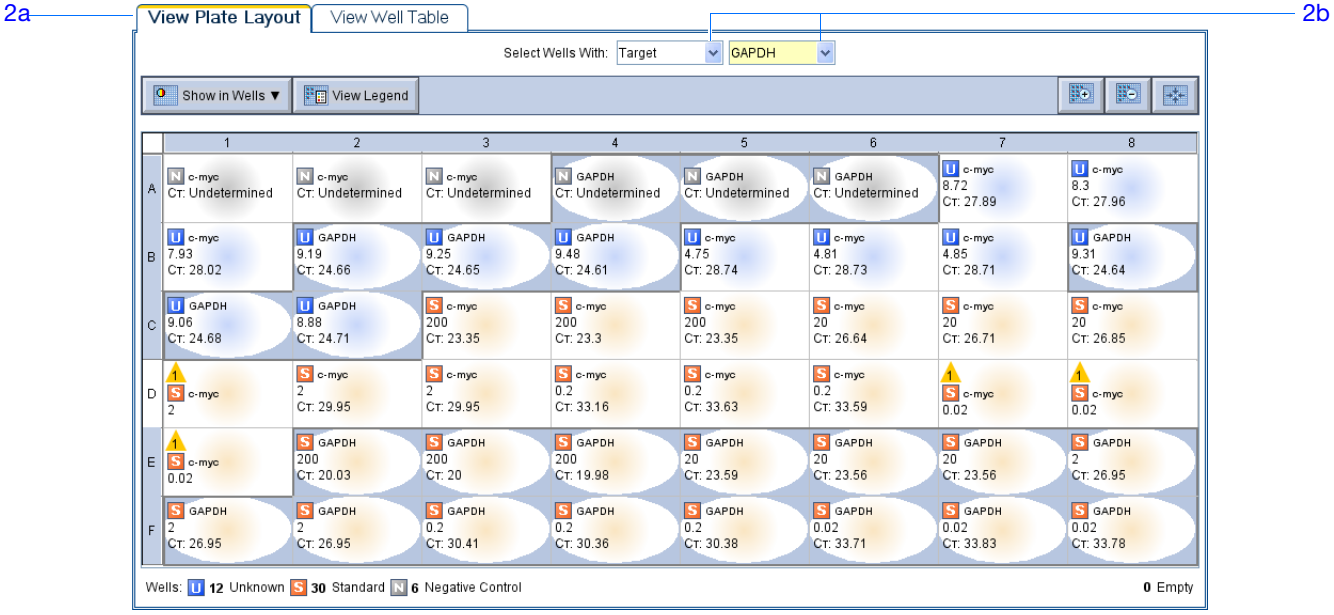
View the Amplification Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

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

2. Display the GAPDH wells in the Amplification Plot screen:
   a. Click the **View Plate Layout** tab.
   b. From the Select Wells With dropdown menus, select **Target**, then **GAPDH**.

Notes

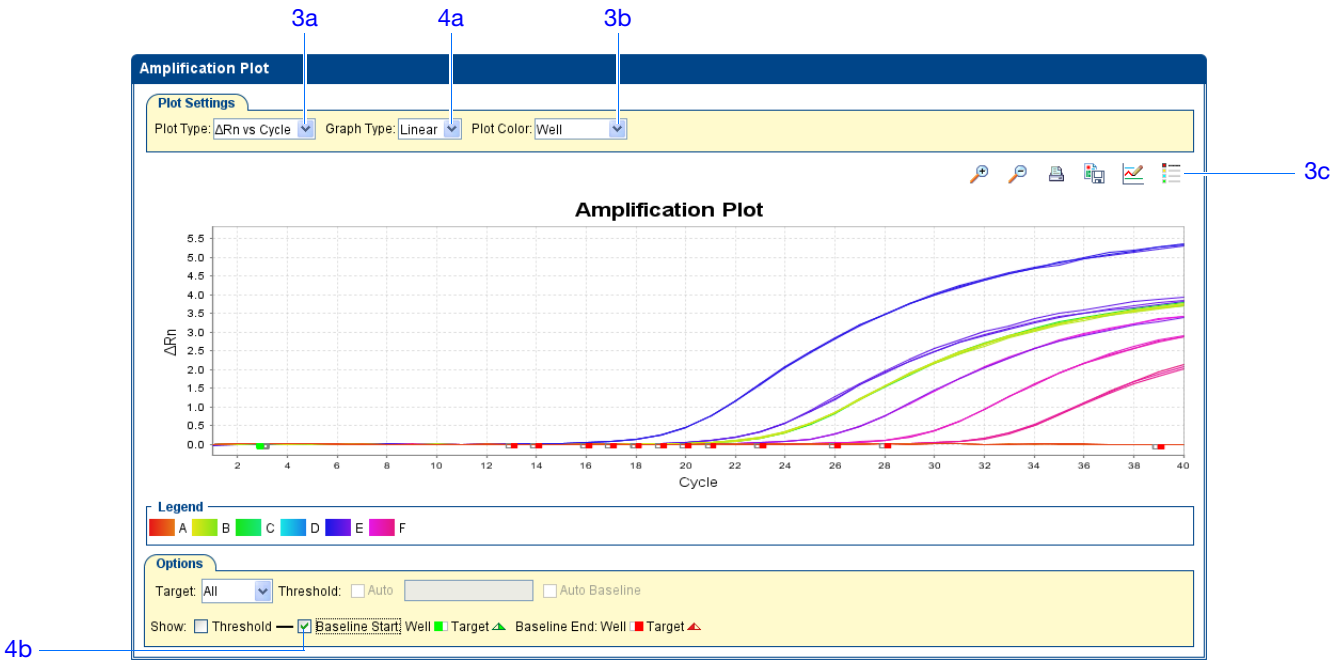


3. In the Amplification Plot screen:
   a. From the Plot Type dropdown menu, select **ΔRn vs Cycle** (default).
   b. From the Plot Color dropdown menu, select **Well** (default).
   c. Click **Show a legend for the plot** (default).

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

4. View the baseline values:
   a. From the Graph Type dropdown menu, select **Linear**.
   b. Select the **Baseline** checkbox 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 fluorescent signal is detected. In the example experiment, the baseline is set correctly.

Notes



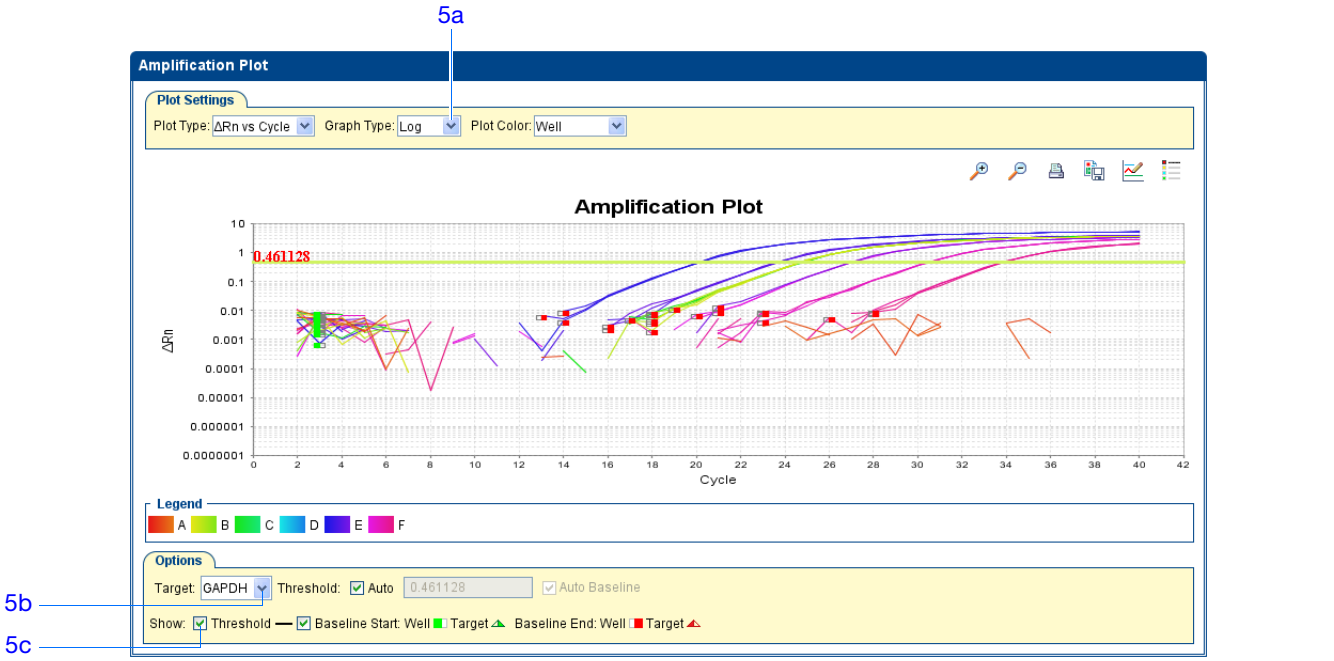
5. View the threshold values:

   a. From the Graph Type dropdown menu, select **Log**.

   b. From the Target dropdown menu, select **GAPDH**.

   c. Select the **Threshold** checkbox to show the threshold.

   d. Verify that the threshold is set correctly. In the example experiment, the threshold is in the exponential phase.

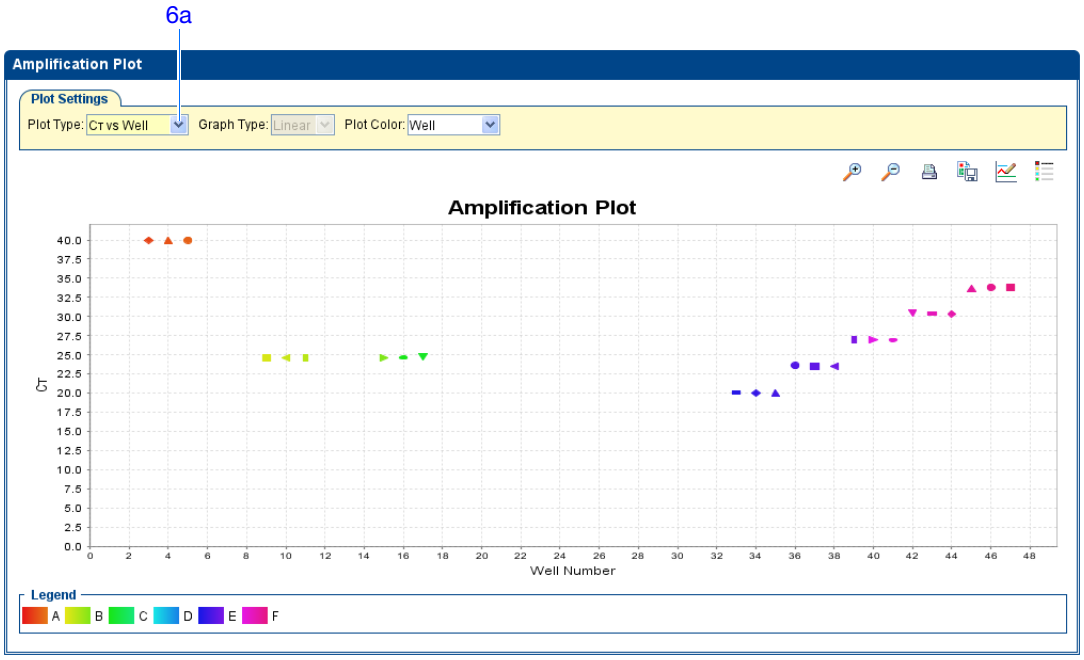


Notes

6. Locate any outliers:

   a. From the Plot Type dropdown menu, select **C_T vs Well**.

   b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for GAPDH.



7. Repeat steps 2 through 6 for the c-myc wells. In the example experiment, there is one outlier for c-myc (well D1). You will omit this well in the troubleshooting section ("Omit Wells from the Analysis" on page 126).

Analysis Guidelines

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

- Outliers
- A typical amplification plot – The StepOne 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:

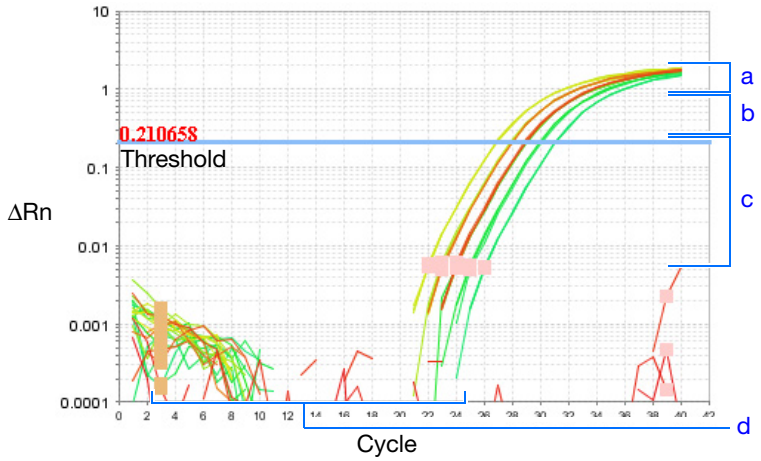
  a. Plateau phase

  b. Linear phase

  c. Exponential (geometric phase)

  d. Baseline

Notes



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 StepOne 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 completes.

- Correct baseline and threshold values – See the threshold examples on page 114 and the baseline examples on page 115.

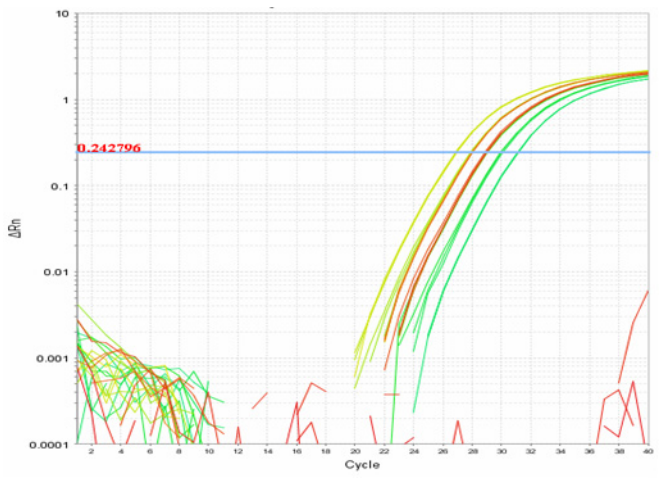
Notes

Threshold Set Correctly

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

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

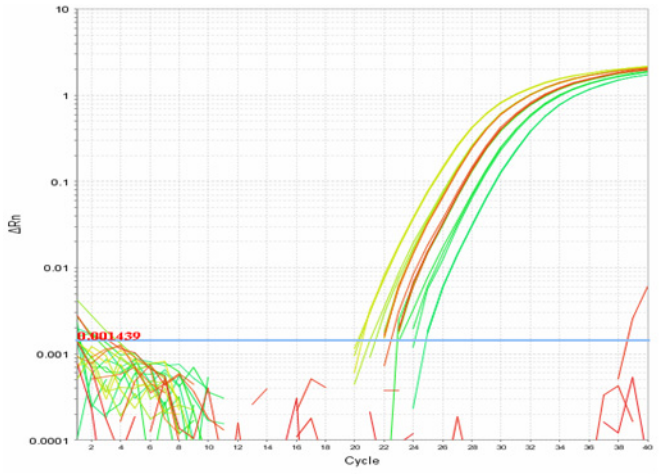
Task	Dyes	Cт	Cт Mean	Cт SD	Quantity	Quantity ...	Quantity ...	Comm
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
UNKNOWN	FAM-NFQ-...	28.96287	28.923796	0.074	2,484.31	2,551.476	126.2	
UNKNOWN	FAM-NFQ-...	28.838797	28.923796	0.074	2,697.054	2,551.476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

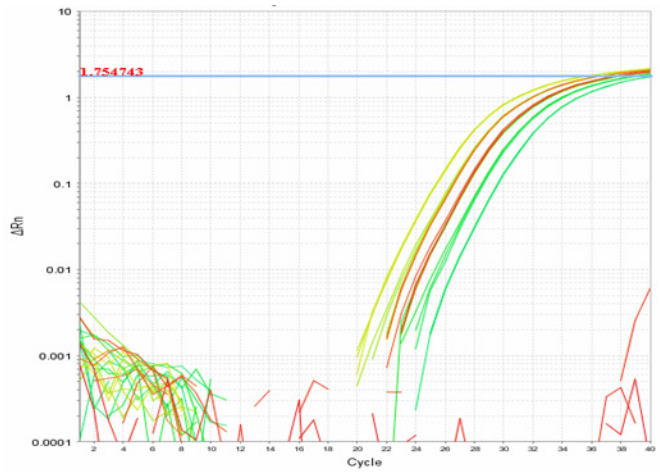
Task	Dyes	Cт	Cт Mean	Cт SD	Quantity	Quantity ...	Quantity ...
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	38.453182					
UNKNOWN	FAM-NFQ-...	22.85404	22.761744	0.252	2,314.852	2,472.463	400.435
UNKNOWN	FAM-NFQ-...	22.476973	22.761744	0.252	2,927.722	2,472.463	400.435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



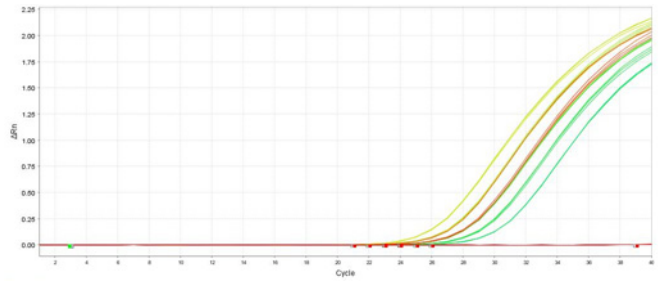
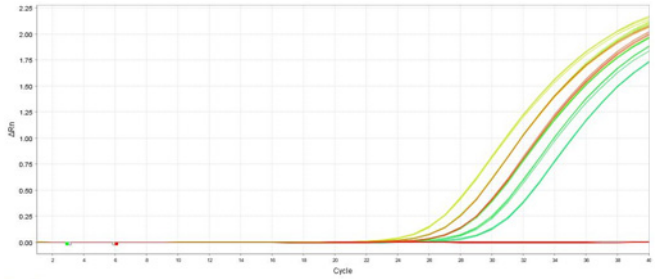
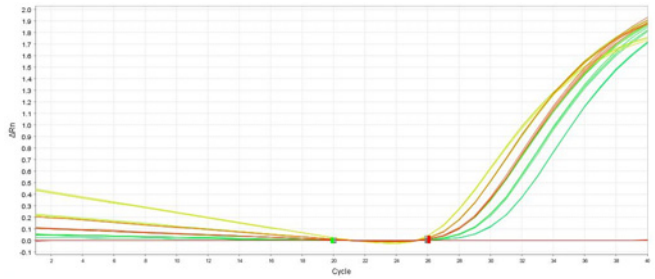
Threshold Set Too High

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	Cт	Cт Mean	Cт 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 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 126).

  Or

- Manually adjust the baseline and/or threshold (see "View the Analysis Settings" on page 122).

For More Information

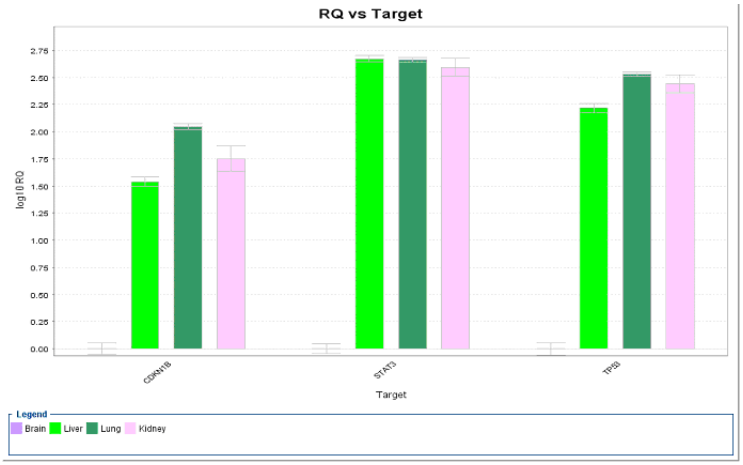
For more information on the Amplification Plot screen, access the StepOne Software Help by clicking or pressing **F1**.

Notes

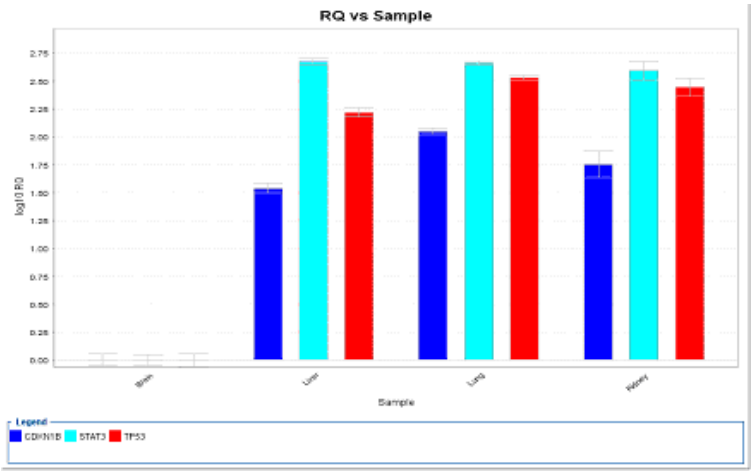
View the Gene Expression Plot and Well Table

The Gene Expression Plot screen displays the results of relative quantitation calculations in the gene expression profile. There are two plots 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 the following graph types: linear, log10, Ln, log2.



- **RQ vs Sample** – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.



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 Well Table

1. From the Experiment Menu pane, select **Analysis** ▸ **Gene Expression**.

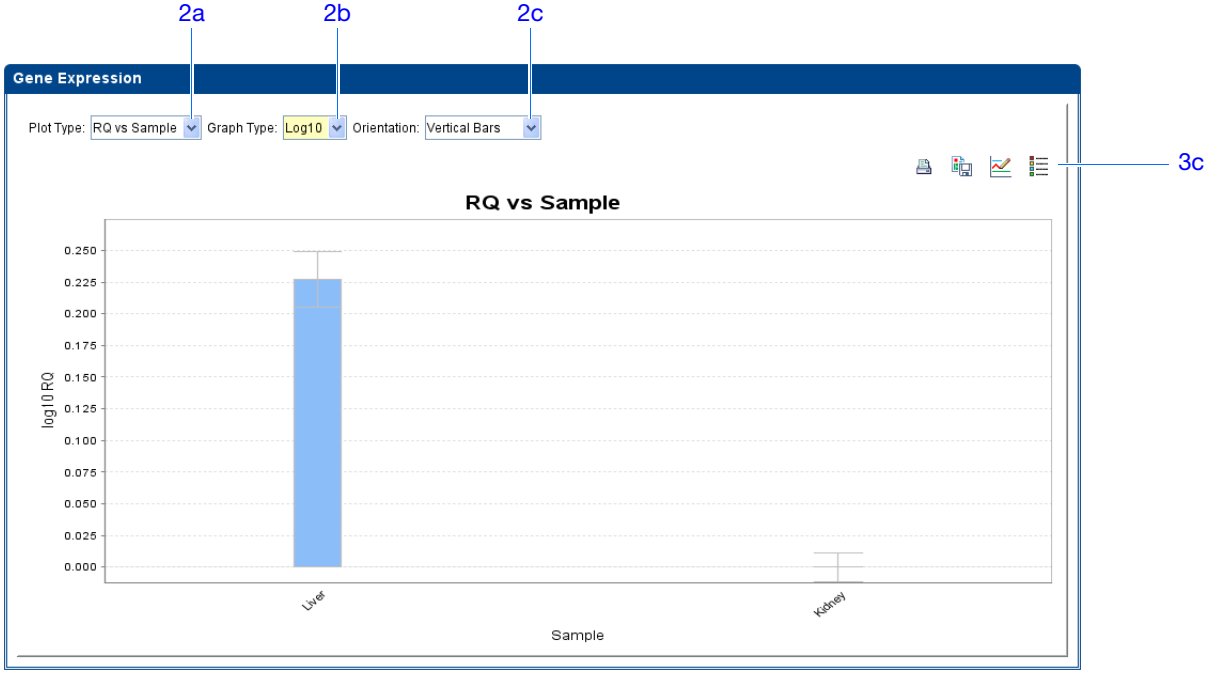
   Note: If no data are displayed, click **Analyze**.

2. In the Gene Expression Plot screen:
   a. From the Plot Type dropdown menu, select **RQ vs Sample**.
   b. From the Graph Type dropdown menu, select **Log10**.
   c. From the Orientation dropdown menu, select **Vertical Bars**.

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

   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 liver is displayed relative to its expression level in the reference sample (kidney). Since 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).



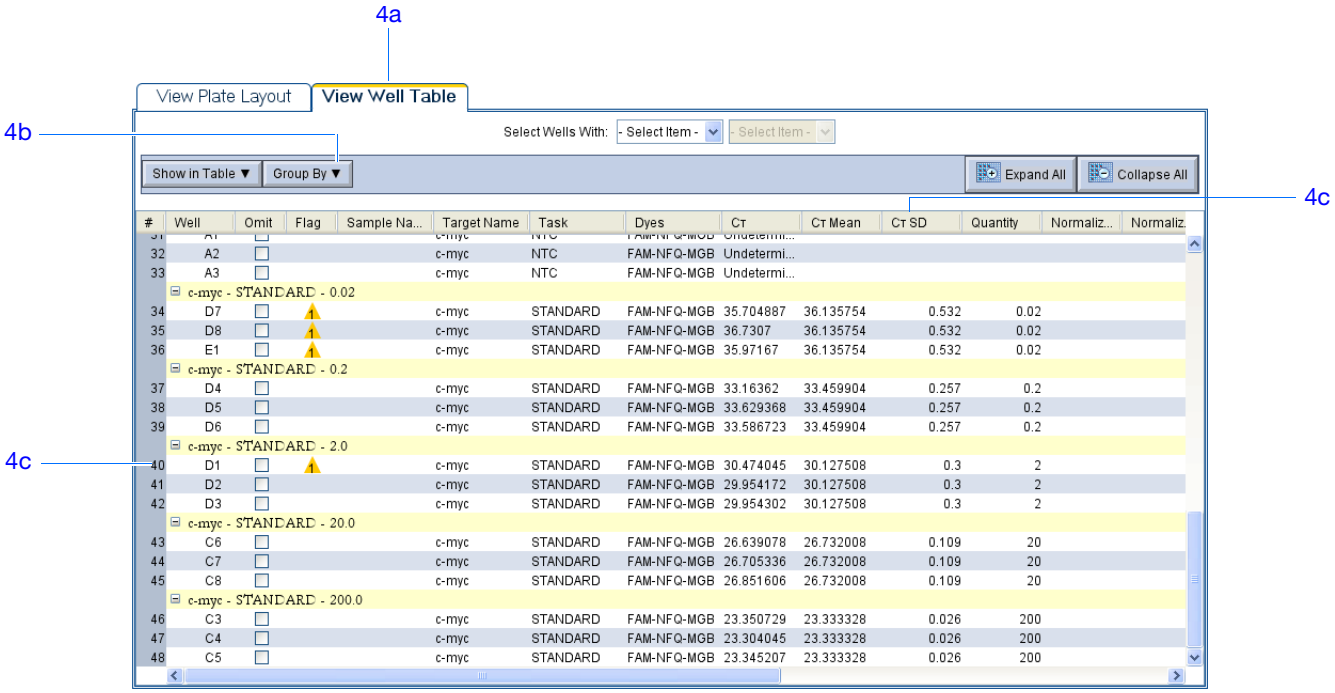
4. View the Well Table:

Notes

a. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**, then select the **View Well Table** tab.

b. From the Group By dropdown menu, 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 D1). You will omit this well in the troubleshooting section ("Omit Wells from the Analysis" on page 126).



Note: To show/hide columns in the Well Table, select/deselect the column name from the Show in Table dropdown menu.

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 126).

For More Information

For more information on the Gene Expression Plot screen or Well Table, access the StepOne 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 plate layout
- Create slides
- Print a report
- Export data

For information on performing these procedures, access the StepOne Software Help by clicking or pressing **F1**.

Notes

Notes

Section 5.2 Troubleshoot (If Needed)

This section covers:

Notes

View the Analysis Settings

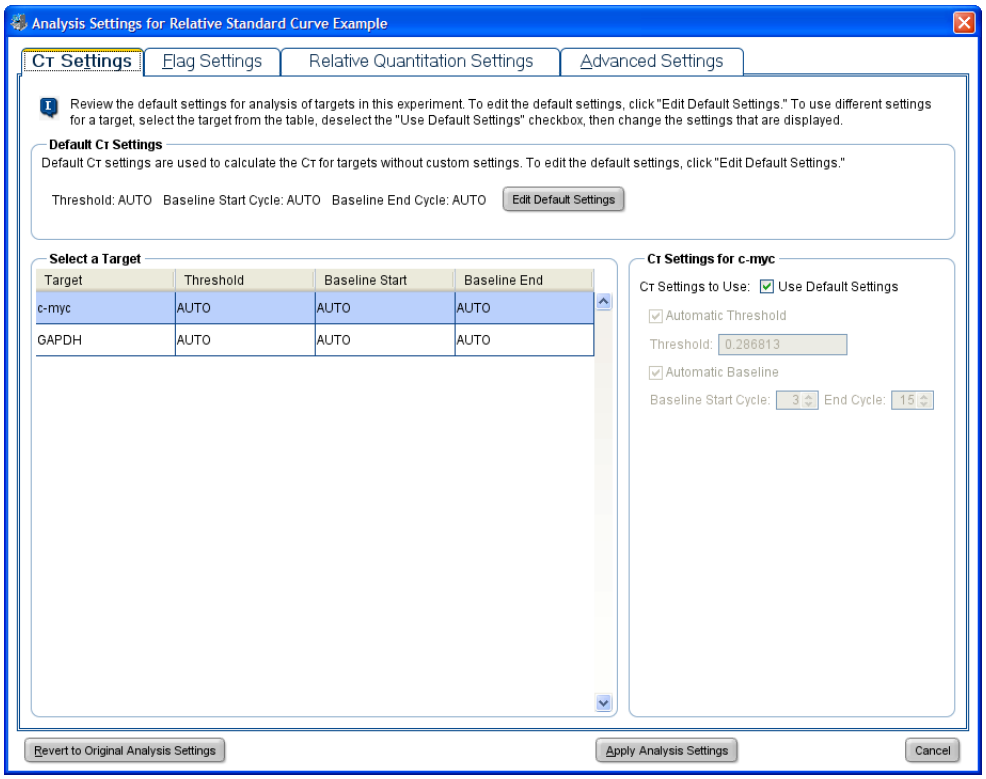
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, relative quantitation, and advanced options. If the default analysis settings in the StepOne software are not suitable for your experiment, you can change the settings in the Analysis Settings dialog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

About the Example Experiment

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

View the Analysis Settings

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis Settings** to open the Analysis Settings dialog box.
3. In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Relative Quantitation Settings
   - Advanced Settings



Notes

Analysis Guidelines

Unless you have already determined the optimal settings for your experiment, use the default analysis settings in the StepOne 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 StepOne software.
- **Relative Quantitation Settings** – Use this tab to:
  - Change the reference sample and/or endogenous control.
  - 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.

For More Information

For more information on the analysis settings, access the StepOne 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 StepOne software flags, and includes the flag frequency and location for the open experiment.

About the Example Experiment

In the relative standard curve example experiment, you review the QC Summary screen for any flags triggered by the experiment data:

- Wells D7, D8, and E1 produced data that triggered the HIGHSD flag.
- Well D1 produced data that triggered the OUTLIERRG flag.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis** ▸  **QC Summary**.

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

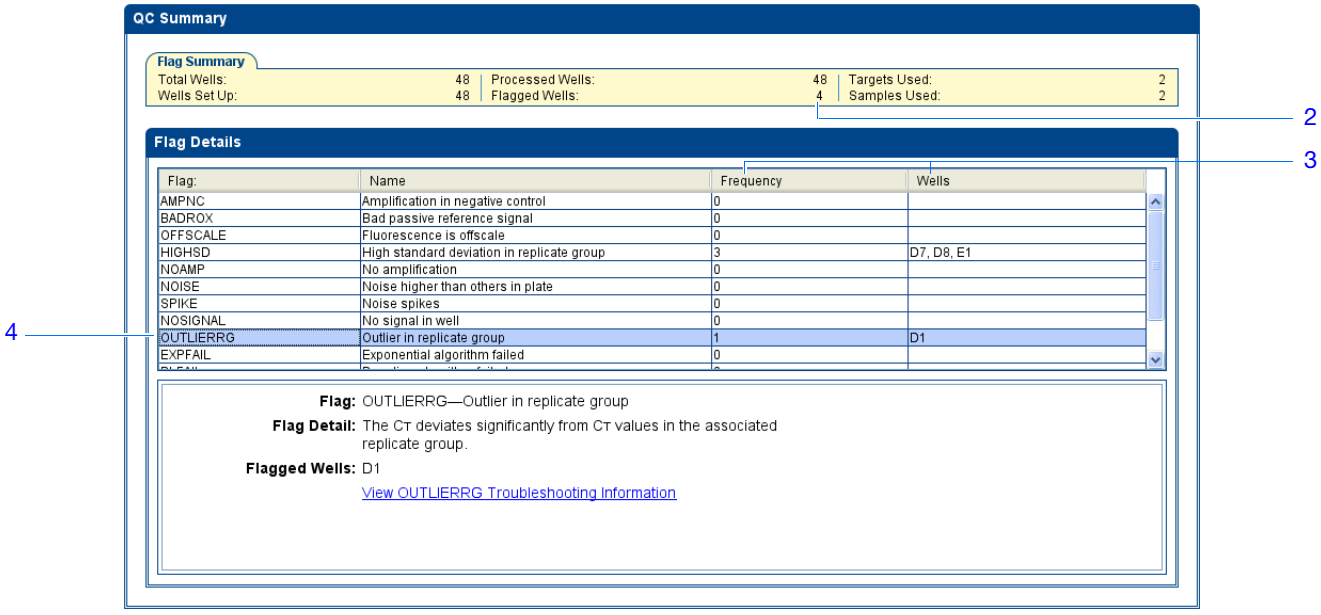
2. Review the Flags Summary. In the example experiment, there are 4 flagged wells.

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 HIGHSD flag appears 3 times, in wells D7, D8, and E1.
   - The OUTLIERRG flag appears 1 time, in well D1.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the experiment.

4. For each flag that appears in the experiment, click the flag row to display detailed information about the flag. In the example experiment:
   a. The HIGHSD flag (wells D7, D8, and E1) indicates high standard deviation in the replicate group. This is expected for C_T values >35 due to a low amount of target. The wells do not need to be removed from the analysis.
   b. The OUTLIERRG flag (well D1) indicates an outlier in replicate group. Proceed to "Omit Wells from the Analysis" on page 126 to remove well D1.

Notes



Possible Flags

For relative standard curve experiments, the flags listed below may be triggered 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 only displayed 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

Notes

Analysis Guidelines

When you analyze your own relative standard curve experiment:

- Click each flag in the Flag Details table with a frequency >0 to display detailed information 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 StepOne software.

For More Information

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

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 relative standard curve example experiment, you use the Well Table to remove well D1 from the analysis. Well D1 is flagged with the OUTLIERRG flag (see "View the QC Summary" on page 124).

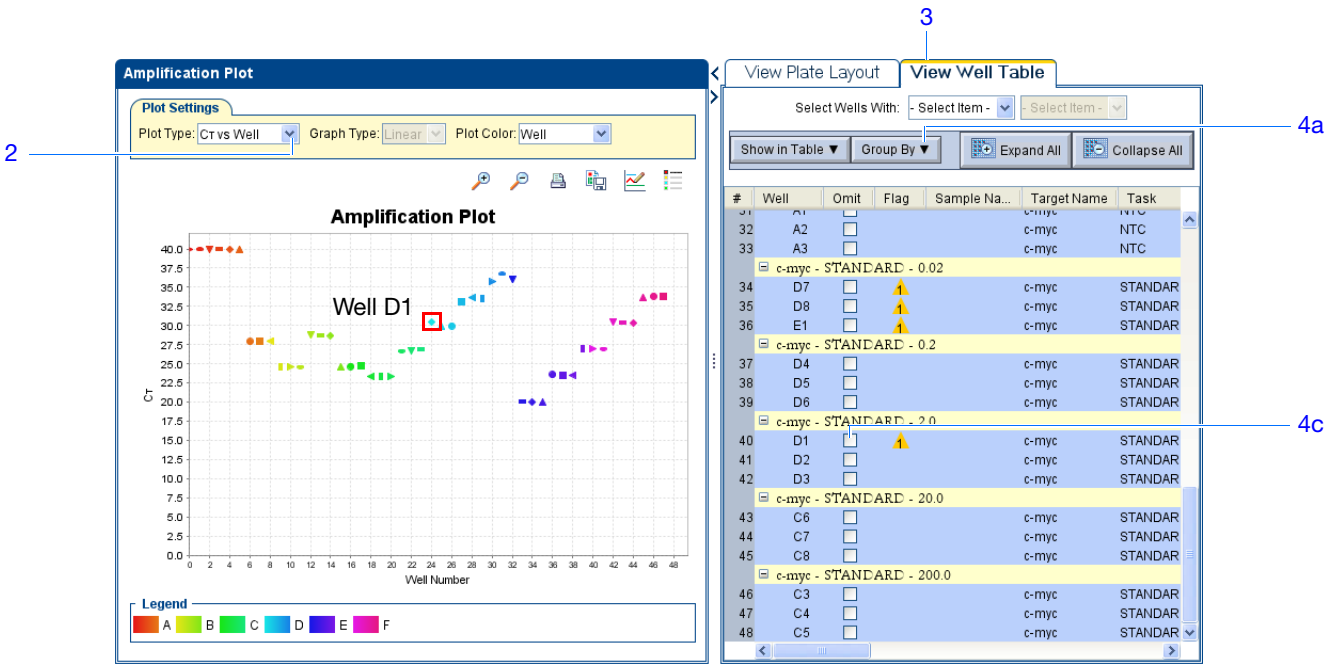
Omit Wells

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

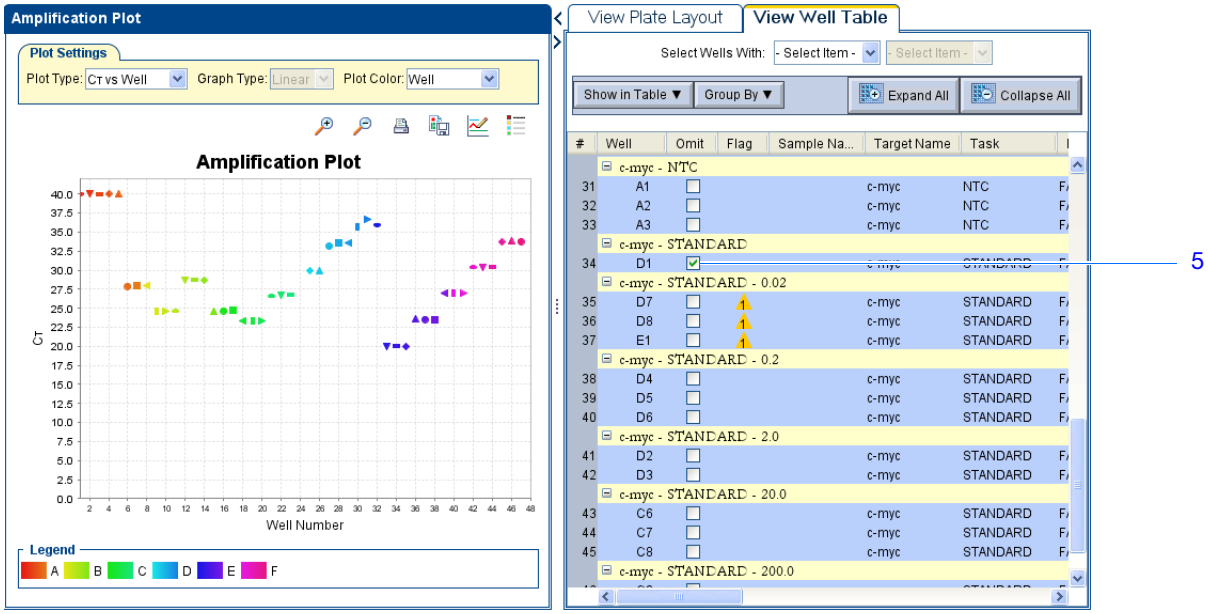
   Note: If no data are displayed, click **Analyze**.

2. In the Amplification Plot screen, select **C_T vs Well** from the Plot Type dropdown menu.
3. Select the **View Well Table** tab.
4. In the Well Table:
   a. From the Group By dropdown menu, select **Replicate**.
   b. Look for any outliers in the replicate group (be sure they are flagged). In the example experiment, well D1 is an outlier.
   c. Select the **Omit** checkbox next to well D1.

Notes



5. Click **Analyze** to reanalyze the experiment data with well D1 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. Follow the "Omit Wells" procedure above to remove the outliers in your experiment.

Notes

For More Information

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

1. Click 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 run.

About the Example Experiment

In the relative standard curve 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 Multicomponent Plot

1. From the Experiment Menu 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. Click 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.

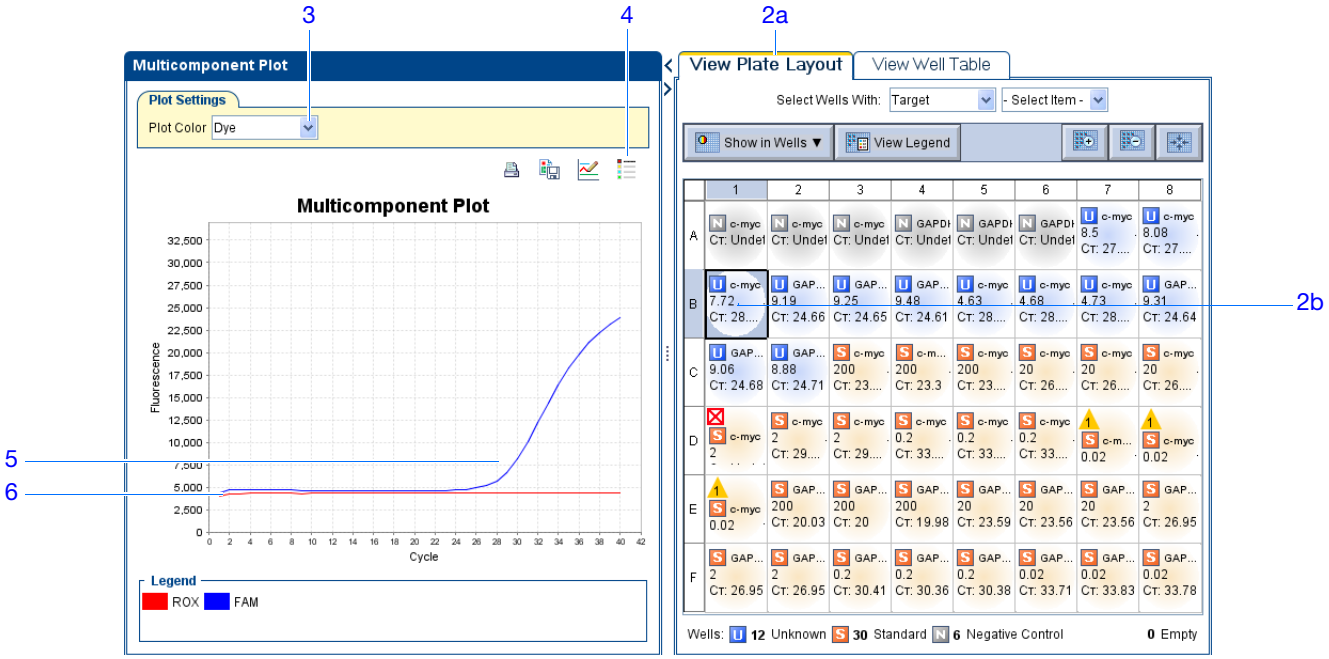
3. From the Plot Color dropdown menu, select **Dye**.
4. Click **Show a legend for the plot** (default).

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

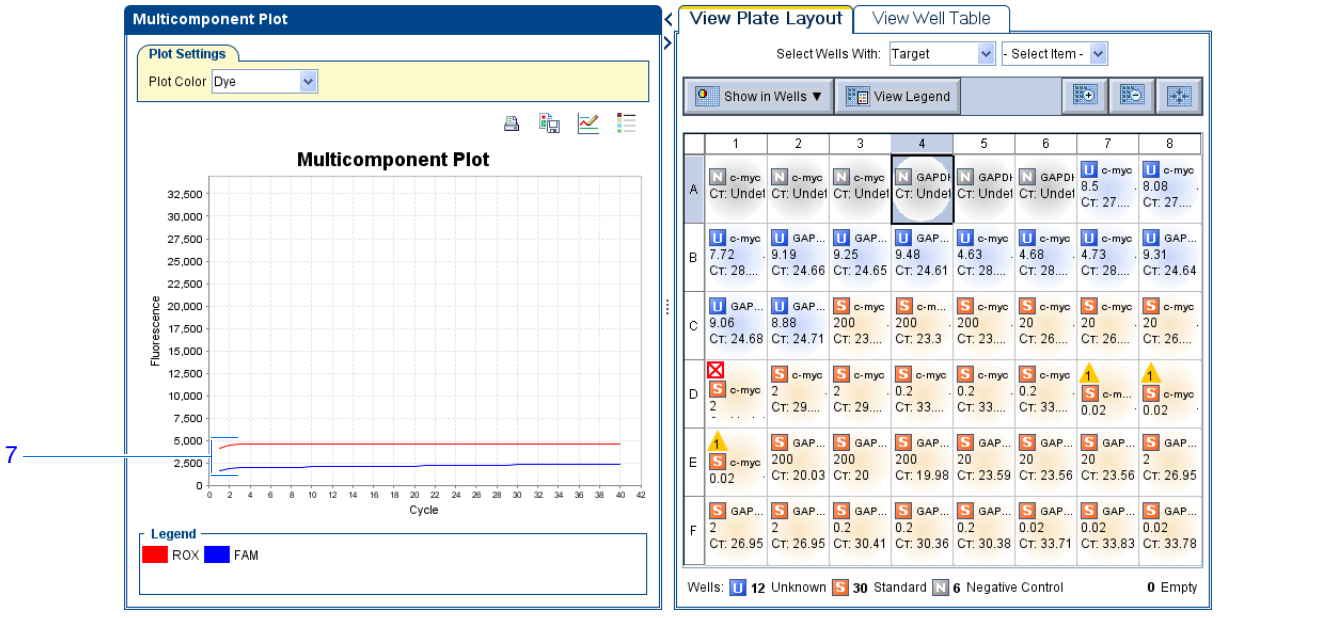
Notes

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

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



7. Select the negative control wells one at time and check for amplification. In the example experiment, there is no amplification 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 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 fluorescent signal.
- 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, access the StepOne Software Help by clicking or pressing **F1**.

View the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (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 Raw Data Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

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

2. Display all 48 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the View Plate Layout tab.

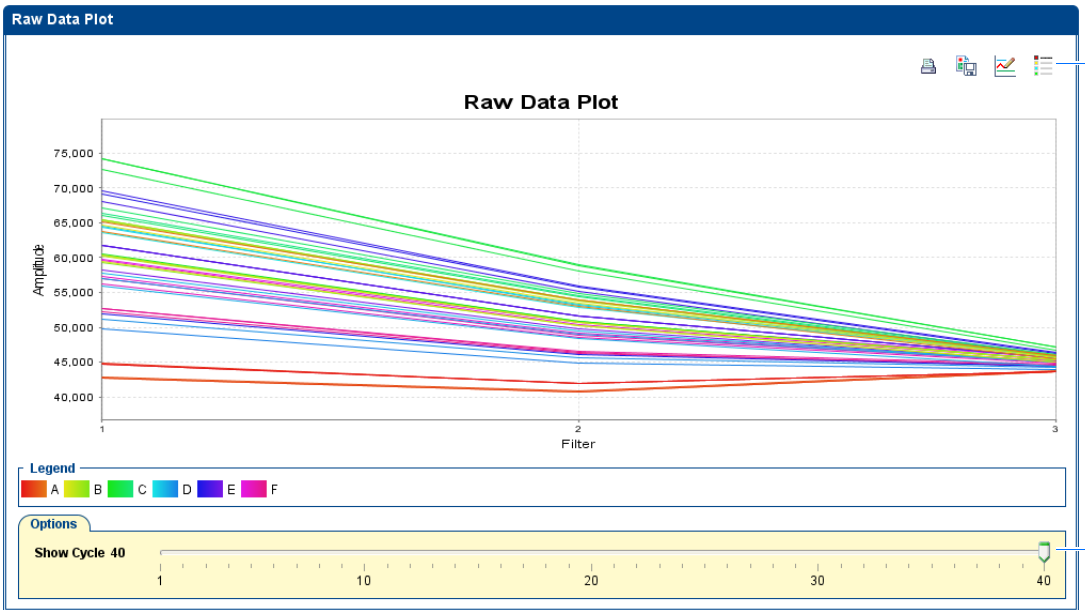
3. Click **Show a legend for the plot** (default).

   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.

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.

Notes



The filters are:

StepOne system			StepOnePlus system	
Filter	**Dye**		**Filter**	**Dye**
1	FAM™ dye		1	FAM™ dye
	SYBR® Green dye			SYBR® Green dye
2	JOE™ dye		2	JOE™ dye
	VIC® dye			VIC® dye
3	ROX™ dye		3	TAMRA™ dye
				NED™ dye
			4	ROX™ 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, access the StepOne Software Help by clicking or pressing **F1**.

Notes

Notes

6 Design the Comparative C_T Experiment

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

Notes

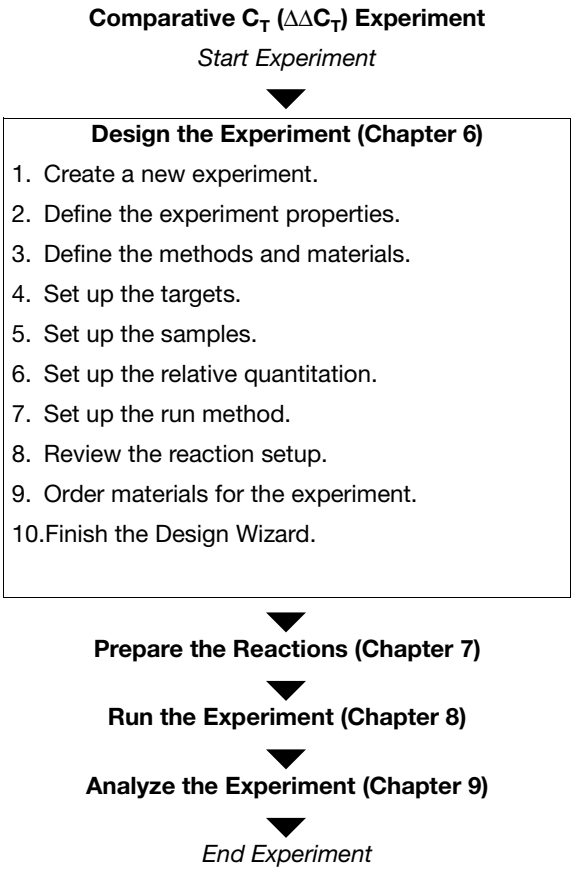
Chapter Overview

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

Example Experiment Workflow

The workflow for designing the example experiment provided with this getting started guide is shown below.

Note: Design the example experiment using the Design Wizard in the StepOne software. When you design your own experiments, you can select alternate workflows (see "Using This Guide With Your Own Experiments" on page 11).



Notes

Create a New Experiment

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

Log In to the Software and Create an Experiment

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

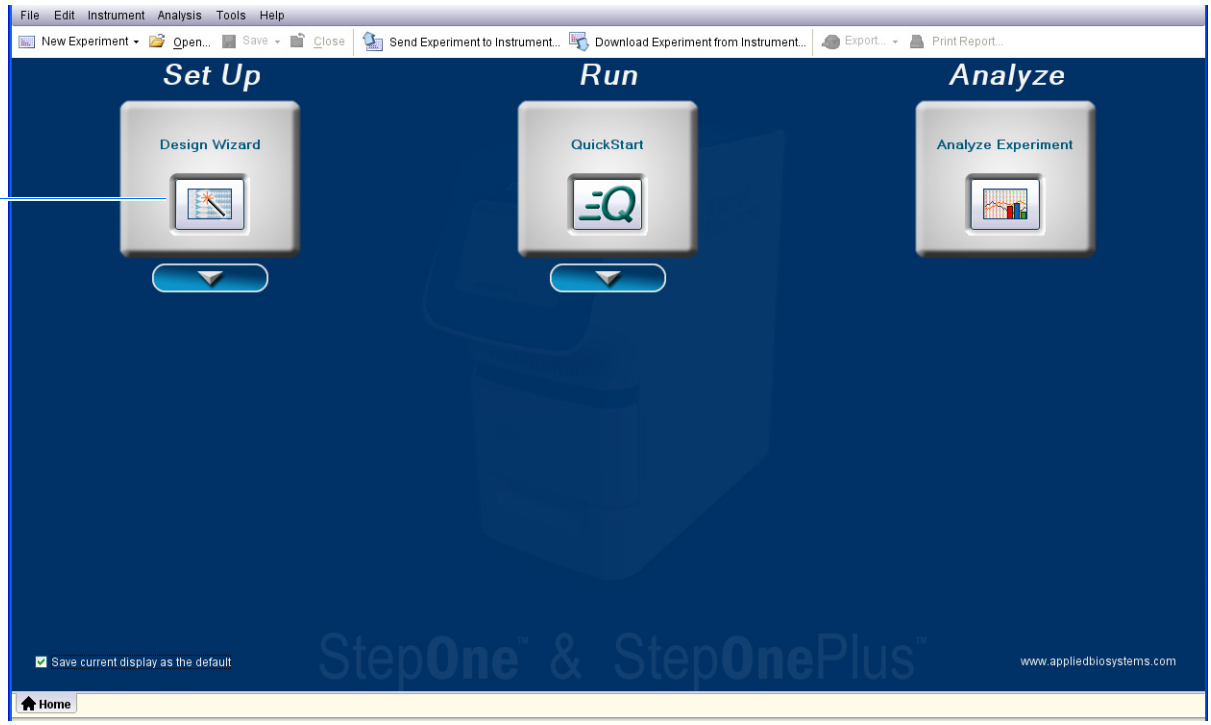
   where *<software name>* is the current version of the StepOne software.

2. In the Login dialog box, create a user name:

   a. In the User Name field, enter **EXAMPLEUSER**. You cannot enter spaces in the User Name field.

   b. Click **OK**.

3. From the Home screen, click (Design Wizard) to open the Design Wizard.



Notes

4. See "Software Elements" on page 137 for information on navigating within the Design Wizard.

Design Guidelines

When you design your own experiment, you can do one of the following in the Login dialog box:

- Log in as a new user – In the User Name field, enter a user name, then click **OK**.

  Note: You cannot use the following characters in the User Name field: space, forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), or semicolon (;).

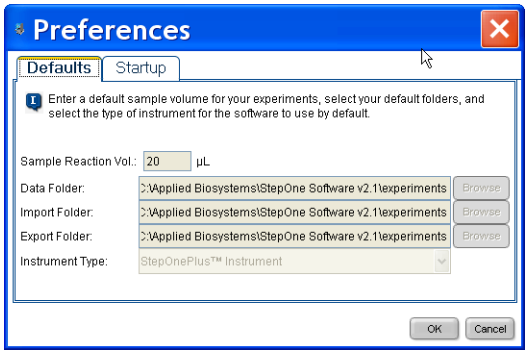
- Log in as an existing user – From the User Name dropdown menu, select an existing user, then click **OK**.
- Log in anonymously – Click **Log in as Guest**.

Applied Biosystems recommends that you log in with a user name. If you log in with a user name, you can set preferences in the software. The next time you log in to the software with the same user name, the software uses the preferences you set as the defaults.

IMPORTANT! If you log in to the software as a Guest, you cannot set preferences.

You can set preferences as follows:

- **Save as the default checkboxes** – Select the **Save as the default** checkboxes as needed. The Save as the default checkboxes appear on the Home screen, on the Export Data dialog box, and on several Analysis screens.
- **Preferences dialog box** – Select **Tools** ▸ **Preferences** to open the Preferences dialog box. In the Defaults and/or Startup tabs, change the preferences as desired.



For More Information

For more information, access the StepOne Software Help by clicking ? or pressing **F1**.

Notes

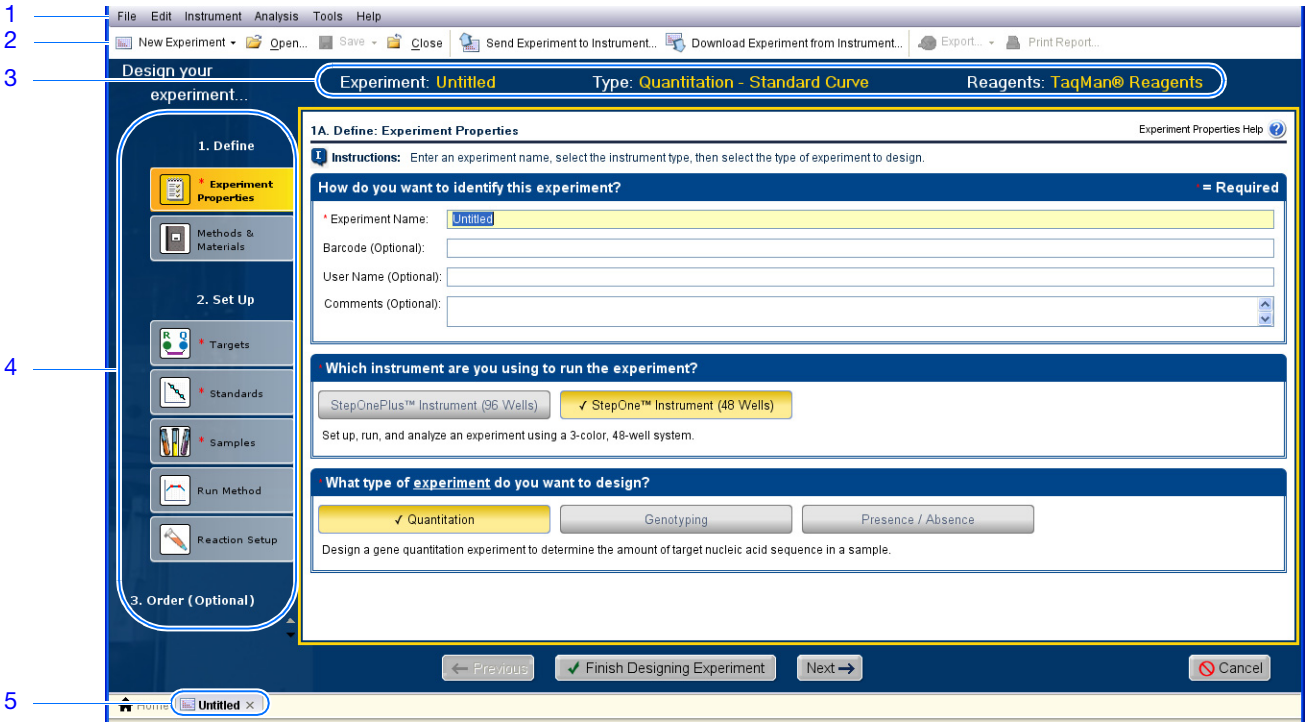
Software Elements

The StepOne software elements for the Design Wizard are illustrated below.

1. Menu bar – Displays the menus available in the software:
   - File
   - Edit
   - Instrument
   - Analysis
   - Tools
   - Help
2. Toolbar – Displays the tools available in the software:
   - New Experiment
   - Open
   - Close
   - Send Experiment to Instrument
   - Download Experiment from Instrument
3. Experiment header – Displays the experiment name, experiment type, and reagents for the open experiment.
4. Navigation pane – Provides links to all screens in the Design Wizard:
   - Experiment Properties
   - Methods & Materials
   - Targets
   - Relative Quantitation Settings
   - Samples
   - Run Method
   - Reaction Setup
   - Materials List

   Note: The Design Wizard initially displays the Quantitation - Standard Curve experiment type. The available Design Wizard screens may change when you select a different experiment type. For example, the Relative Quantitation Settings screen is not displayed until you select the relative standard curve or comparative C_T ($\Delta\Delta C_T$) experiment type.
5. Experiment tab(s) – Displays a tab for each open experiment.

Notes


1
2
3
4
5
File Edit Instrument Analysis Tools Help
New Experiment Open... Save Close Send Experiment to Instrument... Download Experiment from Instrument... Export... Print Report...
Design your experiment...
Experiment: Untitled
Type: Quantitation - Standard Curve
Reagents: TaqMan® Reagents
1. Define
* Experiment Properties
Methods & Materials
2. Set Up
* Targets
* Standards
* Samples
Run Method
Reaction Setup
3. Order (Optional)
1A. Define: Experiment Properties
Experiment Properties Help
Instructions: Enter an experiment name, select the instrument type, then select the type of experiment to design.
How do you want to identify this experiment?
* = Required
* Experiment Name: Untitled
Barcode (Optional):
User Name (Optional):
Comments (Optional):
* Which instrument are you using to run the experiment?
StepOnePlus™ Instrument (96 Wells)
✓ StepOne™ Instrument (48 Wells)
Set up, run, and analyze an experiment using a 3-color, 48-well system.
* What type of experiment do you want to design?
✓ Quantitation
Genotyping
Presence / Absence
Design a gene quantitation experiment to determine the amount of target nucleic acid sequence in a sample.
Previous
✓ Finish Designing Experiment
Next
Cancel
Untitled

Notes

Define the Experiment Properties

On 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 instrument selected to run the experiment is the StepOne instrument.
- A MicroAmp® Fast Optical 48-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 updates with the experiment name you entered.

2. Leave the Barcode field empty.

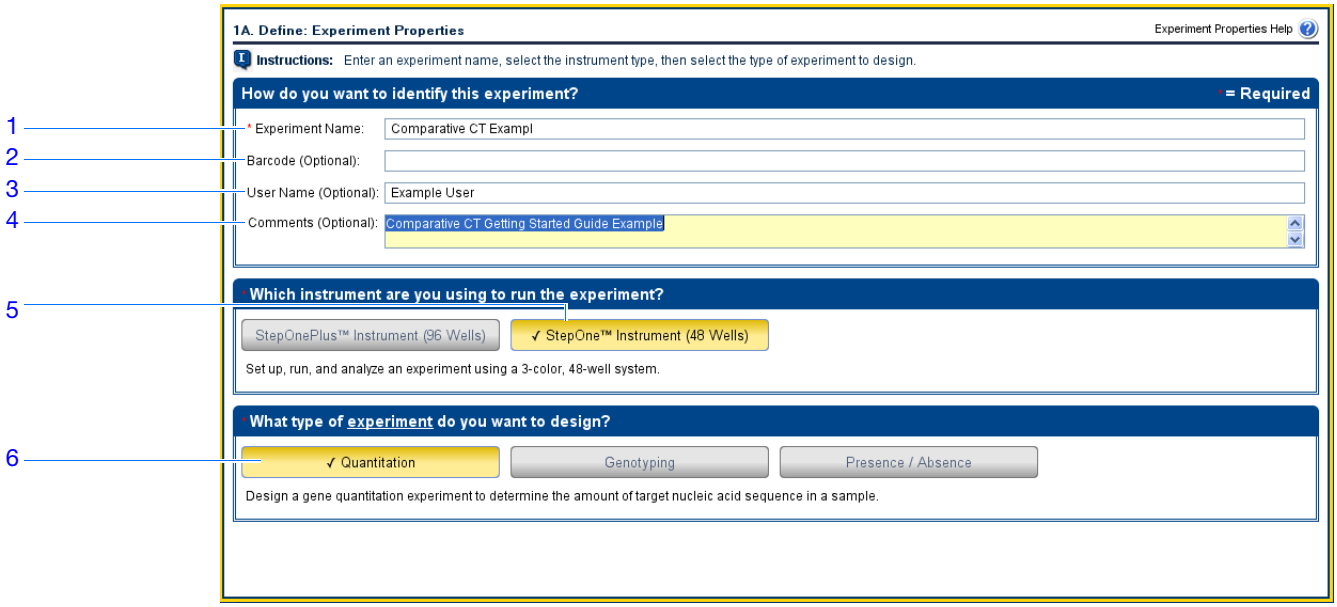
   Note: The MicroAmp Fast Optical 48-Well Reaction Plate does not have a barcode.

3. Click the **User Name** field, then enter **Example User**.
4. Click the **Comments** field, then enter **Comparative CT Getting Started Guide Example**.
5. Select **StepOne™ Instrument (48 Wells)**.

   Note: The example experiment was created for a StepOne instrument. You can create the example experiment for a StepOnePlus instrument; however, your reaction plate layout will differ from the layout shown in this guide. The software displays a 48-well reaction plate layout for the StepOne instrument and a 96-well reaction plate layout for the StepOnePlus instrument. To create the example experiment for a StepOnePlus instrument, select **StepOnePlus™ Instrument (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:
  - The experiment name is used as the default file name.
  - Enter a name that is descriptive and easy to remember. You can enter up to 100 characters in the Experiment Name field.

  Note: You cannot use the following characters in the Experiment Name field: forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), semicolon (;), and sign (&), percent sign (%), dollar sign ($), at sign (@), circumflex (^), left parenthesis (( ), right parenthesis ( )), or exclamation point (!).

  IMPORTANT! If you run the instrument in standalone mode from the instrument touchscreen, you cannot enter more than 32 characters in the Experiment Name field and you cannot include spaces in the name.

- (Optional) If you use a MicroAmp® Fast Optical 96-Well Reaction Plate, enter a barcode to identify the barcode on the PCR reaction plate. You can enter up to 100 characters in the Barcode field.

  Note: The MicroAmp Fast Optical 48-Well Reaction Plate does not have a barcode.

- (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.

Notes

- Select the instrument you are using to run the experiment:
  - **StepOne™ Instrument (48 Wells)**
  - **StepOnePlus™ Instrument (96 Wells)**

Note: You can use StepOne Software v2.1 or later to design experiments for both the StepOne and StepOnePlus instruments. The instrument you select in the Experiment Properties screen affects the reaction plate layout and materials list.

Note: To set the default instrument type, select **Tools ▸ Preferences**, then select the **General** tab (default). From the Default Instrument Type dropdown menu, select the appropriate instrument. To change the default instrument type, you must be logged into the software with a user name, not as a Guest. For more information, see the "Design Guidelines" for logging in on page 136.

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

For More Information

For more information on:

- Completing the Experiment Properties screen, access the StepOne Software Help by clicking or pressing **F1**.
- Consumables, see "Supported Consumables" on page 4.
- Quantitation experiments, refer to the *Real-Time PCR Systems Reagent Guide*.

Define the Methods and Materials

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

About the Example Experiment

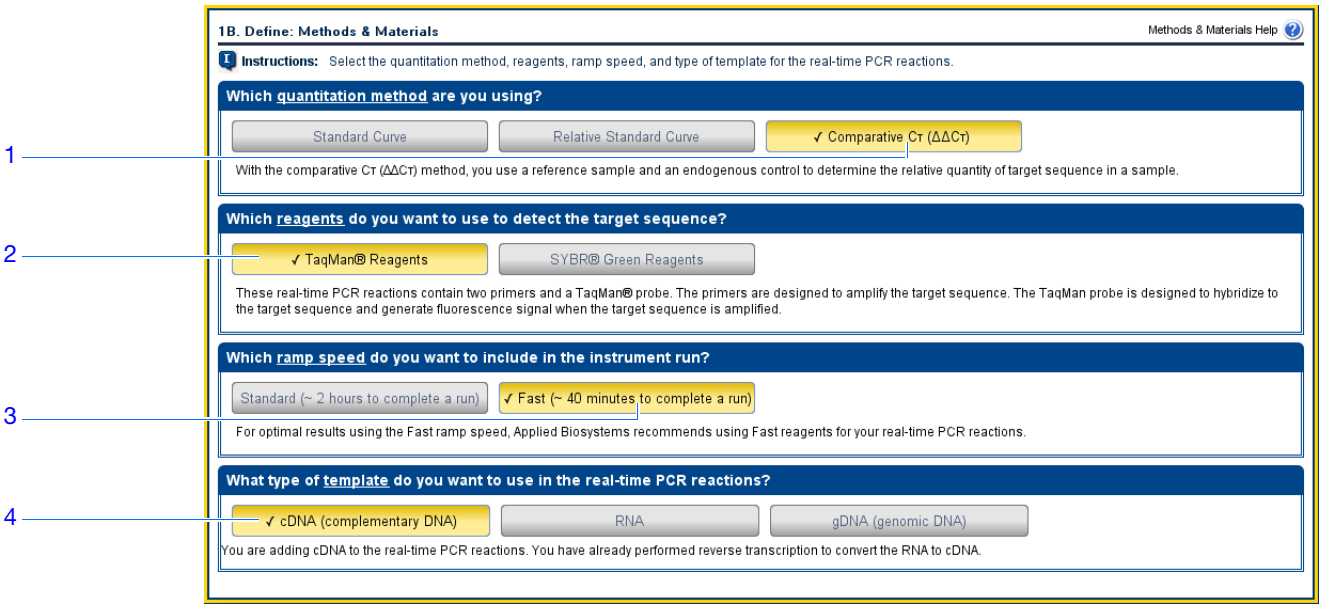
In the comparative C_T example experiment:

- The comparative C_T quantitation method is used.
- TaqMan® reagents are used.
- The Fast ramp speed is used in the instrument run.
- cDNA (prepared from total RNA isolated from liver, kidney, and brain tissues) is the template type. Before using cDNA template, you must first perform reverse transcription to convert the RNA to cDNA (see "Prepare the Template" on page 167).

Complete the Methods & Materials Screen

1. Select **Comparative C_T ($\Delta\Delta C_T$)** as the quantitation method.
2. Select **TaqMan® Reagents** for the reagents.
3. Select **Fast (~ 40 minutes 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 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 generate fluorescence when the target is amplified.

    IMPORTANT! Applied Biosystems does not recommend the use of TAMRA™ dye as a reporter or quencher with the StepOne™ system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus™ system.

  - 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 generates fluorescence

Notes

when it binds to double-stranded DNA. SYBR Green dye is often part of the SYBR Green master mix that is added to the reaction. If you use SYBR Green dye, select the **Include Melt Curve** checkbox to perform melt curve analysis of the amplified target.

Note: You can use other fluorescence-based reagents on the StepOne and StepOnePlus systems, but you must design your experiment using Advanced Setup instead of the Design Wizard.

- Select the appropriate ramp speed for the instrument run:
  - Select **Fast (~ 40 minutes to complete a run)** if you use fast reagents for the PCR reactions.
  - Select **Standard (~ 2 hours to complete a run)** if you use 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.

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 & Materials screen, access the StepOne Software Help by clicking or pressing **F1**.
- Determining PCR efficiencies, access the StepOne Software Help by clicking or pressing **F1**. Within the Help, search as follows:
  a. Click 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 260.
- Using the relative standard curve quantitation method, see Chapters 2 to 5 of this guide.
- Using the standard curve quantitation method, refer to the *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments*.
- TaqMan and SYBR Green reagents, refer to the *Real-Time PCR Systems Reagent Guide*.

Notes

- PCR, including singleplex vs. multiplex PCR and 1-step vs. 2-step RT PCR, refer to the *Real-Time PCR Systems Reagent Guide*.

Set Up the Targets

On 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 target you are studying. For the example experiment, this is TP53 (a transcription factor that regulates other genes).
- The Target 2 assay is set up for the endogenous control. For the example experiment this is human glyceraldehyde-3-phosphate (GAPDH). GAPDH serves as the endogenous control because its expression levels tend to be relatively stable.

Complete the Targets Screen

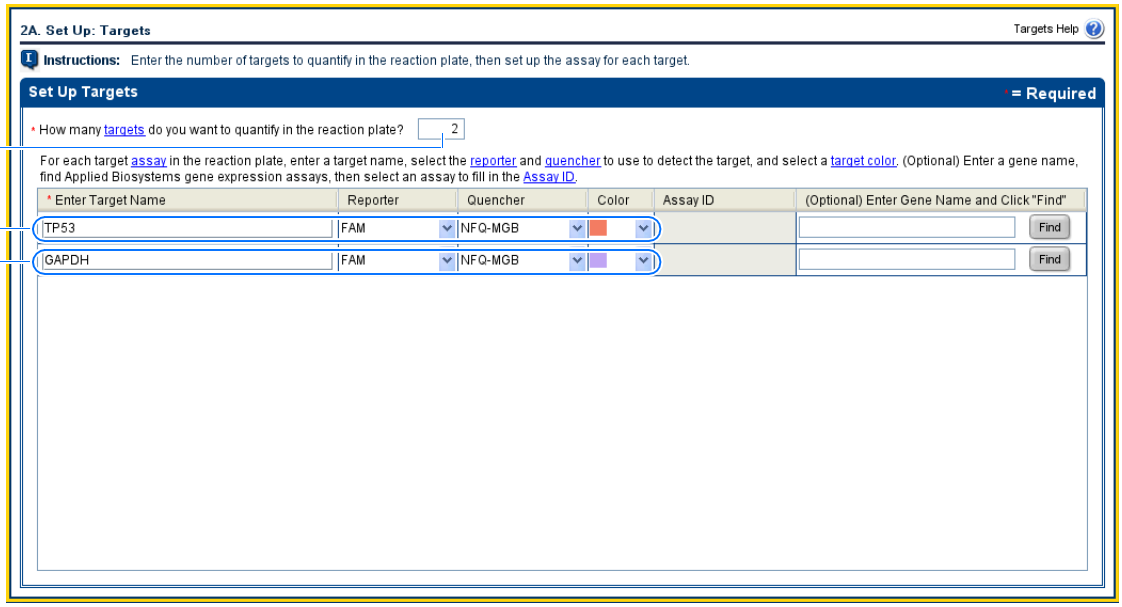
1. Click the **How many targets do you want to quantify in the reaction plate?** field, then enter **2**.

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

2. Set up the Target 1 assay:
   a. Click the **Enter Target Name** cell, then enter **TP53**.
   b. From the Reporter dropdown menu, select **FAM** (default).
   c. From the Quencher dropdown menu, select **NFQ-MGB** (default).
   d. Leave the default in the Color field.
3. Set up the Target 2 assay:
   a. Click the **Enter Target Name** cell, then enter **GAPDH**.
   b. From the Reporter dropdown menu, select **FAM** (default).
   c. From the Quencher dropdown menu, select **NFQ-MGB** (default).
   d. Leave the default in the Color field.
4. Click **Next >**.

Note: For all targets, leave the (Optional) Enter Gene Name field blank. You can search for the gene/assay ID when you order your materials (see "Order Materials for the Experiment" on page 157).

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 spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.
- Select the reporter dye used in the target assay. In the Methods & Materials screen on page 141, 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 & Materials screen on page 141, if you selected:
  - TaqMan® Reagents, select the quencher attached to the 3′ end of the TaqMan probe.

Notes

– SYBR® Green Reagents, select **None**.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA dye as a reporter or quencher with the StepOne system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus system.

For More Information

For more information on:

- Completing the Targets screen, access the StepOne 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*.

Set Up the Samples

On 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:

- Three samples are used: cDNA prepared from total RNA isolated from liver, kidney, and brain tissues. The samples contain unknown quantities of TP53 (target) and GAPDH (endogenous control).
- Three replicates are used. The replicates are identical reactions, containing identical reaction components and volumes.
- Six negative controls are used. The negative control reactions contain water instead of sample and should not amplify. The software automatically includes three negative controls for each target assay.

Complete the Samples Screen

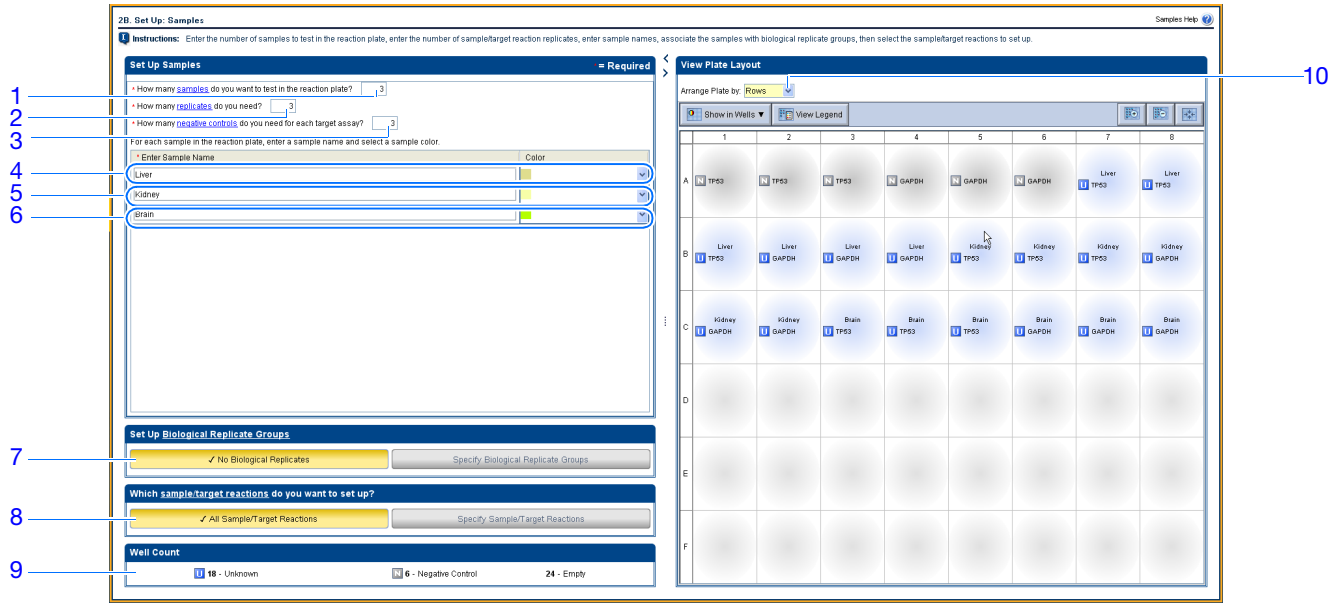
1. Click the **How many samples do you want to test in the reaction plate?** field, then enter **3**.

   Note: The number of rows in the samples table updates 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. Leave the default in the Color field.

Notes

5. Set up Sample 2:
   a. Click the **Enter Sample Name** field, then enter **Kidney**.
   b. Leave the default in the Color field.
6. Set up Sample 3:
   a. Click the **Enter Sample Name** field, then enter **Brain**.
   b. Leave the default in the Color field.
7. Select **No Biological Replicates**.
8. Select **All Sample/Target Reactions** to test all targets in all samples.
9. In the Well Count pane, confirm there are:
   - 18 Unknown wells U
   - 6 Negative control wells N
   - 24 Empty wells
10. In the View Plate Layout tab, from the Arrange Plate by dropdown menu, select **Rows** (default).
11. Click **Next >**.



Notes

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.
- The software automatically includes three negative controls for each target assay.
- Set up the biological replicates groups:
  - Select **No Biological Replicates** if you do not want to include biological replicate groups in the experiment.
  - Select **Specify Biological Replicate Groups** to include biological replicate groups in the experiment. For more information on biological replicate groups, see Chapter 10.
- 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.

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.

- If you are running the experiment on a StepOnePlus instrument and plan to edit the Run Method (page 150) to set a different temperature for one or more of the VeriFlex blocks, you need to:

  a. Design your experiment using Advanced Setup instead of the Design Wizard.

  b. In the Plate Setup screen, select the **Assign Targets and Samples** tab, select the **View Plate Layout** tab, then select the **Enable VeriFlex™ Block** checkbox.

IMPORTANT! If you do not select the **Enable VeriFlex™ Block** checkbox in the Plate Setup screen, you will not be able to set a different temperature for one or more of the VeriFlex blocks in the Run Method screen (page 150).

For More Information

For more information on:

- Completing the Samples screen, access the StepOne Software Help by clicking or pressing **F1**.
- Using Advanced Setup, see "Advanced Setup Workflow" on page 260.

Notes

Set Up the Relative Quantitation Settings

On 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.
- GAPDH is used as the endogenous control.

Complete the Relative Quantitation Settings Screen

1. From the Which sample do you want to use as the reference sample? dropdown menu, select **Brain**.
2. From the Which target do you want to use as the endogenous control? dropdown menu, select **GAPDH**.
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 146). 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 144). 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.

Notes

For More Information

For more information on:

- Completing the Relative Quantitation Settings screen, access the StepOne 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*.

Set Up the Run Method

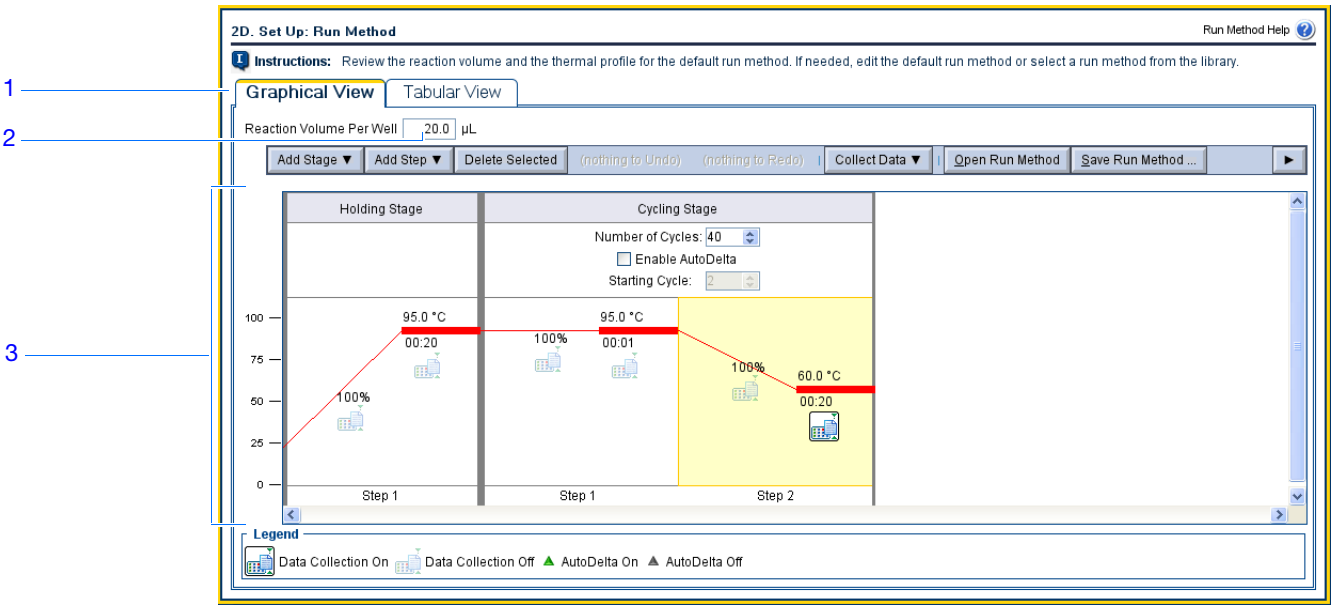
On the Run Method screen, review the reaction volume and the thermal profile for the default run method. If needed, you can edit the default run method or replace it with one from the Run Method library.

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. Click either the **Graphical View** tab (default) or **Tabular View** tab.
2. Make sure the Reaction Volume Per Well field displays **20 µL**.
3. Make sure the thermal profile displays the holding and cycling stages shown below.
4. Click **Next >**.



Design Guidelines

When you design your own comparative C_T experiment:

- Enter a number from 10 to 30 for the reaction volume/well. The StepOne and StepOnePlus systems support reaction volumes from 10 to 30 µL.

Notes

- 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 StepOne software.
- If you are running the experiment on a StepOnePlus instrument and you want to set a different temperature for one or more of the VeriFlex blocks, you need to:

  a. Design your experiment using Advanced Setup instead of the Design Wizard.

  b. In the Plate Setup screen (page 148), select the **Assign Targets and Samples** tab, select the **View Plate Layout** tab, then select the **Enable VeriFlex™ Block** checkbox.

  IMPORTANT! If you do not select the **Enable VeriFlex™ Block** checkbox in the Plate Setup screen, you will not be able to set a different temperature for one or more of the VeriFlex blocks in the Run Method screen.

  c. In the Run Method screen, select the **Graphical View** tab.

  d. For each VeriFlex™ block you want to change, click the temperature, then enter the desired value.

Note: You can set a different temperature for one or more of the VeriFlex blocks, or set each of the VeriFlex blocks to the same temperature. If neighboring VeriFlex blocks are not set to the same temperature, the temperature difference must be between 0.1 and 5.0 °C. The maximum temperature is 99.9 °C.

For More Information

For more information on:

- The Run Method library or on completing the Run Method screen, access the StepOne Software Help by clicking or pressing **F1**.
- Setting temperatures for the VeriFlex blocks, access the StepOne Software Help by clicking or pressing FI.
- Using Advanced Setup, see "Advanced Setup Workflow" on page 260.

Notes

Review the Reaction Setup

On 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.

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

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 20 µL.
- The excess reaction volume is 10%.
- The reaction components are:
  - TaqMan® Fast Universal PCR Master Mix (2X)
  - TP53 Assay Mix (20X)
  - GAPDH Assay Mix (20X)
  - Sample
  - Water
- The diluted sample concentration is 5.0 ng/µL.
- The sample stock concentration is 100 ng/µL.

Complete the Reaction Setup Screen

Complete the Reaction Mix Calculations Tab for the TP53 Assay

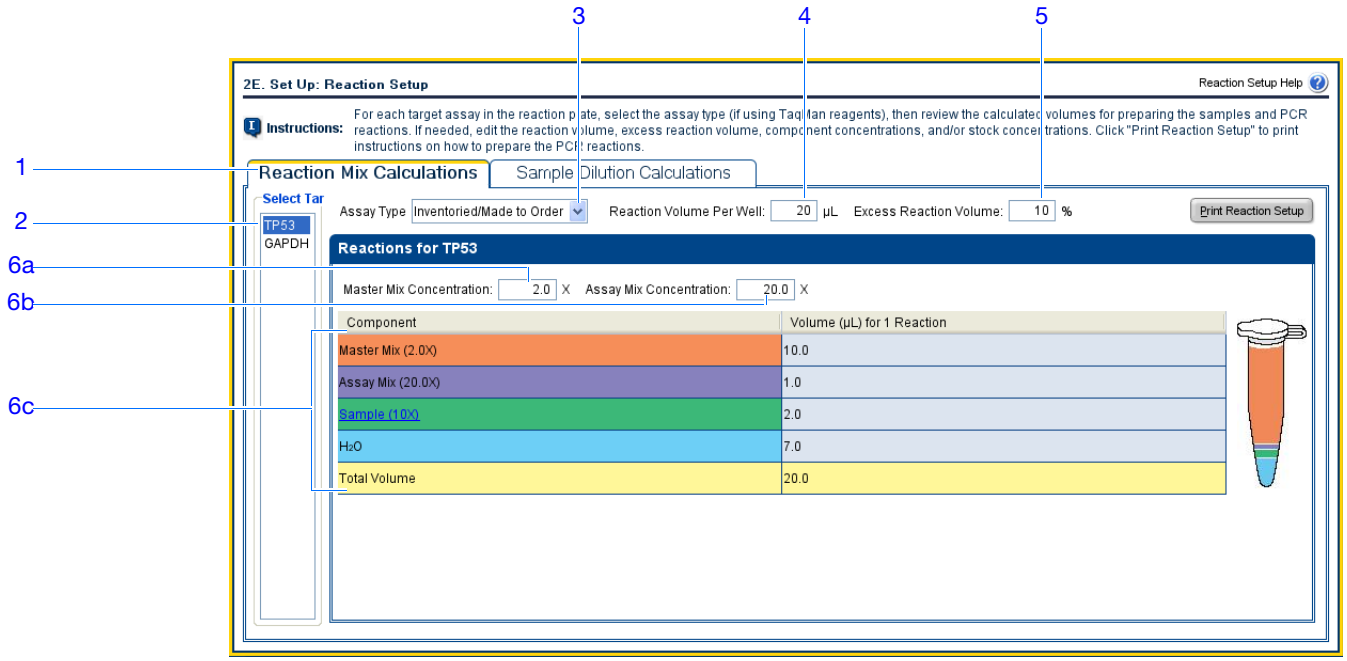
1. Select the **Reaction Mix Calculations** tab (default).
2. From the Select Target pane, select **TP53**.
3. From the Assay Type dropdown menu, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **20 µL**.
5. Make sure the Excess Reaction Volume field displays **10%**.
6. In the Reactions for TP53 pane:
   a. Make sure the Master Mix Concentration field displays **2.0X**.
   b. Make sure the Assay Mix Concentration field displays **20.0X**.
   c. Review the components and calculated volumes for the PCR reactions:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	10.0
Assay Mix (20.0X)	1.0

Notes

Component	Volume (µL) for 1 Reaction
Sample (10X)	2.0‡
H_2O	7.0
Total Volume	20.0

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



Complete the Reaction Mix Calculations Tab for the GAPDH Assay

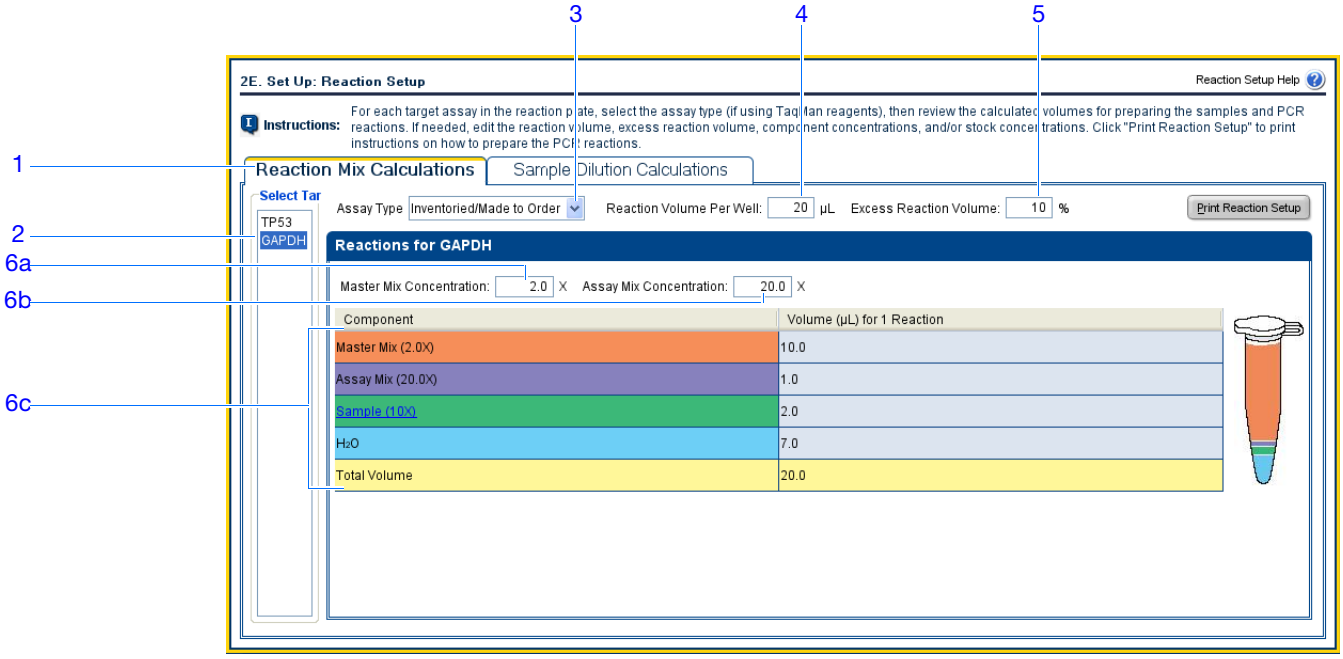
1. Select the **Reaction Mix Calculations** tab (default).
2. From the Select Target pane, select **GAPDH**.
3. From the Assay Type dropdown menu, select **Inventoried/Made to Order**.
4. Make sure the Reaction Volume Per Well field displays **20 µL**.
5. Make sure the Excess Reaction Volume field displays **10%**.
6. In the Reactions for GAPDH pane:
   a. Make sure the Master Mix Concentration field displays **2.0X**.
   b. Make sure the Assay Mix Concentration field displays **20.0X**.

Notes

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

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	10.0
Assay Mix (20.0X)	1.0
Sample (10X)	2.0‡
H_2O	7.0
Total Volume	20.0

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



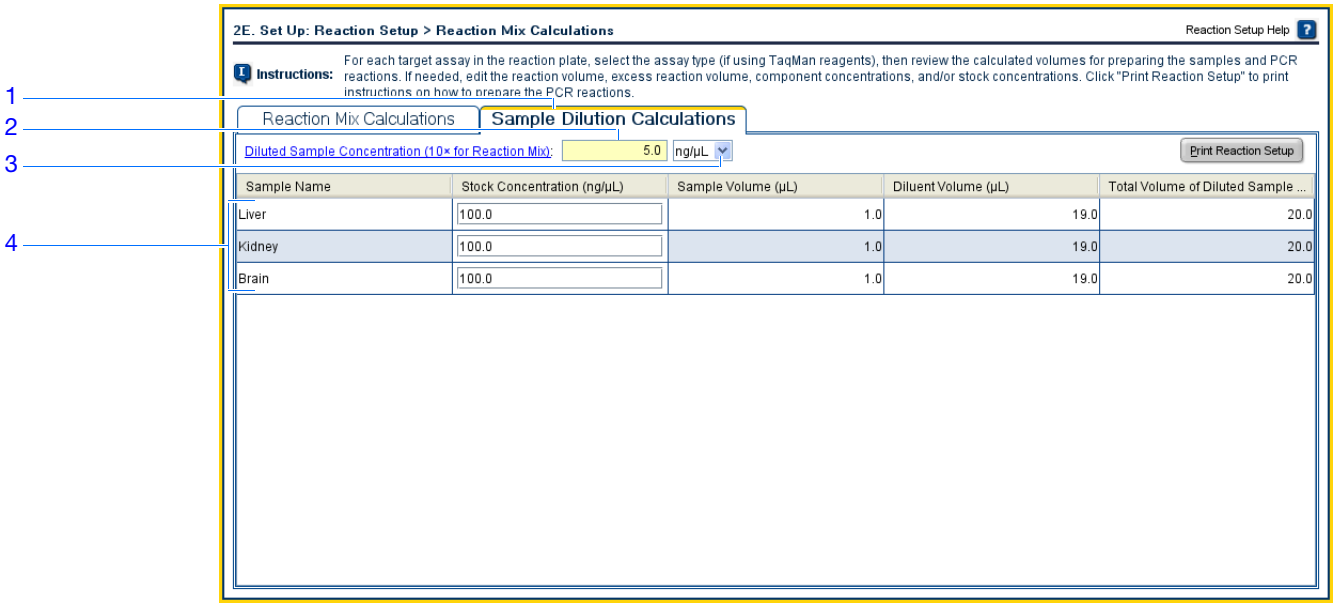
Complete the Sample Dilution Calculations Tab

1. Select the **Sample Dilution Calculations** tab.
2. Click the **Diluted Sample Concentration (10× for Reaction Mix)** field, then enter **5.0**.
3. From the unit dropdown menu, select **ng/µL** (default).

Notes

4. Review the calculated volumes for the sample dilutions:

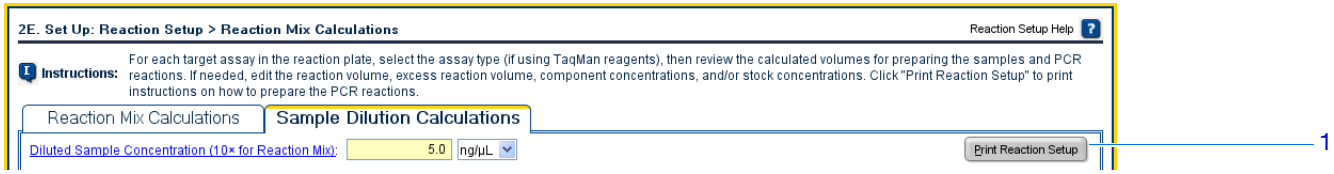
Sample Name	Stock Concentration (ng/µL)	Sample Volume (µL)	Diluent Volume (µL)	Total Volume of Diluted Sample (µL)
Liver	100.0	1.0	19.0	20.0
Kidney	100.0	1.0	19.0	20.0
Brain	100.0	1.0	19.0	20.0



Print Reaction Setup Instructions

Print detailed reaction setup instructions, then save the instructions for Chapter 7, "Prepare the Comparative CT Reactions."

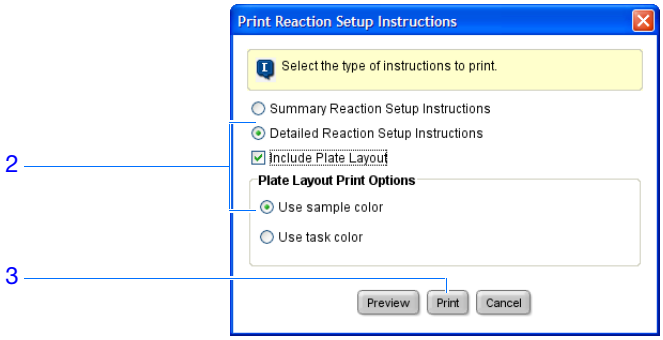
1. Click **Print Reaction Setup**.



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

3. Click **Print** to send the reaction setup instructions to your printer.

Notes



4. Click **Next >**.

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 30 for the reaction volume/well. The StepOne and StepOnePlus systems support 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, access the StepOne Software Help by clicking or pressing **F1**.
- Applied Biosystems assays, refer to the:
  - *TaqMan® Gene Expression Assays Protocol*

Notes

– *Custom TaqMan® Gene Expression Assays Protocol*.

Order Materials for the Experiment

On 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: To access the Applied Biosystems Store, you need to have an Internet connection. 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 StepOne 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® Fast Optical 48-Well Reaction Plate
- MicroAmp® 48-Well Optical Adhesive Film
- MicroAmp® 96-Well Support Base
- TaqMan® Fast Universal PCR Master Mix (2✕), No AmpErase® UNG
- TP53 Assay Mix: Hs00153340_m1 (RefSeq NM_000546.2)
- GAPDH Assay Mix: Human GAPD (GAPDH) Endogenous Control kit (PN 4333764T)

Note: The example experiment was created for a StepOne instrument. If you selected the StepOnePlus instrument in the Experiment Properties screen (page 139), the 96-well consumables (for example, the MicroAmp® Fast Optical 96-Well Reaction Plate) are listed in place of the 48-well consumables.

Complete the Ordering Materials Screen

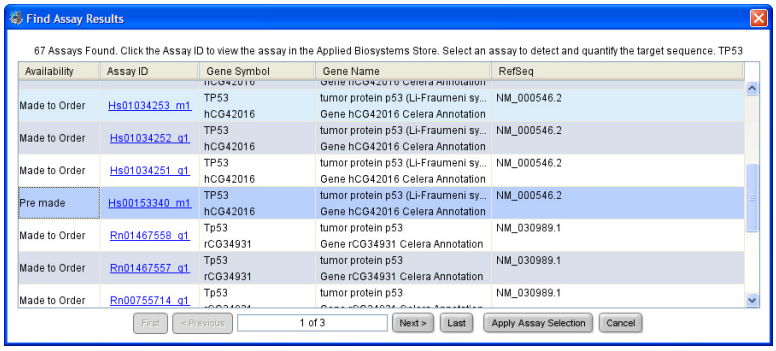
1. Find the target assay on the Applied Biosystems Store:

   Note: To access the Applied Biosystems Store, you need to have an Internet connection.

   a. Click the **Enter Gene Name** field, enter **TP53**, then click **Find Assay**.

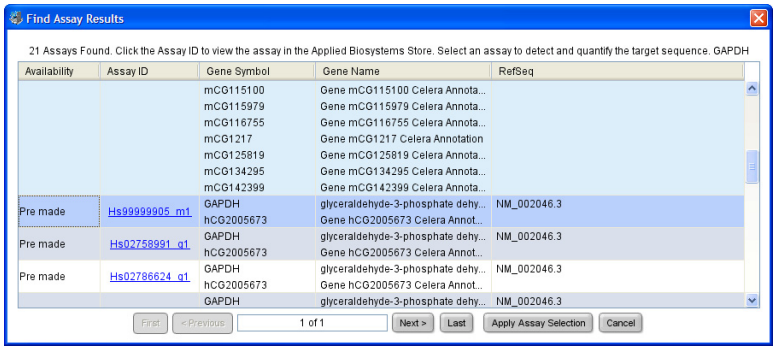
   b. In the Find Assay Results dialog box, select the **Hs00153340_m1** row, then click **Apply Assay Selection**.

Notes



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

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



Note: The Human GAPD (GAPDH) Endogenous Control kit (PN 4333764T) was used in the example experiment. However, it is not available through the StepOne software Find Assay feature and must be ordered separately. You can order the kit separately or you can select one of the Applied Biosystems GAPDH assays listed in the Find Assay Results dialog box (as illustrated in the example above).

2. From the Display dropdown menu, select **All Items** (default), then review the recommended materials. If needed, use the scroll bar at right to see all items.

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

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

4. (Optional) Create a shopping list:

   a. Select the checkbox next to each of the following items:
      - MicroAmp® Fast Optical 48-Well Reaction Plate
      - MicroAmp® 48-Well Optical Adhesive Film

Notes

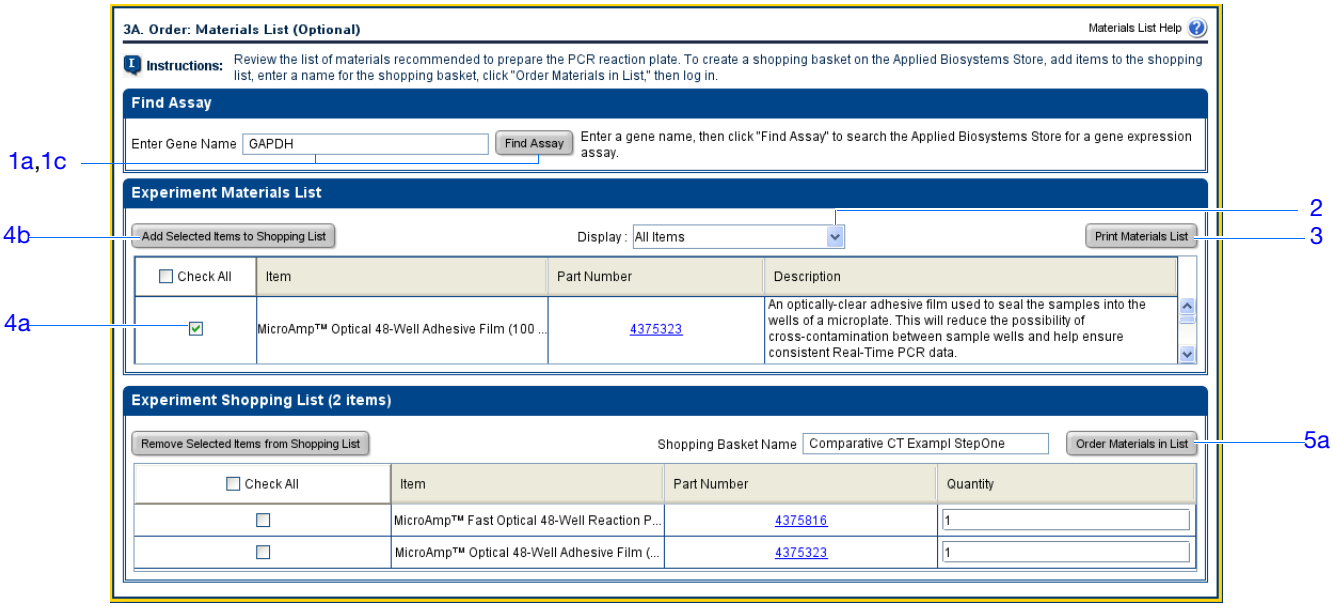
- MicroAmp® 96-Well Support Base
- TaqMan® Fast Universal PCR Master Mix (2✕), No AmpErase® UNG
- Hs00153340_m1 (TP53 Assay Mix)
- GAPDH Assay Mix: Human GAPD (GAPDH) Endogenous Control kit (PN 4333764T)

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

5. (Optional) Create a shopping basket on the Applied Biosystems Store:

Note: To access the Applied Biosystems Store, you need to have an Internet connection. 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.

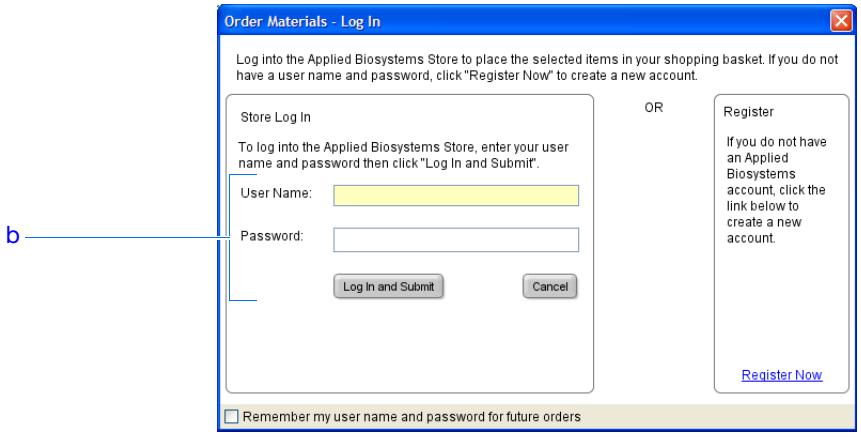
a. Check that the Experiment Shopping List contains the desired materials and that the quantities are correct, then click **Order Materials in List**.



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

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

Notes



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 161.

Design Guidelines

When you design your own comparative C_T experiment:

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

Desktop Operating System	Microsoft® Internet Explorer	Adobe® Acrobat® Reader
Windows® XP (Service Pack 2 or Service Pack 3)	v6.x	v4.0 or later
Windows® Vista	v7.x or later	v4.0 or later

Note: Make sure that cookies and Java Script are turned on for the web site to function correctly.

For More Information

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

Notes

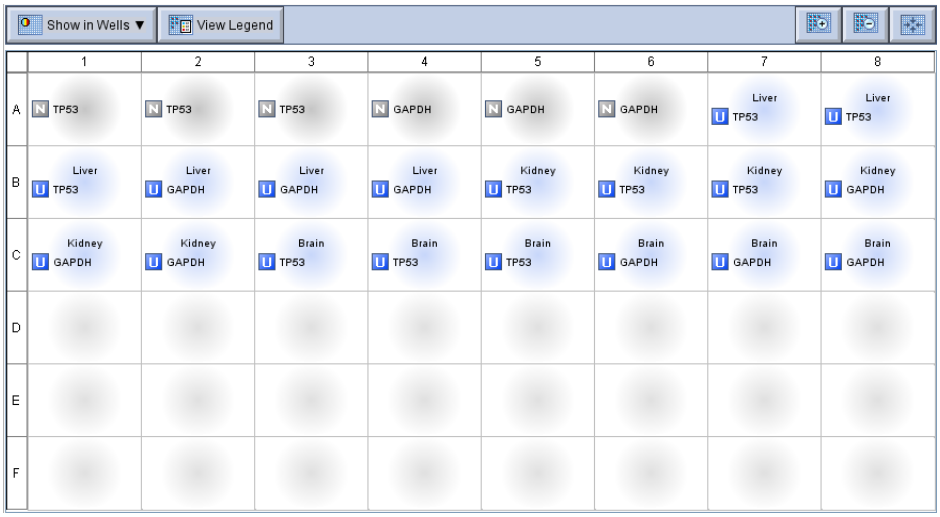
Finish the Design Wizard

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

About the Example Experiment

The StepOne 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.



Note: The example experiment was created for a StepOne instrument. If you selected the StepOnePlus instrument in the Experiment Properties screen (page 139), your reaction plate layout will differ from the layout shown above. The software displays a 96-well reaction plate layout for the StepOnePlus instrument. For an example of the 96-well reaction plate layout, see page 14.

- The experiment is saved as is and closed.

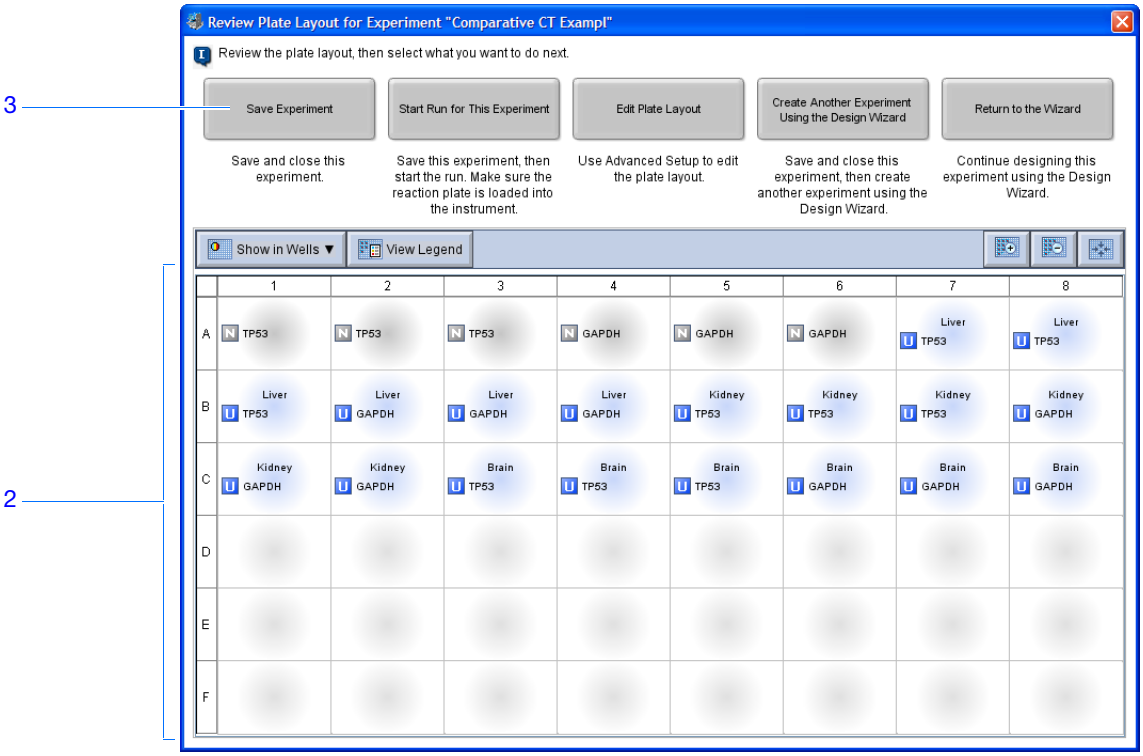
Note: For the example experiment, do not perform the run at this time.

Finish the Design Wizard

1. At the bottom of the StepOne software screen, click **Finish Designing Experiment**.
2. In the Review Plate for Experiment window, review the plate layout. Make sure there are:
   - 18 Unknown wells U
   - 6 Negative control wells N
   - 24 Empty wells

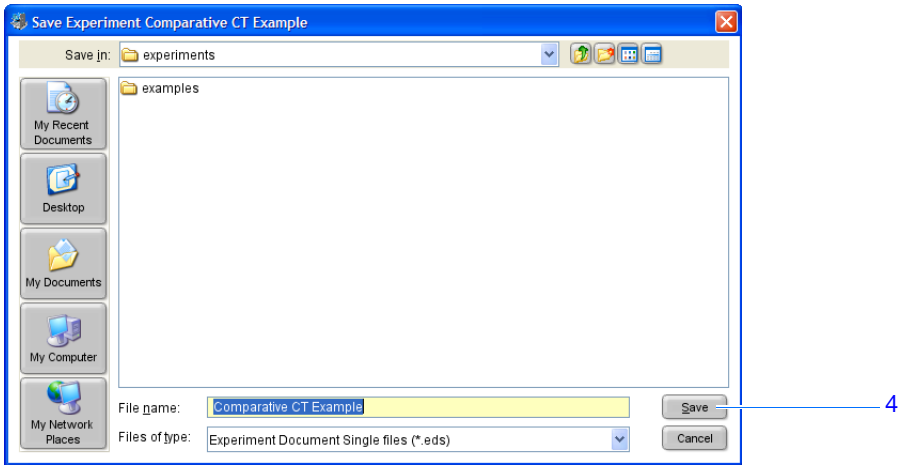
   Note: If the plate layout is incorrect, click **Return to the Wizard** and check your entered values.
3. Click **Save Experiment**.

Notes



4. In the Save Experiment dialog box, click **Save** to accept the default file name and location. The example experiment is saved and closed, and you are returned to the Home screen.

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 comparative C_T experiment:

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

Click	If you want 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. • (*StepOnePlus instrument only*) Set a different temperature for one or more of the VeriFlex blocks using Advanced Setup.
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 the <*drive*>:\Applied Biosystems\<*software name*>\experiments folder. 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 **General** tab (default). In the Default Data Folder field, browse to the desired location.

Note: To change the default save location, you must be logged in to the software with a user name, not as a Guest. For more information, see the "Design Guidelines" for logging in on page 136.

For More Information

For more information on using Advanced Setup, see "Advanced Setup Workflow" on page 260.

Notes

Notes

7 Prepare the Comparative C_T Reactions

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

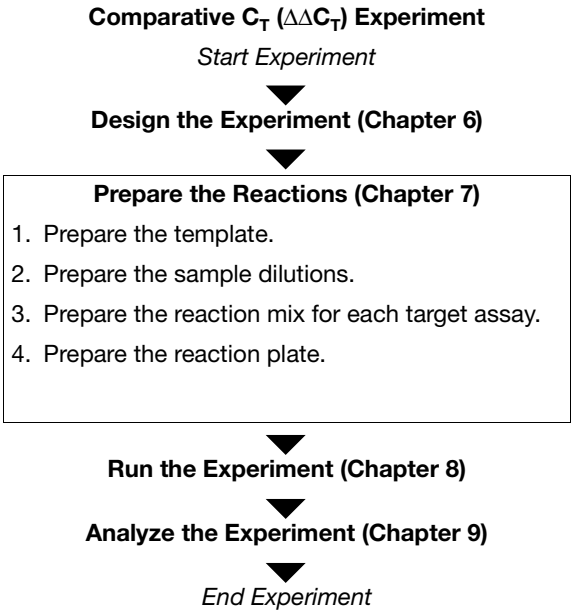
Notes

Chapter Overview

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

Example Experiment Workflow

The workflow for preparing the PCR reactions for the example experiment provided with this getting started guide is shown below.



Notes

Prepare the Template

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

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 Ambion® starter packs listed below 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 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
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

Notes

- Total RNA isolated from liver, kidney, and brain 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 is used to perform the example experiment. For a list of other kits you can use to perform your own experiment, see "Preparation Guidelines" on page 169.

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 one of the following kits:

Kit	Part Number
High Capacity RNA-to-cDNA Kit (50 reactions)	4387406
High Capacity RNA-to-cDNA Master Mix (200 reactions)	4390778
High-Capacity cDNA Reverse Transcription Kit (200 reactions)	4368814
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	4374966

  - **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 protocol for the kit you select:
  - *High Capacity RNA-to-cDNA Kit Protocol*
  - *High Capacity RNA-to-cDNA Master Mix Protocol*
  - *High-Capacity cDNA Reverse Transcription Kits Protocol*

  Note: The *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits 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 StepOne™ software ("Complete the Sample Dilution Calculations Tab" on page 154).

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 StepOne software. The total reaction volume is 20 µL/reaction, so the sample volume is 2 µL/reaction.
- The stock concentration is 100 ng/µL. After diluting the sample according to the Sample Dilutions Calculations table, the sample will have a concentration of 5.0 ng/µL. This is a 10× concentration when adding 2 µL to the final reaction mix volume of 20 µL. You will have 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	1.0	19.0	20.0
Kidney	100.0	1.0	19.0	20.0
Brain	100.0	1.0	19.0	20.0

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

Tube	Sample Name	Diluent Volume (µL)
1	Liver	19.0
2	Kidney	19.0

Notes

Tube	Sample Name	Diluent Volume (µL)
3	Brain	19.0

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

Tube	Sample Name	Sample Volume (µL)
1	Liver	1.0
2	Kidney	1.0
3	Brain	1.0

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 StepOne 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. For Fast reagents, Applied Biosystems recommends 10 ng.
- 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*.

Prepare the Reaction Mix

Prepare the reaction mix using the components and volumes that were calculated by the StepOne software ("Complete the Reaction Mix Calculations Tab for the TP53 Assay" on page 152 and "Complete the Reaction Mix Calculations Tab for the GAPDH Assay" on page 153).

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 174).

Notes

About the Example Experiment

For the comparative C_T example experiment:

- The reaction mix components are:
  - TaqMan® Fast Universal PCR Master Mix (2X)
  - TP53 Assay Mix (20X)
  - GAPDH Assay Mix (20X)
  - Water
- The volumes calculated in the software for both target assays are:

Component	Volume (µL) for 1 Reaction
Master Mix (2.0X)	10.0
Assay Mix (20.0X)	1.0
H_2O	7.0
Total Volume	18.0

Note: The sample is not added at this time.

Required Materials

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

Prepare the Reaction Mix

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

1. Label an appropriately sized tube for each reaction mix:
   - **TP53 Reaction Mix**
   - **GAPDH Reaction Mix**
2. For the TP53 assay, add the required volumes of each component to the TP53 Reaction Mix tube:

Component	Volume (µL) for 1 Reaction	Volume (µL) for 12 Reactions (Plus 10% Excess)
TaqMan® Fast Universal PCR Master Mix (2X)	10.0	132.0
TP53 Assay Mix (20X)	1.0	13.2
Water	7.0	92.4
Total Reaction Mix Volume	18.0	237.6

Notes

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 12 Reactions (Plus 10% Excess)
TaqMan® Fast Universal PCR Master Mix (2X)	10.0	132.0
GAPDH Assay Mix (20X)	1.0	13.2
Water	7.0	92.4
Total Reaction Mix Volume	18.0	237.6

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.

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*

Notes

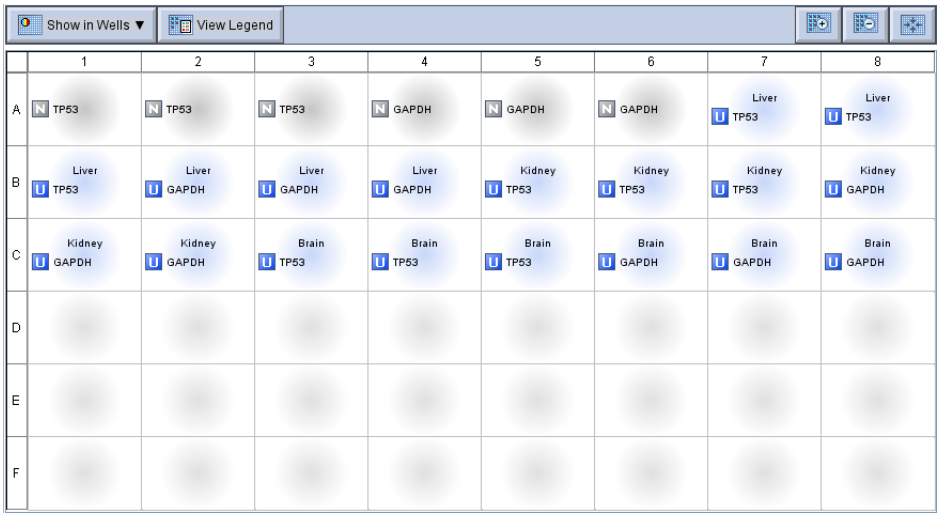
Prepare the Reaction Plate

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

About the Example Experiment

For the comparative C_T example experiment:

- A MicroAmp® Fast Optical 48-Well Reaction Plate is used.
- The reaction volume is 20 µL/well.
- The reaction plate contains:
  - 18 Unknown wells U
  - 6 Negative control wells N
  - 24 Empty wells
- The plate layout automatically generated by the StepOne software is used:



Note: The example experiment was created for a StepOne instrument. If you selected the StepOnePlus instrument in the Experiment Properties screen (page 139), your reaction plate layout will differ from the layout shown above. The software displays a 96-well reaction plate layout for the StepOnePlus instrument. For an example of the 96-well reaction plate layout, see page 14.

Required Materials

- Microcentrifuge tubes
- Pipettors
- Pipette tips
- TP53 reaction mix (from page 172)
- GAPDH reaction mix (from page 172)
- Water
- Samples (from page 170)

Notes

- MicroAmp® Fast Optical 48-Well Reaction Plate
- MicroAmp® Optical 48-Well 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	TP53 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 20 µL of the negative control reaction to the appropriate wells in the reaction plate.

2. 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.

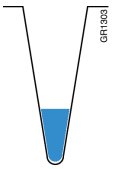
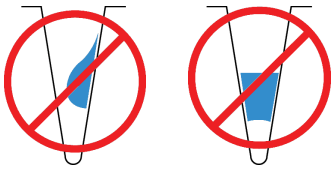
Tube	Unknown Reaction	Reaction Mix	Reaction Mix Volume (µL)	Sample	Sample Volume (µL)
1	TP53 Liver	TP53 reaction mix	59.4	Liver	6.6
2	TP53 Kidney	TP53 reaction mix	59.4	Kidney	6.6
3	TP53 Brain	TP53 reaction mix	59.4	Brain	6.6
4	GAPDH Liver	GAPDH reaction mix	59.4	Liver	6.6
5	GAPDH Kidney	GAPDH reaction mix	59.4	Kidney	6.6
6	GAPDH Brain	GAPDH reaction mix	59.4	Brain	6.6

   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 20 µL of the unknown (sample) reaction to the appropriate wells in the plate.

Notes

3. Seal the reaction plate with optical adhesive film.
4. Centrifuge the reaction plate briefly to remove air bubbles.
5. Confirm 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(s) 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

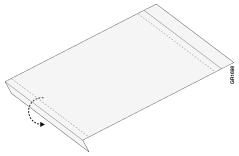
6. Until you are ready to perform the run, place the reaction plate on ice in the dark.

Preparation Guidelines

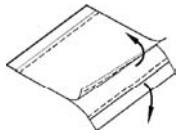
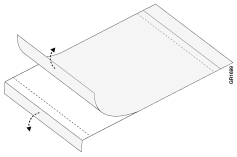
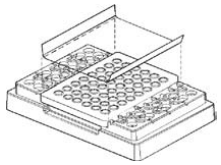
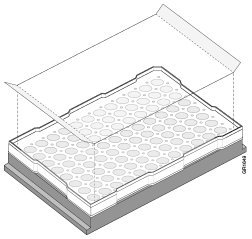
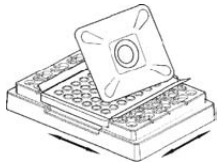
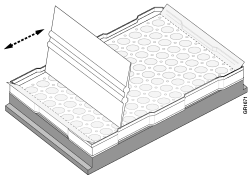
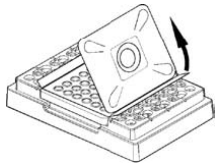
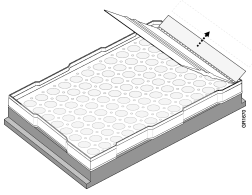
When you prepare your own comparative C_T experiment:

- Make sure you use the appropriate consumables.
- Make sure the arrangement of the PCR reactions matches the plate layout displayed in the StepOne 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 to seal your reaction plates, seal each reaction plate as follows:

Action	Example	
	StepOne™ System	StepOnePlus™ System
1. Place the reaction plate onto the center of the 96-well base. Be sure the reaction plate is flush with the top surface of the 96-well base.		
2. Load the reaction plate as desired.		
3. Remove a single optical adhesive film (film) from the box. • *For the StepOne system reaction plate,* bend both end-tabs upward. Hold the film backing side up. • *For the StepOnePlus system reaction plate*, fold back one of the end-tabs. Hold the film backing side up.		

Notes

Action	Example	
	StepOne™ System	**StepOnePlus™ System**
4. In one swift 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 will not affect results. Haziness will disappear when the film comes into contact with the heated cover in the instrument.		
5. Holding the film by the end-tabs, lower the film onto the reaction plate (adhesive side facing the reaction plate). Be sure the film is completely covering all wells of the reaction plate.		
6. 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.		
7. 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.		
8. To ensure a tight, evaporation-free seal: a. Repeat step 6. b. 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, evaporation-free seal.		
9. 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 260.

Notes

Notes

8 Run the Comparative C_T Experiment

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

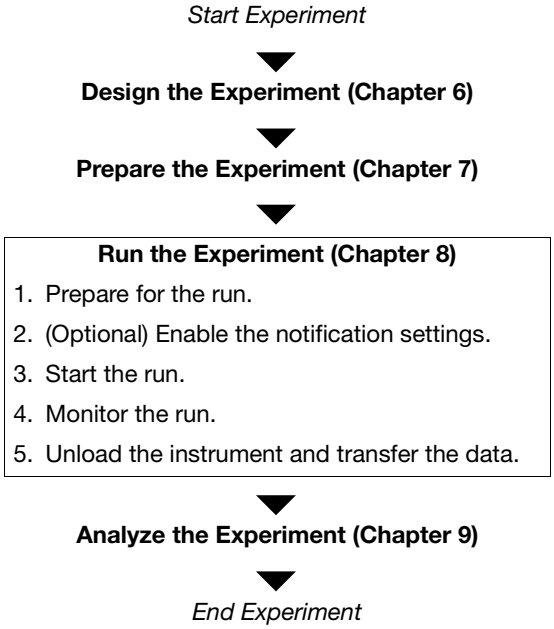
Notes

Chapter Overview

This chapter explains how to perform a run on the Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems.

Example Experiment Workflow

The workflow for running the example experiment provided with this getting started guide is shown below.



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 StepOne™ or StepOnePlus™ instrument.

Open the Example Experiment

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

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

2. In the Login dialog box, select **EXAMPLEUSER** from the User Name dropdown menu, then click **OK**.

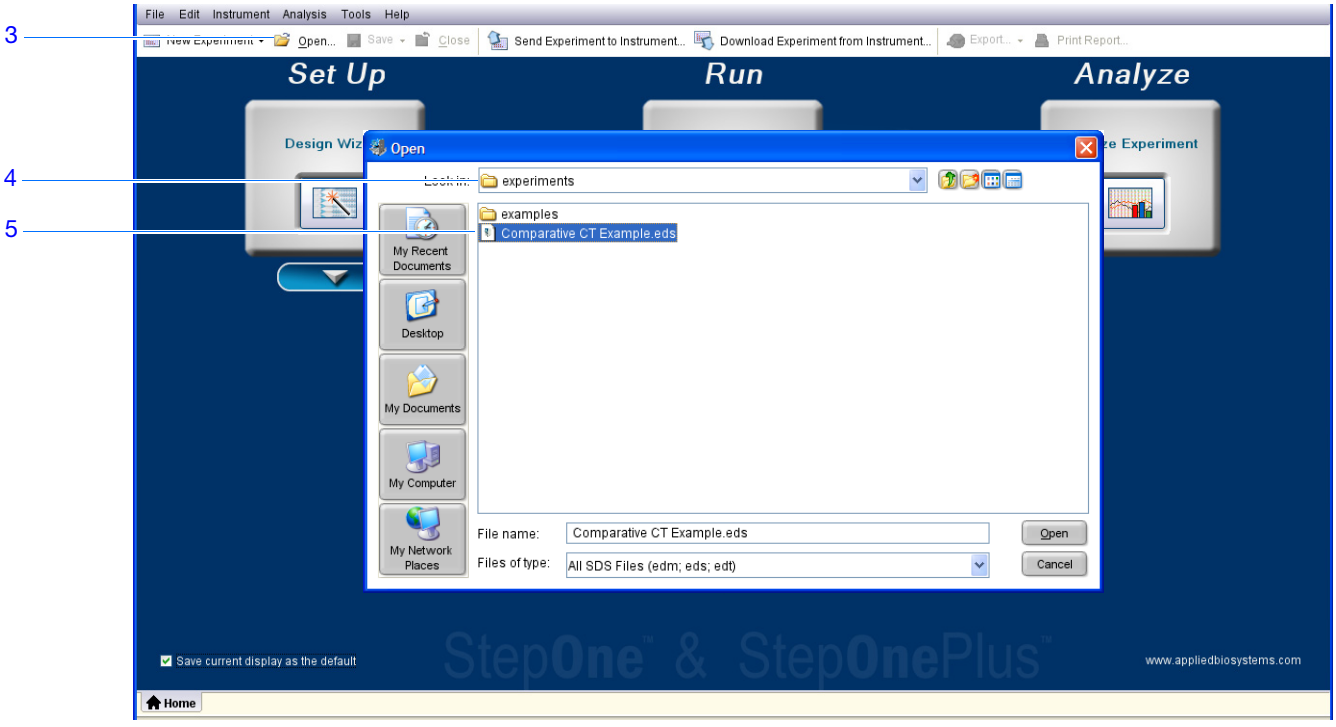
   Note: EXAMPLEUSER is the user name you created when designing the comparative C_T experiment (page 135).

3. From the Home screen, click **Open**.

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

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

5. Double-click **Comparative CT Example** to open the example experiment file 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 78
- "(Optional) Enable the Notification Settings" on page 79
- "Start the Run" on page 82
- "Monitor the Run" on page 86
- "Unload the Instrument and Transfer the Data" on page 93

Be sure to select the **Comparative CT Example.eds** file rather than the **Relative Standard Curve Example.eds** file.

Notes

9 Analyze the Comparative C_T Experiment

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

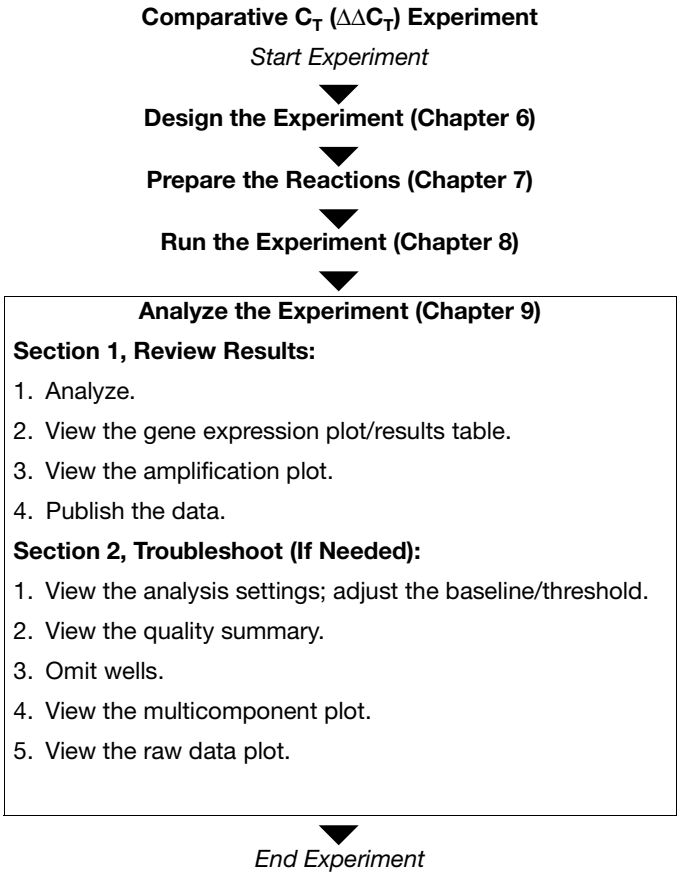
Notes

Chapter Overview

The StepOne™ software analyzes the data using the comparative C_T ($\Delta\Delta C_T$) 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. If you receive questionable results, Section 2 of this chapter explains how to perform some troubleshooting steps.

Example Experiment Workflow

The workflow for analyzing the example experiment provided with this getting started guide is shown below.



Notes

Section 9.1 Review Results

This section covers:

Notes

Analyze the Experiment

The StepOne 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 installs with the StepOne software. The data file was created with the same design parameters provided in Chapter 6, then run and analyzed on a StepOne™ instrument.

You can find the data file for the example experiment on your computer:

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

where:

- <*drive*> is the computer hard drive on which the StepOne software is installed. The default installation drive for the software is the D drive.
- <*software name*> is the current version of the StepOne software.

Analyze the Example Experiment

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

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

2. In the Login dialog box, select **EXAMPLEUSER** from the User Name dropdown menu, then click **OK**.

   Note: EXAMPLEUSER is the user name you created when you designed the comparative C_T experiment (page 135).

3. From the Home screen, click **Open**.

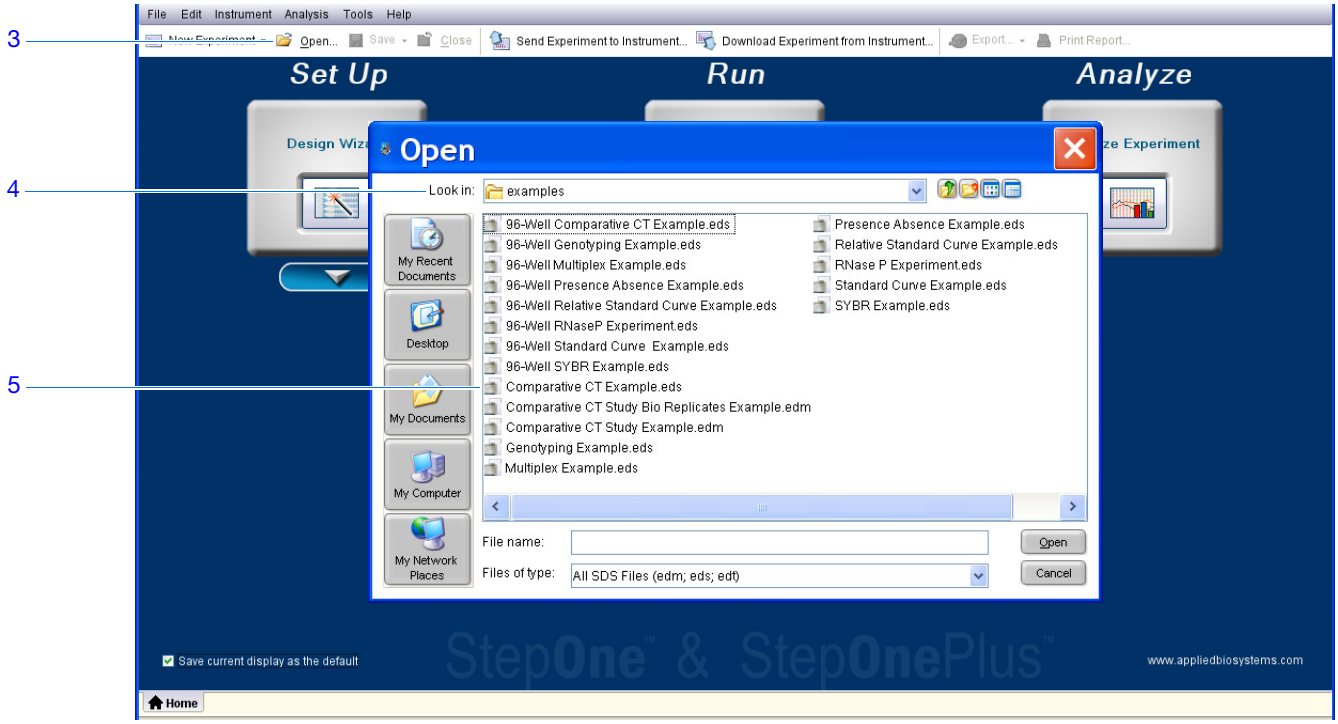
4. In the Open dialog box, navigate to the **examples** folder:

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

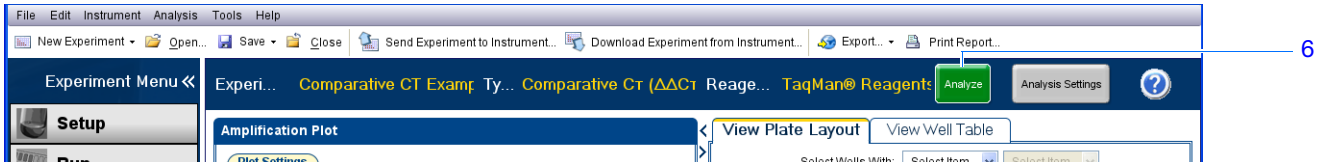
5. Double-click **Comparative CT Example** to open the example experiment data file.

   Note: The examples folder contains several data files; be sure to select **Comparative CT Example**. For information on the other data files, see "Data Files in the Examples Folder" on page 18.

Notes



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



7. See "Software Elements" on page 188 and "Navigation Tips" on page 190 for information on navigating within the analysis screens.

Guidelines

When you analyze your own comparative C_T experiment:

- Immediately after a run, the StepOne 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**.

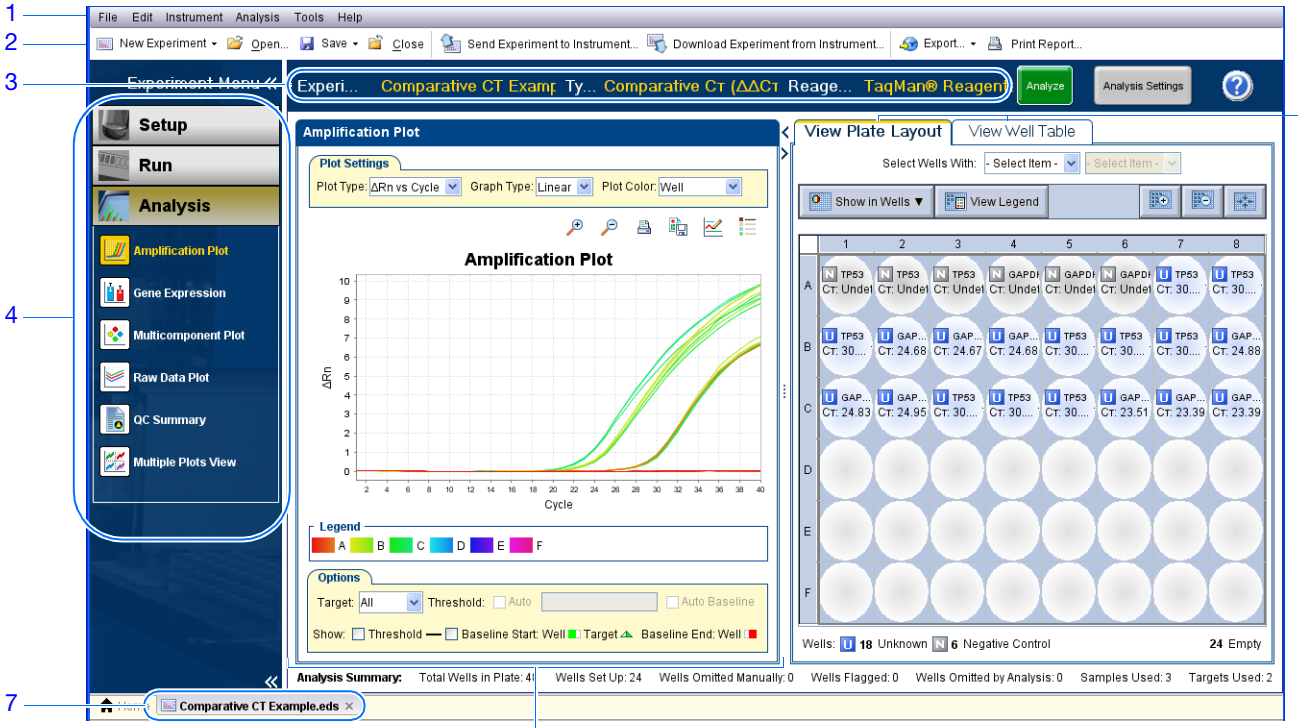
Notes

Software Elements

The StepOne software elements for the analysis screens are illustrated below.

1. Menu bar – Displays the menus available in the software:
   - File
   - Edit
   - Instrument
   - Analysis
   - Tools
   - Help
2. Toolbar – Displays the tools available in the software:
   - New Experiment
   - Open
   - Save
   - Close
   - Send Experiment to Instrument
   - Download Experiment from Instrument
   - Export
   - Print Report
3. Experiment header - Displays the experiment name, experiment type, and reagents for the open experiment.
4. Experiment Menu pane – Provides links to the following software screens:
   - Setup screens
   - Run screens
   - Analysis screens:
     - Amplification Plot
     - Gene Expression
     - Multicomponent Plot
     - Raw Data Plot
     - QC Summary
     - Multiple Plots View
5. Plot pane – Displays the selected analysis screen for the open experiment.
6. View tabs – Displays the plate layout or Well Table for the open experiment
7. Experiment tab(s) – Displays a tab for each open experiment.

Notes


1
2
3
4
5
6
7
File Edit Instrument Analysis Tools Help
New Experiment Open... Save Close Send Experiment to Instrument... Download Experiment from Instrument... Export... Print Report...
Experiment Menu
Setup
Run
Analysis
Amplification Plot
Gene Expression
Multicomponent Plot
Raw Data Plot
QC Summary
Multiple Plots View
Comparative CT Example.eds
Analyze
Analysis Settings
Amplification Plot
Plot Settings
Plot Type: ΔRn vs Cycle
Graph Type: Linear
Plot Color: Well
Cycle
Legend
Options
Target: All
Threshold:
Auto Baseline
Show: Threshold Baseline Start: Well Target Baseline End: Well
View Plate Layout
View Well Table
Select Wells With:
Show in Wells
View Legend
Wells: 18 Unknown 6 Negative Control 24 Empty
Analysis Summary: Wells Set Up: 24 Wells Omitted Manually: 0 Wells Flagged: 0 Wells Omitted by Analysis: 0 Samples Used: 3 Targets Used: 2

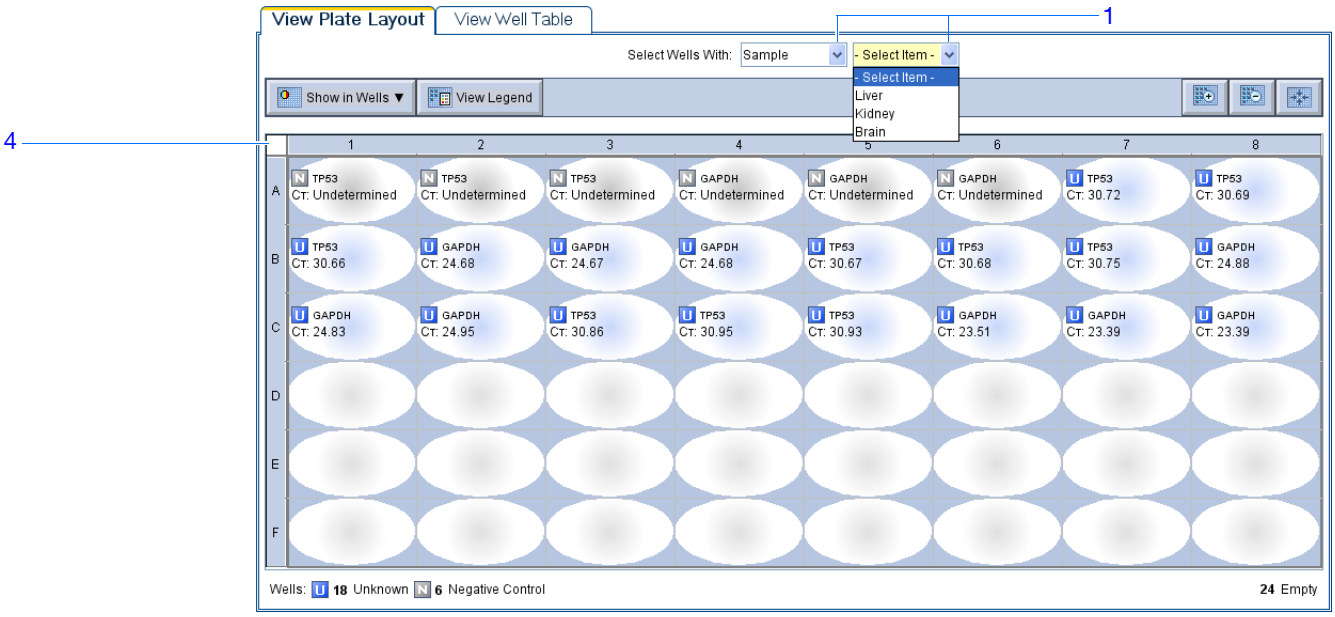
Notes

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 dropdown menus: 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 and drag over the desired wells, press **CTRL+click**, or press **Shift+click** in the plate layout.
4. To select all 48 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:

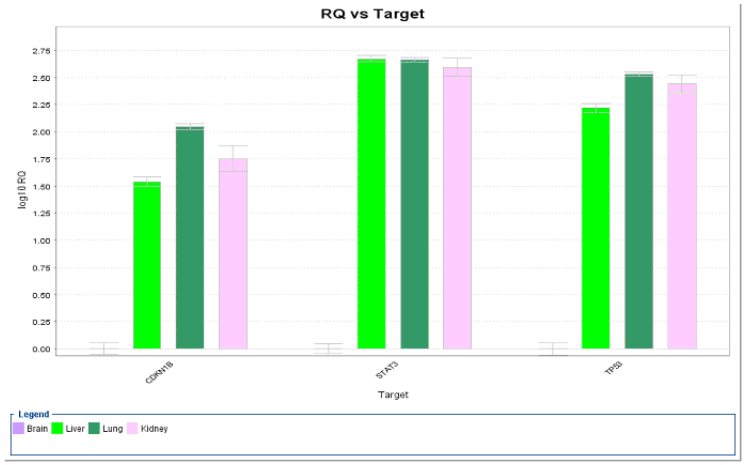
1. From the Experiment Menu pane, select **Analysis** ▸ **Multiple Plots View**.
2. To display four plots, click **Show plots in a 2 × 2 matrix**.
3. To display two plots in rows, click **Show plots in two rows**.
4. To display two plots in columns, click **Show plots in two columns**.
5. To display a specific plot, select the plot from the dropdown menu above each plot display.

Notes

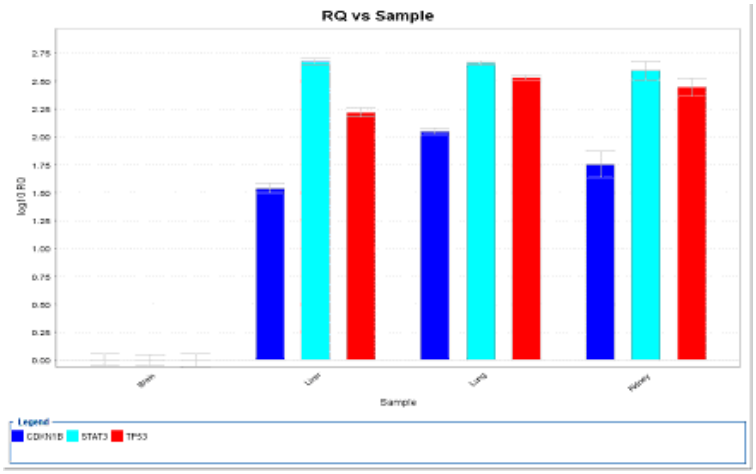
View the Gene Expression Plot and Well Table

The Gene Expression Plot screen displays the results of relative quantitation calculations in the gene expression profile. There are two plots 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 the following graph types: linear, log10, Ln, log2.



- **RQ vs Sample** – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample.You can view the plot as the following graph types: linear, log10, Ln, log2.



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 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.

View the Gene Expression Plot and Well Table

1. From the Experiment Menu pane, select **Analysis** ▸ **Gene Expression**.

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

2. In the Gene Expression Plot screen:
   a. From the Plot Type dropdown menu, select **RQ vs Sample**.
   b. From the Graph Type dropdown menu, select **Log10**.
   c. From the Orientation dropdown menu, select **Vertical Bars**.

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

   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 TP53 in liver and kidney is displayed relative to its expression level in the reference sample (brain). Since 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).

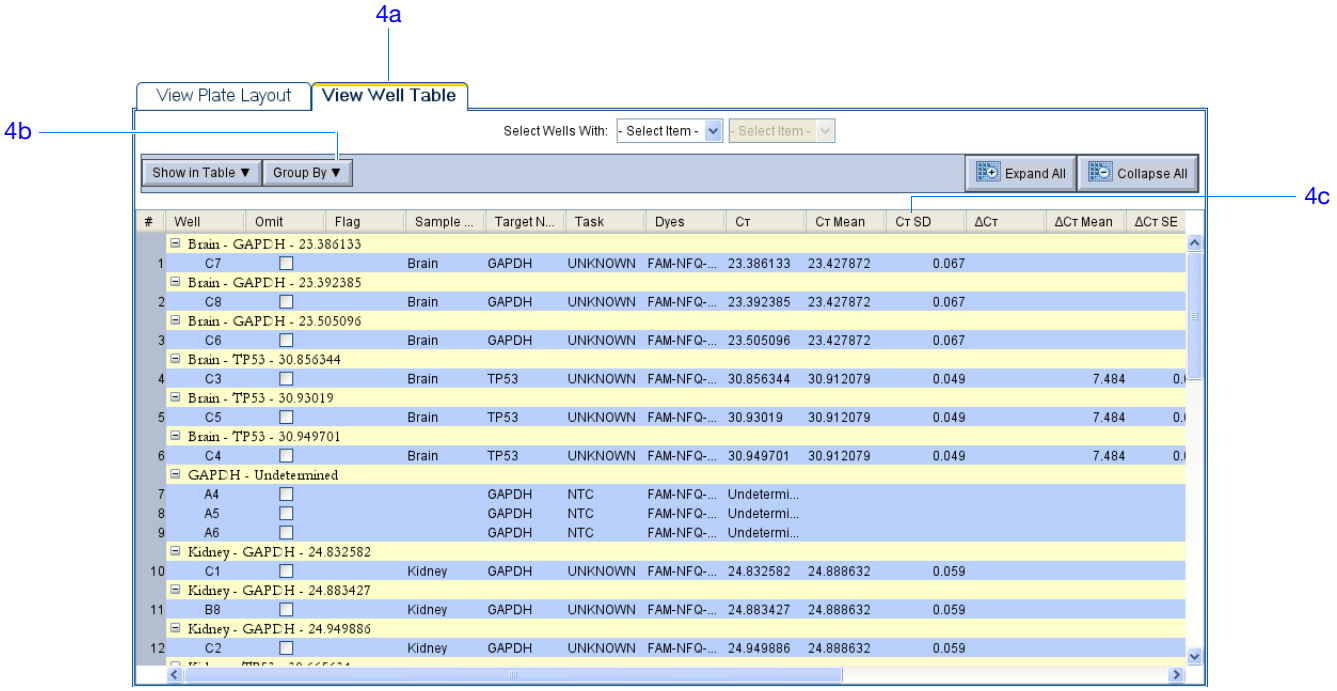
Notes

4. View the Well Table:

   a. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**, then select the **View Well Table** tab.

   b. From the Group By dropdown menu, 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.



Note: To show/hide columns in the Well Table, select/deselect the column name from the Show in Table dropdown menu.

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 208).

For More Information

For more information on the Gene Expression Plot screen or Well Table, access the StepOne 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. There are three plots available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal 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 signal from the reporter dye normalized to the fluorescence signal 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 viewed as the following graph types: linear or log10.

About the Example Experiment

In the comparative C_T example experiment, you review each target in the Amplification Plot screen for:

- Correct baseline and threshold values
- Outliers

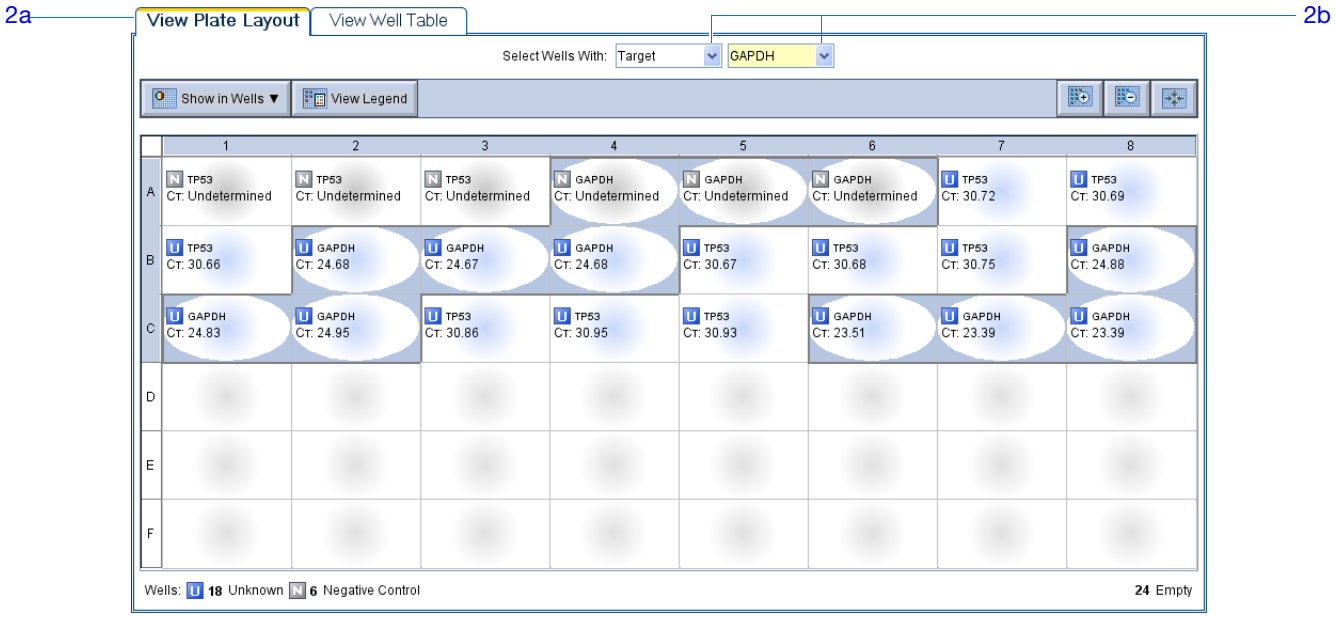
View the Amplification Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

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

2. Display the GAPDH wells in the Amplification Plot screen:
   a. Click the **View Plate Layout** tab.
   b. From the Select Wells With dropdown menus, select **Target**, then **GAPDH**.

Notes

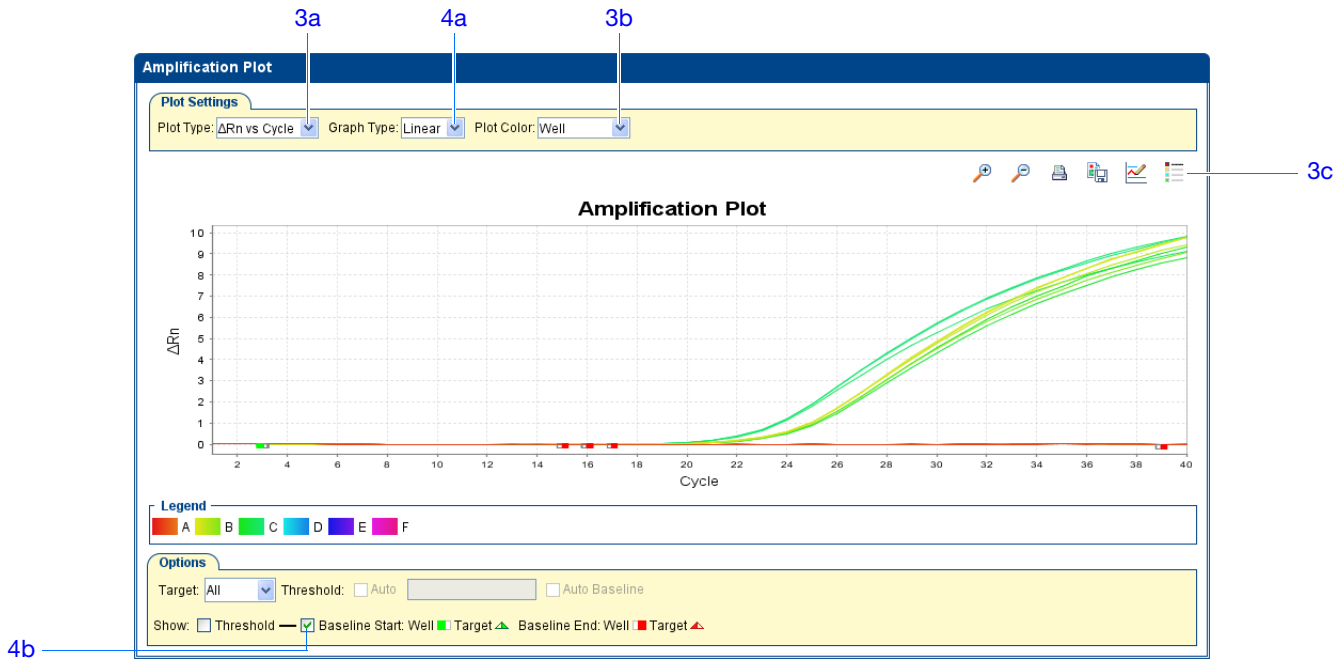


3. In the Amplification Plot screen:
   a. From the Plot Type dropdown menu, select **ΔRn vs Cycle** (default).
   b. From the Plot Color dropdown menu, select **Well** (default).
   c. Click **Show a legend for the plot** (default).

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

4. View the baseline values:
   a. From the Graph Type dropdown menu, select **Linear**.
   b. Select the **Baseline** checkbox 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 fluorescent signal is detected. In the example experiment, the baseline is set correctly.

Notes



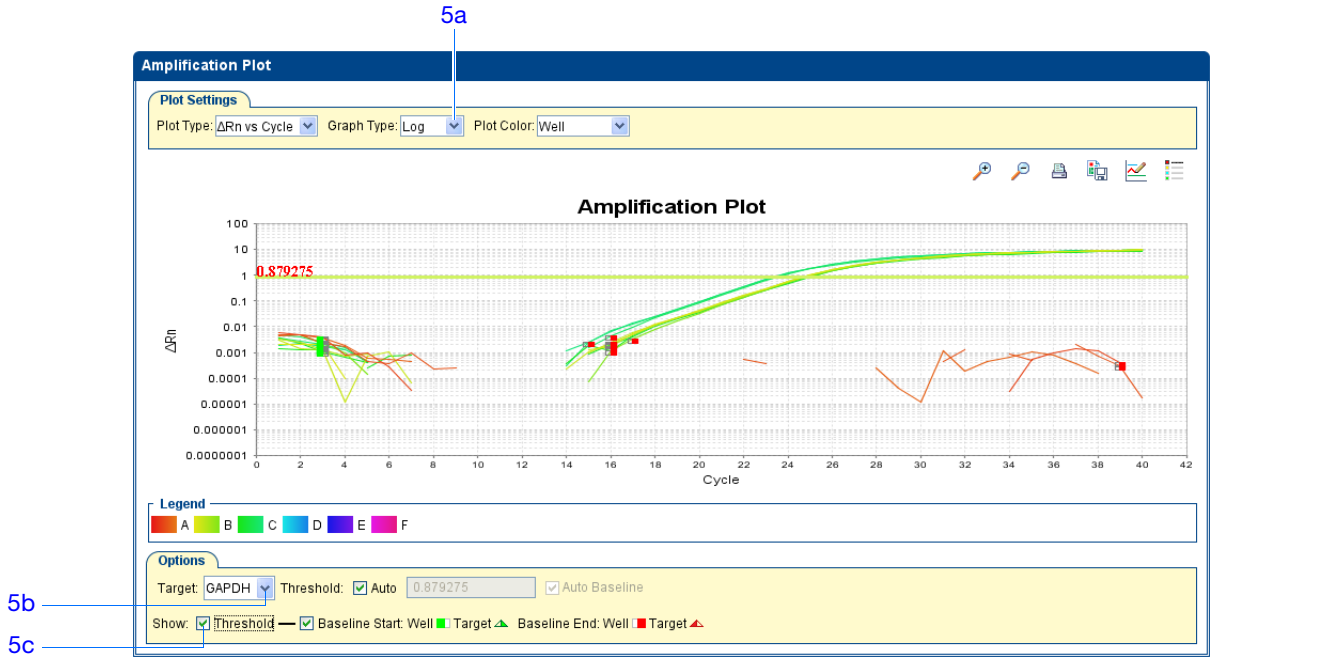
5. View the threshold values:

   a. From the Graph Type dropdown menu, select **Log**.

   b. From the Target dropdown menu, select **GAPDH**.

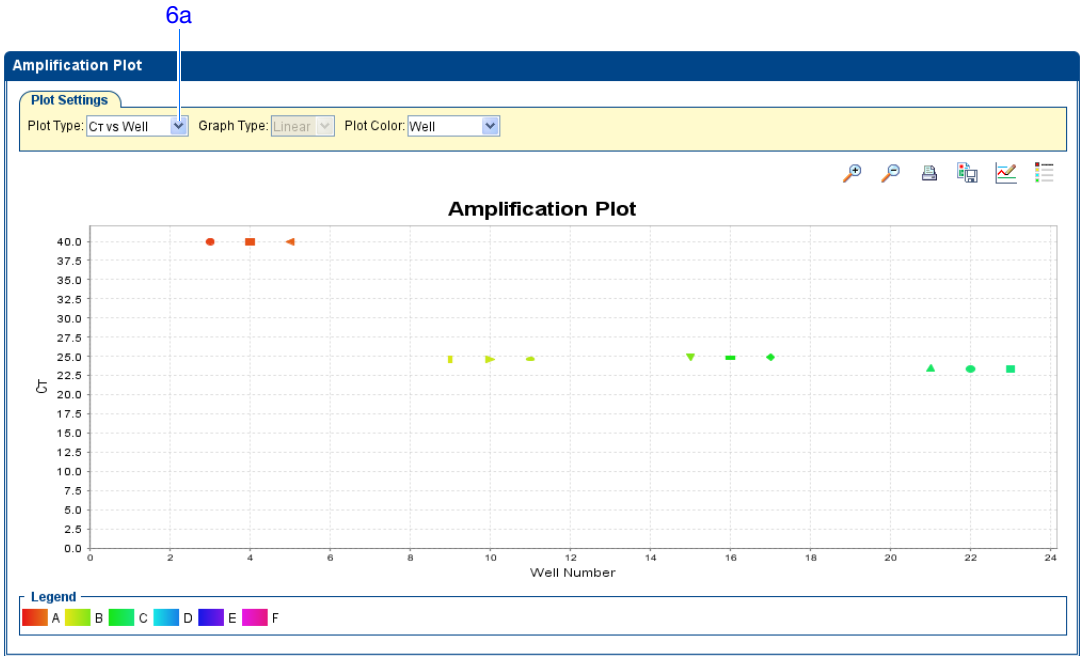
   c. Select the **Threshold** checkbox to show the threshold.

   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. From the Plot Type dropdown menu, select **C_T vs Well**.
   b. Look for outliers from the amplification plot. In the example experiment, there are no outliers for GAPDH.



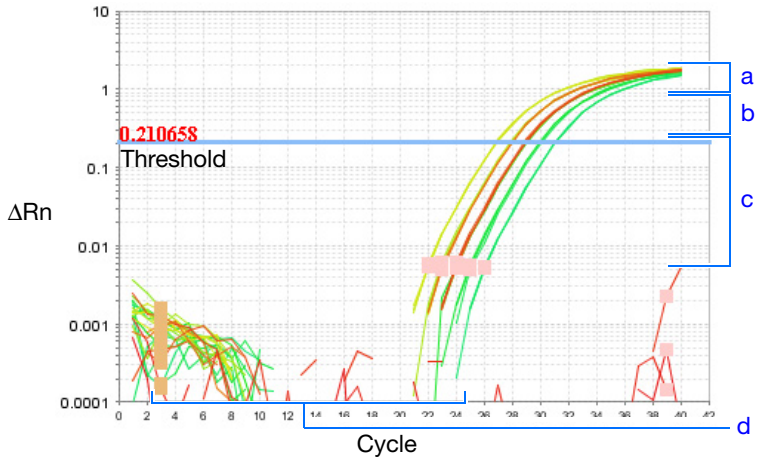
7. Repeat steps 2 through 6 for the TP53 wells. In the example experiment, there are no outliers for TP53.

Analysis Guidelines

When you analyze your own comparative C_T experiment, look for:

- Outliers
- A typical amplification plot – The StepOne 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

Notes



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 StepOne 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 completes.

- Correct baseline and threshold values – See the threshold examples on page 200 and the baseline examples on page 201.

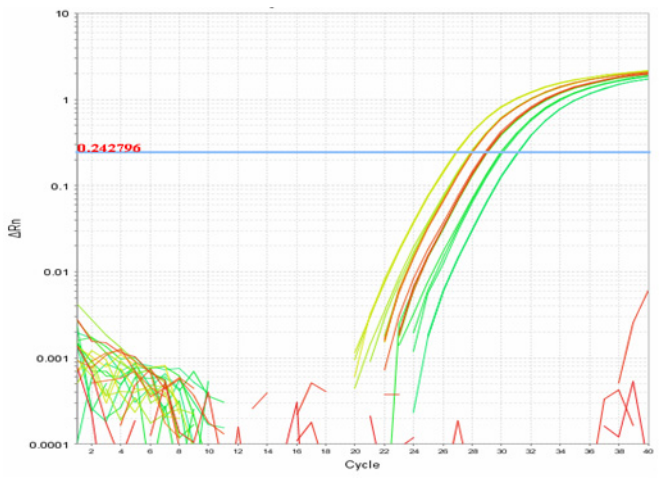
Notes

Threshold Set Correctly

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

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

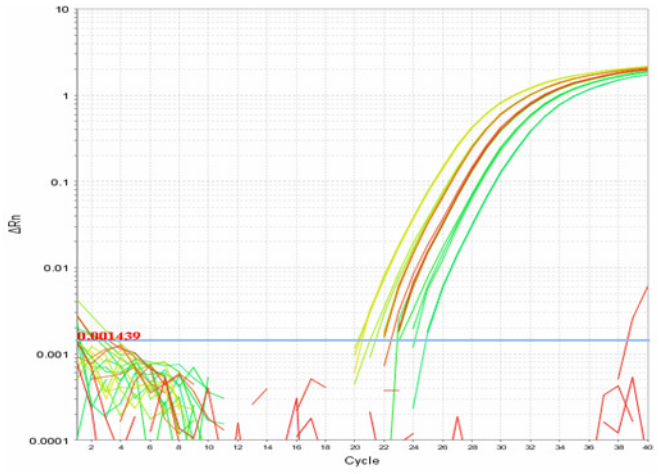
Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...	Comm
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
NTC	FAM-NFQ-...	Undetermi...						
UNKNOWN	FAM-NFQ-...	28.96287	28.923796	0.074	2,484.31	2,551.476	126.2	
UNKNOWN	FAM-NFQ-...	28.838797	28.923796	0.074	2,697.054	2,551.476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

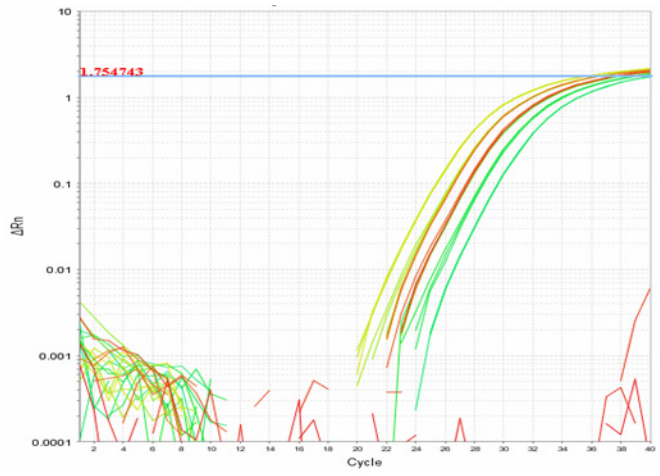
Task	Dyes	CT	CT Mean	CT SD	Quantity	Quantity ...	Quantity ...
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	Undetermi...					
NTC	FAM-NFQ-...	38.453182					
UNKNOWN	FAM-NFQ-...	22.85404	22.761744	0.252	2,314.852	2,472.463	400.435
UNKNOWN	FAM-NFQ-...	22.476973	22.761744	0.252	2,927.722	2,472.463	400.435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



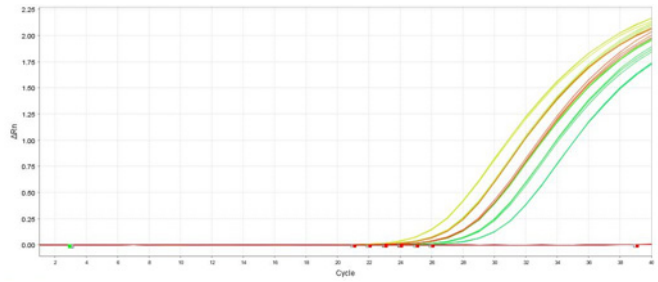
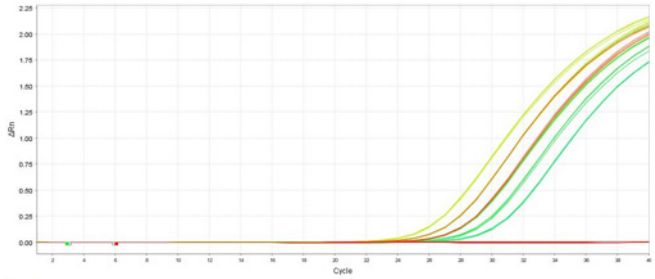
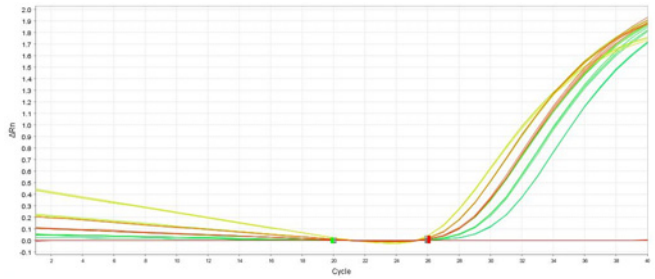
Threshold Set Too High

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 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 208).

  or

- Manually adjust the baseline and/or threshold (see "View the Analysis Settings" on page 204).

For More Information

For more information on the Amplification Plot screen, access the StepOne 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 plate layout
- Create slides
- Print a report
- Export data

For information on performing these procedures, access the StepOne Software Help by clicking  or pressing **F1**.

Notes

Section 9.2 Troubleshoot (If Needed)

This section covers:

Notes

View the Analysis Settings

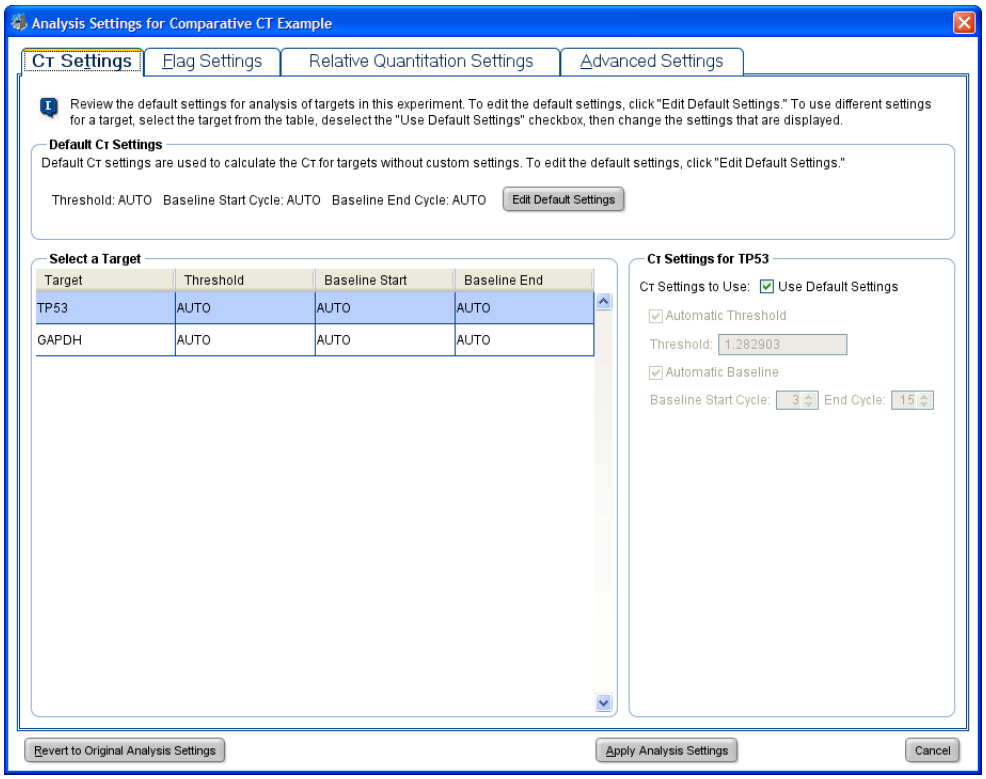
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, relative quantitation, and advanced options. If the default analysis settings in the StepOne software are not suitable for your experiment, you can change the settings in the Analysis Settings dialog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

1. From the Experiment Menu pane, select **Analysis**.
2. Click **Analysis Settings** to open the Analysis Settings dialog box.
3. In the example experiment, the default analysis settings are used for each tab:
   - C_T Settings
   - Flag Settings
   - Relative Quantitation Settings
   - Advanced Settings



Notes

Analysis Guidelines

Unless you have already determined the optimal settings for your experiment, use the default analysis settings in the StepOne 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 StepOne software.
- **Relative Quantitation Settings** – Use this tab to:
  - Change the reference sample and/or endogenous control.
  - Correct the amplification efficiency. You can enter a percentage value between 1% and 150%. When you set an assay to have a value that differs from 100% efficiency, the software uses the relative standard curve algorithm.
  - (For multiplex reactions) Specify the ΔC_T value at which to reject replicates (outlier rejection).
  - 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.

For More Information

For more information on the analysis settings, access the StepOne 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 StepOne 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 triggered by the experiment data. In the example experiment, no flags have been triggered.

View the QC Summary

1. From the Experiment Menu pane, select **Analysis** ▸ **QC Summary**.

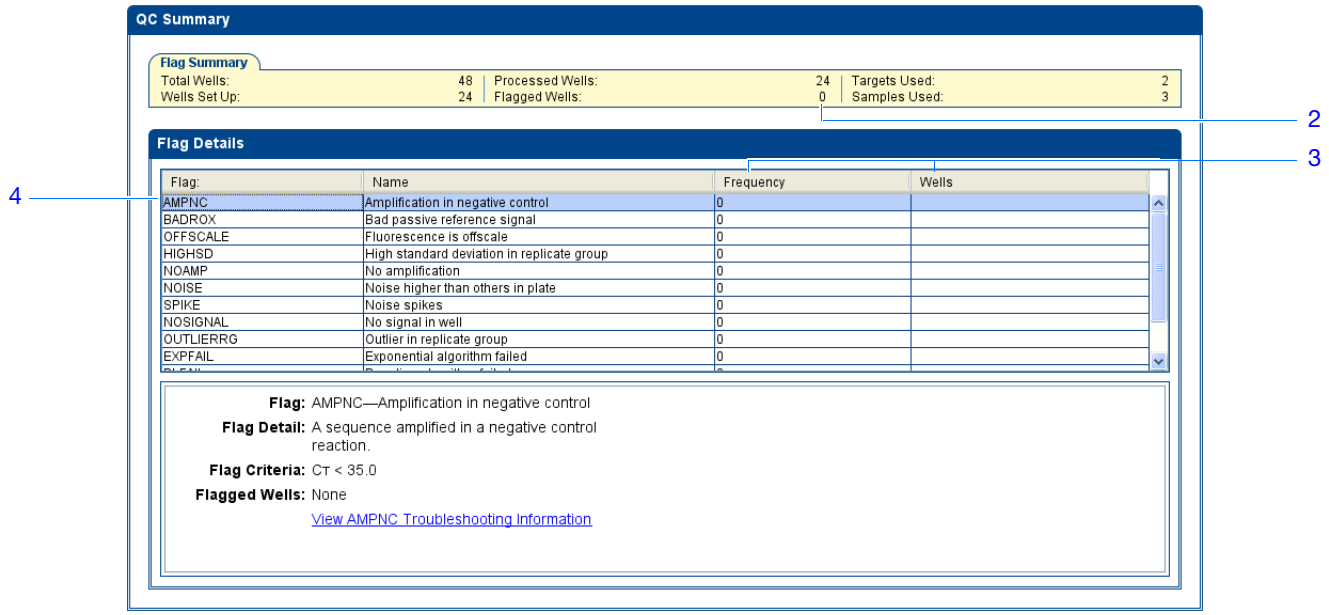
   Note: If no data are displayed, click **Analyze**.

2. Review the Flags Summary. In the example experiment, there are 0 flagged wells.

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 0 for all flags.

   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 detailed information about the flag.



Notes

Possible Flags

For comparative C_T experiments, the flags listed below may be triggered 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 only displayed 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 comparative C_T experiment:

- Click each flag in the Flag Details table with a frequency >0 to display detailed information 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 StepOne software.

For More Information

For more information on the QC Summary screen or on flag settings, access the StepOne 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

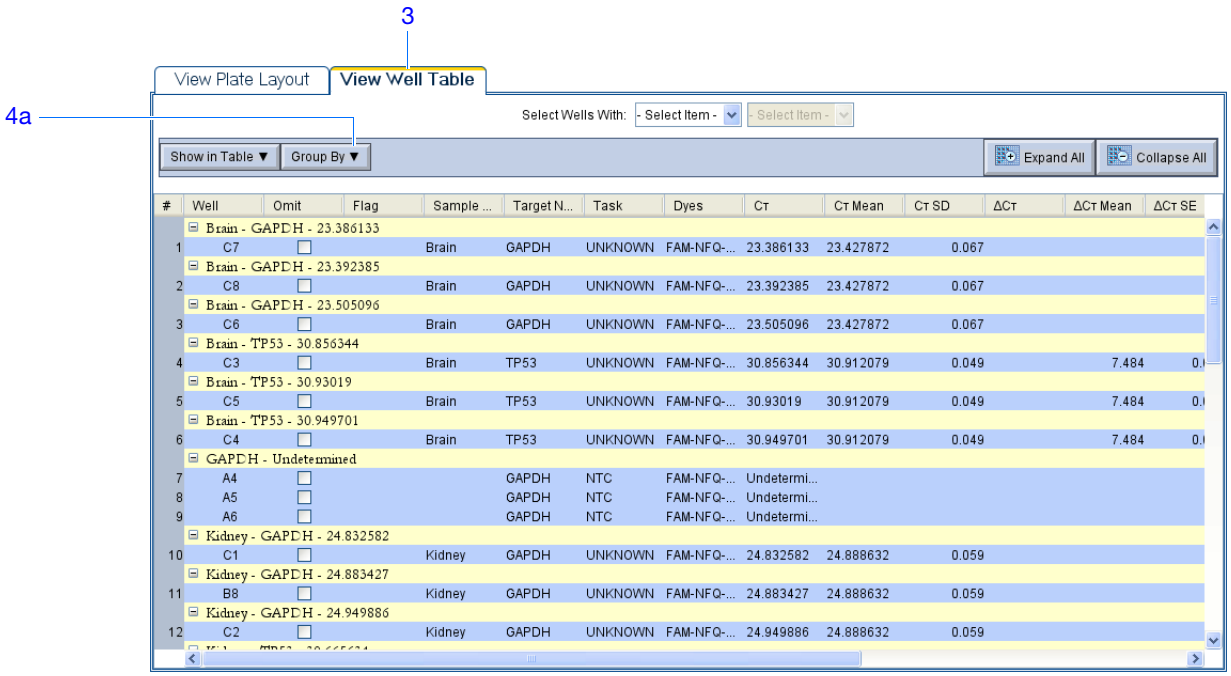
In the comparative C_T example experiment, there are no outliers; no wells need to be removed from analysis.

Omit Wells

1. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

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

2. In the Amplification Plot screen, select **C_T vs Well** from the Plot Type dropdown menu.
3. Select the **View Well Table** tab.
4. In the Well Table:
   a. From the Group By dropdown menu, 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 - GAPDH - 23.386133												
1	C7			Brain	GAPDH	UNKNOWN	FAM-NFQ-...	23.386133	23.427872	0.067			
	Brain - GAPDH - 23.392385												
2	C8			Brain	GAPDH	UNKNOWN	FAM-NFQ-...	23.392385	23.427872	0.067			
	Brain - GAPDH - 23.505096												
3	C6			Brain	GAPDH	UNKNOWN	FAM-NFQ-...	23.505096	23.427872	0.067			
	Brain - TP53 - 30.856344												
4	C3			Brain	TP53	UNKNOWN	FAM-NFQ-...	30.856344	30.912079	0.049		7.484	0.
	Brain - TP53 - 30.93019												
5	C5			Brain	TP53	UNKNOWN	FAM-NFQ-...	30.93019	30.912079	0.049		7.484	0.
	Brain - TP53 - 30.949701												
6	C4			Brain	TP53	UNKNOWN	FAM-NFQ-...	30.949701	30.912079	0.049		7.484	0.
	GAPDH - Undetermined												
7	A4				GAPDH	NTC	FAM-NFQ-...	Undetermi...					
8	A5				GAPDH	NTC	FAM-NFQ-...	Undetermi...					
9	A6				GAPDH	NTC	FAM-NFQ-...	Undetermi...					
	Kidney - GAPDH - 24.832582												
10	C1			Kidney	GAPDH	UNKNOWN	FAM-NFQ-...	24.832582	24.888632	0.059			
	Kidney - GAPDH - 24.883427												
11	B8			Kidney	GAPDH	UNKNOWN	FAM-NFQ-...	24.883427	24.888632	0.059			
	Kidney - GAPDH - 24.949886												
12	C2			Kidney	GAPDH	UNKNOWN	FAM-NFQ-...	24.949886	24.888632	0.059			

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. From the Experiment Menu pane, select **Analysis** ▸ **Amplification Plot**.

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

2. In the Amplification Plot screen, select **C_T vs Well** from the Plot Type dropdown menu.
3. Select the **View Well Table** tab.
4. In the Well Table:
   a. From the Group By dropdown menu, select **Replicate**.
   b. Look for any outliers in the replicate group (be sure they are flagged).
   c. Select the **Omit** checkbox 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, access the StepOne Software Help by clicking or pressing **F1**. Within the Help, search for the omit well topics:

1. Click 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 run.

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. From the Experiment Menu 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 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.

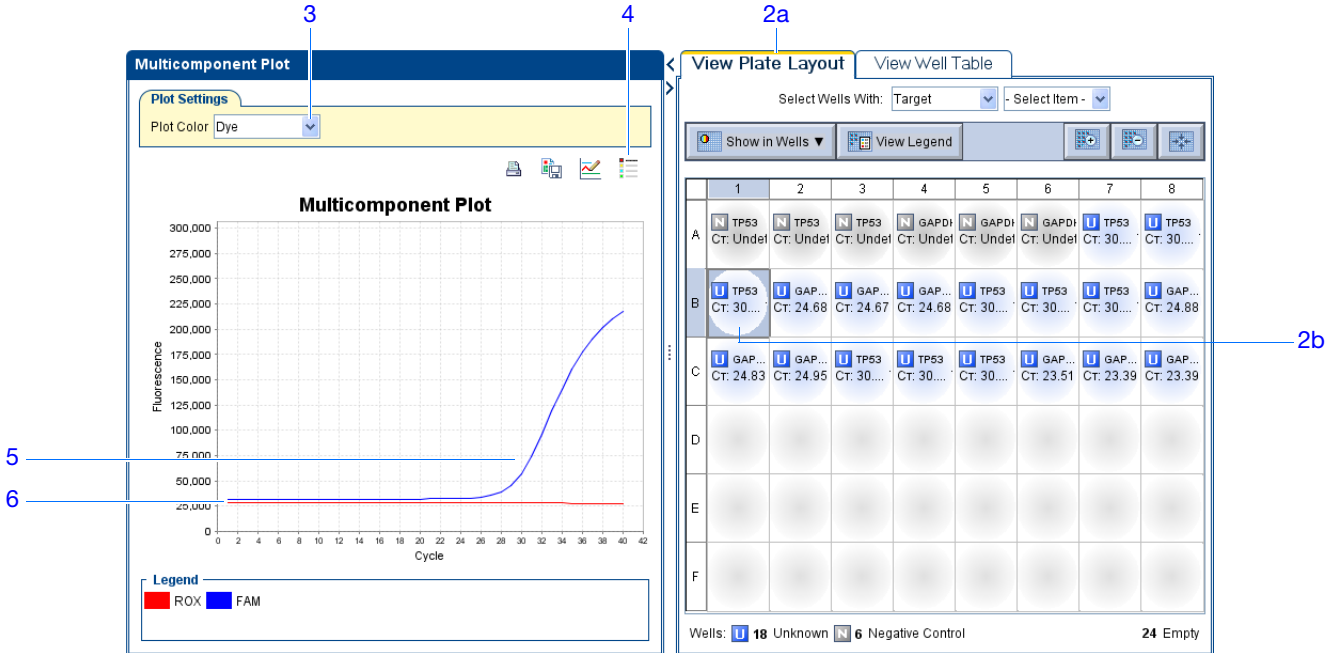
3. From the Plot Color dropdown menu, select **Dye**.

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

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

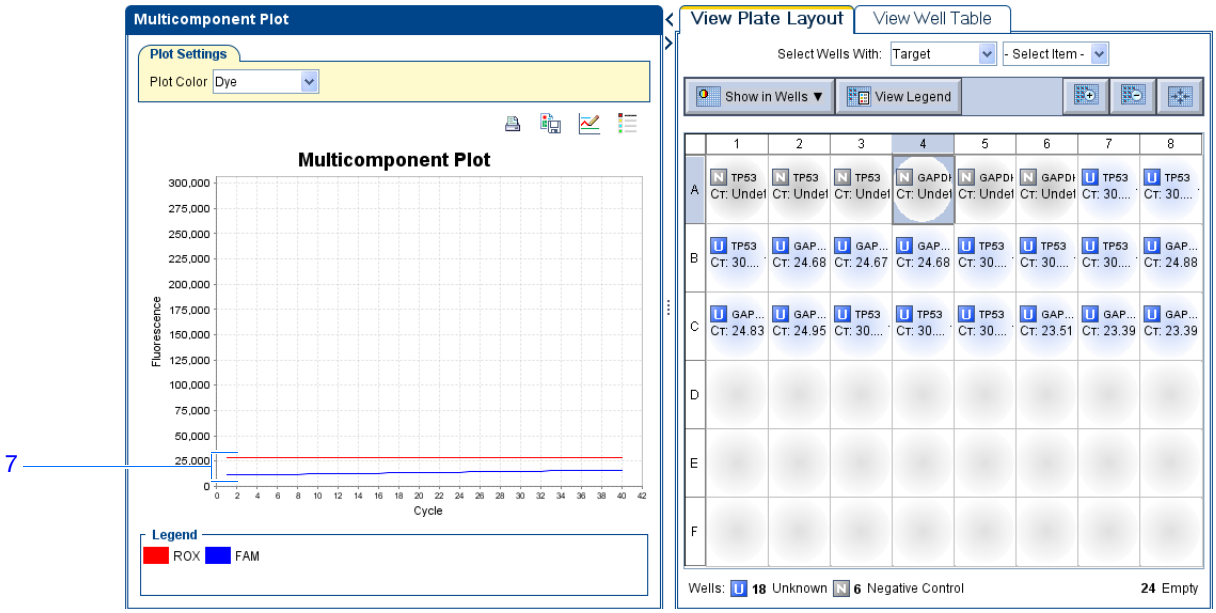
5. Check the FAM dye signal. In the example experiment, the FAM dye signal increases throughout the PCR process, which indicates normal amplification.

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



Notes

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



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 fluorescent signal.
- 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, access the StepOne Software Help by clicking or pressing **F1**.

Notes

View the Raw Data Plot

The Raw Data Plot screen displays the raw fluorescence signal (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 Raw Data Plot

1. From the Experiment Menu pane, select **Analysis** ▸ **Raw Data Plot**.

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

2. Display all 48 wells in the Raw Data Plot screen by clicking the upper left corner of the plate layout in the View Plate Layout tab.

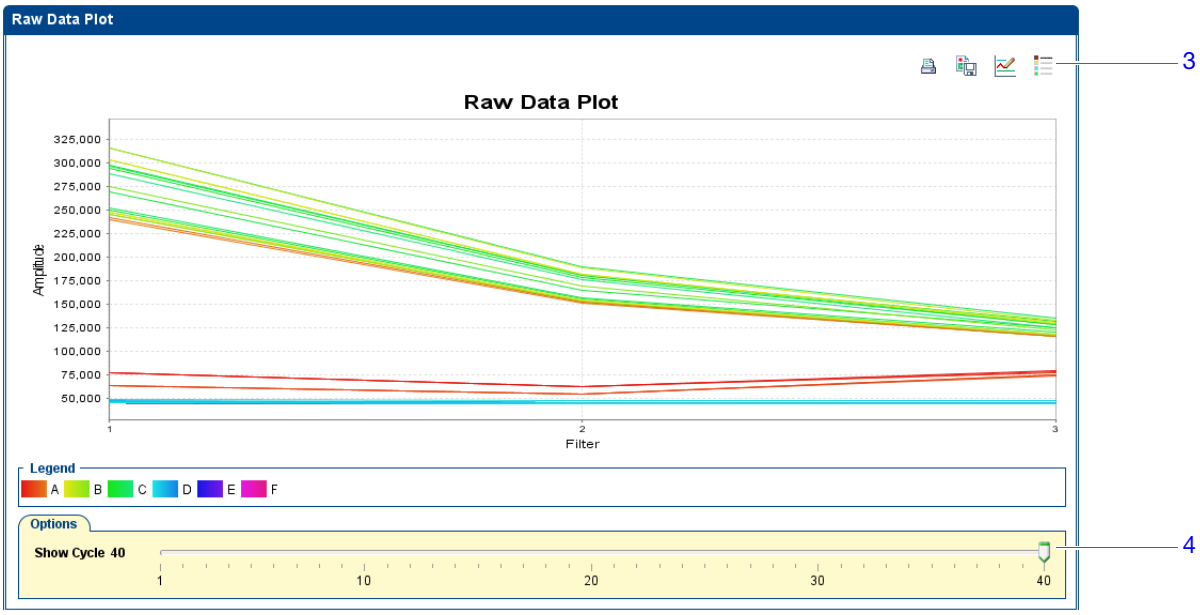
3. Click **Show a legend for the plot** (default).

   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.

4. Click and drag the Show Cycle pointer from cycle 1 to cycle 40. In the example experiment, there is a stable increase in signal from filter 1, which corresponds to the FAM™ dye filter.

Notes



The filters are:

StepOne system			StepOnePlus system	
Filter	**Dye**		**Filter**	**Dye**
1	FAM™ dye		1	FAM™ dye
	SYBR® Green dye			SYBR® Green dye
2	JOE™ dye		2	JOE™ dye
	VIC® dye			VIC® dye
3	ROX™ dye		3	TAMRA™ dye
				NED™ dye
			4	ROX™ 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, access the StepOne Software Help by clicking or pressing **F1**.

Notes

Notes

10 Design and Analyze a Study

This chapter covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

Notes

Chapter Overview

The StepOne™ software can combine the analysis of experiments that use the comparative C_T ($\Delta\Delta C_T$) quantitation method into a comparative C_T study. This chapter explains how to design and analyze multiple experiments as a study.

About Studies

In a comparative C_T study, you can...	You cannot...
• Specify the endogenous control(s) and reference sample for the study. • Set individual efficiency values for each target. • 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 individual comparative C_T experiments.

Note: For more information about methods of calculating relative quantitation, refer to the *Real-Time PCR Systems Reagent Guide*.

Notes

Example Study Workflow

The workflow for analyzing the example studies provided with this getting started guide is shown below.

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

Start Experiment

▼

Design the Experiment (Chapter 6)

▼

Prepare the Reactions (Chapter 7)

▼

Run the Experiment (Chapter 8)

▼

Analyze the Experiment (Chapter 9)

▼

Design and Analyze a Study (Chapter 10)

1. Design a study:
   a. Create a study.
   b. Define study properties.
   c. Define replicates.
2. Analyze a study:
   a. View the analysis settings.
   b. View the amplification plot.
   c. View the gene expression plot.
   d. View the replicate results data and well results data.
   e. View the multicomponent plot.
   f. View the quality summary.
   g. Compare the analysis settings.
   h. (If needed) Omit wells from the analysis.
3. Publish the data.

▼

End Experiment

Notes

Design a Study

This section explains how to use the StepOne software to set up a comparative C_T example study. It also describes Applied Biosystems recommended best practices as you design the study.

Create a New Study

To create a new study in the StepOne software:

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

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

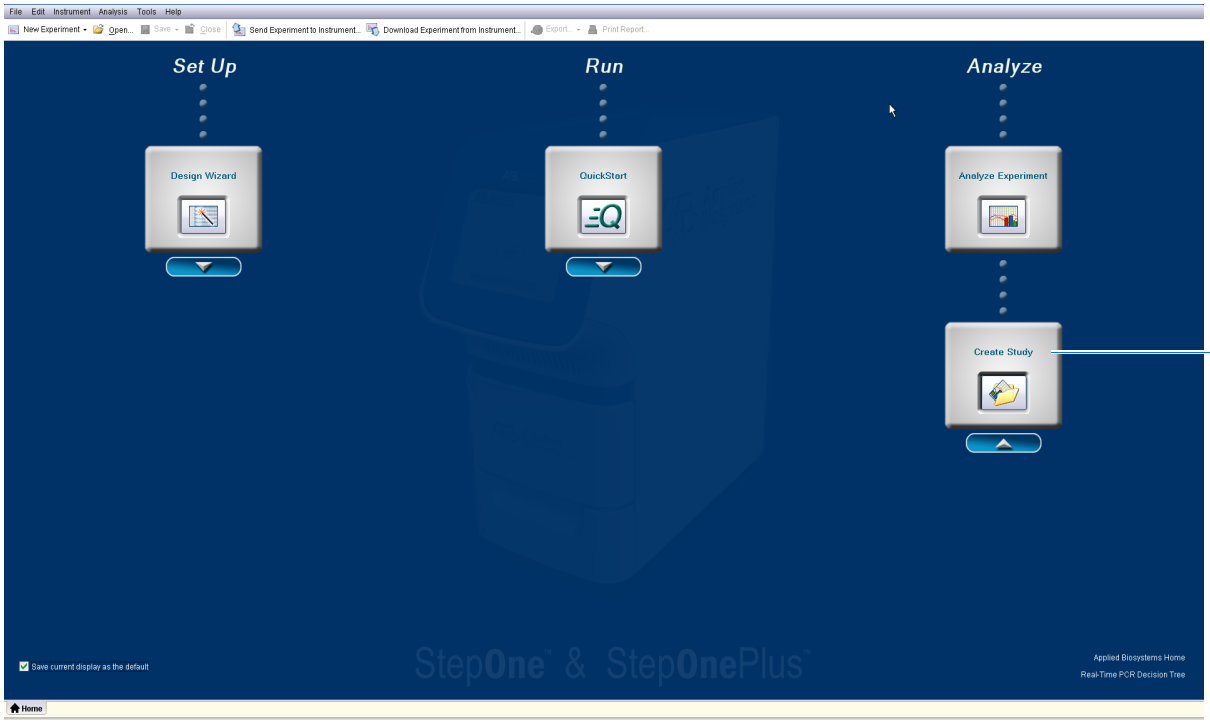
2. In the Login dialog box, select **EXAMPLEUSER** from the User Name dropdown menu, then click **OK**.

   Note: EXAMPLEUSER is the user name you created when you designed the comparative C_T experiment (page 135).

3. From 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



4. See "Software Elements" on page 220 for information on navigating within a study.

Design Guidelines

When you design your own study, you can do one of the following in the Login dialog box:

- Log in as a new user – In the User Name field, enter a user name, then click **OK**.

  Note: You cannot use the following characters in the User Name field: space, forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), or semicolon (;).

- Log in as an existing user – From the User Name dropdown menu, select an existing user, then click **OK**.
- Log in anonymously – Click **Log in as Guest**.

Applied Biosystems recommends that you log in with a user name. If you log in with a user name, you can set preferences in the software. The next time you log in to the software with the same user name, the software uses the preferences you set as the defaults.

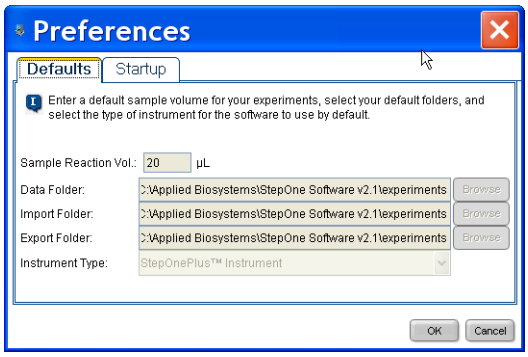
IMPORTANT! If you log in to the software as a Guest, you cannot set preferences.

You can set preferences as follows:

- **Save as the default checkboxes** – Select the **Save as the default** checkboxes as needed. The Save as the default checkboxes appear on the Home screen, on the Export Data dialog box, and on several Analysis screens.

Notes

- **Preferences dialog box** – Select **Tools** ▸ **Preferences** to open the Preferences dialog box. In the Defaults and/or Startup tabs, change the preferences as desired.

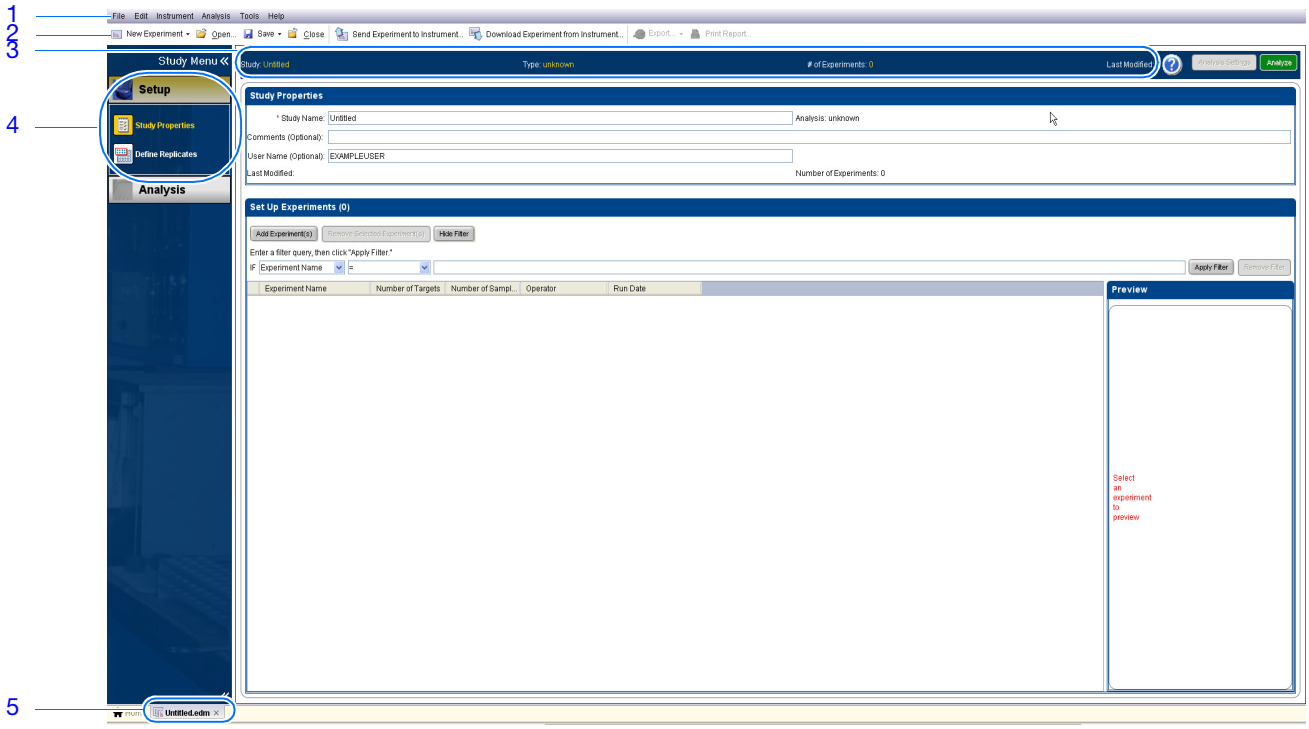


Software Elements

The StepOne software elements for the study are illustrated below.

1. Menu bar – Displays the menus available in the software:
   - File
   - Edit
   - Instrument
   - Analysis
   - Tools
   - Help
2. Toolbar – Displays the tools available in the software:
   - New Experiment
   - Open
   - Save
   - Close
   - Send Experiment to Instrument
   - Download Experiment from Instrument
3. Study header – Displays the study name, the number of experiments in the study, and the date/time that the study was last modified.
4. Study Menu pane – Provides links to the following Setup screens:
   - Study Properties
   - Define Replicates
5. Study/experiment tab(s) – Displays a tab for each open study or experiment.

Notes


1
2
3
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5
File Edit Instrument Analysis Tools Help
New Experiment
Open...
Save
Close
Send Experiment to Instrument...
Download Experiment from Instrument...
Export...
Print Report...
Study Menu
Setup
Study Properties
Define Replicates
Analysis
Study: Untitled
Type: unknown
# of Experiments: 0
Last Modified:
Analysis Settings
Analyze
Study Properties
* Study Name: Untitled
Analysis: unknown
Comments (Optional):
User Name (Optional): EXAMPLEUSER
Last Modified:
Number of Experiments: 0
Set Up Experiments (0)
Add Experiment(s)
Remove Selected Experiment(s)
Hide Filter
Enter a filter query, then click "Apply Filter."
IF Experiment Name =
Apply Filter
Remove Filter
Experiment Name
Number of Targets
Number of Sampl...
Operator
Run Date
Preview
Select an experiment to preview
Untitled.edm

Notes

Define Study Properties

In the Study Properties screen, enter identifying information for the study, then add experiments to the study.

About the Example Study

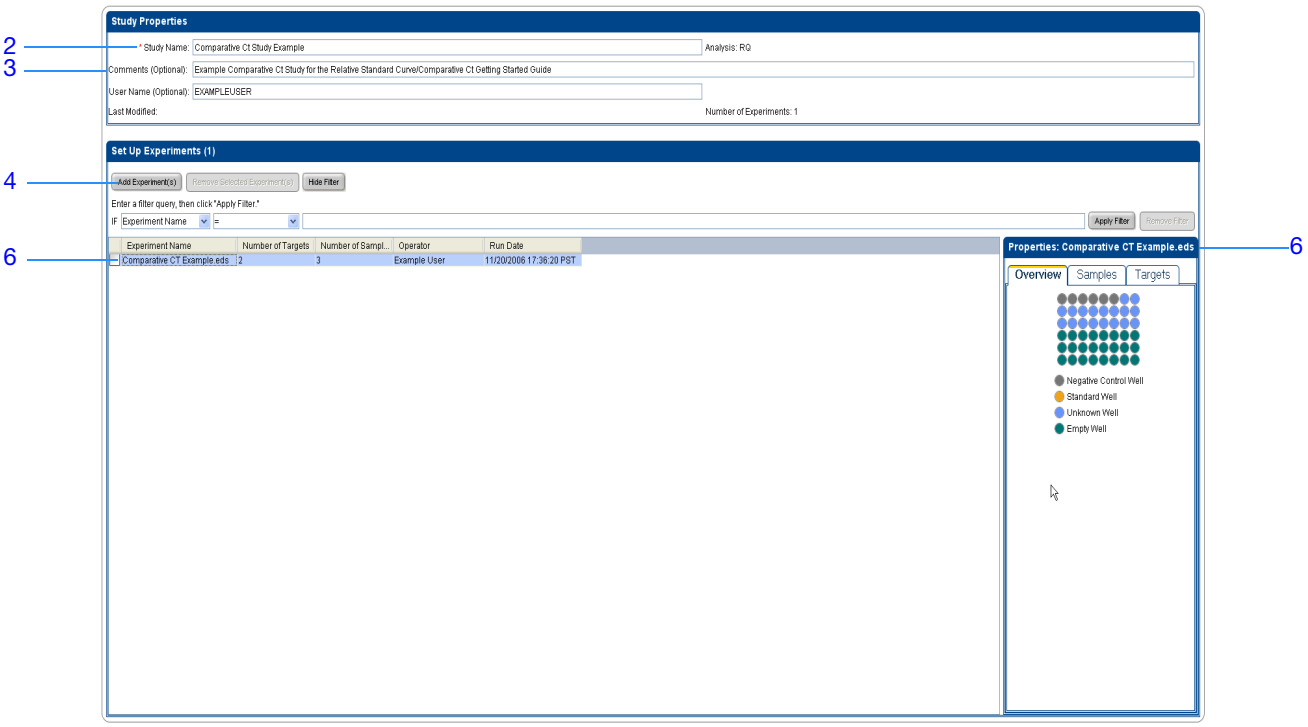
In the example study, the:

- Study is identified as an example.
- Experiment that you add to the study is the same example experiment that you analyzed in Chapter 9 of this guide.

Complete the Study Properties Screen

1. In the Study Menu 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. In the Set Up Experiments pane, click **Add Experiment(s)**.
5. In the Open dialog box, open the **Comparative CT Example.eds** file at:

   <drive>:\Applied Biosystems\<*software name*>\experiments\examples
6. In the Set Up Experiments table, select the **Comparative CT Example** experiment. The StepOne software displays the details of the experiment in the Properties pane.



Notes

Design Guidelines

When you design your own comparative C_T study:

- Enter a study name that is descriptive and easy to remember. You can enter up to 100 characters in the Study Name field. The study name is used as the default file name.

  Note: You cannot use the following characters in the Study Name field: forward slash (/), backslash (\), greater than sign (>), less than sign (<), asterisk (*), question mark (?), quotation mark ("), vertical line (|), colon (:), semicolon (;), and sign (&), percent sign (%), dollar sign ($), at sign (@), circumflex (^), left parenthesis (( ), right parenthesis ( )), or exclamation point (!).

- (Optional) Enter comments to describe the study. You can enter up to 1000 characters in the Comments field.
- Use the default user name, or enter a new user name, to identify the owner of the study. You can enter up to 100 characters in the User Name field.

  Note: The User Name field is automatically populated with the name you selected at log in.

- You can add an unlimited number of comparative C_T experiments (reaction plates) to the study. To add experiments to a study, note the following:
  - Each experiment in the study must:
    - Have one or more common endogenous control(s). The endogenous control(s) must be present on each reaction plate within the study.
    - Have identical thermal cycling parameters (the same number of steps, cycles, sample volume, and emulation mode). The StepOne software cannot combine in the same study experiments that use Fast and standard thermal cycling conditions.
    - Have been run *and analyzed* on the same type of instrument (that is, you cannot add experiments from a StepOne instrument and experiments from a StepOnePlus instrument to the same study).
    - Have the same passive reference.
  - As the default, the StepOne software assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment that you added to the study.
  - If experiments that contain biological replicate groups are added to a study, the StepOne software automatically merges the matching biological groups.
  - The StepOne software automatically analyzes a study after you add more than one experiment 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.
- 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.

Notes

- Filter the experiments added to the study to simplify the list for easier review. See "How to Filter the Experiments Added to the Study" below.

How to Filter the Experiments Added to the Study

To narrow your search for an experiment, define and apply a filter:

1. In the left-most dropdown menu, select an experiment attribute to query.
2. In the center dropdown menu, select an operator for the query.
3. In the right-most field, enter the condition to look for, then click **Apply Filter**.

Note: After you apply 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 Study Properties screen – Open the StepOne Software Help by clicking or pressing **F1**.
- Quantitation experiments – Refer to the *Real-Time PCR Systems Reagent Guide*.

Notes

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. Including biological replicates can give insight into any natural variation that is in the population.

About the Example Study

The comparative C_T example study does not contain biological replicate groups. You will create and apply the biological group "LiverGroup" as an exercise.

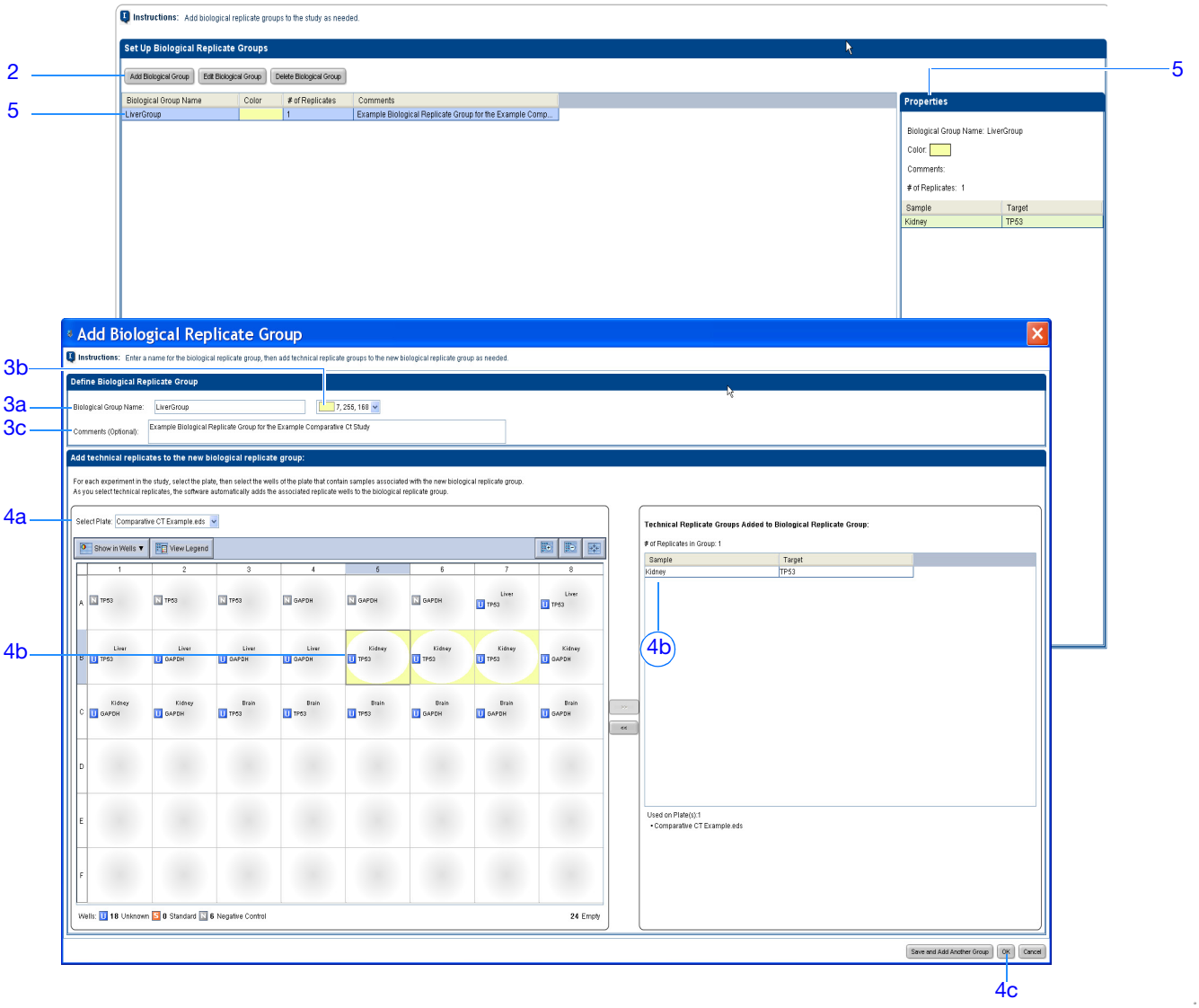
Complete the Define Replicates Screen

1. In the Study Menu pane, select **Setup** ▸  **Define Replicates**.
2. Click **Add Biological Group** to open the Add Biological Replicate Group dialog box.
3. Define the biological replicate group:
   a. Click the **Biological Group Name** field, then enter **LiverGroup**.

      Note: In the example study, the LiverGroup 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**.
4. Add technical replicates:
   a. From the Select Plate dropdown menu, select **Comparative CT Experiment.eds**.
   b. In the plate layout, select well **B5**, then click >> to add the technical replicate wells that are associated with the selected well to the biological group.
   c. Click **OK**.
5. Select the **LiverGroup** group that you just added to the study. The StepOne software displays the details of the biological group in the Properties pane.

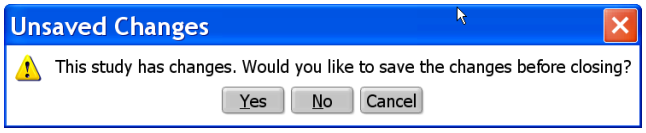
Notes



6. Click **Analyze**, then close the study:

   a. Select **File** ▸ **Close**.

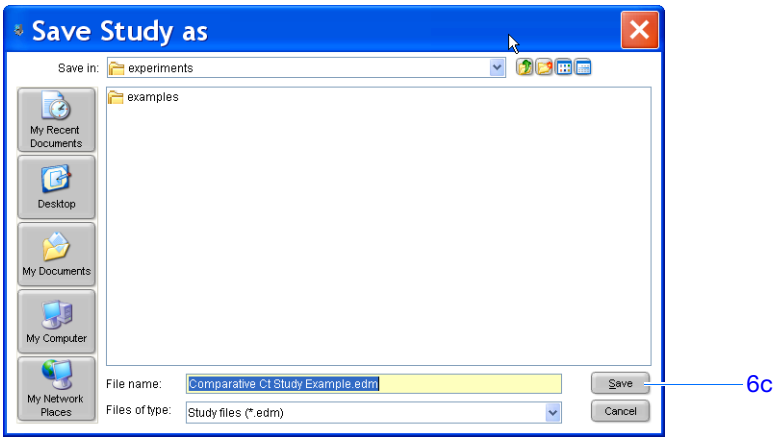
   b. At the prompt, click **Yes**.



Notes

c. In the Save Study as dialog box, click **Save** to accept the default file name and location. The example study is saved and closed, and you are returned to the Home screen.

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



Design Guidelines

When you design your own comparative C_T study:

- Enter a biological replicate group name that is descriptive and easy to remember. You can enter up to 100 characters in the Biological Group Name field. You cannot use the following characters in the Biological Group Name field: / \ > < * ? " | : ;
- (Optional) Enter comments to describe the biological replicate group. You can enter up to 1000 characters in the Comments field.
- You can 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 wells.
- You can 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" on page 228.
- You can remove an existing biological replicate group. See "Remove Biological Replicate Groups" on page 228.

IMPORTANT! After you remove a biological replicate group from a study, you cannot restore it.

Notes

Edit a Biological Replicate Group

1. Open the study of interest.
2. In the Study Menu pane, select **Setup** ▸  **Define Replicates**.
3. Click **Edit Biological Group** to open the Edit Biological Replicate Group dialog box.
4. Edit the group information:
   a. From the Biological Group Name dropdown menu, select the group to edit.
   b. To change the group name, click **Edit**, enter a new name, then click **OK**.
   c. To change the color, select a color from the dropdown menu.
   d. (Optional) Enter comments.
5. From the Select Plate dropdown menu, select the experiment of interest.
6. Add samples to the biological group:
   a. In the Plate Layout, select the well(s) of the plate that contain samples to add to the biological group.
   b. Click >> to add the samples that are associated with the selected wells to the biological group.
7. Remove samples from the biological group:
   a. In the Technical Replicate Groups Added to Biological Replicate Group pane, select a sample. You can select only one sample at a time.
   b. Click << to remove the sample from the biological group.
8. Repeat steps 5 through 7 for the other experiments in the study.
9. Click **OK** to save the changes and return to the study; click **Cancel** to exit the dialog box without saving the changes.

Remove Biological Replicate Groups

1. Open the study of interest.
2. In the Study Menu pane, select **Setup** ▸  **Define Replicates**.
3. In the Define Replicates screen, select the group to remove, then click **Delete Biological Group**.
4. Click **Yes** to remove the group from the study; click **No** to keep the group in the study.

Notes

Analyze the Study

This section explains how to use the StepOne 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 does not demonstrate the use of biological replicate groups. However, an additional example study that uses biological replicate groups is provided with the StepOne software. You can find this study file on your computer at: *<drive>*:\Applied Biosystems*<software name>*\experiments\examples\Comparative CT Study Bio Replicates Example.edm.

About the Example Study

For the comparative C_T example study, use the data file that is installed with the StepOne software. The data file was created with the same design parameters that are provided in "Design a Study" on page 218.

You can find the data file for the example study on your computer at:

<drive>:\Applied Biosystems*<software name>*\experiments\examples\Comparative CT Study Example.edm

where:

<drive> is the computer hard drive on which the StepOne software is installed. The default installation drive for the software is the D drive.

<software name> is the current version of the StepOne software.

Open the Example Study

IMPORTANT! If you use the same computer to collect run data and to analyze data, Applied Biosystems recommends that you do not analyze studies during a run.

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

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

2. In the Login dialog box, select **EXAMPLEUSER** from the User Name dropdown menu, then click **OK**.

   Note: EXAMPLEUSER is the user name you created when you designed the comparative C_T experiment (page 135).

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

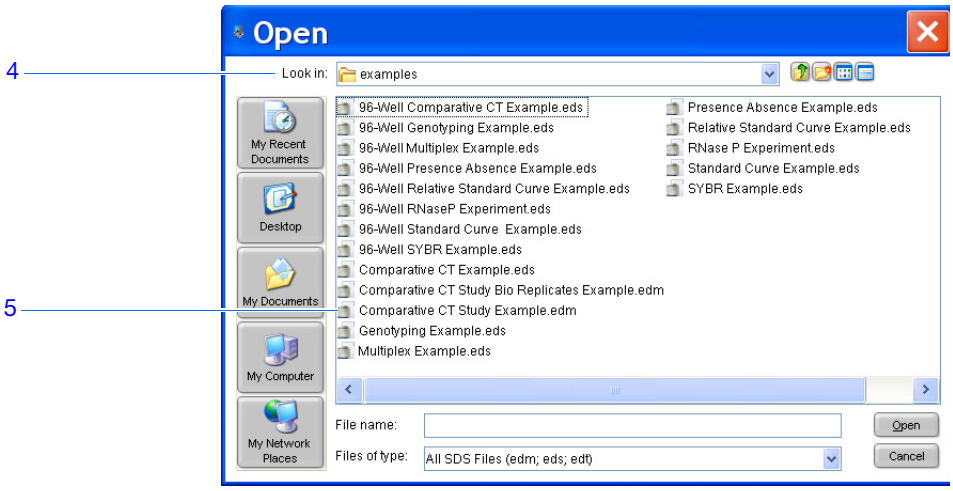
4. In the Open dialog box, navigate to the **examples** folder at:

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

Notes

5. 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**.



6. See "Software Elements" below and "Navigation Tips" on page 232 for information on navigating within a study.

Software Elements

The StepOne software elements for the study are illustrated below.

1. Menu bar – Displays the menus available in the software:
   - File
   - Edit
   - Instrument
   - Analysis
   - Tools
   - Help

2. Toolbar – Displays the tools available in the software:
   - New Experiment
   - Open
   - Save
   - Close
   - Send Experiment to Instrument
   - Download Experiment from Instrument
   - Export
   - Print Report

Notes

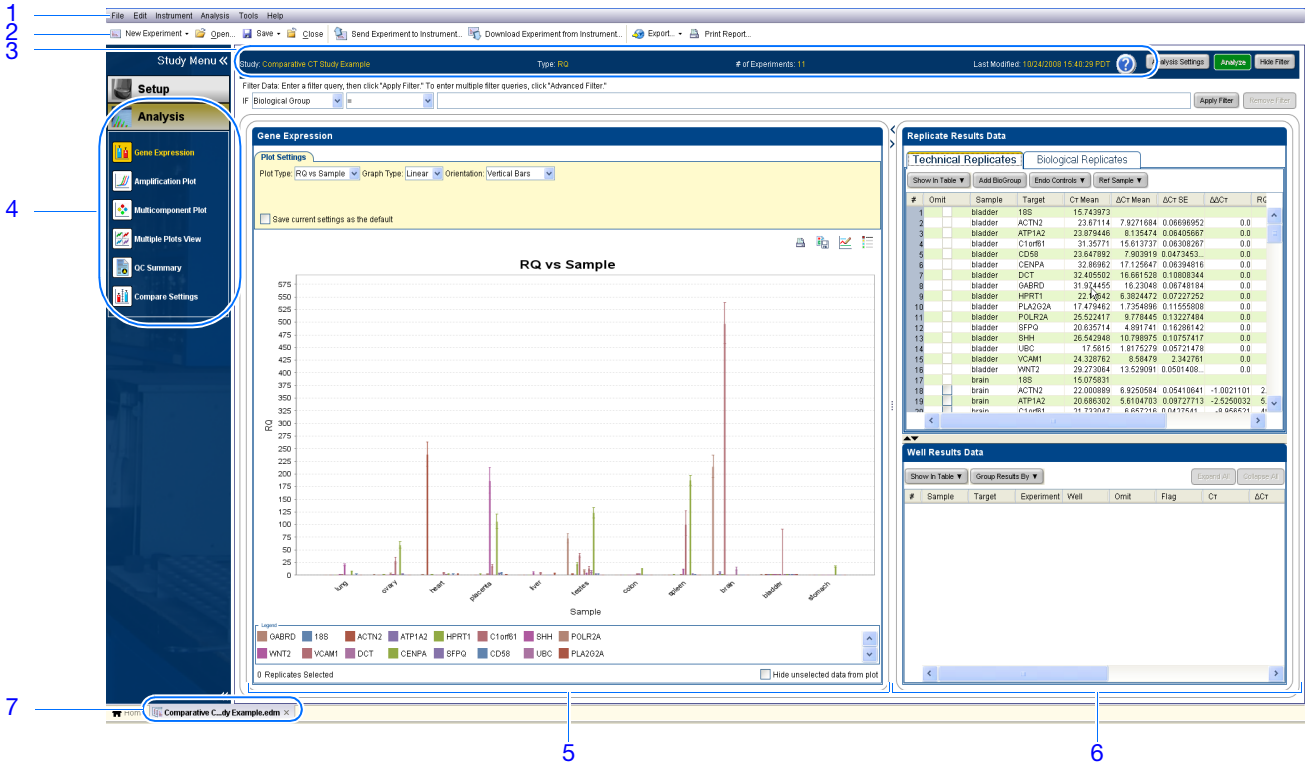
3. Study header – Displays the study name, the number of experiments in the study, and the date/time that the study was last modified.

4. Study Menu pane – Provides links to the following Analysis screens:
   - Gene Expression
   - Amplification Plot
   - Multicomponent Plot
   - Multiple Plots View
   - QC Summary
   - Compare Settings

5. Plot pane – Displays the selected analysis screen for the open study.

6. Replicate Results Data pane – For the open study, displays results data for the technical replicate groups (Technical Replicates tab) and, if present, the biological replicate groups (Biological Replicates tab).

7. Study/experiment tab(s) – Displays a tab for each open study or experiment.



Notes

Navigation Tips

How to Filter the Data Displayed in the Analysis Screens

To reduce the data displayed in the analysis screens, use the filter function at the top of the analysis screen to define and apply a filter:

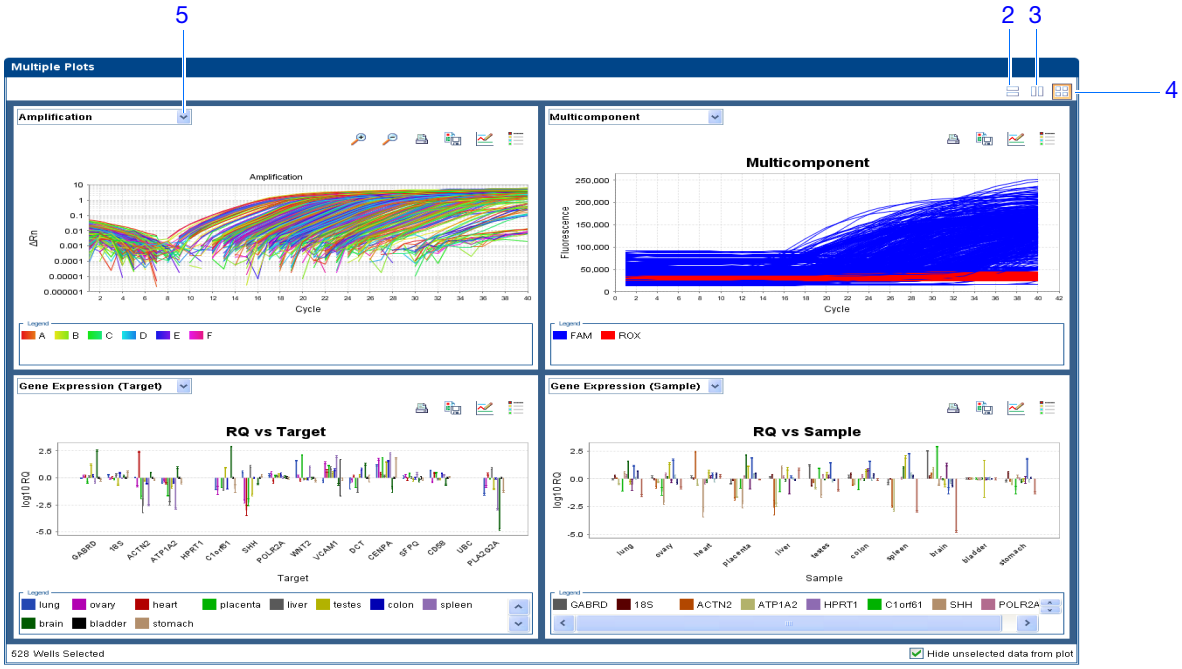
1. In the left-most drop-down list, select an attribute to query.
2. In the center drop-down list, select an operator for the query.
3. In the right-most field, enter the condition to look for, then click **Apply Filter**.

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.

How to Display Multiple Plots

Use the Multiple Plots View screen to display up to four plots simultaneously. To navigate within the Multiple Plots View screen:

1. From the Study Menu pane, select **Analysis** ▸ **Multiple Plots View**.
2. To display two plots in rows, click **Show plots in two rows**.
3. To display two plots in columns, click **Show plots in two columns**.
4. To display four plots, click **Show plots in a 2 × 2 matrix**.
5. To display a specific plot, select the plot from the dropdown menu above each plot display.

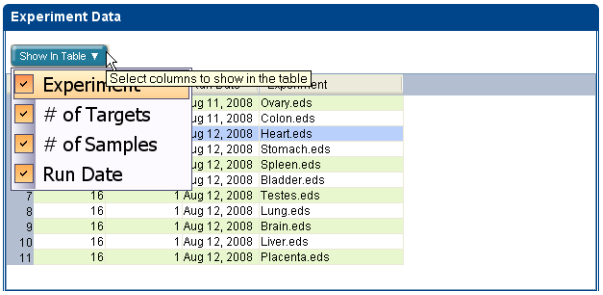


Notes

How to Use the Experiment Data and Well Results Data Panes

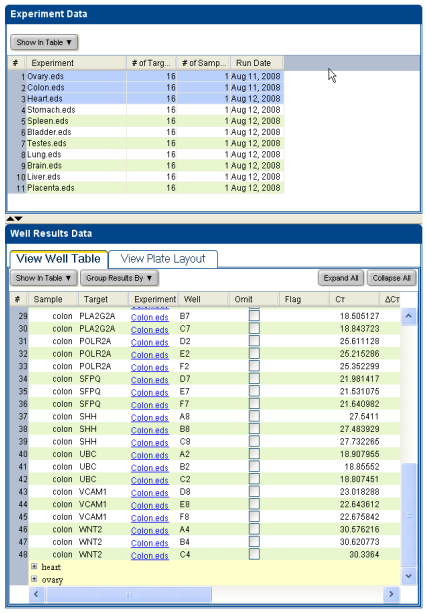
The Experiment Data and Well Results Data panes appear in the Amplification Plot, Multicomponent Plot, and Multiple Plots View screens.

- To display or hide columns in the Experiment Data pane: From the Show In Table dropdown menu, select or deselect one or more options: **Experiments**, **# of Targets**, **# of Samples**, **Run Date**.



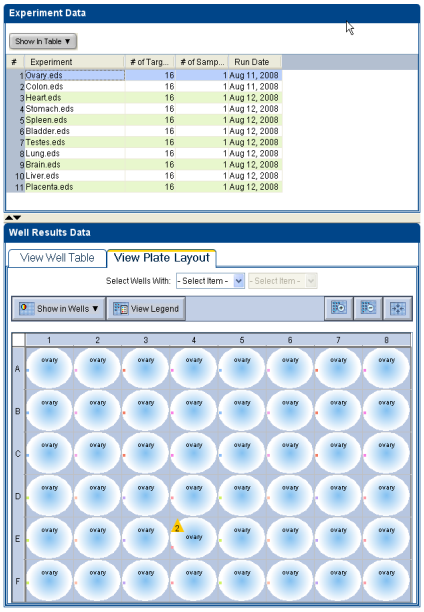
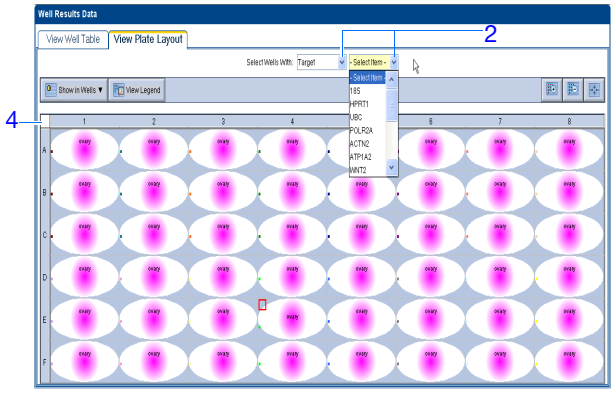
- To display a subset of the study data in the plots: Select one or more rows in the Experiment Data pane or the Well Results Data pane, then select **Hide unselected data from plot** to display data only from the selected rows.

The Experiments Data pane lists each reaction plate (experiment) that is added to a study. The data that are displayed in the Well Results Data pane depend on which tab you select in the Experiment Data pane:

Tab	Description	Illustration
View Well Table tab	When you select one or more experiments in the Experiment Data pane, the well table displays the wells that make up the selected experiment(s).	

10

Notes

Tab	Description	Illustration
View Plate Layout tab	When you select one experiment in the Experiment Data pane, the plate layout displays the plate layout for the selected experiment. **Note:** If you select more than one experiment in the Experiment Data pane, only the plate layout for the first experiment is displayed in the View Plate Layout tab.	
	Using the Plate Layout tab: 1. To select wells of a specific type, use the Select Wells With dropdown menus: 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 and drag over the desired wells, press **CTRL+click**, or press **Shift+click** in the plate layout. 4. To select all wells, click the upper left corner of the plate layout.	

Notes

View the Analysis Settings

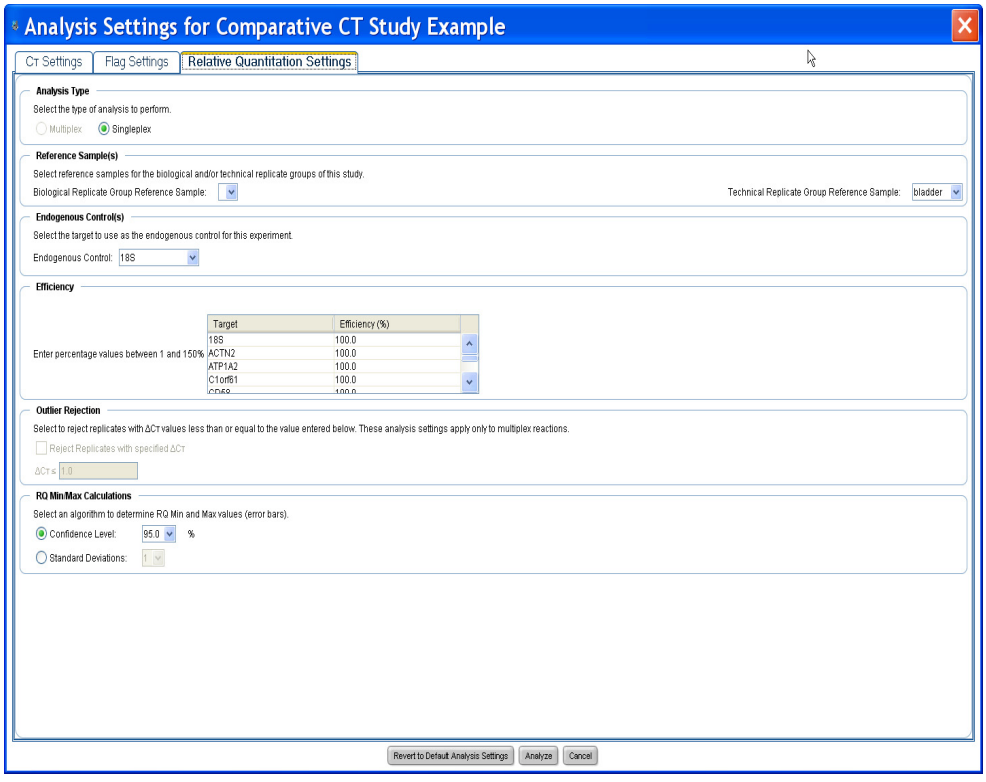
The Analysis Settings dialog box displays the analysis settings for the threshold cycle (C_T), flags, and relative quantitation. If the default analysis settings in the StepOne software are not suitable for your study, you can change the settings in the Analysis Settings dialog box, then reanalyze your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

About the Example Study

In the comparative C_T example study, the default analysis settings are used without changes.

View the Analysis Settings

1. From the Study Menu pane, select **Analysis**.
2. Click **Analysis Settings** to open the Analysis Settings dialog box.
3. Select the **Relative Quantitation Settings** tab, then view the default reference sample and endogenous control. In the example study, the default reference sample is bladder and the default endogenous control is 18S.
4. Select the **C_T Settings** tab, then the **Flag Settings** tab. In the example study, the default analysis settings are used in each tab.
5. Click **Analyze**.



Notes

Analysis Guidelines

Unless you have already determined the optimal settings for your study, use the default analysis settings in the StepOne software. If the default settings are not suitable for your study, 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 StepOne software.
- **Relative Quantitation Settings** – Use this tab to:
  - Change the reference sample and/or endogenous control. As the default, the StepOne software assigns the endogenous control and reference sample for a study based on the analysis settings of the first experiment added to it.
  - Correct the amplification efficiency. You can enter a percentage value between 1% and 150%. When you set an assay to have a value that differs from 100% efficiency, the software uses the relative standard curve algorithm.
  - (For multiplex reactions) Specify the ΔC_T value at which to reject replicates (outlier rejection).
  - Select the algorithm to use to determine RQ Min/Max values (confidence level or standard deviations).

For More Information

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

Notes

View the Amplification Plot

The Amplification Plot screen displays post-run amplification of the samples of each experiment that is added to a study. Three plots are available:

- **ΔRn vs Cycle** – ΔRn is the magnitude of normalized fluorescence signal 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 signal from the reporter dye normalized to the fluorescence signal 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 viewed as the following graph types: linear or log10.

About the Example Study

In the comparative C_T example study, you review each target in the Amplification Plot screen for correct baseline and threshold values.

View the Amplification Plot

1. From the Study Menu pane, select **Analysis** ▸ **Amplification Plot**.

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

2. In the Experiment Data pane, select all of the experiments (click and drag to select all rows in the table).

3. In the Amplification Plot pane, set the parameters for the plot:

   a. From the Plot Type dropdown menu, select **ΔRn vs Cycle**.

   b. From the Plot Color dropdown menu, 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.

   d. From the Target dropdown menu, select **18S** to highlight all 18S wells in the study.

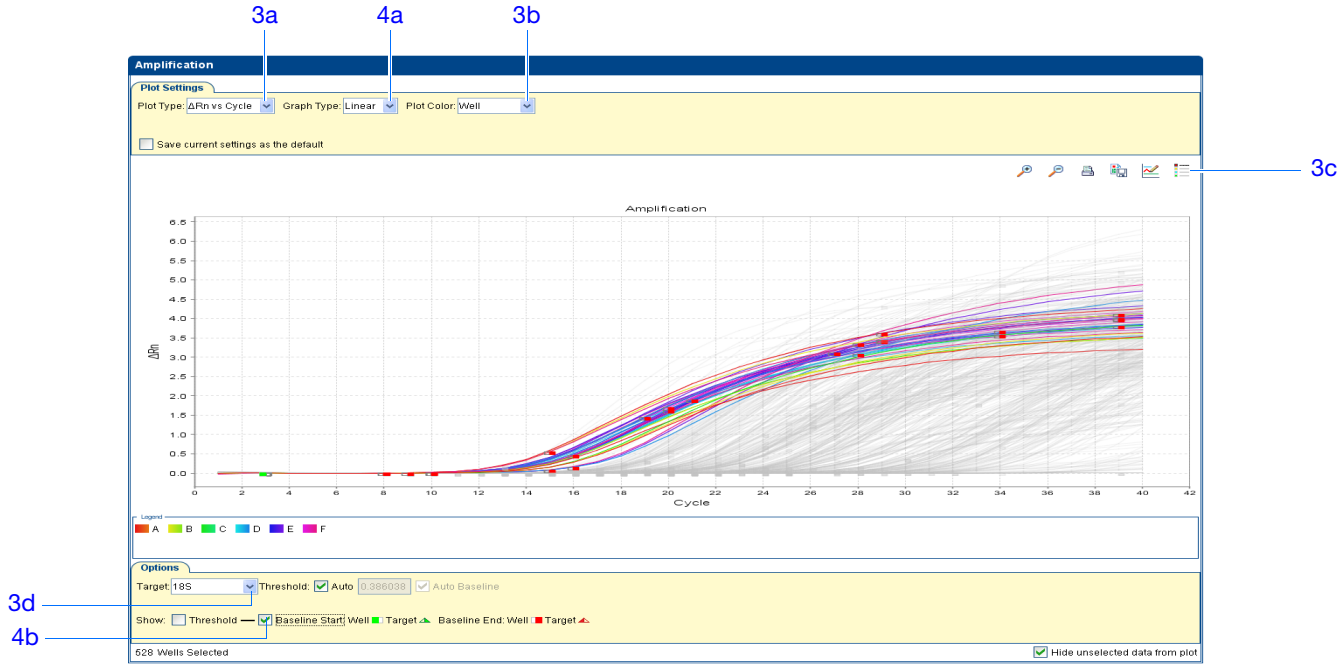
4. View the baseline values:

   a. From the Graph Type dropdown menu, select **Linear**.

   b. Select the **Show Baseline** checkbox to show the start cycle and end cycle.

Notes

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 study, the baseline is set correctly.



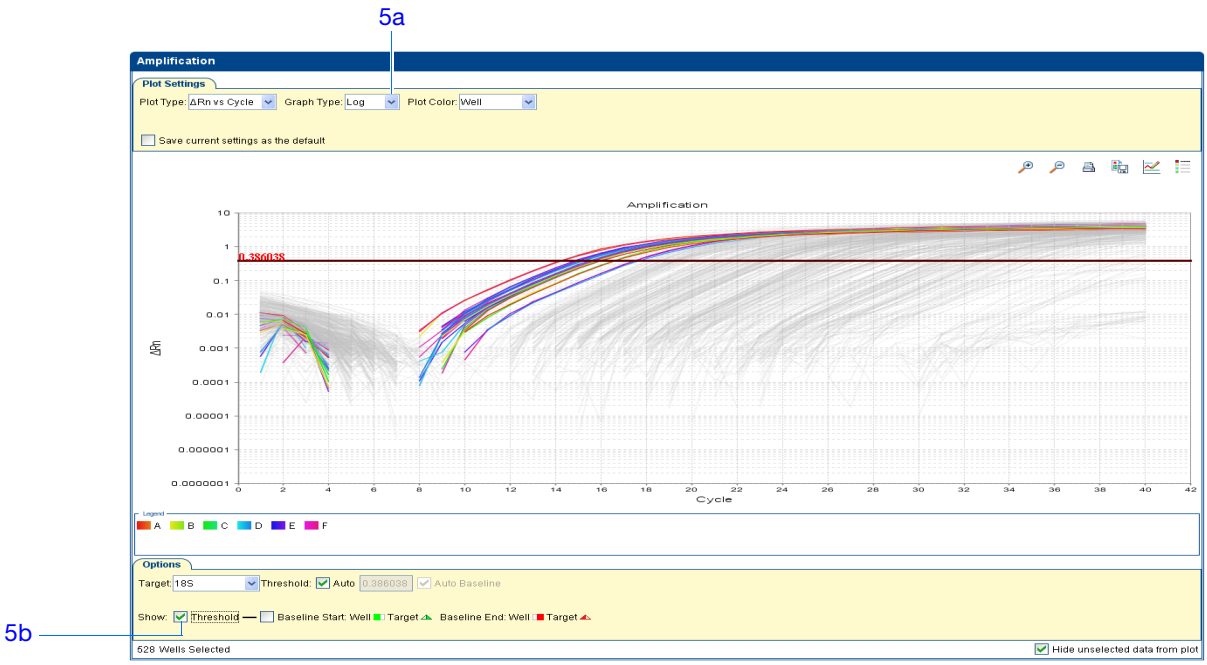
5. View the threshold values:

a. From the Graph Type dropdown menu, select **Log**.

b. Deselect the **Show Baseline** checkbox, then select the **Show Threshold** checkbox to show the threshold.

c. Verify that the threshold is set correctly. In the example study, the threshold is in the exponential phase.

Notes



6. Repeat steps 3 through 5 for the remaining targets.

Analysis Guidelines

When you analyze your own comparative C_T study, look for:

- A typical amplification plot – See the amplification plot example on pages 198 to 199.
- Correct baseline and threshold values – See the threshold examples on page 200 and the baseline examples on page 201.

If your study does not meet the guidelines above, you can:

- Manually adjust the baseline and/or threshold. See "View the Analysis Settings" on page 235.
- Omit individual wells from the analysis. See "(If Needed) Omit Wells from the Analysis" on page 257.

Note: For tips on navigating within the analysis screens, see "Navigation Tips" on page 232.

For More Information

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

Notes

View the Gene Expression Plot

The Gene Expression Plot screen displays the results of the relative quantitation calculations in the gene expression profile. Three 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 the following graph types: linear, log10, Ln, log2.
- **RQ vs Sample** (displayed only when the Technical Replicates tab is selected in the Replicate Results Data pane) – Groups the relative quantitation (RQ) values by sample. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.
- **RQ vs BioGroup** – (displayed only when the Biological Replicates tab is selected in the Replicate Results Data pane) – Groups the relative quantitation (RQ) values by biological replicate group. Each target is plotted for each sample. You can view the plot as the following graph types: linear, log10, Ln, log2.

Note: The Comparative CT Example Study.edm does not contain biological replicate groups.

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.

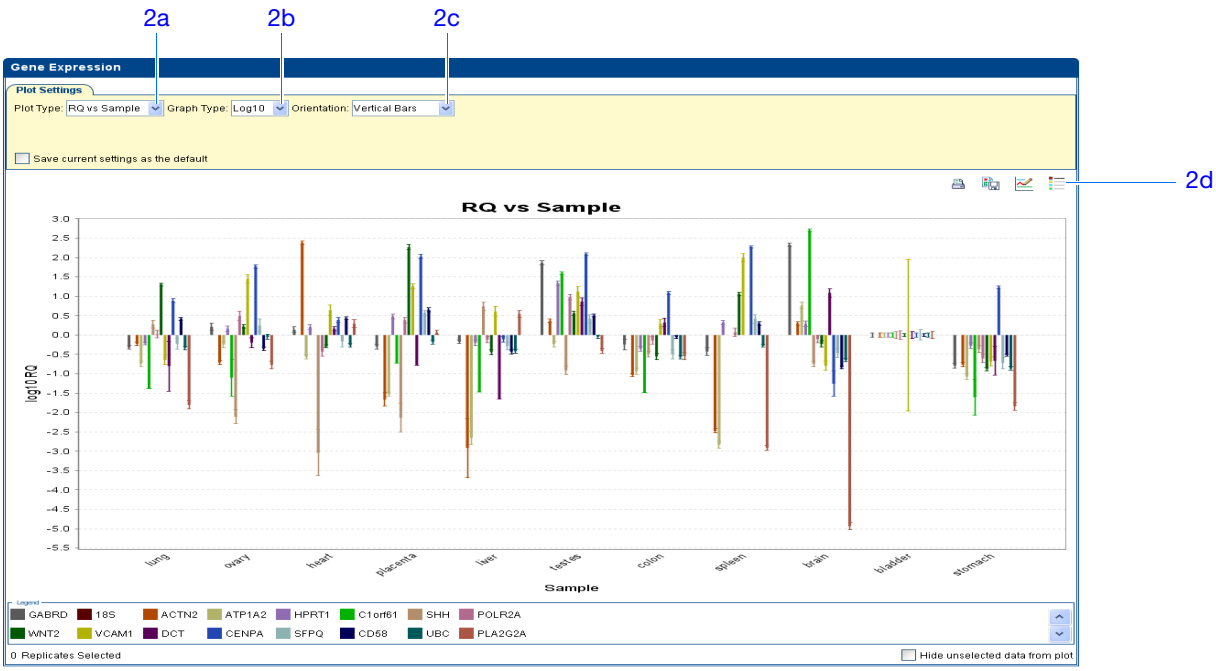
View the Gene Expression Plot

1. From the Study Menu pane, select **Analysis** ▸ **Gene Expression**.
2. In the Gene Expression Plot pane, set the parameters for the plot:
   a. In the Plot Type dropdown menu, select **RQ vs Sample**.
   b. In the Graph Type dropdown menu, select **Log10**.
   c. In the Orientation dropdown menu, select **Vertical Bars**.
   d. 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 study, the expression levels of multiple targets in samples are displayed relative to the expression levels 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

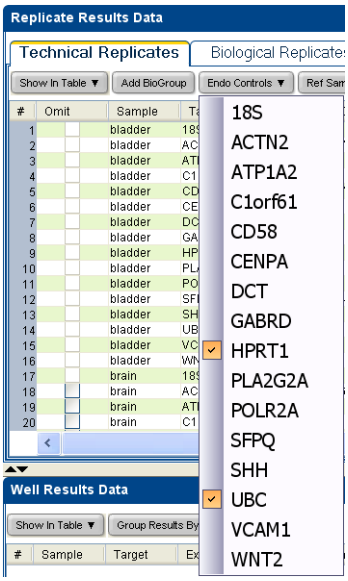


3. Select multiple endogenous controls:

   a. In the Replicate Results Data pane, select the **Technical Replicates** tab.

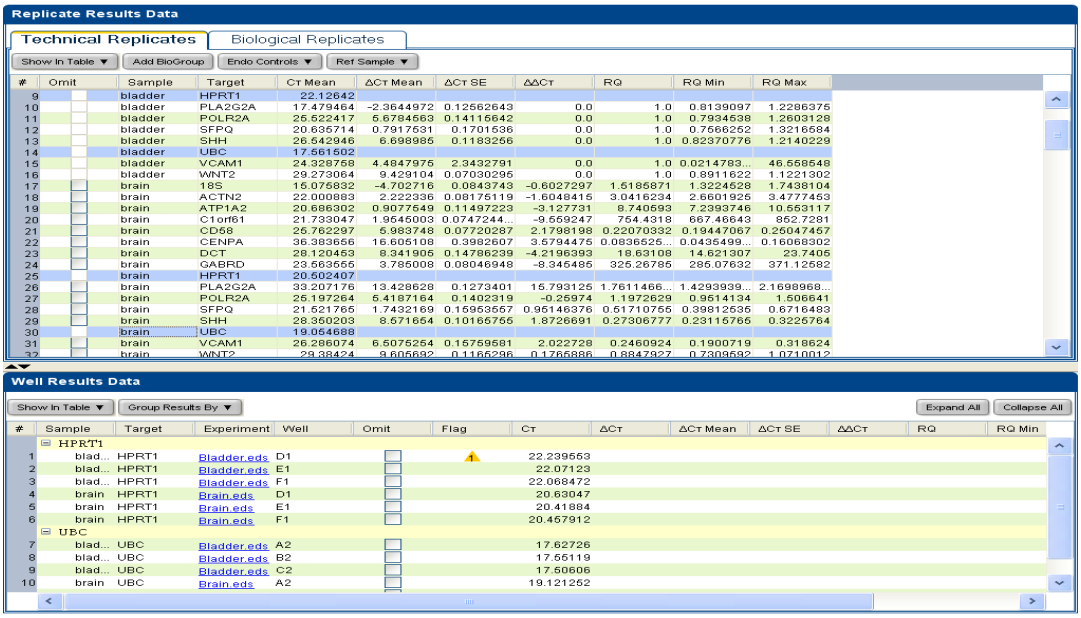
   b. From the Endo Controls dropdown menu, select **HPRT1** and **UBC**, then deselect **18S**.

Note: You can also select the endogenous controls in the Analysis Settings dialog box. See "View the Analysis Settings" on page 235.



Notes

4. Click **Analyze**. In the example study, all samples for the endogenous controls **HPRT1** and **UBC** have an no values (with the exception of the C_T Mean value), and the RQ values for the remaining samples change. For example, in the example study, the RQ value for the ACTN2/brain sample changes from ~2 (with 18S as the endogenous control) to ~3 with the new endogenous controls.



Analysis Guidelines

When you analyze your own comparative C_T study, 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, you can omit outliers. See "Omit Replicates from the Analysis" on page 247.

Note: To display a subset of the study data in the Gene Expression Plot pane, select one or more rows in the Technical Replicates tab or the Biological Replicates tab, then select **Hide unselected data from plot** to display data only from the selected rows. For more tips on navigating within the analysis screens, see "Navigation Tips" on page 232.

For More Information

For more information on:

- The Gene Expression Plot screen, access the StepOne Software Help by clicking or pressing **F1**.
- Calculating Relative Quantitation Values – Refer to the *User Bulletin #2: Relative Quantitation of Gene Expression*.

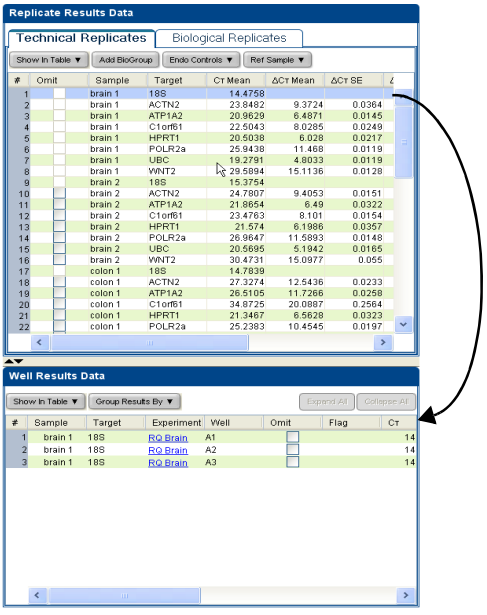
Notes

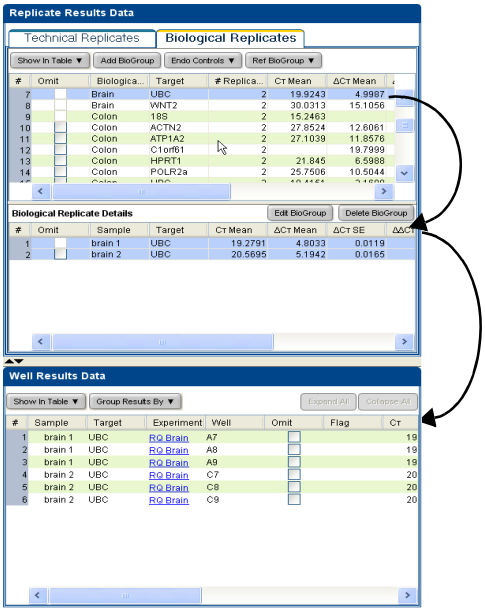
View the Replicate Results Data and the Well Results Data

The Replicate Results Data pane lists each reaction plate (experiment) that is added to a study. The results of the study are arranged by technical or biological replicate association.

The data that are displayed in the Well Results Data pane depend on which tab you select in the Replicate Results Data pane:

Technical Replicates Tab	Biological Replicates Tab
This tab arranges the results of the relative quantitation analysis by technical replicate group. The StepOne 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 Results Data pane displays the wells that make up the selected technical replicate group.	This tab arranges the results of the relative quantitation analysis by biological replicate group. The StepOne software displays the results for each biological group as a row in the table (each row displays a biological sample with its target). You can view the members of a biological replicate group by selecting the appropriate row from the table. When a row is selected: • The Biological Replicate Details table displays the technical replicate groups that make up the selected biological replicate group. • The Well Results Data pane displays the individual members of the technical replicate groups that make up the selected biological replicate group.





Notes

Column Descriptions

The table below provides definitions for the column headings that appear in the tables in the Technical Replicates and Biological Replicates tabs.

Column	Description
ΔC_T	The calculated ΔC_T value for the replicate group associated with the test sample. **Note:** The ΔC_T value is calculated only for multiplex experiments and is calculated at the well level (that is, the individual technical replicate level) by subtracting the target C_T value from the endogenous control C_T value.
ΔC_T Mean	The arithmetic average of the technical replicate C_T values for the sample replicate group. **Note:** The ΔC_T Mean value is calculated at the reaction plate level and represents the mean difference between the target C_T values and the endogenous control C_T values for all the technical replicates for that sample that are present on the plate.
ΔC_T SE	The sample standard deviation of the sample replicate group level C_T values. **Note:** The ΔC_T SE value is calculated differently for multiplex and singleplex experiments. For multiplex experiments, the calculation is at the well level. For singleplex experiments, the calculation combines the plate-level C_T value variation between the target and the endogenous control.
$\Delta\Delta C_T$	The calculated $\Delta\Delta C_T$ value for the replicate group associated with the reference sample.
# Replicates	The number of biological replicate groups in the study.
Biological Group	The name of the biological replicate group.
C_T	Threshold cycle; the PCR cycle number at which the fluorescence meets the threshold in the amplification plot.
C_T Mean	The arithmetic average of the technical replicate C_T values.
Experiment	The name of the experiment file (for example, heart.eds).
Flag	The number of QC flags that the well generated as listed in the ▲ symbol.
Omit (Replicate Results Data pane)	Indicates the omission status of the members of the associated technical or biological replicate group(s): • 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.
Omit (Well Results Data pane)	Indicates the omission status of the well. A check mark (✓) indicates that the well has been removed from the analysis.
RQ	The calculated relative level of gene expression for the replicate group that is associated with the test sample.
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

Column	Description
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.
Sample	The sample associated with the data displayed in the row.
Target	The target assay associated with the data displayed in the row.
Well	The location of the well in the reaction plate.

About the Example Study

In the comparative C_T example study, you review the Replicate Results Data pane and the Well Results Data pane to evaluate the precision of the replicate groups and view related RQ information.

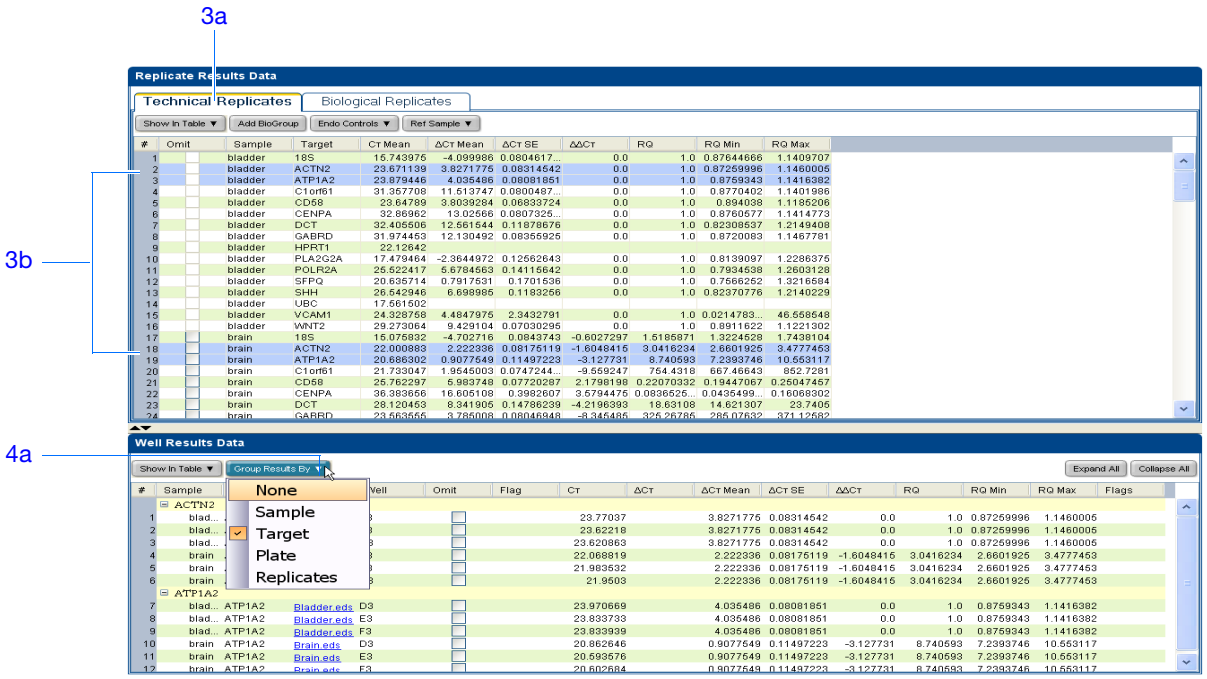
View the Results Data

1. From the Study Menu pane, select **Analysis** ▸ **Gene Expression**.
2. Click < at the top left of the Replicate Results Data pane.
3. View the technical replicates:
   a. Click the **Technical Replicates** tab. The table displays the results by technical replicate group.
   b. In the Technical Replicates table, select the following groups:
      - Bladder/ACTN2 (row 2)
      - Bladder/ATP1A2 (row 3)
      - Brain/ACTN2 (row 18)
      - Brain/ATP1A2 (row 19)

   The Well Results Data pane displays all wells that make up the selected groups.
4. View the values in the Well Results Data pane:
   a. From the Group Results By dropdown menu, select **Target**.
   b. View the C_T, ΔC_T Mean, and ΔC_T SE values to evaluate the precision of the replicate groups. In the example study, the low ΔC_T SE values indicate these replicates have good precision.

Note: See page 244 for a description of each column in the tables. To show or hide columns in a table, select or deselect the column name from the Show In Table dropdown menu.

Notes



Analysis Guidelines

When you analyze your own 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.
- View all wells for a technical or biological replicate group by selecting the appropriate row in the table. When a row is selected, the Well Results Data pane displays the wells that make up the group. **Ctrl+click** to select multiple rows.
- 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.
- Add biological replicate groups by clicking **Add BioGroup**.
- Omit biological or technical replicates from the analysis. See "Omit Replicates from the Analysis" on page 247.

Note: The Comparative CT Study Example.edm file does not demonstrate the use of biological replicate groups. However, an additional example study that uses biological replicate groups is provided with the StepOne software. You can find this study file on your computer at: *<drive>*:\Applied Biosystems*<software name>*\experiments\examples\Comparative CT Study Bio Replicates Example.edm.

Notes

Omit Replicates from the Analysis

To omit a technical or biological replicate from the analysis:

1. From the Study Menu pane, select **Analysis** ▸ **Gene Expression**.
2. 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 corresponding checkbox in the Omit column.
4. Click **Analyze** when you finish omitting wells.

IMPORTANT! You cannot omit *all* technical replicates that belong to a reference sample or a reference biological group, or that serve as the endogenous control for a study.

Note: You can also omit the biological replicates in the Biological Replicate Details table at the bottom of the Biological Replicates tab.

For More Information

For more information on:

- The Gene Expression Plot screen, access the StepOne 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 Multicomponent Plot

The Multicomponent Plot screen displays the complete spectral contribution of each dye over the duration of the PCR run in a selected well of any experiment that is added to the study.

About the Example Study

In the comparative C_T example study, 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 Multicomponent Plot

1. From the Study Menu pane, select **Analysis** ▸ **Multicomponent Plot**.
2. In the Experiment Data pane, select the **Ovary.eds** experiment.
3. Display the unknown wells one at a time in the Multicomponent Plot pane:
   a. Click the **View Plate Layout** tab.
   b. Select one well in the plate layout; the well is shown in the Multicomponent Plot pane.

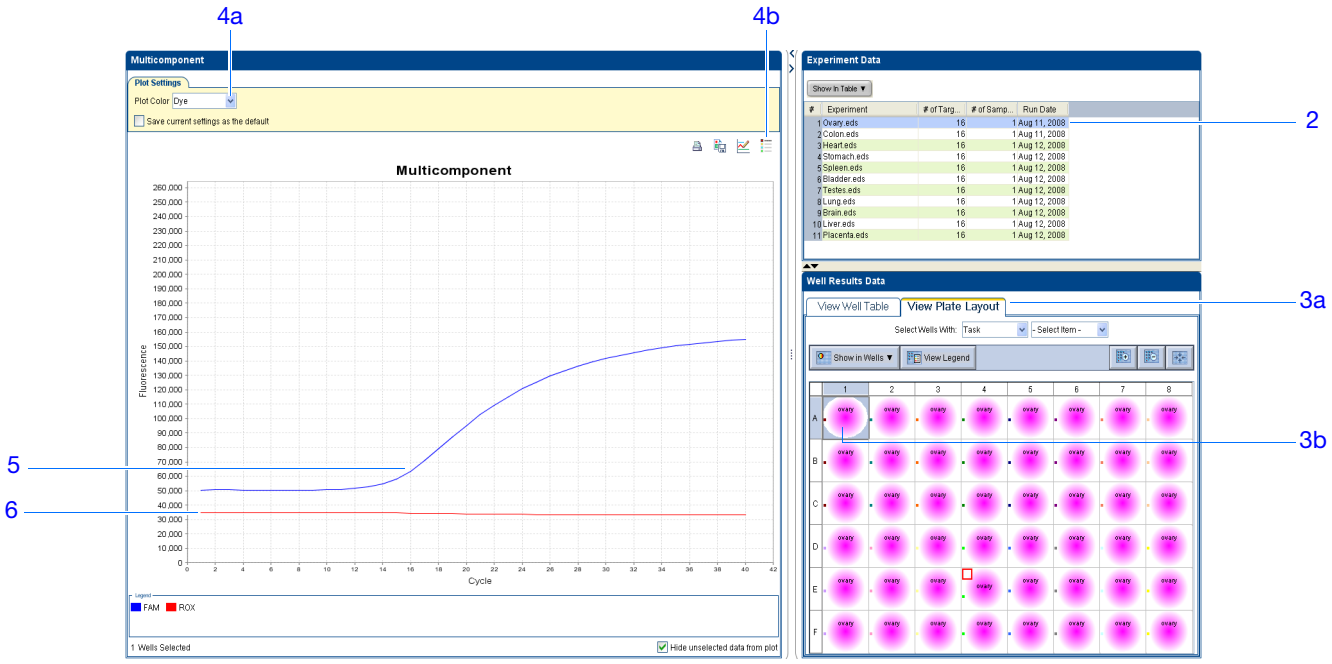
   Note: If you select multiple wells, the Multicomponent Plot pane displays the data for all selected wells simultaneously.

4. In the Multicomponent Plot pane, set the parameters for the plot:
   a. From the Plot Color dropdown menu, select **Dye**.
   b. 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. Check the FAM dye signal. In the example study, the FAM dye signal increases throughout the PCR process, which indicates normal amplification.
6. Check the ROX dye signal. In the example study, the ROX dye signal remains constant throughout the PCR process, which indicates typical data.
7. Repeat steps 2 through 6 for the remaining experiments in the study.

Notes



Analysis Guidelines

When you analyze your own comparative C_T study, 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 fluorescence – 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.

If your study does not meet the guidelines above, you can omit individual wells from the analysis. See "(If Needed) Omit Wells from the Analysis" on page 257.

Note: For tips on navigating within the analysis screens, see "Navigation Tips" on page 232.

For More Information

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

Notes

View the QC Summary

The QC Summary screen displays a list of the StepOne software flags, and it includes the flag frequency and location for any experiment that is added to a study.

About the Example Study

In the comparative C_T example study, you review the QC Summary screen for any flags generated by the study data. In the example study, several wells produced data that generated flags.

View the QC Summary

1. From the Study Menu pane, select **Analysis** ▸  **QC Summary**.

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

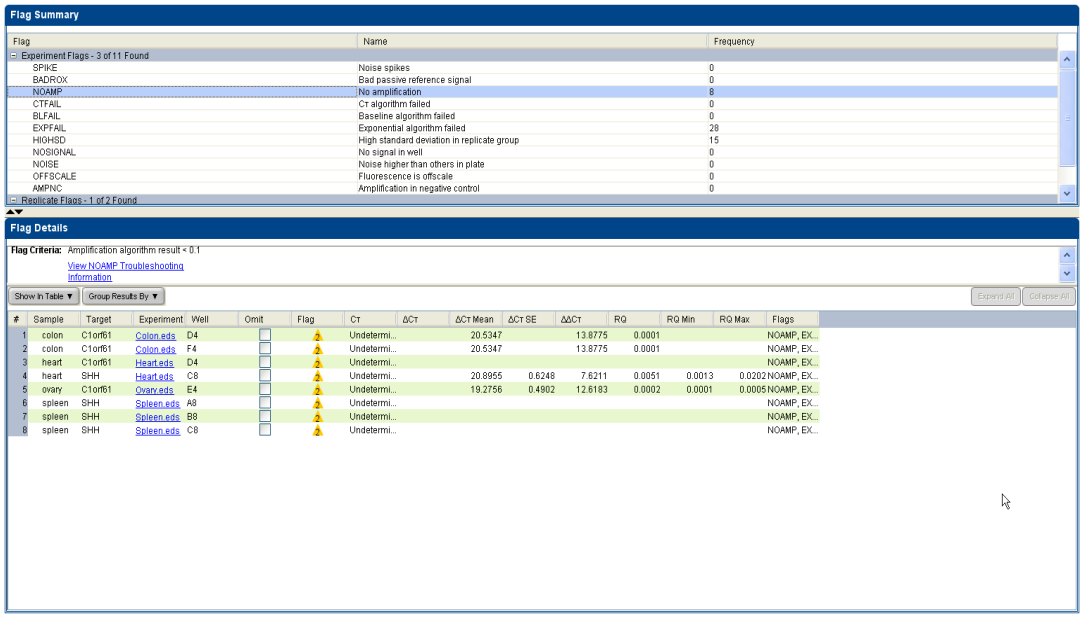
2. In the Flags Summary table, look in the Frequency column to determine which flags appear in the study. In the example study:
   - The NOAMP flag appears 8 times.
   - The EXPFAIL flag appears 28 times.
   - The HIGHSD flag appears 15 times.
   - The OUTLIERRG flag appears 9 times.

   Note: A 0 displayed in the Frequency column indicates that the flag does not appear in the study.

3. For each flag that appears in the study, click the flag row to display details about the flag in the Flag Details table. In the example study, the:
   - NOAMP flag indicates that the sample did not amplify (low- or non-expressing targets).
   - EXPFAIL flag indicates that the automatic C_T algorithm failed, and the software cannot identify the exponential region of the amplification plot.
   - HIGHSD flag indicates high standard deviation in the replicate group. This is expected for C_T values >35 due to a low amount of target. The wells do not need to be removed from the analysis.
   - OUTLIERRG flag indicates an outlier in the replicate group.

4. Consider removing the NOAMP, EXPFAIL, and/or OUTLIERRG wells from the analysis. See "(If Needed) Omit Wells from the Analysis" on page 257.

Notes



Possible Flags

For comparative C_T studies, the flags listed below may be generated by the study data.

If a flag does not appear in the study, its frequency is 0. If the frequency is >0, the flag appears somewhere in the study, and the associated 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
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

Notes

Analysis Guidelines

When you analyze your own comparative C_T study:

- In the Flag Summary table, click each flag that has a frequency >0 to display details about the flag in the Flag Details table. If needed, click the troubleshooting link in the Flag Details table to view information on correcting the flag.

  Note: In the Flag Details table, the numbers on each flag symbol indicate the number of flags generated for that well. For example,  indicates that two flags have been generated for that well.

- 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 StepOne software.
- You can omit individual wells from the analysis. See "(If Needed) Omit Wells from the Analysis" on page 257.

For More Information

For more information on the QC Summary screen, access the StepOne Software Help by clicking or pressing **F1**.

Compare Analysis Settings

Use the Compare Settings screen to perform a side-by-side comparison of analysis settings for a comparative C_T study. You can change one or more of the analysis settings, then compare the new results with the previous results. For example, you can compare the effects of:

- Using multiple endogenous controls versus a single endogenous control.
- Changing the amplification efficiency of a specific target versus keeping it at 100%.

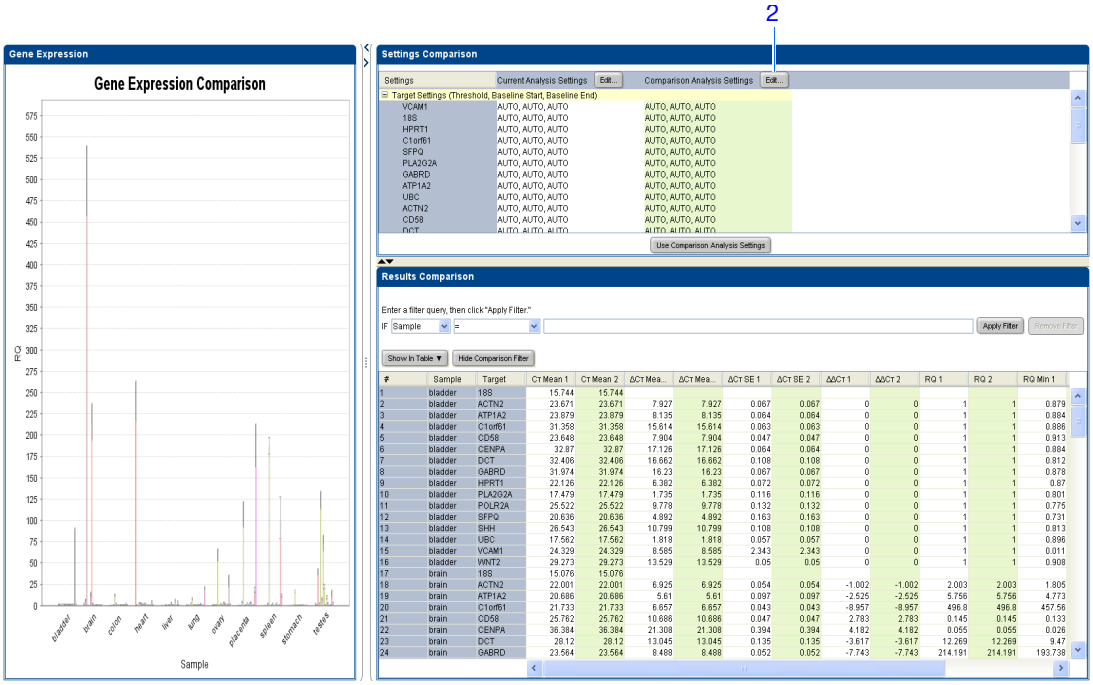
About the Example Study

In the comparative C_T example study, you change the endogenous control to UBC, then compare results.

Complete the Compare Settings Screen

1. From the Study Menu pane, select **Analysis** ▸  **Compare Settings**. When the Compare Settings screen is initially displayed (that is, before you make any changes):
   - In the Settings Comparison pane, the white column is titled "Current Analysis Settings," the green column is titled "Comparison Analysis Settings," and the Use Comparison Analysis Settings button is under the green column.
   - In the Results Comparison pane, values in the white columns and values in the green columns are the same.
   - The Gene Expression Comparison plot is the same plot that is displayed in the Gene Expression screen (**Analysis** ▸  **Gene Expression**).
2. In the Settings Comparison pane, click **Edit** in the green column to open the Comparison Analysis Settings dialog box.

Notes

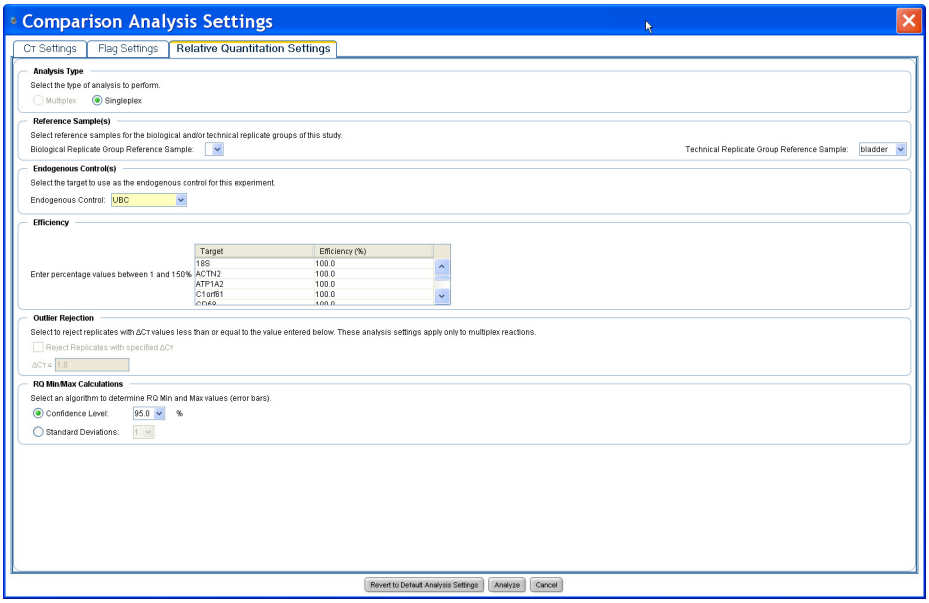


3. In the Comparison Analysis Settings dialog box, change the endogenous control:

   a. Select the **Relative Quantitation Settings** tab.

   b. In the Endogenous Control(s) pane, select **UBC** from the Endogenous Control dropdown menu.

   c. Click **Analyze** to analyze the data and close the dialog box.

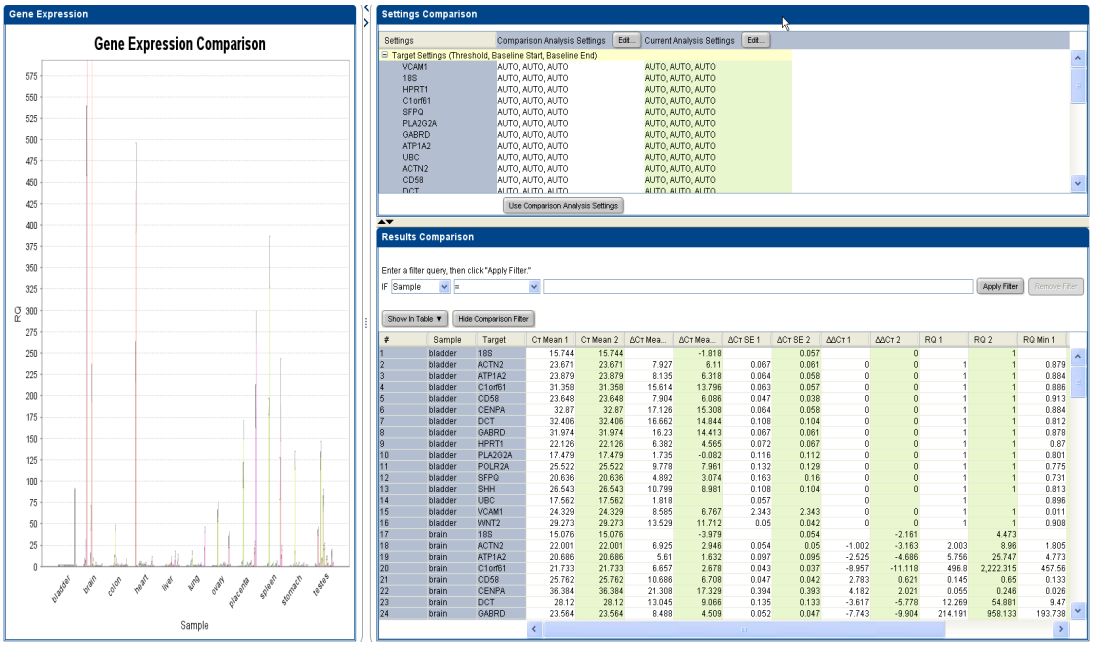


Notes

4. In the Settings Comparison pane, click **Use Comparison Analysis Settings** under the green column, then compare the results:
   - In the Settings Comparison pane, the white column is titled "Comparison Analysis Settings," the green column is titled "Current Analysis Settings," and the Use Comparison Analysis Settings button is under the white column.
   - In the Results Comparison pane, values in the white columns are based on the default analysis settings, and values in the green columns are based on the modified analysis settings. In the example experiment, changing the endogenous control to UBC affects the RQ values. To view the RQ values, scroll to the left. If desired, you can click and drag the RQ column headings so that they appear first in the table.
   - The Gene Expression Comparison plot displays the default analysis settings (the values from the white columns).

   Note: The Gene Expression Comparison plot has limited functions. For example, you cannot change to log scale and you cannot view by target.

Note: The default analysis settings are the settings automatically made by the software when the study is initially analyzed.



5. From the Study Menu pane, select **Analysis** ▸  **Gene Expression** to view the gene expression plot using the modified analysis settings.

Note: In the Gene Expression screen you can view the modified data in log scale, by target, and so on. See "View the Gene Expression Plot" on page 240.

Notes

6. (Optional) View the data in the other analysis screens. All other analysis screens for the study display the data using the modified analysis settings.

7. Close the study. You can do one of the following:
   - Save your changes before closing the study.
   - Close the study without saving your changes. If you do not save your changes, the software reverts to the default analysis settings the next time you open the study.

Analysis Guidelines

When you analyze your own comparative C_T study:

- Edit the comparison analysis settings as desired. For information on editing the settings, see "View the Analysis Settings" on page 235.
- After making your first round of changes to the analysis settings, you can continue making changes using one of the following methods:
  - (Recommended) Revert to the saved analysis settings, then make new changes. To do this: In the Settings Comparison pane, click **Use Comparative Analysis Settings** (now under the white column) to revert to the saved analysis settings, then repeat steps 2 through 6 above. This method ensures that you do not lose the saved analysis settings.

    Note: If you have made changes, but have not saved them, the software reverts to the default analysis settings when you click **Use Comparative Analysis Settings**. The default analysis settings are the settings automatically made by the software when the study is initially analyzed.

  - Continually compare new settings with previous settings. To do this: In the Settings Comparison pane, alternate clicking **Edit** in the white and green columns, then repeat steps 3 through 6 above. This method does not allow you to return to your saved settings; subsequent comparisons are made with the previous analysis settings, building upon any changes that you have already made.
- You can revert to the default analysis settings. See "Revert to the Default Analysis Settings" below.

Revert to the Default Analysis Settings

IMPORTANT! The default analysis settings are defined by the software. If you make changes to the analysis settings and save the study, the saved changes are lost when you revert to the default analysis settings.

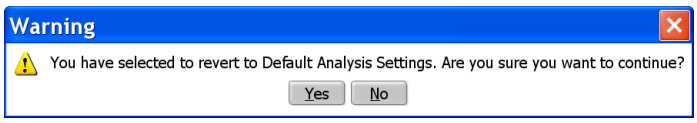
1. In the Settings Comparison pane, click **Edit** next to the settings you want to revert to the default: *Current Analysis Settings* or *Comparison Analysis Settings*.

Notes

2. In the Analysis Settings dialog box:

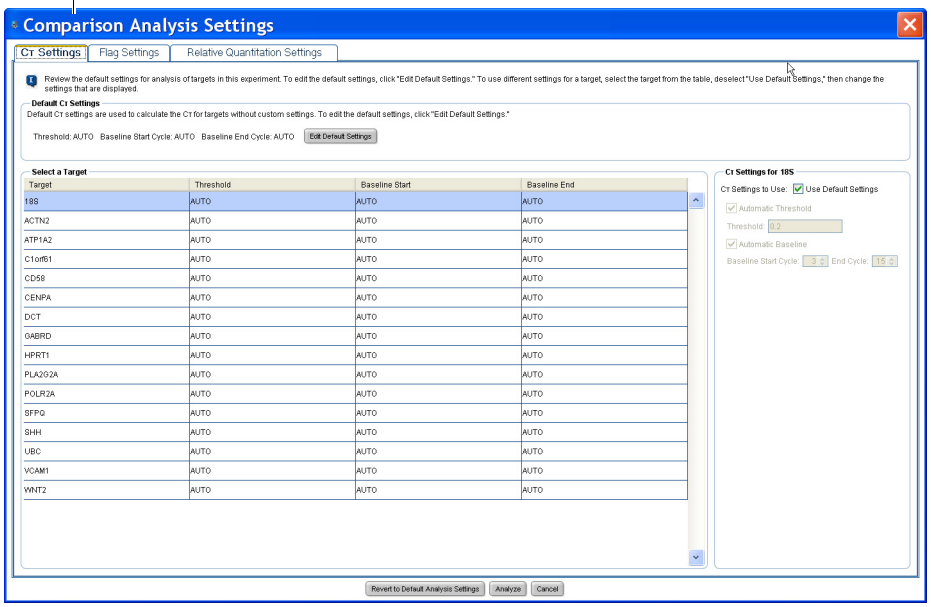
   a. Click **Revert to Default Analysis Settings**.

   b. At the prompt, click **Yes**.



   c. Click **Analyze** to analyze the data and close the dialog box.

This dialog box is titled Comparison Analysis Settings or Current Analysis Settings, depending on the Edit button you clicked in step 1.



3. In the Settings Comparison pane, click **Use Comparison Analysis Settings**. In the Results Comparison pane, values for the settings you selected to edit in step 1 ("Current Analysis Settings" or "Comparison Analysis Settings") are generated according to the default analysis settings.

For More Information For more information on the Compare Settings screen, access the StepOne Software Help by clicking or pressing **F1**.

Notes

(If Needed) Omit Wells from the Analysis

You can use the Well Table to omit individual wells from the analysis. To omit a well:

1. From the Study Menu pane, select one of the following analysis screens:
   - **Analysis** ▸ **Amplification Plot**
   - **Analysis** ▸ **Multicomponent Plot**
   - **Analysis** ▸ **Multiple Plots View**
2. In the Experiment Data pane, select the experiment that contains the well to omit.
3. In the Well Results Data pane, click the **View Well Table** tab, then select the checkbox in the Omit column for the well to omit.

IMPORTANT! You cannot omit all technical replicates that belong to a reference sample or a reference biological group, or that serve as the endogenous control.

For More Information

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

Publish the Data

You can publish the study data in the same way that you publish experiment data. See "Publish the Data" on page 202 for more information.

Notes

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Alternate Experiment Workflows

This appendix covers:

Note: For more information about any of the topics discussed in this guide, access the Help from within Applied Biosystems StepOne™ Real-Time PCR Software by pressing **F1**, clicking in the toolbar, or selecting **Help ▸ StepOne Software Help**.

Notes

Advanced Setup Workflow

When you create an experiment using Advanced Setup in the StepOne™ software, you can set up the experiment according to your own design.

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

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

2. Log in with a user name or as a Guest.

3. From 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.

4. 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 **Run Method**, review the reaction volume and thermal profile, then edit as needed.

   d. Click **Reaction Setup**, review the components and calculated volumes for the PCR reactions, then edit as needed.

   e. (Optional) Click **Materials List**, review the list of materials, then order the materials you need to prepare the reaction plate.

5. Prepare the PCR reactions:

Experiment Type	Action
Relative standard curve	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the standard dilution series. d. Prepare the reaction mix. e. Prepare the reaction plate.
Standard curve	

Notes

Experiment Type	Action
Comparative C_T	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the reaction mix. d. Prepare the reaction plate.
Genotyping	
Presence/absence	

6. Run the experiment:
   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 StepOne software.
   b. From the Experiment Menu, 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).

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	Action
Relative standard curve	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the standard dilution series. d. Prepare the reaction mix. e. Prepare the reaction plate.
Standard curve	
Comparative C_T	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the reaction mix. d. Prepare the reaction plate.
Genotyping	
Presence/absence	

Notes

2. QuickStart the experiment:

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

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

   b. Log in with a user name or as a Guest.

   c. From the Home screen, click **QuickStart**.

   d. Select the **Experiment Properties** tab (default), enter the experiment name, then select the experiment properties.

   e. Select the **Run Method** tab, review the reaction volume and thermal profile, then edit as needed.

3. Run the experiment:

   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.

4. In the StepOne software, complete the plate setup:

Experiment Type	Action
Genotyping	a. Select and complete the **Define SNP Assays and Samples** tab. b. Select and complete the **Assign SNP Assays and Samples** tab.
All other experiments	a. Select and complete the **Define Targets and Samples** tab. b. Select and complete the **Assign Targets and Samples** tab.

5. Analyze the data:

   a. Open the experiment in the StepOne software.

   b. From the Experiment Menu, 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 are useful when you want to create many experiments with the same setup information.

Create a Template

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

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

2. Log in with a user name or as a Guest.

3. 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 260).

4. Select **File** ▸ **Save As Template**.

5. Enter a file name, select a location for the template, then click **Save**.

6. Click **Close**.

Create an Experiment with a Template

1. From the Home screen, click **Template**.

   Note: If you do not see the Template icon, click the arrow beneath the Design Wizard icon to expand the Set Up menu.

2. Locate and select the template you created in step d, then click **Open**. A new experiment is created using the 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 260).

4. Click **Save**, enter a file name, then click **Save** to save the experiment.

Notes

5. Prepare the PCR reactions:

Experiment Type	Action
Relative standard curve	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the standard dilution series. d. Prepare the reaction mix. e. Prepare the reaction plate.
Standard curve	
Comparative C_T	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the reaction mix. d. Prepare the reaction plate.
Genotyping	
Presence/absence	

6. Run the experiment:
   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 StepOne software.
   b. From the Experiment Menu, 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.

Note: Setup data that are exported from experiments in StepOne Software v1.0 or v2.0 can be imported into experiments in StepOne Software v2.1. However, setup data that are exported from experiments in StepOne Software v2.1 cannot be imported into experiments in StepOne Software v1.0 or v2.0.

Export Setup Data

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

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

2. Log in with a user name or as a Guest.
3. 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 260).

4. Select **File** ▸ **Export**.
5. Select the **Export Properties** tab (default), then:
   a. Select **Setup**.
   b. Select **One File** from the dropdown menu.
   c. Enter a name, then select a location for the export file.
   d. Select **(*.txt)** from the File Type dropdown menu.

   IMPORTANT! You cannot export *.xml files.

6. (Optional) Click the **Customize Export** tab, then select the appropriate options.
7. Click **Start Export**,
8. When prompted, click **Close Export Tool**.

Create an Experiment with an Exported Text File

You can import 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 you select contains only reaction plate setup data and that the experiment types match.

Notes

1. Import the reaction plate setup data from an 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 finished, save the file as a tab-delimited text file.
   c. From 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.

   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 plate setup information, the software asks if you want to replace the plate setup with the data from the text file. Click **Yes** to replace the plate setup.

2. Use Advanced Setup to finish setting up your experiment (see page 260).
3. Prepare the PCR reactions:

Experiment Type	Action
Relative standard curve	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the standard dilution series. d. Prepare the reaction mix. e. Prepare the reaction plate.
Standard curve	
Comparative C_T	a. Prepare the template. b. Prepare the sample dilutions. c. Prepare the reaction mix. d. Prepare the reaction plate.
Genotyping	
Presence/absence	

4. Run the experiment:
   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.

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5. Analyze the data:
   a. Open the experiment in the StepOne software.
   b. From the Experiment Menu, 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

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Bibliography

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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 StepOne™ 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 StepOne™ and StepOnePlus™ systems, 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 (CT).

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 signal.

baseline-corrected normalized reporter (ΔRn)

The magnitude of normalized fluorescence signal generated by the reporter:

1. In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal 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 signal 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 replicate

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).

When using biological replicate groups in a comparative C_T study, the values displayed in the Biological Replicates tab are calculated by combining the results of the separate biological samples and treating this collection as a single population (that is, as one sample). For ΔC_T computations (normalizing by the endogenous control) in a singleplex experiment, the separate biological samples are treated as unpaired data when computing variability estimates of the single biological replicate. You can observe individual contributions of the separate biological samples to the single biological replicate results in the Technical Replicates tab.

Note: To view the Biological Replicates and Technical Replicates tabs, from the Study Menu pane, select **Analysis** ▸ **Gene Expression**.

blocked IPC

In presence/absence experiments, a reaction that contains IPC blocking agent, which blocks amplification of the internal positive control (IPC). In the StepOne™ software, the task for the IPC target in wells that contain IPC blocking agent. See also negative control-blocked IPC wells.

calibrator

See reference sample.

chemistry

See reagents.

colocated layout

A system layout in which the StepOne™ or StepOnePlus™ instrument is directly connected to a colocated computer by the yellow cable. In this layout, you can control the instrument with the StepOne™ software on the colocated computer or with the instrument touchscreen.

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 StepOne™ 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.

C_T

See threshold cycle (CT).

custom dye

Dye that is not supplied by Applied Biosystems. Custom dyes may be adapted for use in experiments on the StepOne™ and StepOnePlus™ systems. When using custom dyes, the custom dye should be added to the Dye Library and a custom dye calibration performed.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA™ dye as reporter or quencher with the StepOne™ system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus™ system.

cycle threshold

See threshold cycle (CT).

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 StepOne™ 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 (DRn).

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 StepOne™ 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 StepOne™ 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 signals for the target you are quantifying. Housekeeping genes can be used as endogenous controls. See also housekeeping gene.

When using multiple endogenous controls, the software treats all endogenous controls as a single population, and calculates the experiment-appropriate mean to establish a single value against which the target of interest is normalized. In comparative C_T experiments, the mean calculated is the arithmetic mean of the C_T values. In relative standard curve experiments, the C_T values are converted to relative quantities prior to normalization; the mean calculated is subsequently the geometric mean of the relative quantities.

Note: Arithmetic and geometric means are related and equivalent due to logarithmic transformation of the data.

Variability estimates for multiple endogenous controls are computed separately. The final variability estimate is a pooled combination of the individual variability estimates (similar to computing pooled standard deviations).

endpoint read See post-PCR read.

experiment Refers to the entire process of performing a run using the StepOne™ or StepOnePlus™ systems, including setup, run, and analysis. The types of experiments you can perform using the StepOne and StepOnePlus 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 (:), semicolon (;), and sign (&), percent sign (%), dollar sign ($), at sign (@), circumflex (^), left parenthesis (( ), right parenthesis ( )), or exclamation point (!).

IMPORTANT! If you run the instrument in standalone mode from the instrument touchscreen, you cannot enter more than 32 characters in the Experiment Name field and you cannot include spaces in the name.

experiment type The type of experiment you are performing using the StepOne™ or StepOnePlus™ 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 StepOne™ 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 StepOne™ 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 StepOne™ 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 signal. 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 signal from the reporter dye normalized to the fluorescence signal 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 signal. 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 StepOne™ systems, the grid contains 6 rows and 8 columns. In StepOnePlus™ systems, the grid contains 8 rows and 12 columns.

In the StepOne™ 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 StepOne™ 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 StepOne™ and StepOnePlus™ 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 signal 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.

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA™ dye as reporter or quencher with the StepOne™ system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus™ system.

QuickStart A feature in StepOne™ and StepOnePlus™ systems that allows you to run an experiment without entering plate setup information. QuickStart requires a colocated layout with the instrument powered on and an intact instrument-computer connection.

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 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! Fast reagents are not supported for presence/absence experiments.

raw data plot A plot of raw fluorescence signal (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 StepOne™ and StepOnePlus™ 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 StepOne™ 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.

Remote Monitor — A feature in the StepOne™ software that allows you to monitor a StepOne™ or StepOnePlus™ instrument over the network. With the Remote Monitor, you can monitor the instrument status, send an experiment to the instrument, monitor amplification plots and temperature plots in real time, and download the results to your computer. You cannot operate the StepOne or StepOnePlus instrument using the Remote Monitor.

replicate group — A set of identical reactions in an experiment.

replicates — Total number of identical reactions containing identical components and identical volumes.

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 StepOne™ and StepOnePlus™ 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 StepOne™ or StepOnePlus™ instrument run.

sample The template that you are testing.

Sample DNA (10×) In the StepOne™ 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 StepOne™ software, a collection of samples. The Sample Library contains the sample name and the sample color.

Sample or Standard (10×) In the StepOne™ 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 StepOne™ 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 StepOne™ 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.

spatial calibration Type of StepOne™ and StepOnePlus™ system calibration in which the system maps the positions of the wells in the sample block(s). Spatial 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.

standalone layout A system layout in which the StepOne™ or StepOnePlus™ instrument is *not* connected to a computer by the yellow cable. In this layout, you control the instrument only with the instrument touchscreen, and you use a USB drive or network connection to transfer data between the instrument and computer.

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 StepOne™ 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 StepOne™ 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 StepOne™ 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 StepOne™ 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. With StepOnePlus™ systems, which contain the VeriFlex™ blocks, each step contains 6 temperatures (1 for each VeriFlex block).

study name Entered during study setup, the name that is used to identify the study. Study 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 (:), semicolon (;), and sign (&), percent sign (%), dollar sign ($), at sign (@), circumflex (^), left parenthesis (( ), right parenthesis ( )), or exclamation point (!).

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 StepOne™ or StepOnePlus™ system. Before you use system dyes in your experiments, make sure the system dye calibration is current in the Instrument Maintenance Manager.

System dyes on the StepOne system:

- FAM™ dye
- JOE™ dye
- ROX™ dye
- SYBR® Green dye
- VIC® dye

System dyes on the StepOnePlus system:

- FAM™ dye
- JOE™ dye
- NED™ dye
- ROX™ dye
- SYBR® Green dye
- TAMRA™ dye
- VIC® dye

IMPORTANT! Applied Biosystems does not recommend the use of TAMRA™ dye as reporter or quencher with the StepOne™ system. TAMRA dye may be used as a reporter or quencher with the StepOnePlus™ system.

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 StepOne™ software, a color assigned to a target to identify the target in the plate layout and analysis plots.

Target Library

In the StepOne™ 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 StepOne™ 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 replicate Reactions that contain identical components and volumes, and that evaluate the same sample.

temperature plot In the StepOne™ software, a display of temperatures for the sample, instrument cover, and instrument block during the StepOne™ or StepOnePlus™ instrument run.

template In the Design Wizard of the StepOne™ 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 StepOne™ or StepOnePlus™ 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 CT) or can be set manually (see manual CT).
2. In presence/absence experiments, the level of fluorescence above which the StepOne™ 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).

touchscreen Instrument display that you touch to control the StepOne™ or StepOnePlus™ instrument.

unknown In the StepOne™ 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).

VeriFlex™ Technology

The StepOnePlus™ instrument contains six independently thermally regulated VeriFlex™ blocks, creating up to six different zones for the 96 sample wells. After you enable the VeriFlex blocks in the StepOne™ software, you can set a different temperature for one or more of the VeriFlex blocks.

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.

zone

One of up to six sample temperatures among the 96 wells formed by independently thermally regulated VeriFlex™ blocks during the StepOnePlus™ instrument run. You can set a different temperature for one or more of the VeriFlex blocks, or you can set the same temperature for each of the VeriFlex blocks.

Note: For melt curve steps, you need to set the same temperature for each of the VeriFlex blocks.

zone boundary

The edge of a zone for samples formed by the six independently thermally regulated VeriFlex™ blocks. In the StepOne™ software, the zone boundaries are displayed in the plate layout as thick red lines.

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Worldwide Sales and Support

Applied Biosystems vast distribution and service network, composed of highly trained support and applications personnel, reaches 150 countries on six continents. For sales office locations and technical support, please call our local office or refer to our Web site at **www.appliedbiosystems.com**.

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