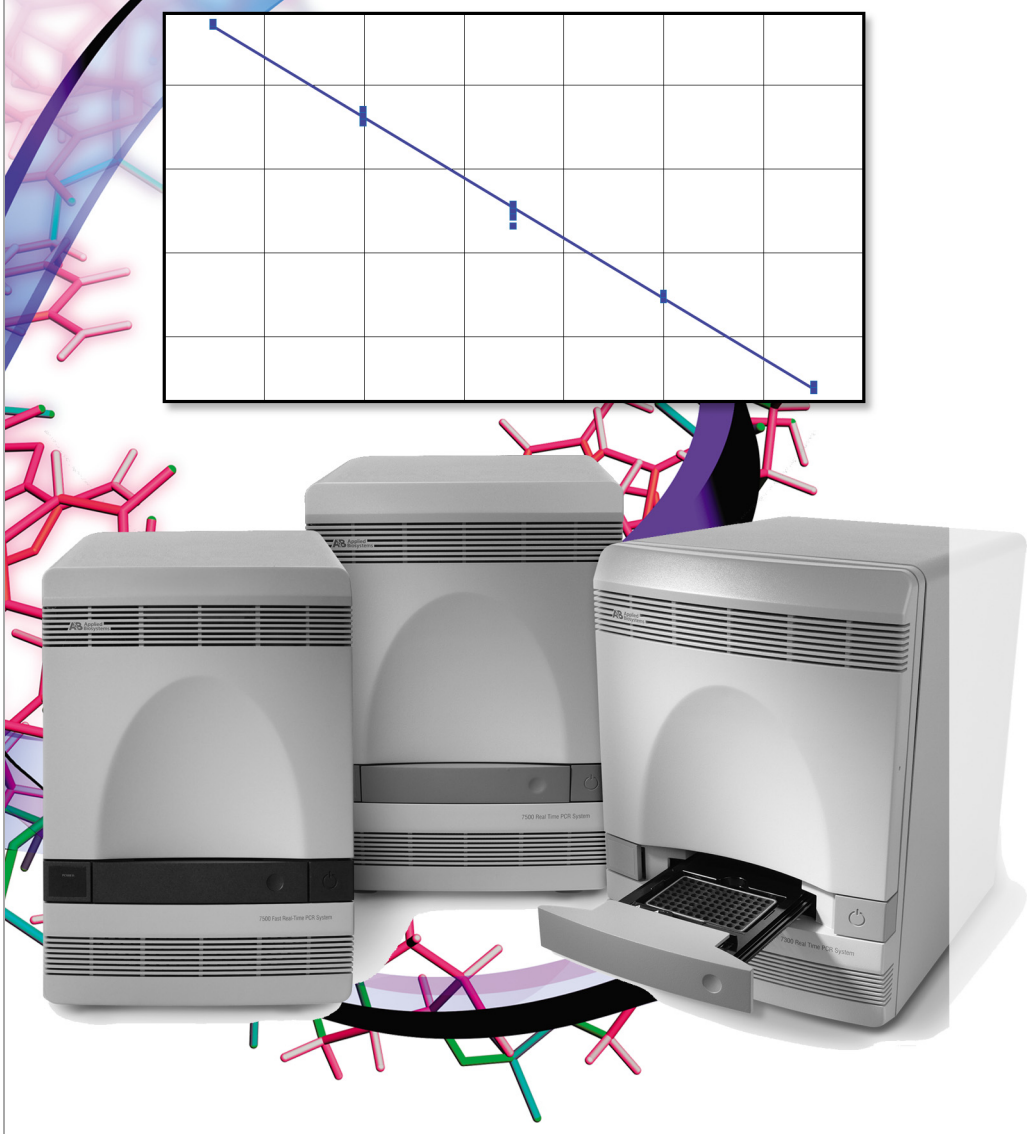
Applied Biosystems

Absolute Quantitation Using Standard Curve Getting Started Guide



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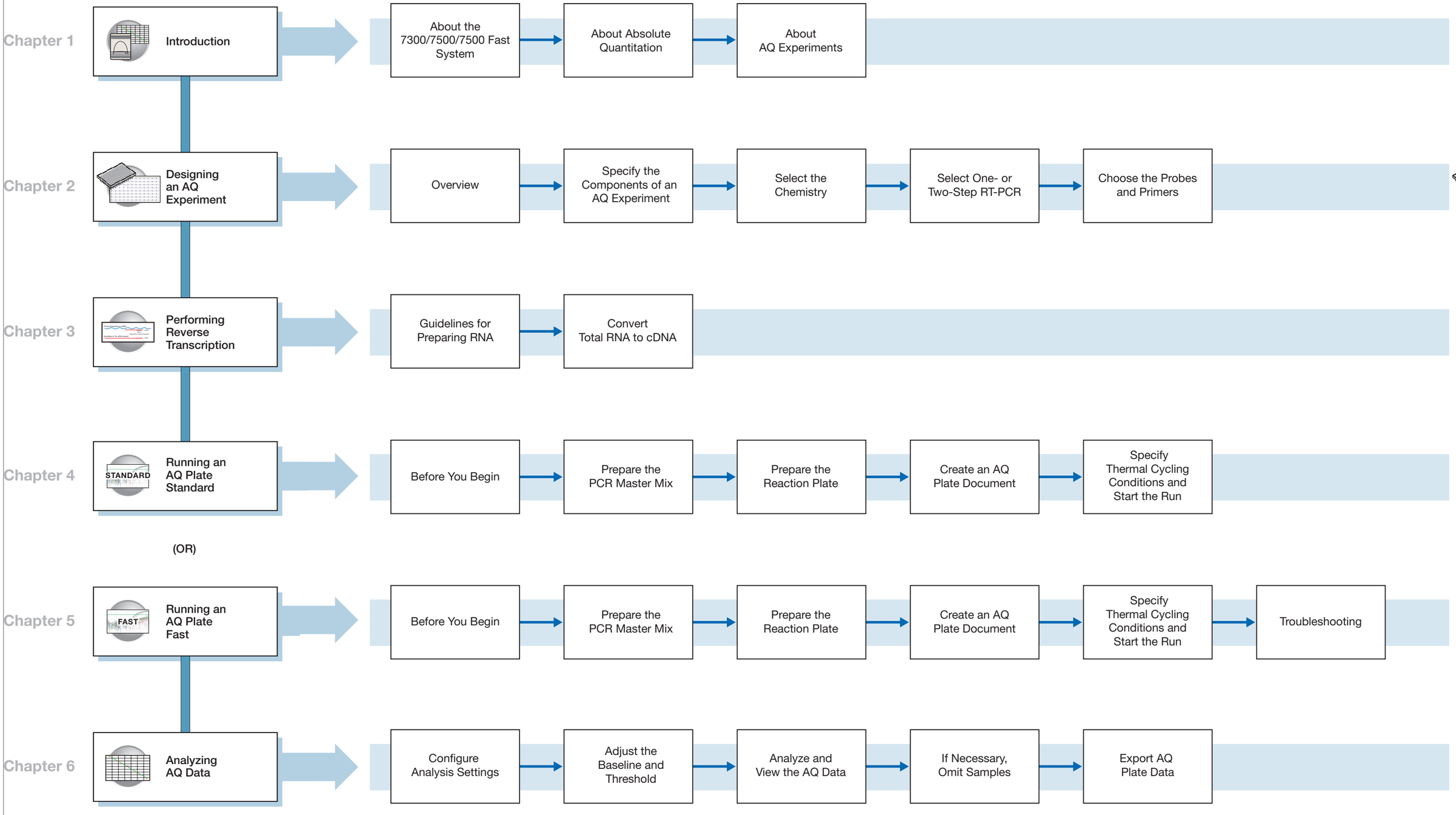
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Preface

How to Use This Guide

Purpose of This Guide

This manual is written for principal investigators and laboratory staff who conduct absolute quantitation assays using the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System (7300/7500/7500 Fast system).

Assumptions

This guide assumes that you have:

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

Text Conventions

This guide uses the following conventions:

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

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

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

  Select **File > Open**.

User Attention Words

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

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

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

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.

How to Obtain More Information

Related Documentation

For more information about using the 7300/7500/7500 Fast system, refer to:

- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Online Help*
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide* (PN 4347822)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Plus/Minus Getting Started Guide* (PN 4347821)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide* (PN 4347824)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System: User Guide for the 21 CFR Part 11 Module in SDS Software v1.4* (PN 4374432)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide* (PN 4347828)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Site Preparation Guide* (PN 4347823)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Performing Fast Gene Quantitation Quick Reference Card* (PN 4362285)
- *Applied Biosystems 7500 Fast Real-Time PCR System Using Expert Mode User Bulletin* (PN 4367499)
- *Applied Biosystems Real-Time PCR Systems Computer Setup Guide* (PN 4365367)
- *Applied Biosystems Real-Time PCR Systems Chemistry Guide* (PN 4348358)
- *TaqMan Universal PCR Master Mix Protocol* (PN 4351891)
- *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol* (PN 4375575)

Accessing the Online Help

Access the Online Help system by clicking  in the toolbar of the SDS software window, or by selecting **Help > Contents and 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

How to Obtain Support

To contact Applied Biosystems Technical Support from North America by telephone, call **1.800.899.5858**.

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:

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

Safety

Safety Alert Words

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

Definitions

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

– 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 to be limited to the most extreme situations.

Except for Important, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard icons that are affixed to Applied Biosystems instruments*.

Examples

The following examples show the use of safety alert words:

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

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

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

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

Good Laboratory Practices

PCR Good Laboratory Practices

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

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
- Sample preparation and PCR setup
- PCR amplification and post-PCR analysis
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully. Do not splash or spray PCR samples.
- Keep reactions and components sealed as much as possible.
- Use positive displacement pipettes or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.

Bibliography

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

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

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.

General Chemical Warnings

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD**. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

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

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

General Warnings

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

General Biohazard Warnings

General Biohazard

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

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **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.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

General Chemical Waste Hazard Warnings

Chemical Waste Hazard

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

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.

Obtaining MSDSs

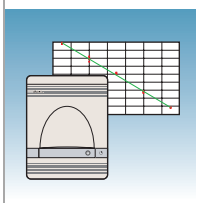
Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store,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

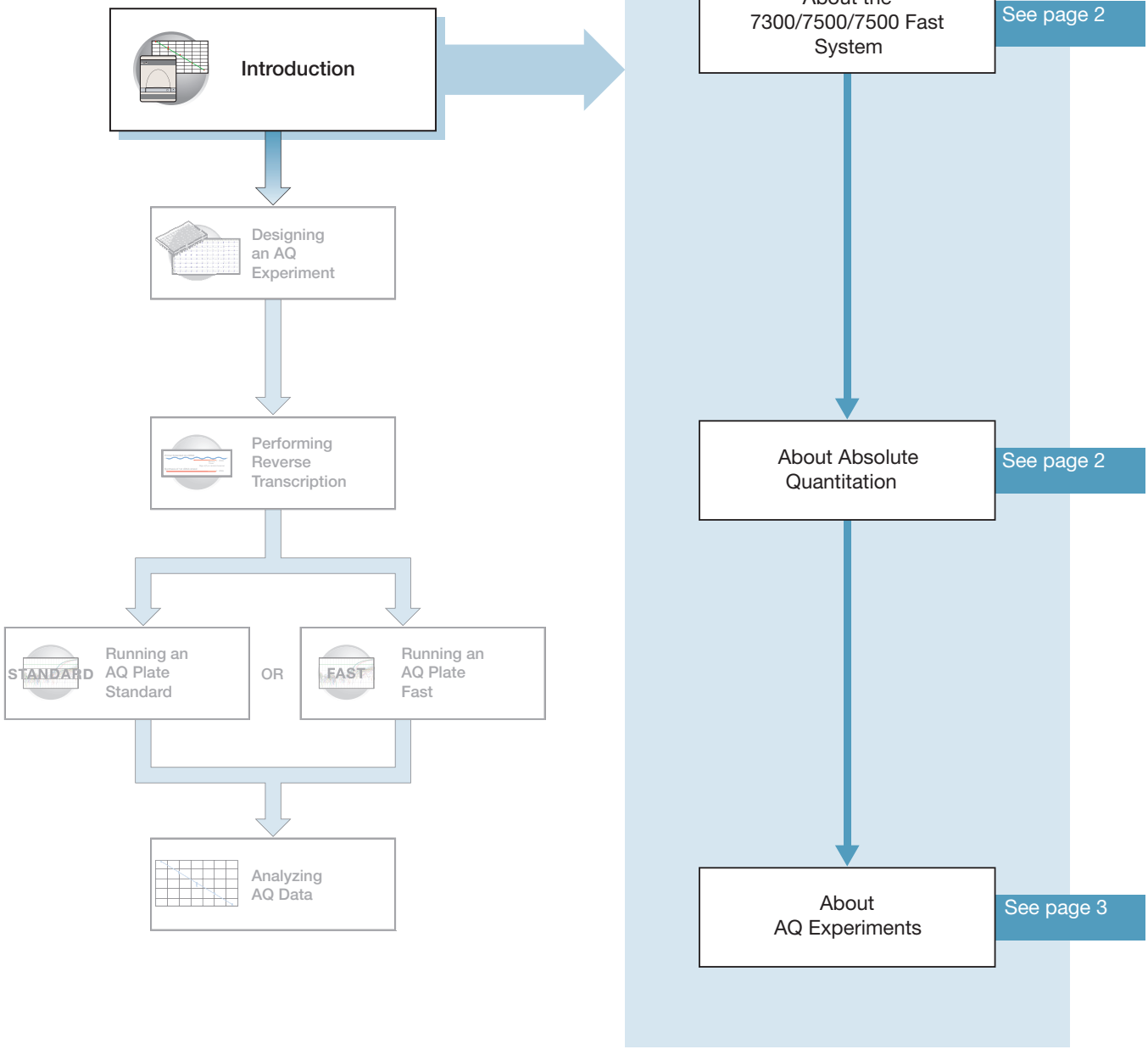
You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

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



Introduction



Notes

About the 7300/7500/7500 Fast System

Description

The Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System uses fluorescent-based PCR chemistries to provide quantitative detection of nucleic acid sequences using real-time analysis and qualitative detection of nucleic acid sequences using end-point and dissociation-curve analysis. The Applied Biosystems 7500 Fast Real-Time PCR System allows the user to perform high-speed thermal cycling giving run times for quantitative real-time PCR applications (such as relative quantitation) in fewer than 40 minutes.

Absolute Quantitation Assay

The 7300/7500/7500 Fast system allows you to perform several assay types using plates in the 96-well format. This guide describes the absolute quantitation (AQ) using standard curve assay.

For more information about the other assay types, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358) and the Online Help for the 7300/7500/7500 Fast System (Online Help).

About Absolute Quantitation

Definition

Absolute quantitation (AQ) is the process that determines the absolute quantity of a single nucleic acid target sequence within an unknown sample.

Real-time PCR Assays

AQ is performed using real-time PCR. In Real-Time PCR you monitor the progress of the PCR as it occurs. Data are collected throughout the PCR process rather than at the end of the PCR process (end-point PCR).

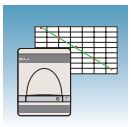
In Real-Time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end of PCR.

Using AQ Plate Documents for Plus/Minus and AD Assays

Although Plus/Minus and Allelic Discrimination (AD) assays are end-point assays, Applied Biosystems recommends that you use the 7300/7500/7500 Fast system to perform amplification and view the real-time PCR results. In the event that an experiment fails, you can study the amplification plots to help determine the cause of the failure.

Use AQ Plate documents to store real-time data for Plus/Minus and AD assays. AQ Plate documents used for troubleshooting Plus/Minus and AD assays do not require standard curves.

Notes



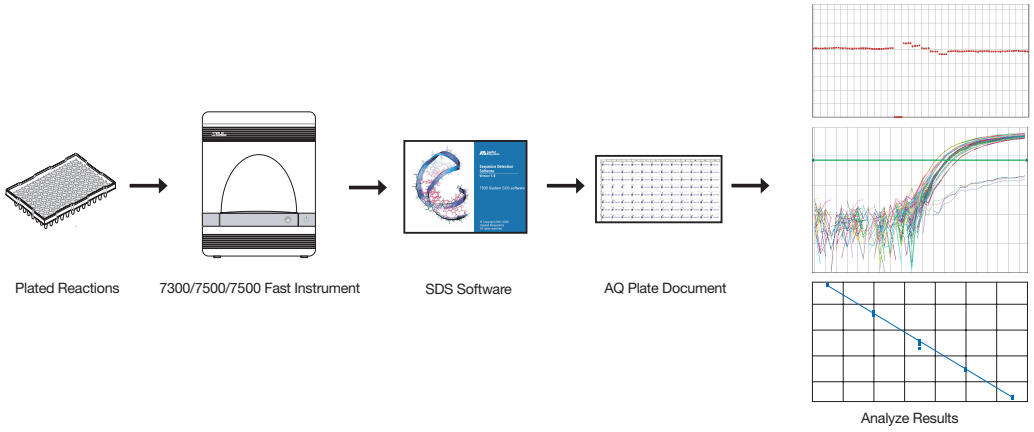
About AQ Experiments

AQ Experiment Workflow

In this document the term "AQ experiment" refers to the entire AQ assay process beginning with generating cDNA from RNA (reverse transcription) through analyzing AQ data. The AQ experiment workflow has several steps, shown in the figure on page iii.

AQ assays use a standard curve to calculate the quantity of an unknown target sequence. The results of AQ experiments are reported in the same units of measure as the standard curve.

The 7300/7500/7500 Fast system stores Real-Time PCR data collected from the reaction plate in an AQ Plate document. Each run consists of a single plate. The 7300/7500/7500 Fast system provides several views for analyzing data.

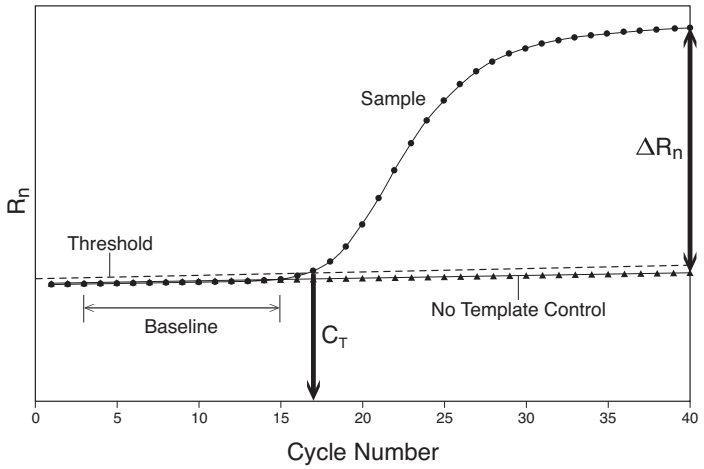


Notes

Terms Used in Quantitation Analysis

Term	Definition
Baseline	The initial cycles of PCR in which there is little change in fluorescence signal.
Threshold	A level of delta R_n—automatically determined by the software or manually set—used for C_T determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the Amplification plot defines the C_T.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold.
No template control (NTC)	A sample that does not contain template. It is used to verify amplification quality.
Nucleic acid target (also called “template”)	Nucleotide sequence that you want to detect and quantitate.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan® probe. The dye provides a fluorescence signal that indicates specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by the specified set of PCR conditions. ($\Delta R_n = R_n$ – baseline)
Standard	A sample of known quantity used to construct a standard curve.
Unknown sample	A sample containing an unknown quantity of template that you want to characterize.

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



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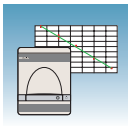


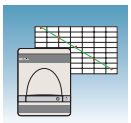
Required User-Supplied Materials

Item	Source
ABI PRISM® 6100 Nucleic Acid PrepStation	Applied Biosystems - (PN 6100-01)
High-Capacity cDNA Reverse Transcription Kit (1000 reactions)	Applied Biosystems - (PN 4368813)
High-Capacity cDNA Reverse Transcription Kit (200 reactions)	Applied Biosystems - (PN 4368814)
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (1000 reactions)	Applied Biosystems - (PN 4374967)
High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	Applied Biosystems - (PN 4374966)
TaqMan® Universal PCR Master Mix	Applied Biosystems - (PN 4304437)
TaqMan® Fast Universal PCR Master Mix (2X) No AmpErase® UNG	Applied Biosystems - (PN 4352042)
TaqMan® One-Step RT-PCR Master Mix	Applied Biosystems - (PN 4309169)
SYBR® Green PCR Master Mix	Applied Biosystems - (PN 4309155)
Power SYBR® Green PCR Master Mix	Applied Biosystems - (PN 4367659)
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	Applied Biosystems - (PN 4306737)
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode (code 128)	Applied Biosystems - (PN 4346906)
MicroAmp™ Optical Adhesive Film (quantity 100)	Applied Biosystems - (PN 4311971)
MicroAmp™ Optical Adhesive Film (quantity 25)	Applied Biosystems - (PN 4360954)
MicroAmp™ Adhesive Film Applicator	Applied Biosystems - (PN 4333183)

Notes

Item	Source
Labeled primers and probes from one of the following sources: • TaqMan® Gene Expression Assays (predesigned primers and probes) – inventoried – non-inventoried • Custom TaqMan® Gene Expression Assays service (predesigned primers and probes) – Small-Scale (20X, 144 × 50 µL reactions) – Medium-Scale (20X, 300 × 50 µL reactions) – Large-Scale (60X, 1160 × 50 µL reactions) • Primer Express® Software (custom-designed primers and probes) – 1-user license – 5-user license	 • Applied Biosystems - (PN 4331182) • Applied Biosystems - (PN 4351372) • Applied Biosystems - (PN 4331348) • Applied Biosystems - (PN 4332078) • Applied Biosystems - (PN 4332079) • Applied Biosystems - (PN 4363991) • Applied Biosystems - (PN 4363993)
6700 Reagent Tubes, 10-mL	Applied Biosystems - (PN 4305932)
Centrifuge with adapter for 96-well plates	Major laboratory supplier (MLS)
Gloves	MLS
Microcentrifuge	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS
Nuclease-free water	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement	MLS
Vortexer	MLS

Notes



Example AQ Experiment

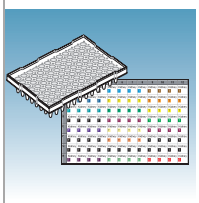
Overview

To better illustrate how to design, perform, and analyze AQ experiments, this section guides you through an example experiment. The example experiment represents a typical AQ experiment setup that you can use as a quick-start procedure to familiarize yourself with the AQ workflow. Detailed steps in the AQ workflow are described in the subsequent chapters of this guide. Included in these chapters are Example Experiment boxes that provide details for some of the related steps in the example experiment. Refer to Appendix E, "Example AQ Experiment," on page 81 for more information. To view the example experiment data file in the SDS software:

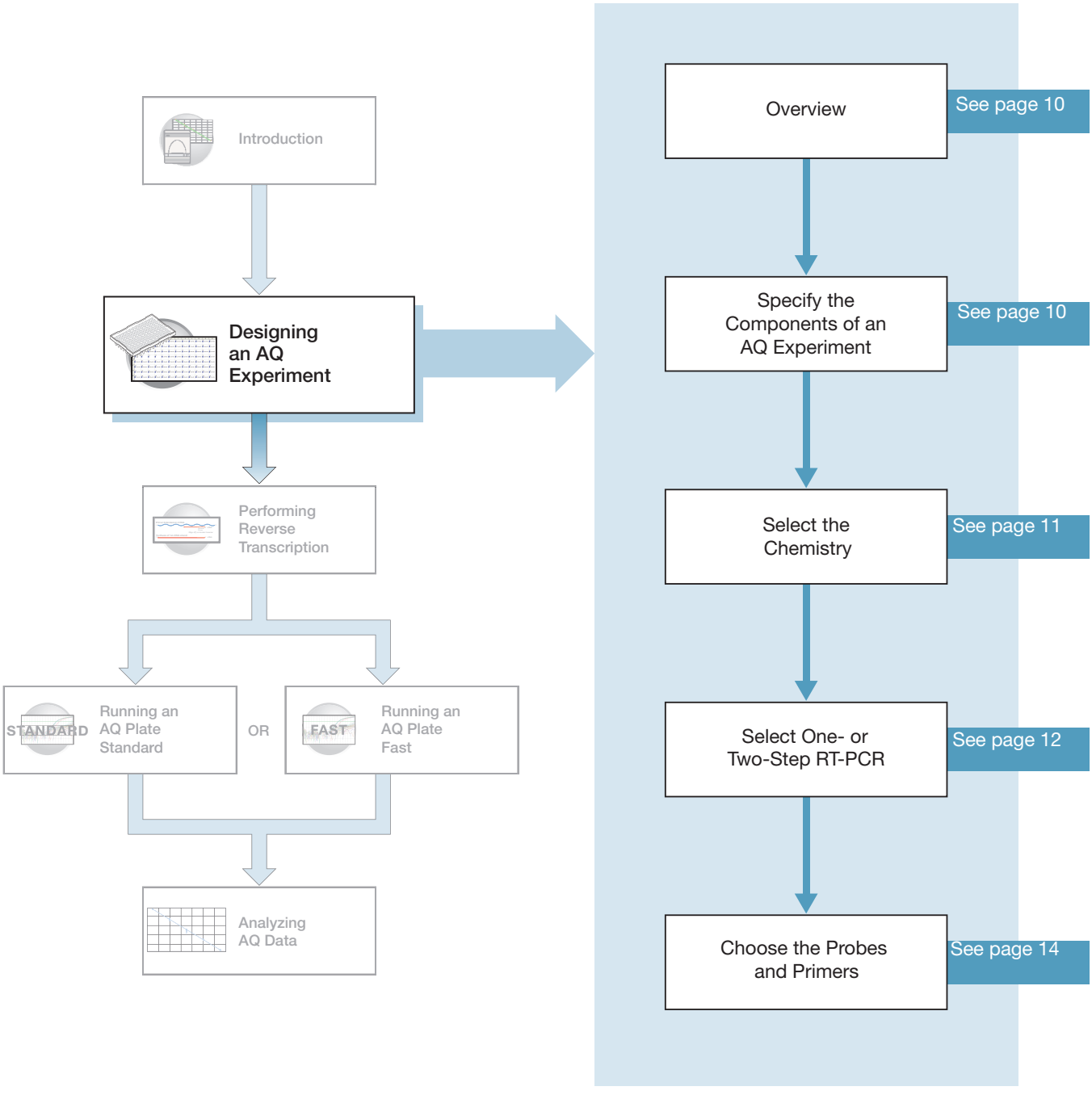
1. Select **File > Open**.
2. Navigate to **Applied Biosystems\SDS Documents\Example Data Files\ EXAMPLE_AQ.sds**, then click **Open**.

Notes

Notes



Designing an AQ Experiment



Notes

Overview

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

Specifying the Components of an AQ Experiment

For each AQ experiment, specify:

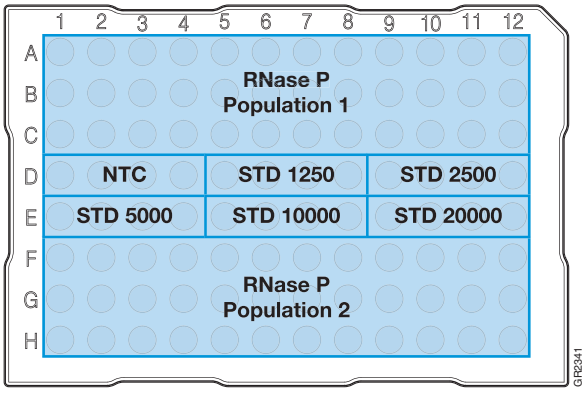
- **An unknown** – The nucleic acid sequence that you are quantitating.
- **Standards** – This guide assumes that you have generated a set of standards for each target sequence that you are quantitating. Appendix B on page 75 provides guidelines for generating standards.
- **Replicate wells** – For absolute quantitation assays, Applied Biosystems recommends the use of three or more replicate reactions per sample to ensure statistical significance.

For more information about these requirements, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358).

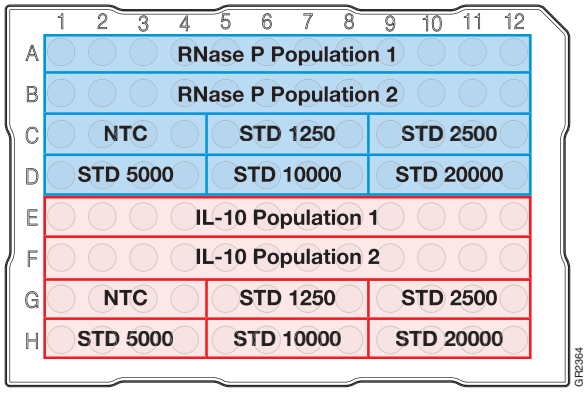
Example Experiment

The example experiment determined the quantity of the RNase P gene in two populations on the 7500 Real Time PCR System. Because a single gene was studied, only one set of standards was required (A). Four replicates of each unknown and standard were performed to ensure statistical significance. In experiments where multiple genes are being studied, a set of standards is required for each gene (B).

A. Single gene in two populations



B. Two genes in two populations



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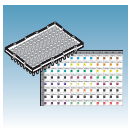
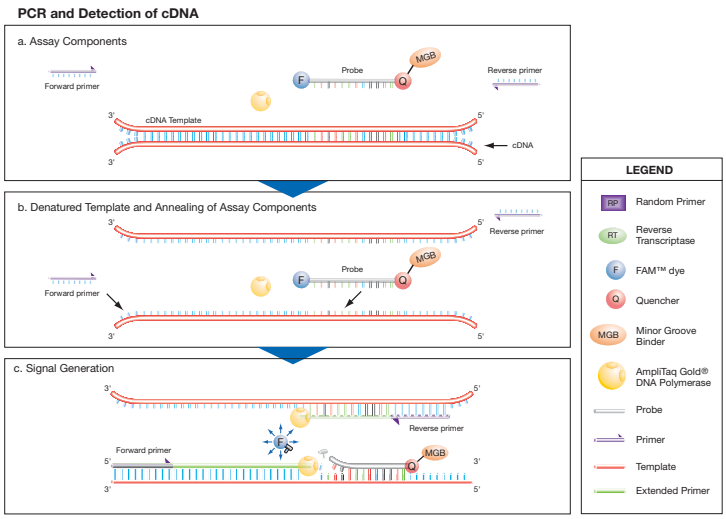
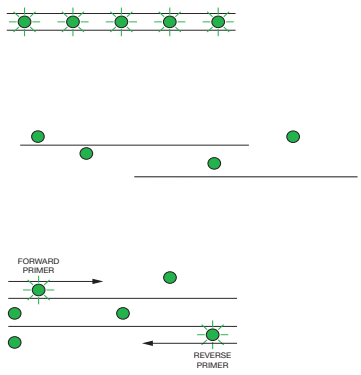


Selecting the Chemistry

About Chemistries

Applied Biosystems offers two types of chemistries that you can use to detect PCR products on real-time instruments, as explained in the following table. Both TaqMan® probe-based and SYBR® Green I dye chemistries can be used for either one- or two-step RT-PCR. For more information about these chemistries, refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358).

Chemistry	Process
TaqMan® reagents or kits **Description** TaqMan® reagent-based chemistry uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles. **Advantages** • Increases specificity with a probe. Specific hybridization between probe and target generates fluorescence signal. • Provides multiplex capability. • Optimized assays available. • Allows 5′-nuclease assay to be carried out during PCR.	
SYBR® Green I reagents **Description** Uses SYBR Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles. **Advantages** • Economical (no probe needed). • Yields a melting profile of distinct PCR yields. • Increases signal fluorescence as amplification product length increases. **Limitations** Binds nonspecifically to all double-stranded DNA sequences. To avoid false positive signals, check for nonspecific product formation using dissociation curve or gel analysis.	 **Step 1: Reaction setup** The SYBR® Green I dye fluoresces when bound to double-stranded DNA. **Step 2: Denaturation** When the DNA is denatured, the SYBR® Green I dye is released and the fluorescence is drastically reduced. **Step 3: Polymerization** During extension, primers anneal and PCR product is generated. **Step 4: Polymerization completed** SYBR® Green I dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the instrument.

Notes

Selecting One- or Two-Step RT-PCR

When performing real-time PCR, you have the option of performing reverse transcription (RT) and PCR in a single reaction (one-step) or in separate reactions (two-step). The reagent configuration you use depends on whether you are performing two-step or one-step RT-PCR:

- Two-step RT-PCR is performed in two separate reactions: First, total RNA is reverse transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. AmpErase® UNG enzyme can be used to prevent carryover contamination.

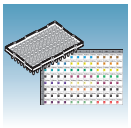
  Users of the 7500 Fast system can use TaqMan® Fast Universal PCR Master Mix or TaqMan® Universal PCR Master Mix for an approximately 40 minute run time or a 2 hour run time, respectively.

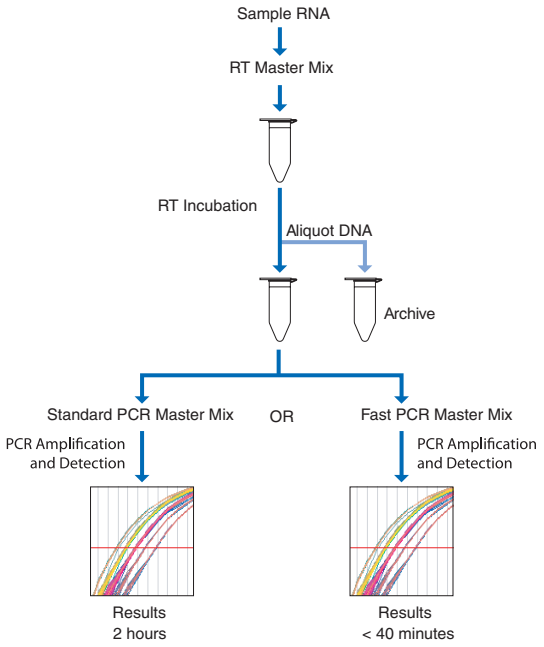
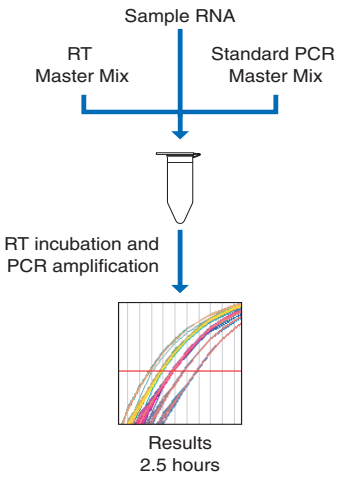
  IMPORTANT! This guide emphasizes AQ experiments that are designed using two-step RT-PCR, but also provides information on one-step RT-PCR. For more information, refer to the *Real-Time PCR Systems Chemistry Guide*.

  Note: TaqMan® Fast Universal PCR Master Mix does not contain AmpErase® UNG enzyme.

- In one-step RT-PCR, RT and PCR take place in one buffer system, which provides the convenience of a single-tube preparation for RT and PCR amplification. However, you can not use Fast PCR Master Mix or the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), to perform one-step RT-PCR. For more information about UNG, refer to the *Real-Time PCR Systems Chemistry Guide*.

Notes



Two-Step RT-PCR	One-Step RT-PCR
	

Recommended Kits for Two-Step RT-PCR			
Chemistry	**Step**	**Reagent**	**Part Number**
TaqMan reagents or kits	RT	High-Capacity cDNA Reverse Transcription Kit (1000 reactions)	4368813
		High-Capacity cDNA Reverse Transcription Kit (200 reactions)	4368814
		High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (1000 reactions)	4374967
		High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	4374966
	PCR	TaqMan® Universal PCR Master Mix	4304437
		TaqMan® Fast Universal PCR Master Mix (2X) No AmpErase® UNG	4352042

Notes

Recommended Kits for Two-Step RT-PCR			
Chemistry	**Step**	**Reagent**	**Part Number**
SYBR® Green I reagents or kits	RT	High-Capacity cDNA Reverse Transcription Kit (1000 reactions)	4368813
		High-Capacity cDNA Reverse Transcription Kit (200 reactions)	4368814
		High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (1000 reactions)	4374967
		High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (200 reactions)	4374966
	PCR	SYBR® Green PCR Master Mix	4309155
		Power SYBR® Green PCR Master Mix	4367659
	RT and PCR	SYBR® Green RT-PCR Reagents	4310179

Example Experiment

Used two-step RT-PCR with the TaqMan reagents and kits indicated in the table above.

Choosing the Probes and Primers

Choose probe and primer sets for your target sequences. Applied Biosystems provides three options for choosing primers and probes:

- **TaqMan® Gene Expression Assays** – Provide you with optimized, ready-to-use TaqMan assays (5′-nuclease) for human, mouse, or rat transcripts. For information on available primer/probe sets, go to:

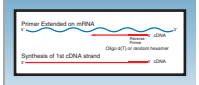
  http://www.allgenes.com
- **Custom TaqMan® Gene Expression Assays** – Designs, synthesizes, formulates, and delivers quality-controlled primer and probe sets. Use this service if the primer-probe set you need is not currently available. To place an order, contact your Applied Biosystems representative.
- **Primer Express® Software** – Helps you design primers and probes for your own quantitation assays. For more information about using this software, refer to the *Primer Express Software v3.0 Getting Started Guide* (PN 4362460).

  Applied Biosystems provides assay design guidelines that have been developed specifically for quantitation assays. When followed, these guidelines provide a reliable system for assay design and optimization. For information about the assay design guidelines, refer to the *Real-Time PCR Systems Chemistry Guide*.

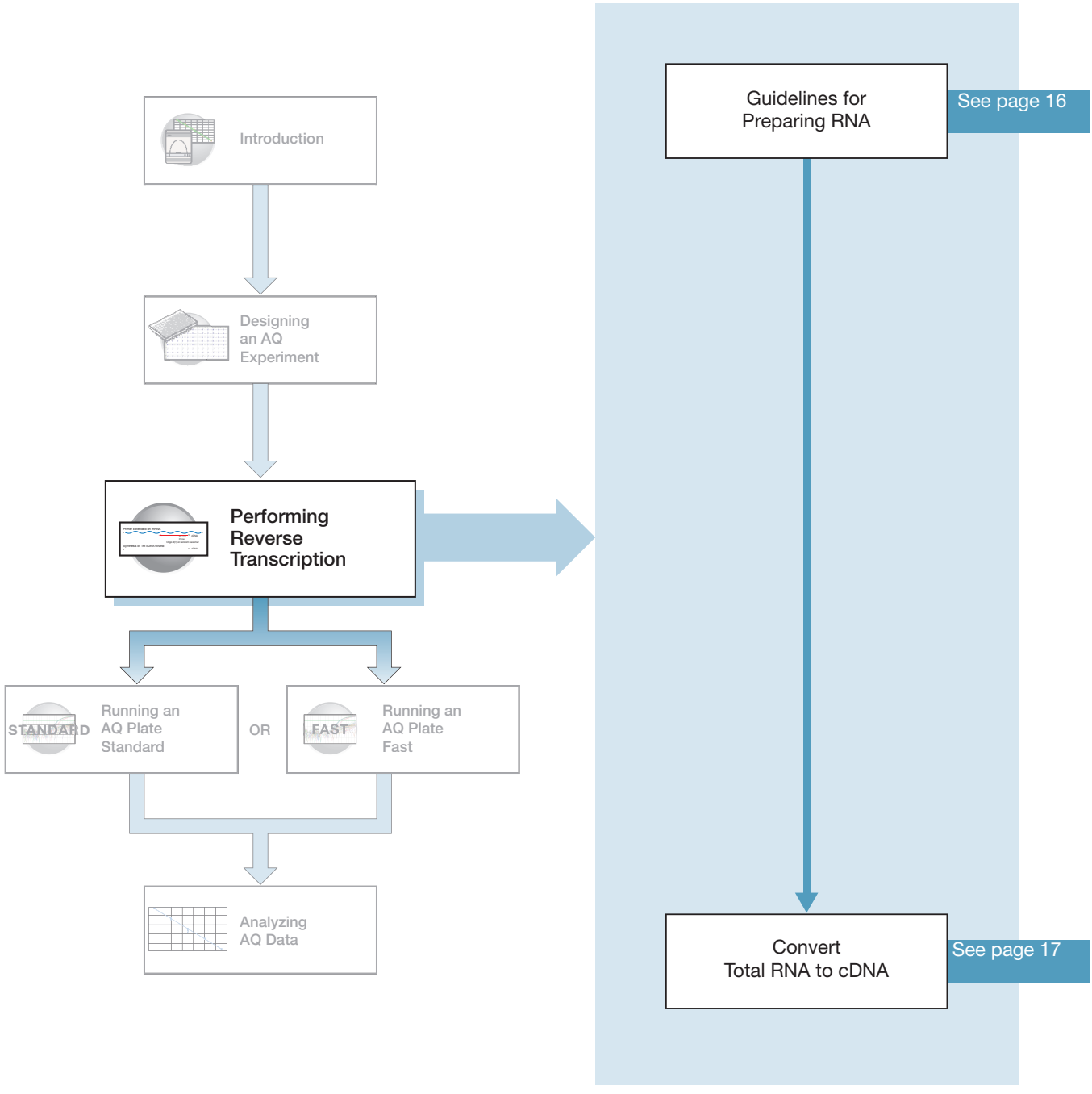
Example Experiment

Primers and probes for RNase P were designed using Primer Express Software.

Notes



Performing Reverse Transcription



Notes

Guidelines for Preparing RNA

Isolating Total RNA

Applied Biosystems supplies several instrument systems, chemistries, and protocols for RNA isolation from a variety of starting materials, such as blood, tissue, cell cultures, and plant material.

System, Chemistry, or Protocol	Source
ABI PRISM® 6100 Nucleic Acid PrepStation	Applied Biosystems (PN 6100-01)
6100 Reagents and Disposables Starter Kit	Applied Biosystems (PN 4328773)
Tempus™ Blood RNA Tube (For collection, stabilization, and isolation of total RNA in whole blood for gene expression analysis using the 6100 PrepStation)	Applied Biosystems (PN 4342792)
Isolation of Total RNA from Whole Blood and from Cells Isolated from Whole Blood Protocol	Applied Biosystems (PN 4332809)
Tempus™ Blood RNA Tube and Large Volume Consumables Protocol	Applied Biosystems (PN 4345218)
Tissue RNA Isolation: Isolation of Total RNA from Plant and Animal Tissue Protocol	Applied Biosystems (PN 4330252)

Quality of RNA

The total RNA you use for AQ experiments should:

- Have an $A_{260/280}$ greater than 1.9
- Be intact when visualized by gel electrophoresis
- Not contain RT or PCR inhibitors

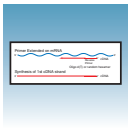
The *High-Capacity cDNA Reverse Transcription Kits Protocol* (PN 4375575) contains additional guidelines for preparing the RNA template.

Adjusting the Starting Concentration of Total RNA

The High-Capacity cDNA Reverse Transcription Kits are optimized to convert up to 2 µg of total RNA to cDNA per 20 µL reaction. Convert enough total RNA so that the final concentration of total RNA converted to cDNA is 10 to 100 ng in 5 µL for each 50-µL PCR reaction.

Note: If you suspect that the RNA contains RNase activity, add RNase Inhibitor to the reverse transcription reaction at a final concentration of 1.0 U/µL.

Notes



Converting Total RNA to cDNA

Using the High-Capacity cDNA Reverse Transcription Kits

Use the High-Capacity cDNA Reverse Transcription Kit to perform the first step (RT) in the two-step RT-PCR method. Follow the manual method for converting total RNA into cDNA, as specified in the *High-Capacity cDNA Reverse Transcription Kits Protocol* (PN 4375575).

IMPORTANT! The protocol is not shipped with the High-Capacity cDNA Reverse Transcription Kit. Download the protocol from

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

To search for the document, select **ABI PRISM® 6100 Nucleic Acid PrepStation** in the Product list box, then click **Search** at the bottom of the page. The protocol is listed under the Protocols heading.

Thermal Profile Parameters for RT

The High-Capacity cDNA Reverse Transcription Kits use the following thermal profile parameters for the RT step.

Step Type	Time	Temperature
HOLD	10 min	25 °C
HOLD	120 min	37 °C
HOLD	5 sec	85 °C

Note: If you are using a standalone thermal cycler, you can add an additional 4 °C HOLD step. For more information, see the *Applied Biosystems High-Capacity cDNA Reverse Transcription Kits Protocol* (PN 4375575).

Note: Thermal cycling conditions for one-step RT-PCR are described on page 30.

Notes

Storing cDNA

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

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

Example Experiment - Standard Plate

For the example experiment, total RNA was extracted from blood. RNA concentration was determined (using A_{260}).

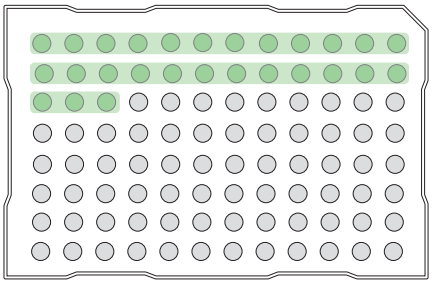
The RT master mix was prepared as follows, using guidelines from the *High-Capacity cDNA Reverse Transcription Kits Protocol* (PN 4375575):

Component	µL/Reaction	µL/27 Reactions‡
10X Reverse Transcription Buffer	2.0	54
25X dNTPs	0.8	21.6
10X random primers	2.0	54
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.0	27
Nuclease-free water	4.2	113.4
Total	10	270

‡ Each RT reaction is 20 µL (see below). If you need 5 µL of cDNA at 50-µL total volume for each of 104 PCR reactions per plate (see "Preparing the PCR Master Mix" on page 20), you need 27 RT reactions. Extra volume is included to account for pipetting losses, as well as extra cDNA for archiving.

The cDNA plate was then prepared by pipetting in each well:

- 10 µL of the RT master mix
- 10 µL of RNA sample



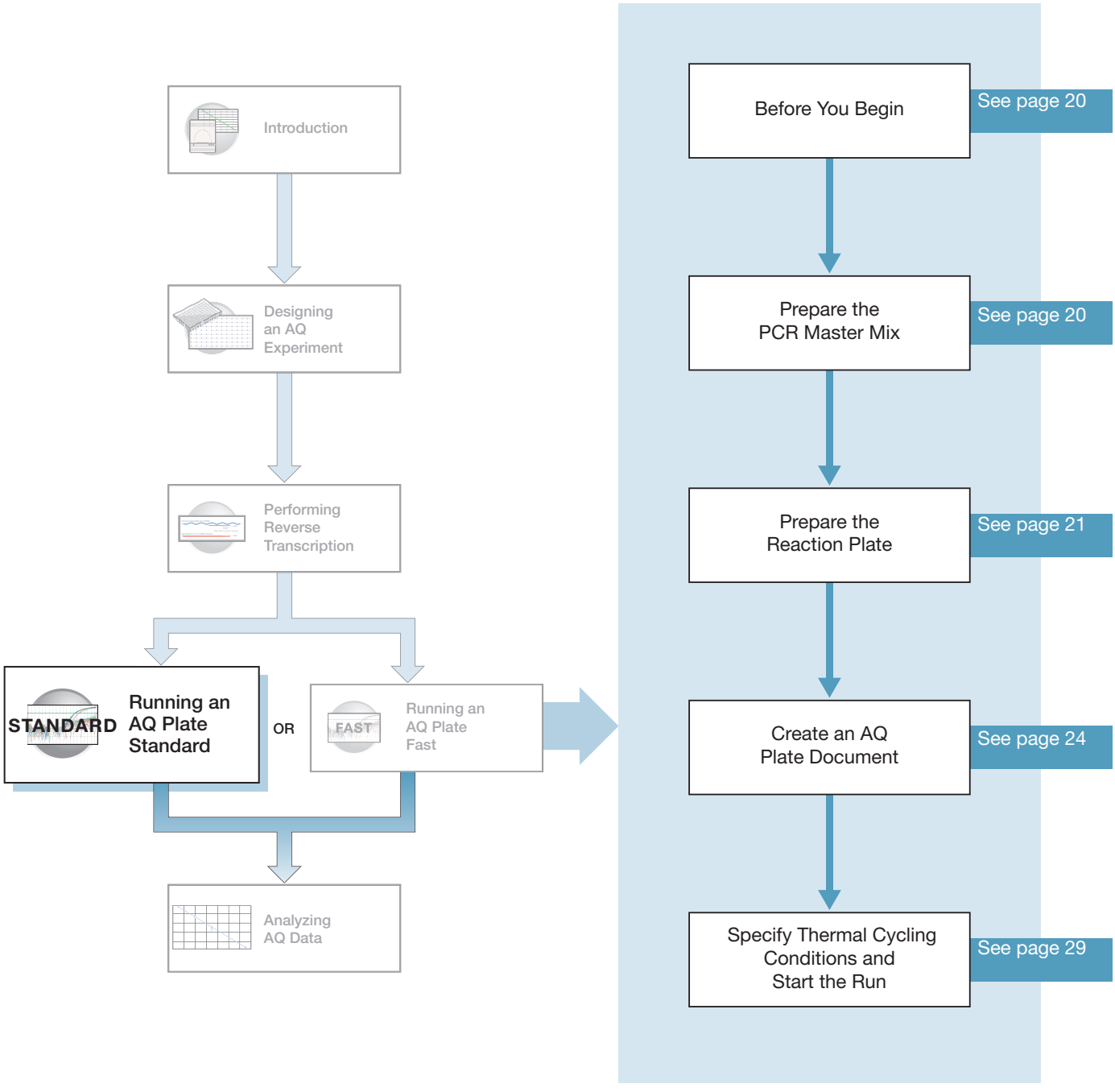
The RNA was then converted to cDNA using the thermal profile parameters for two-step RT-PCR, as described in "Thermal Profile Parameters for RT" on page 17.

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

Notes

STANDARD

Running an AQ Plate – 7300 or Standard 7500 System



4

Notes

Before You Begin

Check that background and pure-dye runs have been performed regularly to ensure optimal performance of the 7300 or Standard 7500 system. For more information about calibrating the 7300/7500 system, refer to the Online Help and the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide*.

Preparing the PCR Master Mix

The second step (PCR) in the two-step RT-PCR procedure is amplifying the cDNA, which you perform using the TaqMan® Universal PCR Master Mix.

Refer to the *TaqMan Universal PCR Master Mix Protocol* (PN 4351891) for details on how to use the reagents. The following table lists the universal assay conditions (volume and final concentration) for using the master mix.

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

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

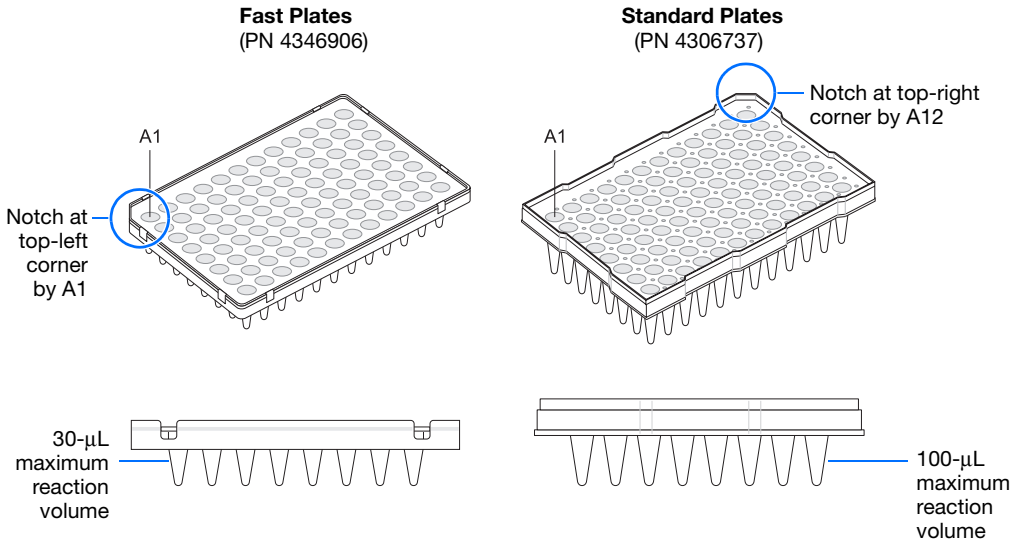
If you design probes and primers using Primer Express® Software, they must be optimized to work with the universal assay conditions, using the volumes listed in the table above. Refer to the *TaqMan Universal PCR Master Mix Protocol* (PN 4351891) for primer optimization. All TaqMan® Gene Expression Assays and Custom TaqMan® Gene Expression Assays are formulated so that the final concentration of the primers and probes are within the recommended values.

Notes

Preparing the Reaction Plate

Standard vs. Fast Plates

IMPORTANT! Make sure that you use the Standard Optical 96-Well Plate on the 7500 Real-Time PCR System. Fast Optical 96-Well Plates will not fit into the standard block correctly and will result in loss of data.



1. Label the reaction plate, ensuring that you include a set of standards for every target sequence. The standards must be on the same plate as the target sequence.

   Note: The arrangement of the reactions (samples and assays) on the plate should match the arrangement (sample names and detectors/markers) in the plate document used for the run.

2. Into each well of the reaction plate, add 50 µL of the appropriate PCR master mix.

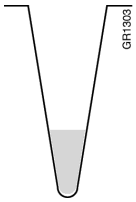
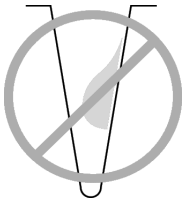
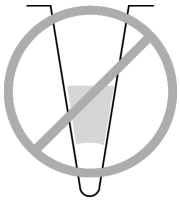
   Note: Prepare reactions containing standards exactly the same way as reactions containing unknowns. Use the same primers and probes, PCR master mix components, and volume, but add to each standard mix a known quantity of template (such as cDNA or plasmid DNA). Prepare all components in a reaction mix prior to adding to the plate wells.

3. Seal the reaction plate with an optical adhesive cover.

4. Centrifuge the plate briefly.

Notes

5. Verify that each reaction is positioned in the bottom of the well.

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

IMPORTANT! Ensure all reaction is positioned correctly in the bottom of the well before starting a run. Failure to do so will impact the quality of data.

6. Keep the reaction plate on ice until you are ready to load it into the 7300/7500 system.

Notes

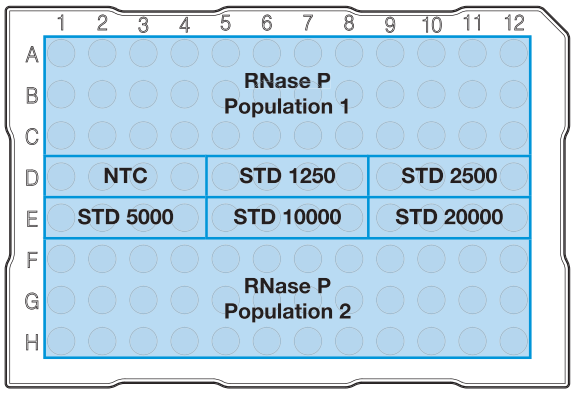
Example Experiment

The PCR master mixes were prepared according to the universal assay conditions.

Reaction Component	µL/Reaction	µL/5 Reactions‡	µL/37 Reactions§	Final Concentration
TaqMan Universal PCR Master Mix (2×)	25.0	125.0	925.0	1×
Forward primer	5.0	25.0	185.0	50 to 900 nM
Reverse primer	5.0	25.0	185.0	50 to 900 nM
TaqMan probe	5.0	25.0	185.0	50 to 250 nM
cDNA sample or template for standards	5.0	25.0	185.0	10 to 100 ng
Nuclease-free water	5.0	25.0	185.0	—
Total	50.0	250.0	1850.0	—

‡ One master mix was prepared for each of the six standards (4 replicates, plus extra volume for pipetting losses).
§ One master mix was prepared for each of the two populations being studied (36 samples, plus extra volume for pipetting losses).

Unknowns (target sequences being quantitated) and standards were arranged on a plate. 50 µL of the appropriate PCR master mix (containing cDNA) was added to each well. The plate was kept on ice until it was loaded in the 7500 system.



4

Notes

Creating an AQ Plate Document

Overview

An AQ plate document stores data collected from an AQ run for a single plate. AQ plate documents also store other information about the run, including sample names and detectors.

Run Setup Requirements

For each AQ plate document that you create, specify detectors, standards, and detector tasks:

- A detector is a virtual representation of a gene-specific nucleic acid primer and probe combination used in assays. You specify which detector to use for each target sequence. Appendix A on page 73 explains how to create detectors.
- A standard is a known amount of a target sequence. You must have a set of standards for each target sequence on the plate.
- A detector task specifies how the software uses the data collected from the well during analysis. You apply one of three tasks to each detector in each well of a plate document.

Task	Symbol	Apply to detectors of...
Unknown	U	Wells that contain target sequences that you are quantitating.
Standard	S	Wells that contain samples of known quantities.
No Template Controls (NTC)	N	Negative control wells that contain PCR reagents, but that lack template.

Notes

Creating an AQ Plate Document

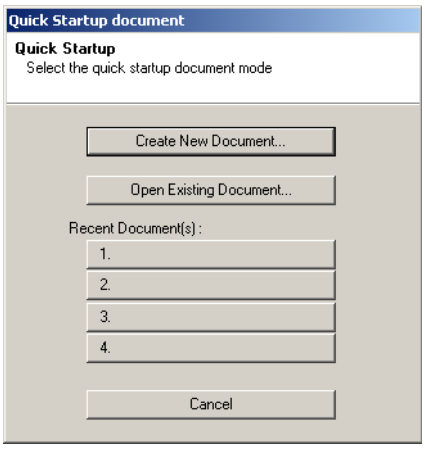
You can enter sample information into a new plate document, copy and paste sample information from existing plate documents, import sample information from existing plate documents, or use a template document to set up new plate documents. This section describes setting up new plate documents. Refer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

Note: The following procedure is illustrated using the example experiment data file (see page 7).

To create a new AQ plate document:

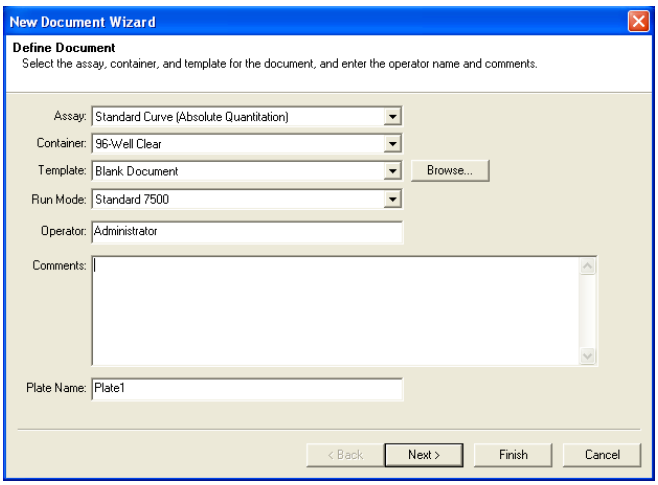
1. Select **Start > All Programs > Applied Biosystems > 7300/7500 System > 7300/7500 System Software** ( ) to start the SDS software.

2. In the Quick Startup document dialog box, select **Create New Document**.



3. In the Assay drop-down list of the New Document Wizard, select **Standard Curve (Absolute Quantitation)**. Accept the default settings for Container and Template (**96-Well Clear** and **Blank Document**). Choose from **Standard 7300, Standard 7500, or 9600 Emulation** Run Modes.

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



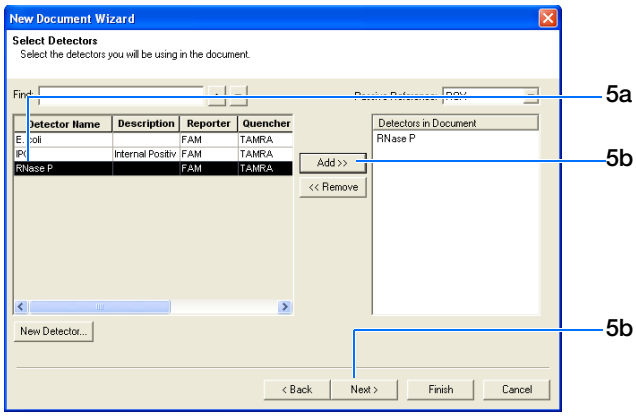
4. Enter a name in the Plate Name field, or accept the default, then click **Next >**.

Notes

5. Select detectors to add to the plate document.

   a. Click to highlight a detector, for example, **RNase P**. (Ctrl-click to select multiple detectors.) If no detectors are listed in the Detector Manager, click **New Detector** to open the New Detector dialog box. For more information about creating new detectors, refer to Appendix A on page 73.

   b. Click **Add >>** to add the detectors to the plate document, then click **Next >**.



   Note: To remove a detector from the Detectors in Document panel, select the detector, then click **Remove**.

6. Specify the detectors and tasks for each well.

   a. Click on a well (or group of wells, for replicates) to select it.

   b. Click on the detector name(s) to select the detector(s) for the well.

   c. Click under the Task column to assign the detector task.

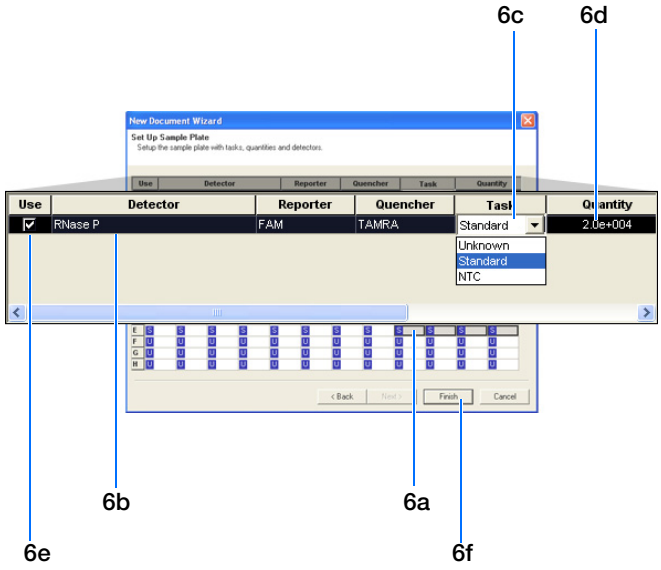
   d. Enter a quantity for wells that contain standards.

   e. Click **Use**.

      The detector task and color are displayed in the selected wells.

   f. Click **Finish**.

      The SDS software creates the plate document.



Notes

7. Enter the sample names.

   a. Click or select **View > Well Inspector**.

   Note: To enter sample names without using the Well Inspector, click-drag to select wells, then type the sample name.

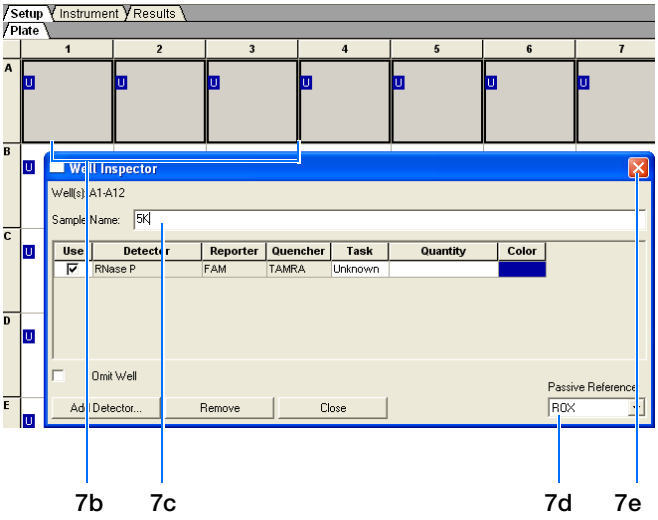
   b. Click a well or click-drag to select replicate wells.

   c. Enter the sample name.

   d. If necessary, change the setting for the Passive Reference dye. (By default, the ROX™ dye is selected.)

   e. Repeat steps b through d until you have specified sample names and passive reference dyes for all the wells on the plate, then click ( ) to close the Well Inspector.

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



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

8. Verify the information on each well in the Setup tab.

4

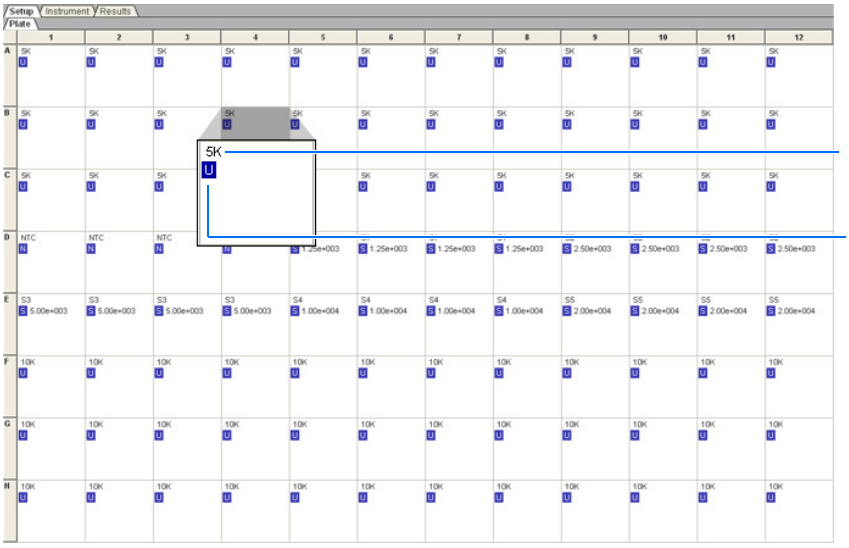
Notes

Example Experiment

The samples being quantitated and the standards were arranged on a single plate. Each well was associated with a detector (indicated by the colored squares). Each well was also assigned a detector task—U (unknown), S (standard), or N (no template control).

Only one detector (named RNase P) was defined because only one gene was being quantitated.

The figure below shows the example AQ plate document after sample names, detectors, and detector tasks were assigned for each well.

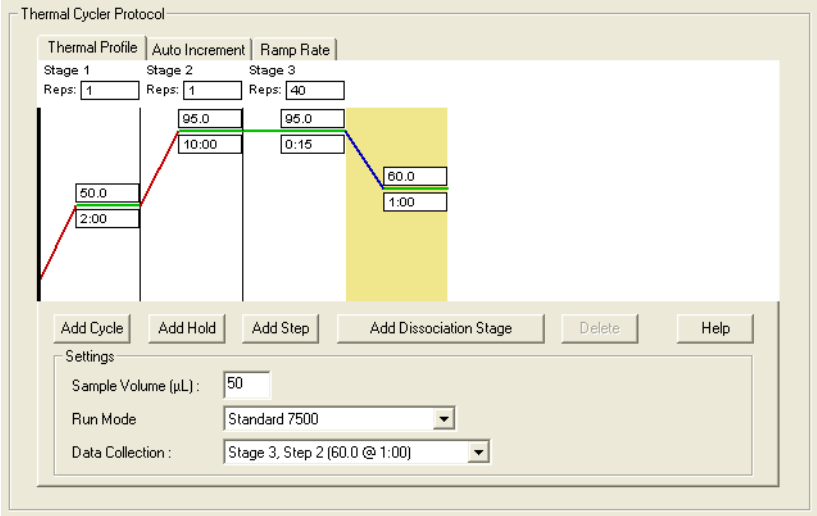


Notes

Specifying Thermal Cycling Conditions and Starting the Run

Default Thermal Cycling Conditions for PCR

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



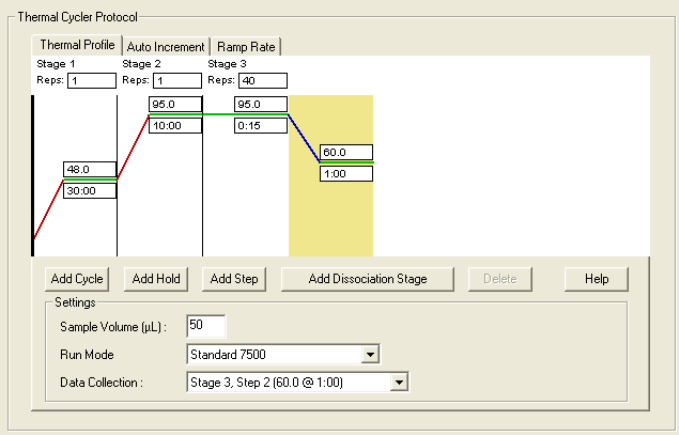
The default thermal cycling conditions for the PCR step of the procedure shown in the following table, Times and Temperatures (Two-step RT-PCR), should appear on the Instrument tab.

Times and Temperatures (Two-step RT-PCR)				
1) RT Step	HOLD	HOLD	HOLD	
	10 min @ 25 °C	120 min @ 37 °C	5 sec @ 85 °C	
2) PCR Step	Initial Steps		PCR (Each of 40 cycles)	
	AmpErase® UNG Activation	AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/Extend
	HOLD	HOLD	CYCLE	
	2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Notes

Thermal Cycling Conditions for One-Step RT-PCR

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



The following table, Times and Temperatures (One-step RT-PCR), shows the thermal cycling conditions for one-step RT-PCR experiments.

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

Notes

To specify thermal cycling conditions and start the run:

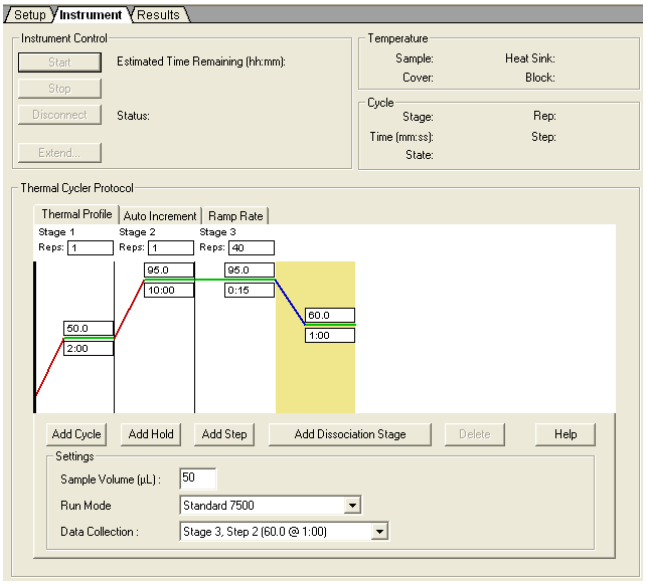
1. Select the **Instrument** tab.

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

2. Verify that:
   - If you are using two-step RT-PCR – The default PCR thermal cycling conditions are set.

     Note: If you are using one-step RT-PCR, use the thermal cycling parameters shown in "Thermal Cycling Conditions for One-Step RT-PCR" on page 30.

   - Sample Volume is 50 µL.
   - Verify desired Run Mode.



Note: If you are using SYBR® Green I reagent chemistry and you want to determine if there is contamination or if you want to determine the dissociation temperature of your amplicons, click **Add Dissociation Stage**. The dissociation stage includes a post-dissociation cooling step. Refer to the Online Help for more information.

Note: In the 7300 instrument, the 9600 Emulation feature is not available.

Notes

3. Select **File** > **Save**, enter a name for the AQ plate document, then click **Save**.

   (Optional) If you want to use this plate document again, save it as a template document. Select **File > Save As**. In the **Save in** drop-down list, navigate to **Applied Biosystems\7300\7500\ 7500 Fast System\Templates**. Type the File name, then select (*.sdt) for **Save as type** to save the file as a template.

4. Load the plate into the instrument.

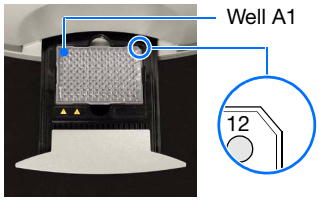
   Note: The A1 position is in the top-left side of the instrument tray.

5. Click **Start**.

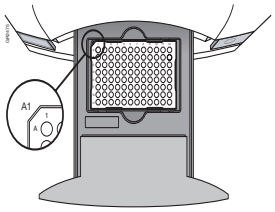
   As the instrument performs the PCR run, it displays real-time status information in the Instrument tab and records the fluorescence emissions.

   After the run, the status values and buttons are grayed-out, the Analysis button is enabled ( ), and a message indicates whether or not the run is successful.

   All data generated during the run are saved to the AQ plate document that you specified in step 3.



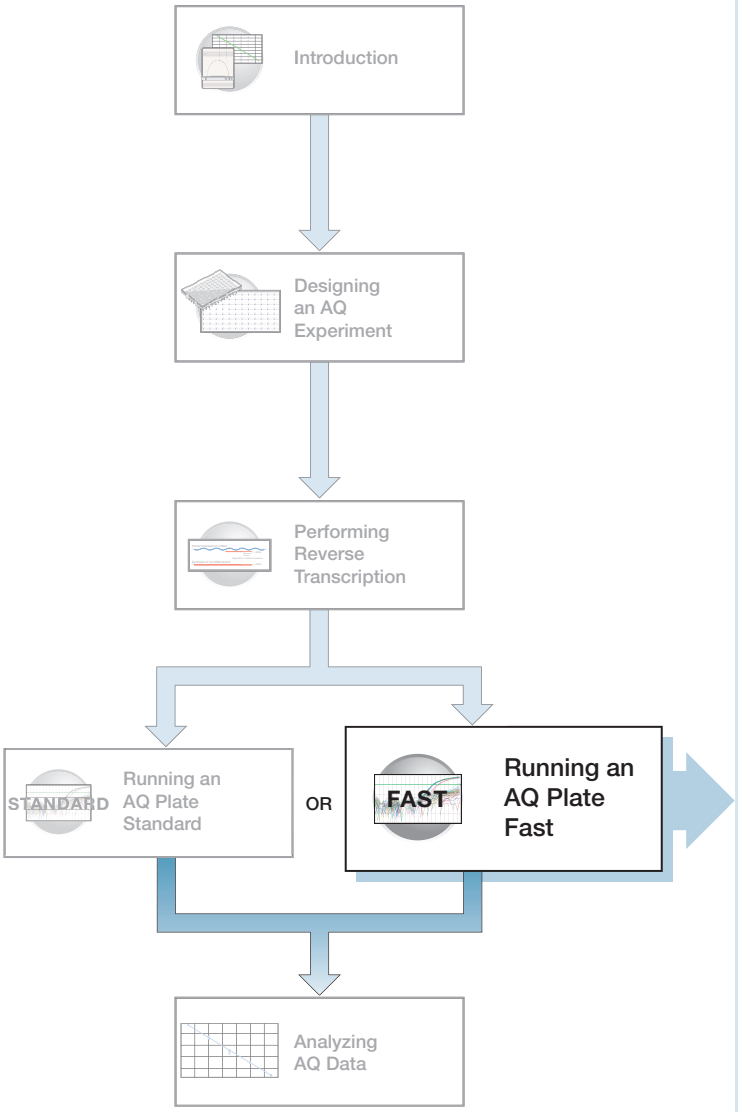
7300/7500 system: Notched top-right corner for standard plates



7500 Fast system: Notched top-left corner for Fast plates

Notes

Running an AQ Plate – 7500 Fast System



Notes

Before You Begin

Check that background and pure-dye runs have been performed regularly to ensure optimal performance of the 7500 Fast system. For more information about calibrating the 7500 Fast system, refer to the Online Help and the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide*.

Preparing the PCR Master Mix

The second step (PCR) in the two-step RT-PCR procedure is amplifying the cDNA, which you perform using the TaqMan® Universal PCR Master Mix reagents.

Users of the 7300/7500 system must use standard *TaqMan Universal PCR Master Mix (2×)* for a 2-hour run time. Users of the 7500 Fast System can choose either the *TaqMan Universal PCR Master Mix (2×)* or *TaqMan Fast Universal PCR Master Mix (2×)* for a run time of fewer than 40 minutes. For further information on the use of Fast Master Mix, refer to *TaqMan Fast Universal PCR Master Mix Protocol* (PN 4351891).

IMPORTANT! If you are using *TaqMan Fast Universal PCR Master Mix*, you must start the run within 2 hours of preparing the plate. The plate can be refrigerated or stored frozen if a run is not started within 2 hours of plate setup.

The *TaqMan Fast Universal PCR Master Mix Protocol* (PN 4351891) explains how to use the reagents in the kit. The following table lists the universal assay conditions (volume and final concentration) for using the master mix.

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

Component	Volume (µL) / 20-µL Reaction
TaqMan® Gene Expression Assay Mix (20×): • Forward PCR primer (18 µM) • Reverse PCR primer (18 µM) • TaqMan® probe (5 µM)	1.0
cDNA template (10 to 100 ng of RNA converted to cDNA + RNase-free water)	9.0‡
TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG	10.0
Total Volume	20.0

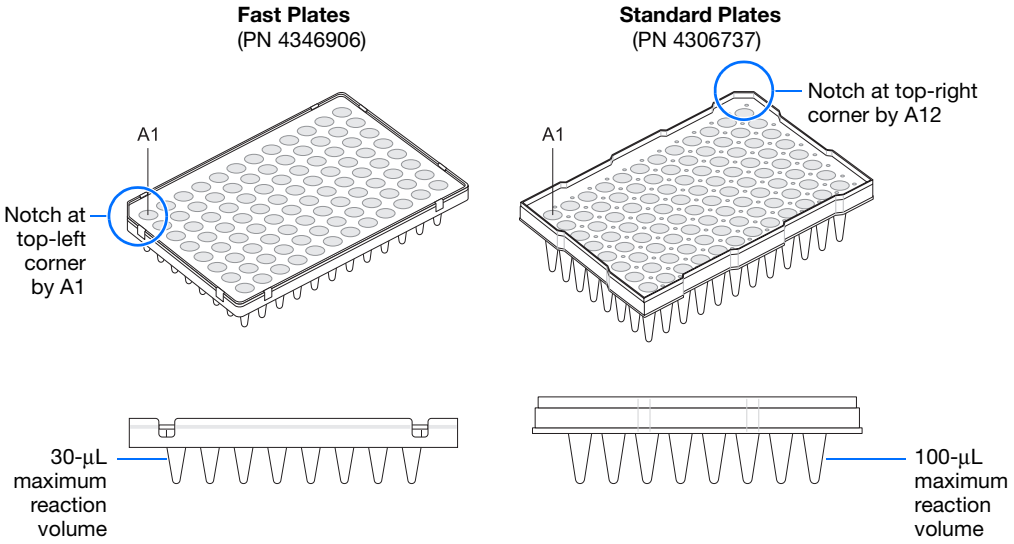
‡ If you choose to use UNG, decrease the volume of cDNA template and RNase-free water to 8.8 µL per 20-µL reaction and add 0.2 µL of UNG stock (1 U/µL).

Notes

Preparing the Reaction Plate

Fast vs. Standard Plates

IMPORTANT! Make sure that you use the Fast Optical 96-Well Plate on the 7500 Fast System. Standard plates will not function properly and may be crushed when using the 96-Well Fast Block.



1. Label the reaction plate, ensuring that you include a set of standards for every target sequence. The standards must be on the same plate as the target sequence.

   Note: The arrangement of the reactions (samples and assays) on the plate should match the arrangement (sample names and detectors/markers) in the plate document used for the run.

2. For the 7500 Fast system, add 20 µL into each well of the low head space reaction plate of the appropriate PCR master mix.

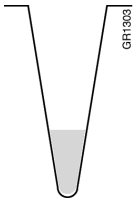
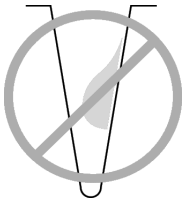
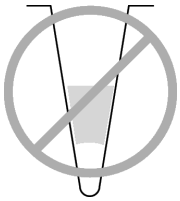
   Note: The reactions containing standards are prepared exactly the same way as reactions containing unknowns. You use the same primers and probes, PCR master mix components, and volume, but add to each standard mix a known quantity of template (such as cDNA or plasmid DNA). All components are prepared in a reaction mix prior to adding to the plate wells.

3. Seal the reaction plate with an optical adhesive cover.

4. Centrifuge the plate briefly.

Notes

5. Verify that each reaction is positioned in the bottom of the well.

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

IMPORTANT! Ensure all reaction is positioned correctly in the bottom of the well before starting a run. Failure to do so will impact the quality of data.

6. Keep the reaction plate on ice until you are ready to load it into the 7500 Fast system.

Notes

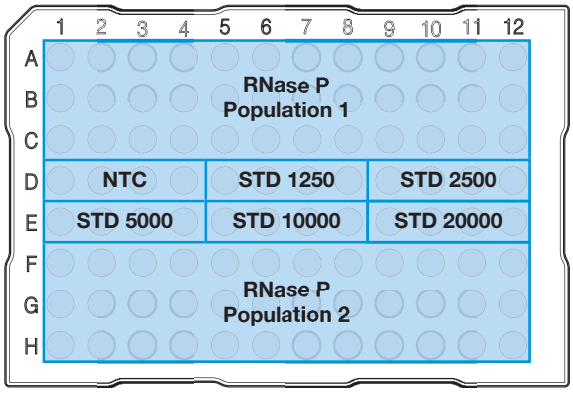
Example Experiment

The PCR master mixes were prepared according to the universal assay conditions.

Reaction Component	µL/Reaction	µL/5 Reactions‡	µL/ 37 Reactions§	Final Concentration
TaqMan Fast Universal PCR Master Mix (2×)	10.0	50.0	370.0	1×
TaqMan® Gene Expression Assay Mix (20×):				
• Forward PCR primer (18 µM)	Equal to 1.0	Equal to 5.0	Equal to 37.0	50 to 900 nM
• Reverse PCR primer (18 µM)				50 to 900 nM
• TaqMan® probe (5 µM)				50 to 250 nM
cDNA sample or template for standards	Equal to 9.0	Equal to 45.0	Equal to 333.0	10 to 100 ng
Nuclease-free water				—
Total	20.0	100.0	740.0	—

‡ One master mix was prepared for each of the six standards (4 replicates, plus extra volume for pipetting losses).
§ One master mix was prepared for each of the two populations being studied (36 samples, plus extra volume for pipetting losses).

Unknowns (target sequences being quantitated) and standards were arranged on a plate. For 7500 Fast system, 20 µL of the appropriate PCR master mix (containing cDNA) was added to each well.The plate was kept on ice until it was loaded in the 7500 Fast system.



5

Notes

Creating an AQ Plate Document

Overview

An AQ plate document stores data collected from an AQ run for a single plate. AQ plate documents also store other information about the run, including sample names and detectors.

Run Setup Requirements

For each AQ plate document that you create, specify detectors, standards, and detector tasks:

- A detector is a virtual representation of a gene-specific nucleic acid probe reagent used in assays. You specify which detector to use for each target sequence. Appendix A on page 73 explains how to create detectors.
- A standard is a known amount of a target sequence. You must have a set of standards for each target sequence on the plate.
- A detector task specifies how the software uses the data collected from the well during analysis. You apply one of three tasks to each detector in each well of a plate document.

Task	Symbol	Apply to detectors of...
Unknown	U	Wells that contain target sequences that you are quantitating.
Standard	S	Wells that contain samples of known quantities.
NTC	N	Negative control wells that contain PCR reagents, but that lack template.

Notes

Creating an AQ Plate Document

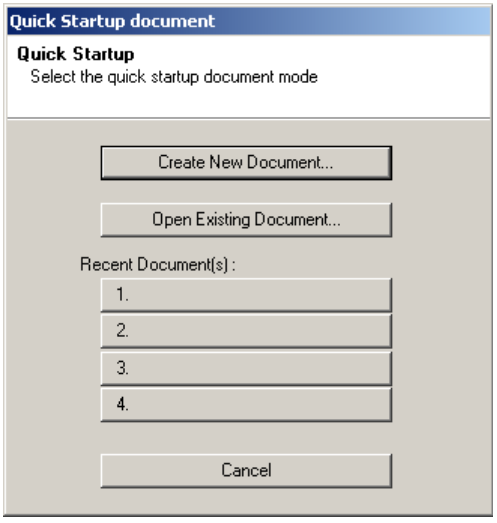
You can enter sample information into a new plate document, copy and paste sample information from existing plate documents, import sample information from existing plate documents, or use a template document to set up new plate documents. This section describes setting up new plate documents. Refer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

Note: The following procedure is illustrated using the example experiment data file (see page 7).

To create a new AQ plate document:

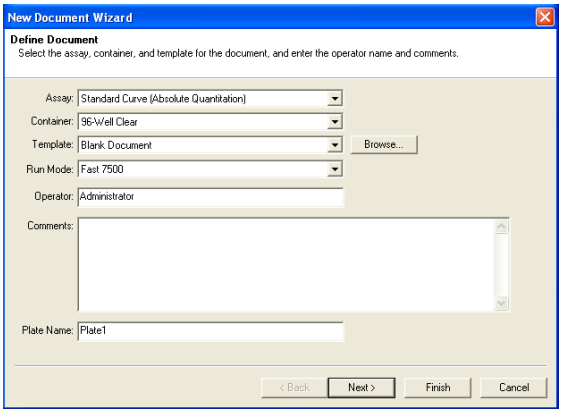
1. Select **Start > All Programs > Applied Biosystems 7500 Fast System > 7500 Fast System Software** ( ) to start the SDS software.

2. In the Quick Startup document dialog box, select **Create New Document**.



3. In the Assay drop-down list of the New Document Wizard, select **Standard Curve (Absolute Quantitation)**. Accept the default settings for Container and Template (**96-Well Clear** and **Blank Document**).

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



4. Enter a name in the Plate Name field, or accept the default and click **Next >**.

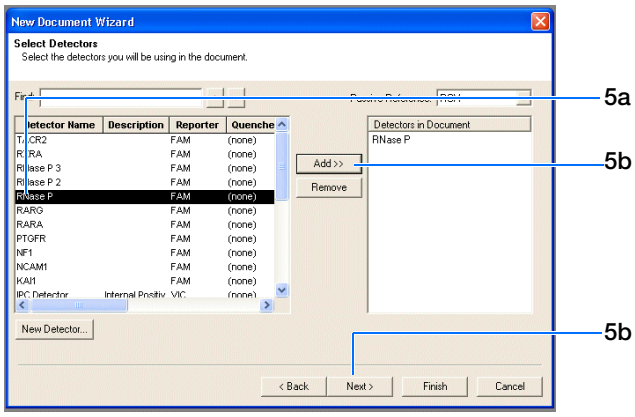
Notes

5. Select detectors to add to the plate document.

   a. Click to select a detector, for example, **RNase P**. (Ctrl-click to select multiple detectors.) If no detectors are listed in the Detector Manager, click **New Detector** to open the New Detector dialog box. For more information about creating new detectors, refer to Appendix A on page 73.

   b. Click **Add >>** to add the detectors to the plate document, then click **Next >**.

   Note: To remove a detector from the Detectors in Document panel, select the detector, then click **Remove**.



6. Specify the detectors and tasks for each well.

   a. Click a well (or group of wells, for replicates) to select it.

   b. Click the detector name(s) to select the detector(s) for the well.

   c. Click under the Task column to assign the detector task.

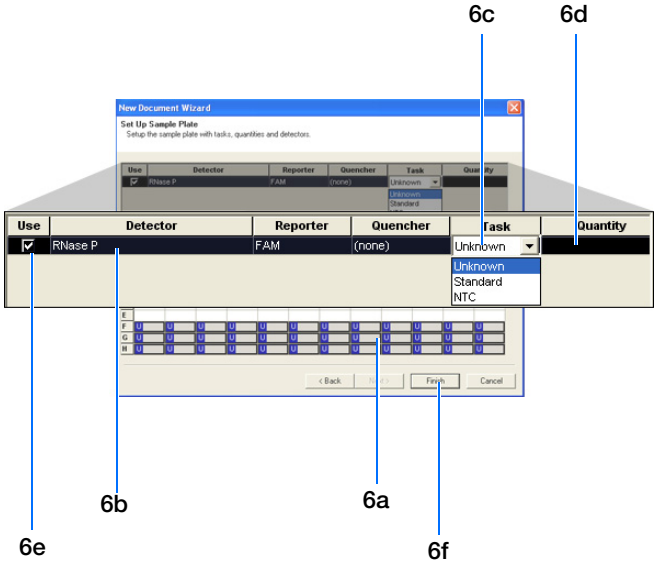
   d. Enter a quantity for wells that contain standards.

   e. Click **Use**.

      The detector task and color are displayed in the selected wells.

   f. Click **Finish**.

      The SDS software creates the plate document.



Notes

7. Enter the sample names.

   a. Click or select **View > Well Inspector**.

   Note: To enter sample names without using the Well Inspector, click-drag to select wells, then type the sample name.

   b. Click well or click-drag to select replicate wells.

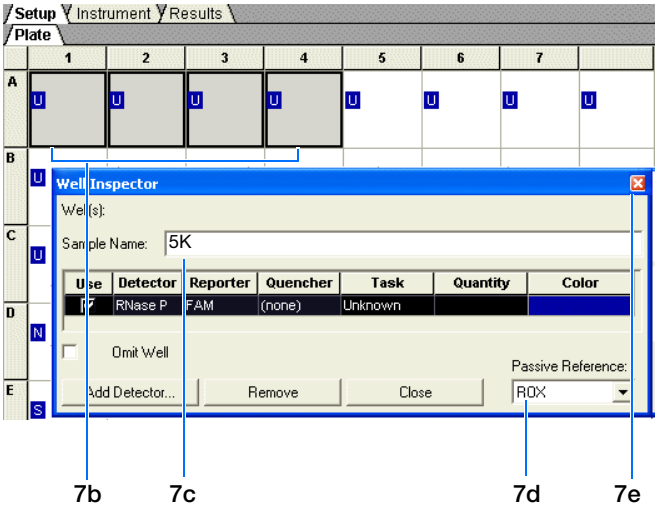
   c. Enter the sample name.

   d. If necessary, change the setting for the Passive Reference dye. (By default, the ROX™ dye is selected.)

   e. Repeat steps b through d until you have specified sample names and passive reference dyes for all the wells on the plate, then click ( ) to close the Well Inspector.

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

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



8. Verify the information on each well in the Setup tab.

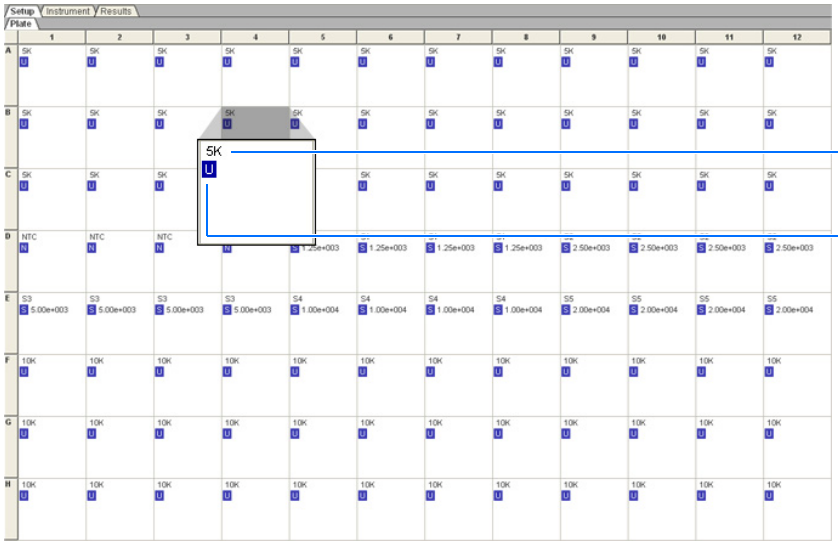
Notes

Example Experiment

The samples being quantitated and the standards were arranged on a single plate. Each well was associated with a detector (indicated by the colored squares). Each well was also assigned a detector task—U (unknown), S (standard), or N (no template control).

Only one detector (named RNase P) was defined because only one gene was being quantitated.

The figure below shows the example AQ plate document after sample names, detectors, and detector tasks were assigned for each well.



Notes

Specifying Thermal Cycling Conditions and Starting the Run

Running Assays Using Fast Thermal Cycling Conditions

- The performance of Fast thermal cycling and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG, has been verified for quantitative applications only and not for endpoint applications, such as allelic discrimination.
- The performance of Applied Biosystems TaqMan Gene Expression Assays and Custom TaqMan® Gene Expression Assays has been verified using the default Fast thermal cycling conditions and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG.
- Most custom 5′ nuclease quantitation assays designed with the Applied Biosystems Assay Design Guidelines will provide comparable performance when run using the default Fast thermal cycling conditions and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG (as compared to running the standard thermal cycling conditions and the TaqMan® 2× Universal PCR Master Mix). If you encounter poor performance, see "Troubleshooting" on page 48.
- When performing multiplex applications (when more than one target is amplified in a single tube), it may be necessary to perform some assay reoptimization. Before performing any multiplex applications, see the troubleshooting information on page 48 for further information.

Expert Mode

Expert mode allows you to select only those filters required for a particular experiment, reducing run times to less than 30 minutes. Observe the following guidelines and for detailed information, refer to *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Using Expert Mode User Bulletin*:

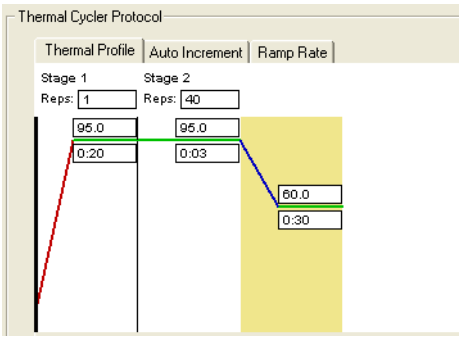
- The default thermal cycling protocol for Fast mode has an extension time of 30 seconds. This extension time has been tested for TaqMan Gene Expression Assays, TaqMan Pre-Developed Assay Reagents and Primer Express Software designed assays that are run using default Fast thermal cycling mode conditions.
- Use of extension times below 30 seconds has been shown to affect the performance of some assays. Validate the performance of assays with extension times of less than 30 seconds.
- Applied Biosystems strongly recommends the use of ROX™ dye to normalize variation caused by pipetting error.
- When using the ROX passive reference dye feature, it is important to select *both* the FAM™ and ROX dye filters.
- Add extension time if you use more than three filters to allow for data collection processes.

Note: The filters are labeled Filter A through E by default, but may be renamed as desired. Select **Tools > Filter Configuration** to open the Filter Naming window.

Notes

Default Thermal Cycling Conditions for PCR

If you selected the two-step RT-PCR method for your AQ experiment (recommended), you have already completed the RT step. At this point in the workflow, you are ready to PCR amplify cDNA. Users of the 7500 Fast System can choose between the standard default and Fast thermal cycling conditions. Expert Mode is disabled by default, and it is only available with the 7500 Fast mode.



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

Fast Default Times and Temperatures (Two-step RT-PCR)				
1) RT Step	HOLD	HOLD	HOLD	
	10 min @ 25 °C	120 min @ 37 °C	5 sec @ 85 °C	
Fast Thermal Cycling Conditions (Fast 7500 users only)				
2) PCR Step	Enzyme Activation		Melt	Anneal/Extend
Fast Conditions	20 sec @ 95 °C		3 sec @ 95 °C	30 sec @ 60 °C
Expert Mode Conditions	20 sec @ 95 °C		3 sec @ 95 °C	20 sec @ 60 °C

Notes

To specify thermal cycling conditions and start the run:

1. Select the **Instrument** tab.

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

2. Verify that:

   - If you are using two-step RT-PCR – The default PCR thermal cycling conditions are set.

     Note: If you are using one-step RT-PCR, set the thermal cycling parameters as shown in "Default Thermal Cycling Conditions for PCR" on page 44.

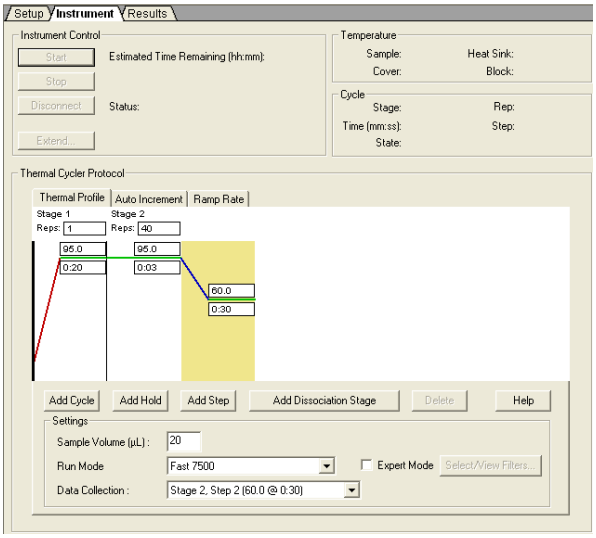
   - Sample Volume is 20 µL (for 7500 Fast).
   - Fast 7500 is selected as the Run Mode.

   Note: If you are using SYBR® Green I dye chemistry and you want to determine if there is contamination or if you want to determine the dissociation temperature of the amplicons, click **Add Dissociation Stage**. The dissociation stage includes a post-dissociation cooling step. Refer to the Online Help for more information. Users of the 7500 Fast System can use SYBR Green I dye with Standard or 9600 Emulation Run Modes.

   IMPORTANT! To enable Expert Mode, continue to step 3. Otherwise skip to step 6.

3. Click the Expert Mode checkbox.

4. Click the **Select/View Filters** button.



Notes

5. Select filters for data collection and click **OK**. If the checkbox next to a filter is checked, that filter is used for data collection.

IMPORTANT! If no filter is selected no data will be collected.

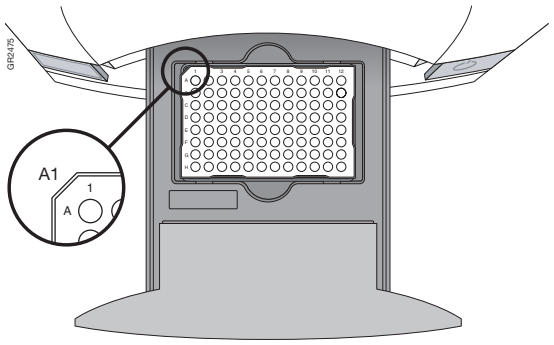
Note: The use of ROX dye is strongly recommended to normalize variation caused by pipetting error. Both the FAM and ROX dye filters must be turned on in order to excite the ROX passive reference dye in Applied Biosystems Master Mix correctly.

Note: For information on changing Filter names, refer to Online Help or the *Applied Biosystems 7500 Fast Real-Time PCR System Using Expert Mode User Bulletin*.

6. Select **File** > **Save As**, enter a name for the AQ plate document, then click **Save**.

   (Optional) If you want to use this plate document again, save it as a template document. Select **File > Save As**. In the **Save in** drop-down list, navigate to **Applied Biosystems\7300\7500\ 7500 Fast System\Templates**. Type the File name, then select (*.sdt) for **Save as type** to save the file as a template.

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



Note: The A1 position is in the top-left side of the instrument tray. The bar code is toward the front of the instrument.

Notes

8. Click **Start**.

   As the instrument performs the PCR run, it displays real-time status information in the Instrument tab and records the fluorescence emissions.

   After the run, the status values and buttons are grayed-out, the Analysis button is enabled ( ), and a message indicates whether or not the run is successful.

   All data generated during the run is saved to the AQ plate document that you specified in step 6.

Notes

Troubleshooting

Troubleshooting		
Observation	**Possible Cause**	**Action**
High C_T values/poor precision or failed PCR reactions	Target is difficult to amplify	• Increase the annealing/extension time in the thermal cycler protocol. • Increase the annealing/extension temperature to 62 °C.
	Insufficient cDNA template is present	Use 10 to 100 ng of cDNA template per 20-µL reaction.
	Quality of cDNA template is poor	1. Quantitate the amount of cDNA template. 2. Test the cDNA template for the presence of PCR inhibitors. 3. Measure $OD_{260/280}$ > 1.8 RNA or 1.9 DNA.
	Sample degradation	Prepare fresh cDNA, then repeat the experiment.
	The TaqMan Universal PCR Master Mix (2X) was used instead of the TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG	Prepare the reactions with the correct Master Mix.
	Primer-dimer formation	To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can load and run it on the 7500 Fast instrument.
Low ΔR_n or R_n values	Extension time is too short	Use the default thermal profile settings (see page 44).
	Primer-dimer formation	To ensure optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can load and run it on the 7500 Fast instrument.

Notes

Troubleshooting		
Observation	**Possible Cause**	**Action**
Run takes more than 40 minutes	Thermal cycler mode is set to Standard or 9600 Emulation	Make sure that the thermal cycler mode is set to Fast (see page 44).
Rn vs. Cycle plot is not displayed	ROX™ dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.
Extremely high ΔR_n or R_n values	ROX dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.
	Evaporation	Make sure that the reaction plate is sealed completely, especially around the edges.
High variability across the reaction plate	ROX dye was not selected as the passive reference when the plate document was set up	Select ROX dye as the passive reference when you set up the plate document.
	Evaporation	Make sure that the reaction plate is sealed completely, especially around the edges.
High variability across replicates	Reaction mix was not mixed well	Mix the reaction mix gently by inversion, then centrifuge briefly before aliquoting to the reaction plate.

Troubleshooting Multiplex Applications

IMPORTANT! Due to the challenging nature of multiplex applications and the complexity that can be encountered, it is impossible to guarantee assay performance. However, the recommendations listed below should be helpful when running multiplex applications using Fast thermal cycling conditions and the TaqMan Fast Universal PCR Master Mix (2×), No AmpErase UNG.

Perform the recommendations in the order listed.

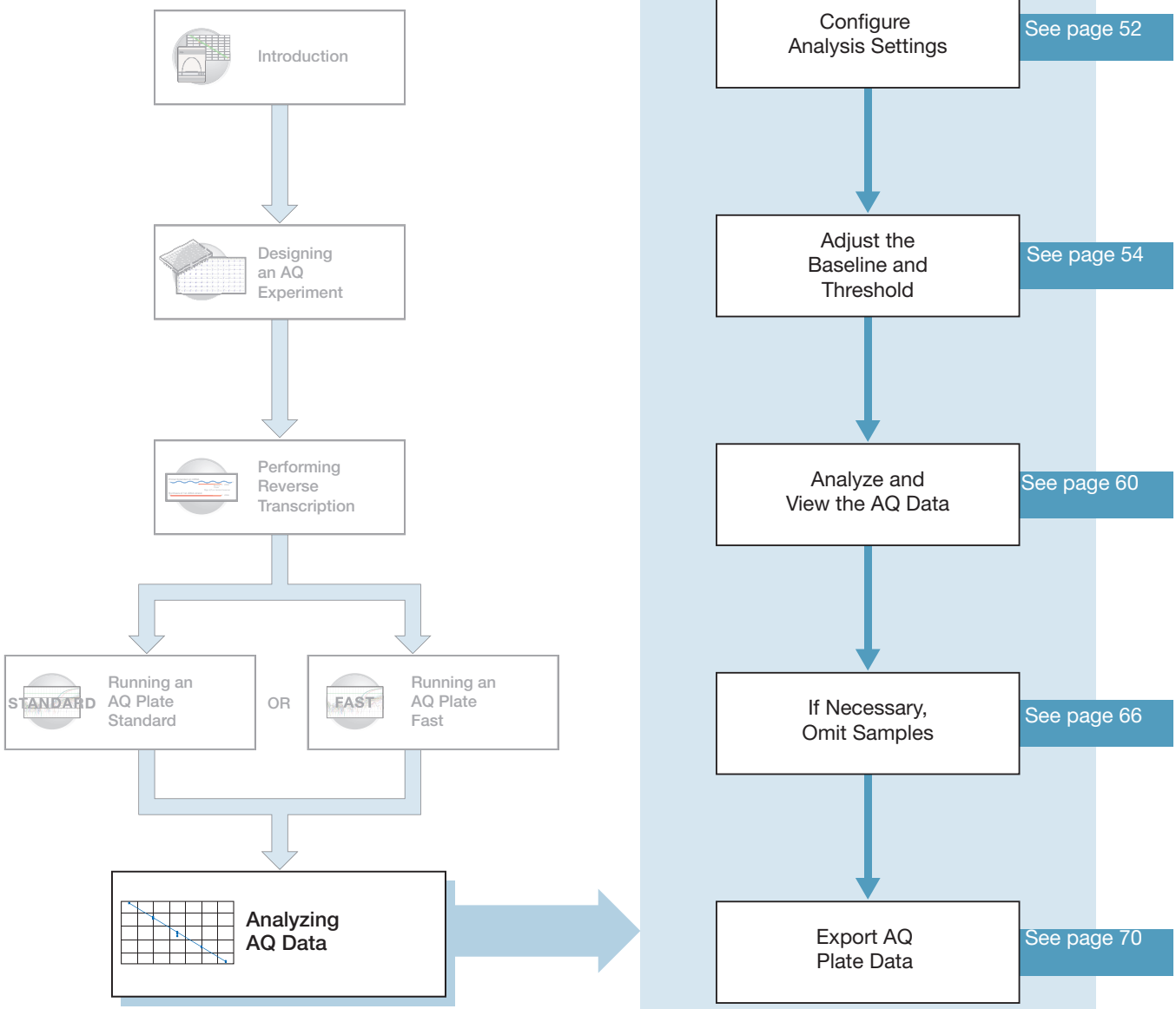
When running multiplex applications:

1. Increase the annealing/extension temperature to 62 °C.
2. If you do not obtain the expected performance by increasing the annealing/extension temperature to 62 °C, increase the annealing/extension time in the thermal cycling protocol by 5 seconds, to 35 seconds.
3. If you do not obtain acceptable performance by increasing both the annealing/extension temperature and time, assay reoptimization may be required. Refer to the *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for more information.

Notes

Notes

Analyzing AQ Data



Notes

Configuring Analysis Settings

Before you can analyze the data, you must specify parameter values for the analysis.

Unless you have already determined the optimal baseline and threshold settings for your experiment, use the automatic baseline and threshold feature of the SDS software (Auto Ct). If the baseline and threshold were called correctly for each well, you can proceed to view the results. Otherwise, you must manually set the baseline and threshold as explained in "Manual Baseline and Threshold Determination" on page 54.

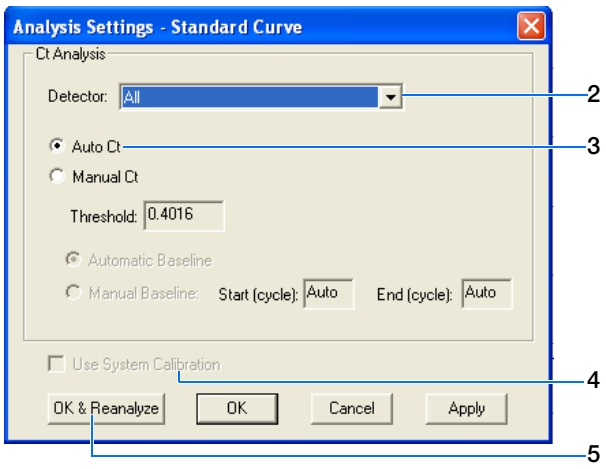
This section describes how to use the auto Ct feature.

To configure analysis settings:

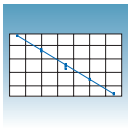
1. Click (**Analysis** > **Analysis Settings**).
2. In the Detectors drop-down list, select **All**.
3. Select **Auto Ct**. The SDS software automatically generates baseline values for each well and threshold values for each detector.

   IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each well, as explained in "Adjusting the Baseline and Threshold" on page 54.

   Alternatively, select Manual Ct and specify the threshold and baseline manually. You can also select Auto baseline and Manual Ct.



Notes



4. (Optional) Select **Use System Calibration** to use the calibration files that are stored on the computer you are currently using.

   Note: If you do not select **Use System Calibration**, the calibration information stored in your plate document is used. This information comes from the computer used for data collection when the plate was run.

   For more information about system calibration files, refer to the Online Help.

5. Click **OK & Reanalyze**.

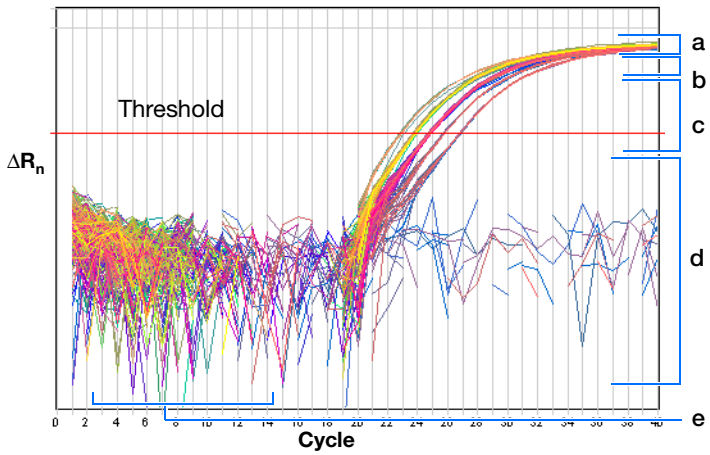
6. Examine the amplification plot, and if necessary manually adjust the baseline and threshold as explained in the following section.

Notes

Adjusting the Baseline and Threshold

Automatic Baseline and Threshold Determination

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



A typical amplification curve has a:

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

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

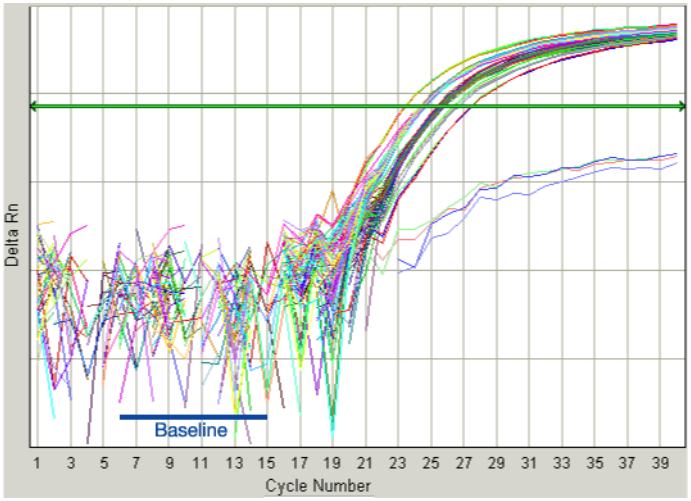
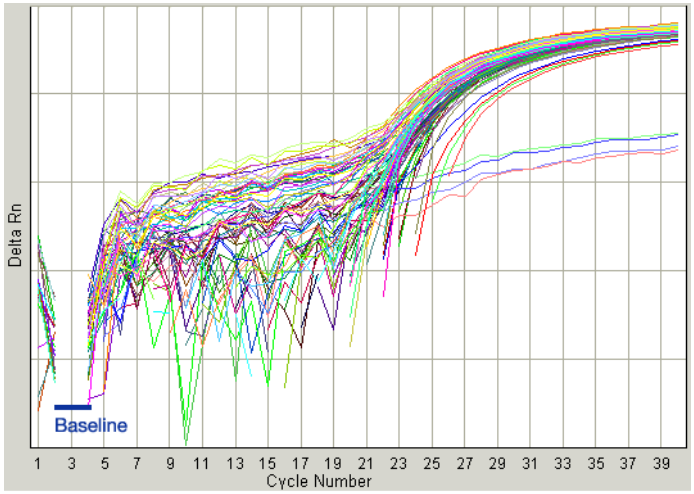
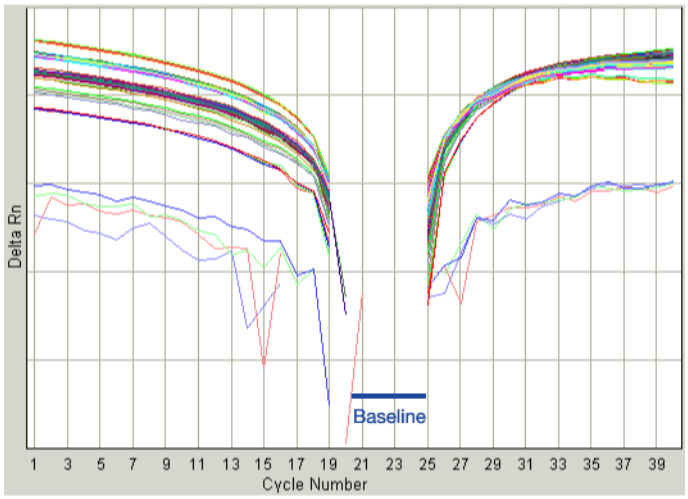
Therefore, Applied Biosystems recommends reviewing all baseline and threshold parameter values after analysis of the study data. If necessary, adjust the values manually as described on page 57.

Manual Baseline and Threshold Determination

If you set the baseline and threshold values manually for any detector in the study, you must perform the procedure on page 57 for each of the detectors.

The following amplification plots show the effects of baseline and threshold settings.

Notes

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

Notes

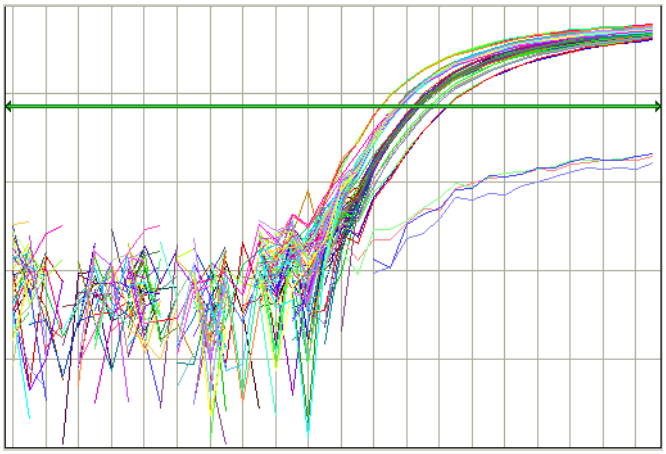
Threshold Set Correctly

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

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

Setup | Instrument | Results
Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered
A1	1-1	RNase P	Unknown	25.51	0.040	4788.97	4732.19	128.616	
A2	1-1	RNase P	Unknown	25.57	0.040	4581.29	4732.19	128.616	
A3	1-1	RNase P	Unknown	25.48	0.040	4877.24	4732.19	128.616	
A4	1-1	RNase P	Unknown	25.54	0.040	4681.28	4732.19	128.616	

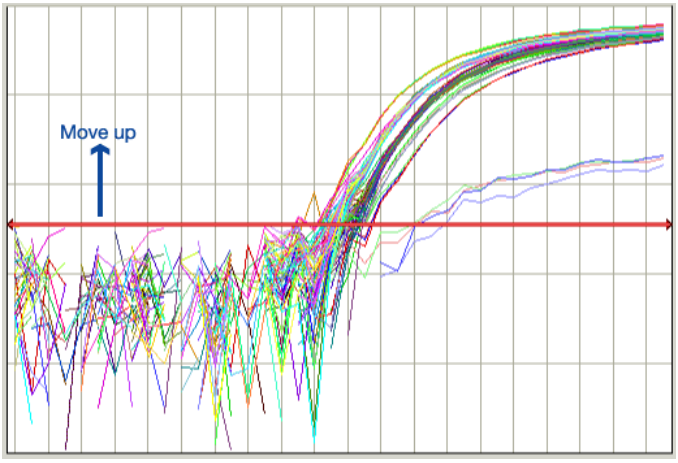


Threshold Set Too Low

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.

Setup | Instrument | Results
Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered
A1	1-1	RNase P	Unknown	20.27	0.576	8146.32	4586.23	2677.676	
A2	1-1	RNase P	Unknown	21.57	0.576	2171.80	4586.23	2677.676	
A3	1-1	RNase P	Unknown	20.73	0.576	5097.34	4586.23	2677.676	
A4	1-1	RNase P	Unknown	21.28	0.576	2929.45	4586.23	2677.676	

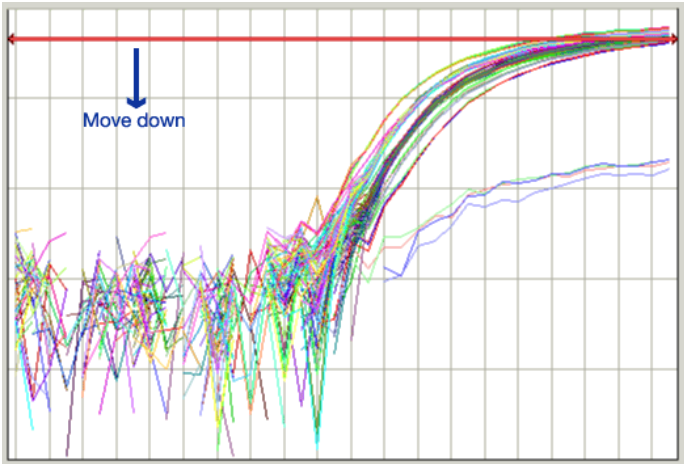


Threshold Set Too High

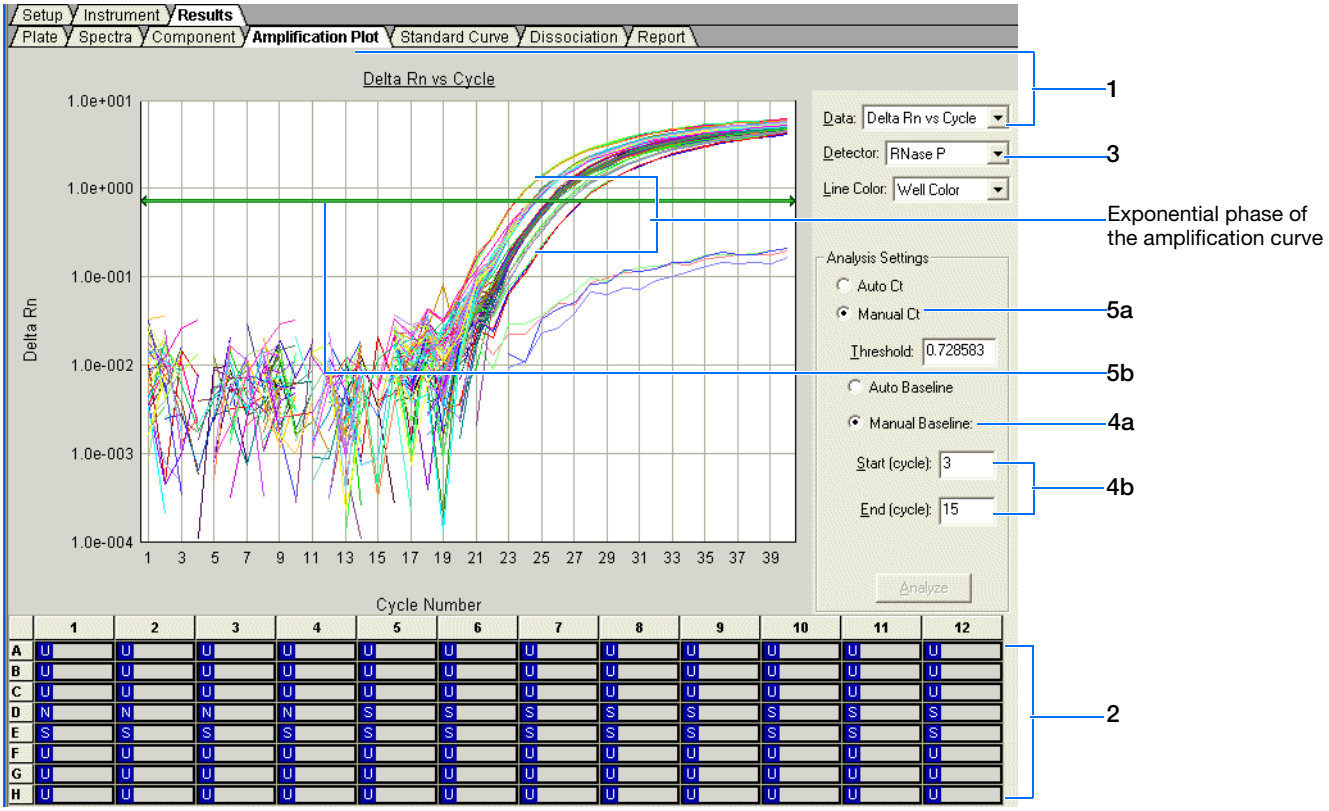
The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the exponential phase of the curve.

Setup | Instrument | Results
Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample	Detector	Task	Ct	StdDev Ct	Qty	Mean Qt	StdDev Qty	Filtered
A1	1-1	RNase P	Unknown	38.24	0.481	4325.50	5015.28	683.698	
A2	1-1	RNase P	Unknown	37.81	0.481	4867.56	5015.28	683.698	
A3	1-1	RNase P	Unknown	37.08	0.481	5960.34	5015.28	683.698	
A4	1-1	RNase P	Unknown	37.78	0.481	4907.72	5015.28	683.698	



Notes



To manually adjust the baseline and threshold:

1. Select the **Amplification Plot** tab, then select **Delta Rn vs Cycle** in the Data drop-down list.
2. Select the wells to display on the plot. (Otherwise, the plot will be empty.)
3. In the Detector drop-down list, select a detector. The SDS software displays the graph for the selected detector and wells.

Notes

4. Set the baseline for the detector.
   a. Under Analysis Settings, select **Manual Baseline**.
   b. Enter values in the Start (cycle) and End (cycle) fields, ensuring that the amplification curve growth begins at a cycle after the End Cycle value.

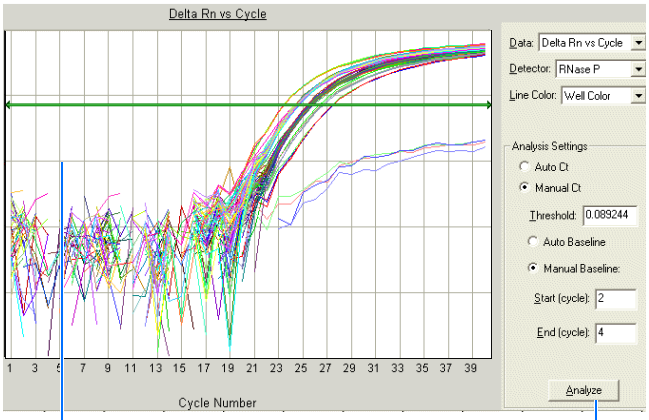
   Note: After you change a baseline or threshold setting for a detector, the Analyze button (Analyze) is enabled, indicating that you must reanalyze the data.

5. Set the threshold for the detector.
   a. Under Analysis Settings, select **Manual Ct**.
   b. Drag the threshold setting bar until the threshold is:
      - Above the background
      - Below the plateau and linear regions of the amplification curve
      - Within the exponential phase of the amplification curve

   The SDS software adjusts the threshold value and displays it in the Threshold field after the analysis.

6. Repeat steps 3 through 4 to set the baseline and threshold values for all remaining detectors in the study.

7. Click **Analysis > Analyze** to reanalyze the data using the adjusted baseline and threshold values.



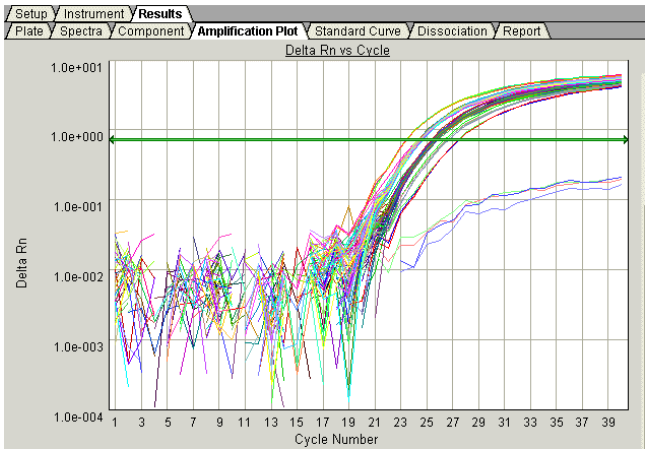
Drag the Threshold bar to adjust the threshold. The bar turns red, indicating that the threshold has been changed.

The Analyze button is enabled after a baseline or threshold setting is changed.

Notes

Example Experiment

The data was first analyzed using the Auto Ct and Auto Baseline settings, resulting in the following amplification plot.



On closer inspection, it appears that the baseline and threshold are called correctly and do not need adjustment:

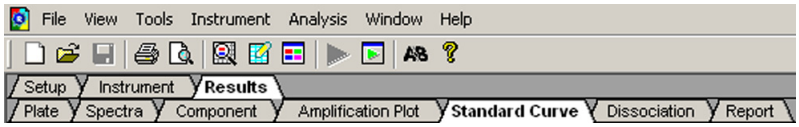
- The amplification curve begins after the maximum baseline.
- The threshold is set in the exponential phase of the amplification curve.

Notes

Analyzing and Viewing the AQ Data

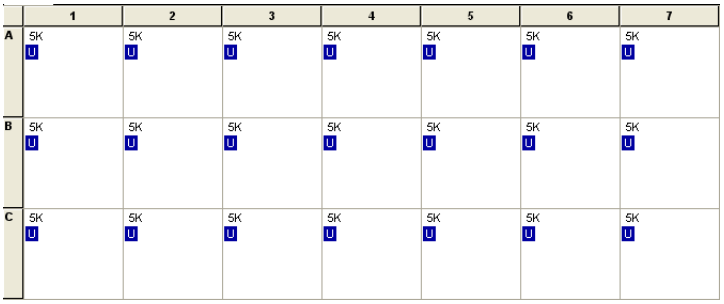
About the Results Tab

In the Results tab, you can view the results of the run and change the parameters. For example, you can omit samples or manually set the baseline and threshold. If you change any parameters, you should reanalyze the data.

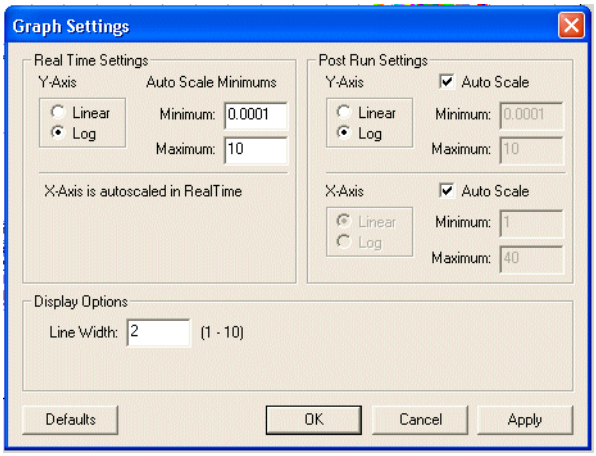


The Results tab has seven secondary tabs, each of which is described below. Details are provided in the Online Help.

- To move between views, click a tab.
- To select all 96 wells on a plate, click the upper-left corner of the plate.



- To adjust graph settings, double-click the y- or x-axis of a plot to display the Graph Settings dialog. The adjustable settings depend on which plot you are viewing.



Notes

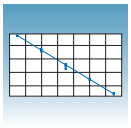
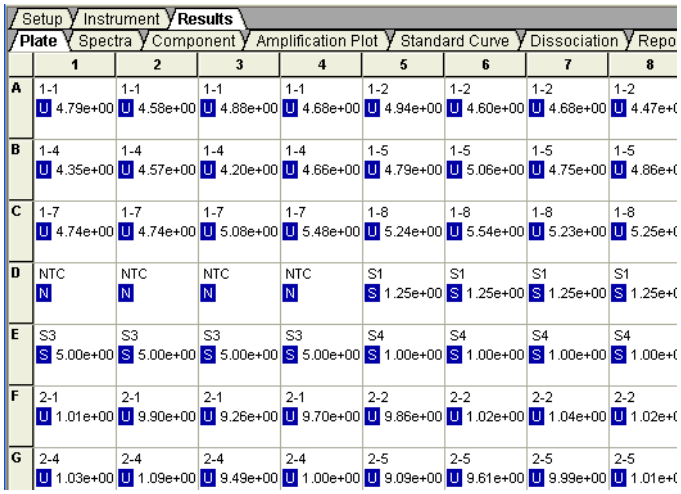


Plate Tab

Displays the results data of each well, including:

- The sample name and detector task and color for each well.
- A calculated value—quantity (default), ΔRn, or Ct. Select **Analysis > Display** to select the value to display.

Note: For detectors without standards, the Plate Tab displays "Undet." (meaning undetermined).

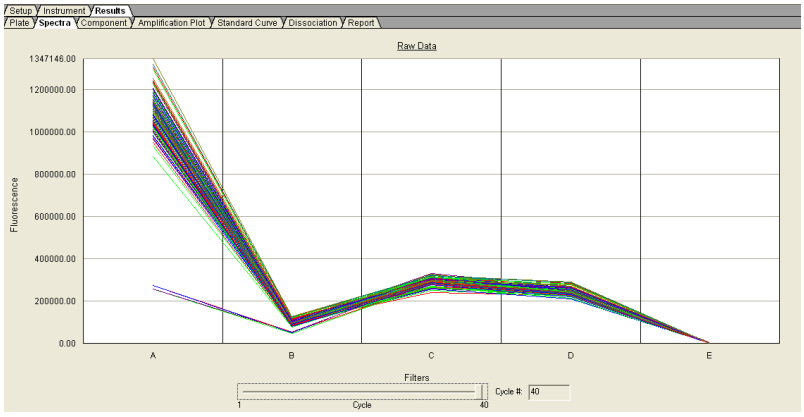


	1	2	3	4	5	6	7	8
A	1-1 U 4.79e+00	1-1 U 4.58e+00	1-1 U 4.88e+00	1-1 U 4.68e+00	1-2 U 4.94e+00	1-2 U 4.60e+00	1-2 U 4.68e+00	1-2 U 4.47e+0
B	1-4 U 4.35e+00	1-4 U 4.57e+00	1-4 U 4.20e+00	1-4 U 4.66e+00	1-5 U 4.79e+00	1-5 U 5.06e+00	1-5 U 4.75e+00	1-5 U 4.86e+0
C	1-7 U 4.74e+00	1-7 U 4.74e+00	1-7 U 5.08e+00	1-7 U 5.48e+00	1-8 U 5.24e+00	1-8 U 5.54e+00	1-8 U 5.23e+00	1-8 U 5.25e+0
D	NTC N	NTC N	NTC N	NTC N	S1 S 1.25e+00	S1 S 1.25e+00	S1 S 1.25e+00	S1 S 1.25e+0
E	S3 S 5.00e+00	S3 S 5.00e+00	S3 S 5.00e+00	S3 S 5.00e+00	S4 S 1.00e+00	S4 S 1.00e+00	S4 S 1.00e+00	S4 S 1.00e+0
F	2-1 U 1.01e+00	2-1 U 9.90e+00	2-1 U 9.26e+00	2-1 U 9.70e+00	2-2 U 9.86e+00	2-2 U 1.02e+00	2-2 U 1.04e+00	2-2 U 1.02e+0
G	2-4 U 1.03e+00	2-4 U 1.09e+00	2-4 U 9.49e+00	2-4 U 1.00e+00	2-5 U 9.09e+00	2-5 U 9.61e+00	2-5 U 9.99e+00	2-5 U 1.01e+0

Spectra Tab

Displays the fluorescence spectra of selected wells.

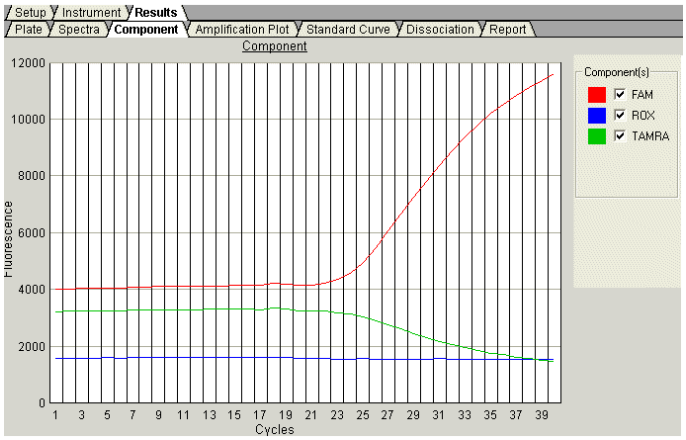
- The Cycles slider allows you to see the spectra for each cycle by dragging it with the pointer.
- The Cycle # text box shows the current position of the slider.



Notes

Component Tab

Displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run. Only the first selected well is shown at one time.



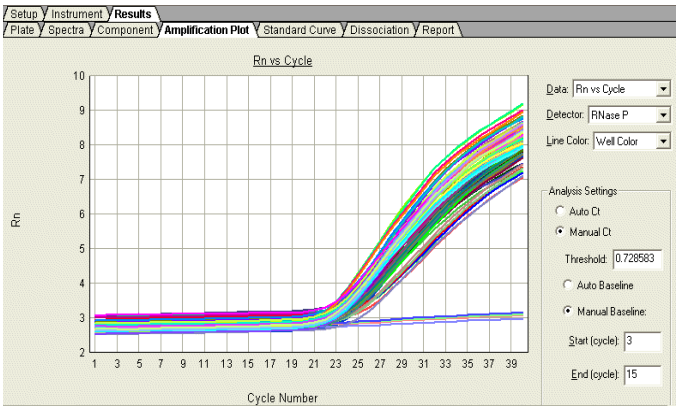
Note: If you are using TaqMan® products, three components (ROX™ dye, reporter dye, and TAMRA™ dye-labeled quencher) are displayed in the Component tab. If you are using TaqMan® MGB products, only two components (ROX and reporter dyes) are displayed.

Amplification Plot Tab

The three Amplification Plots allow you to view post-run amplification of specific samples. The Amplification Plots display all samples in the selected wells.

Rn vs. Cycle (Linear) View

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

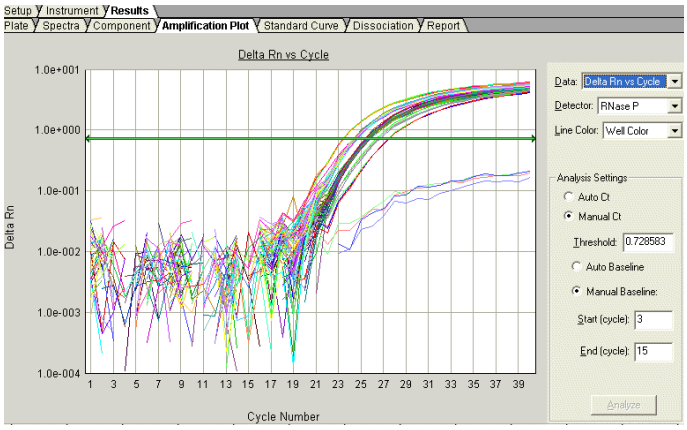


For more information about R_n, refer to the *Real-Time PCR Systems Chemistry Guide*.

Notes

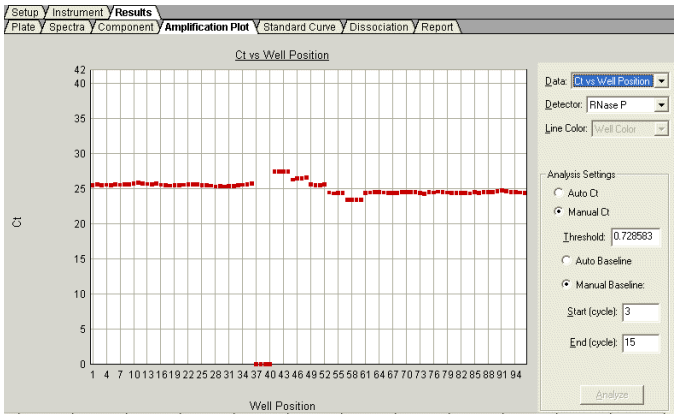
ΔRn vs.Cycle (Log) View

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



Ct vs. Well Position View

Displays threshold cycle (C_T) as a function of well position. You can use this plot to locate outliers in detector data sets (see "Omitting Samples" on page 66 for more information).

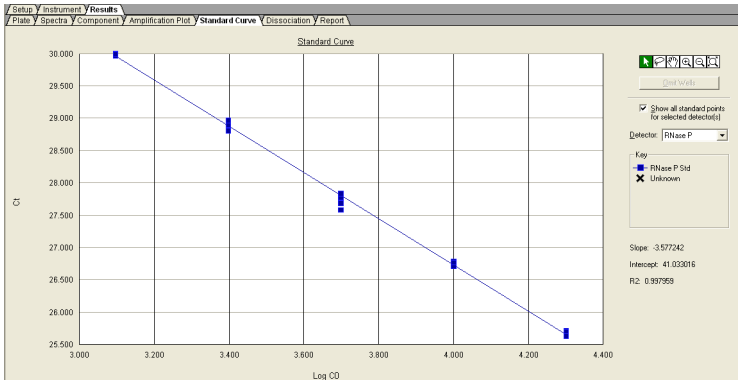


Notes

Standard Curve

Displays the standard curve for samples designated as standards.

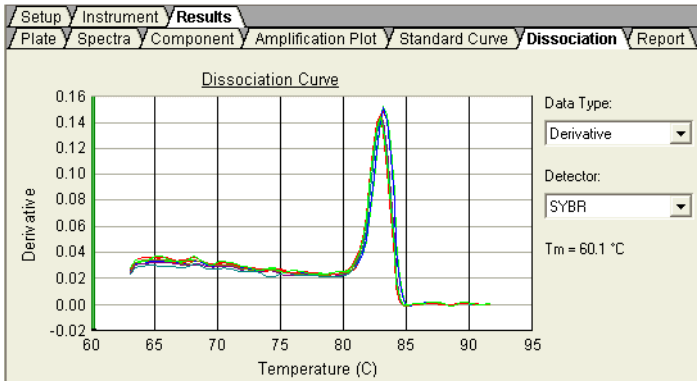
The SDS software calculates the quantity of unknown target sequence from the standard curve for the detector for that target sequence.



Dissociation

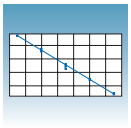
Displays the melting (T_m) curves associated with a dissociation assay. The data are shown when using SYBR® Green dye when either:

- Dissociation Protocol is selected in the Instrument tab
- Dissociation is selected as the assay type



Appendix C on page 77 and the Online Help provide information about dissociation-curve analysis.

Notes



Report

Displays data for selected wells in tabular form. The data columns associated with the report are determined by the assay being run. For AQ assays, the following data columns are available: Well, Sample Name, Detector, Task, Ct, StdDev Ct, Quantity, Mean Qty, StdDev Qty, Filtered, Tm, and three User-Defined columns. Refer to Online Help for information on configuring the User-Defined columns.

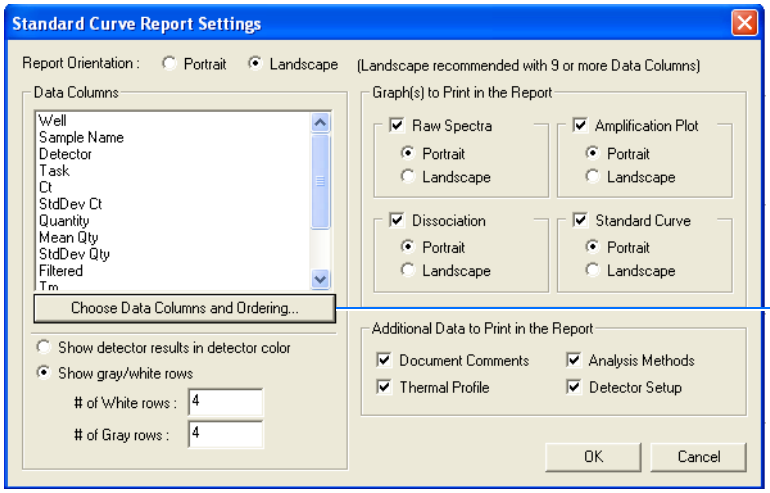
Setup | Instrument | **Results**

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | **Report**

Well	Sample Name	Detector	Task	Ct	St
A1	5K	RNase P	Unknown	28	0.066
A2	5K	RNase P	Unknown	28	0.066
A3	5K	RNase P	Unknown	28	0.066
A4	5K	RNase P	Unknown	28	0.066
A5	5K	RNase P	Unknown	28	0.066
A6	5K	RNase P	Unknown	28	0.066
A7	5K	RNase P	Unknown	28	0.066
A8	5K	RNase P	Unknown	28	0.066
A9	5K	RNase P	Unknown	28	0.066
A10	5K	RNase P	Unknown	28	0.066
A11	5K	RNase P	Unknown	28	0.066
A12	5K	RNase P	Unknown	28	0.066
B1	5K	RNase P	Unknown	28	0.066
B2	5K	RNase P	Unknown	28	0.066
B3	5K	RNase P	Unknown	28	0.066
B4	5K	RNase P	Unknown	28	0.066

Note: To select the column used to sort the data, click the column heading to sort in ascending (first and alternating clicks) or descending alphanumeric order.

The Report Settings dialog box formats the display of the report and how the report will be printed. You have the option (see "Exporting AQ Plate Data" on page 70) to apply these report settings when you export data. Refer to the Online Help for more information about this dialog box.



Click **Chose Data Columns and Ordering** for more report options.

6

Notes

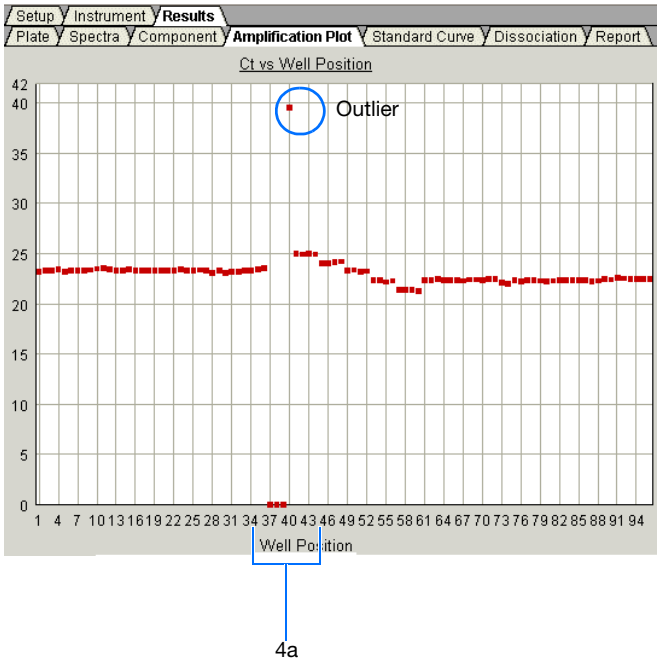
Omitting Samples

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

To ensure precision, carefully view replicate groups for outliers. You can remove outliers manually using the C_T vs. Well Position Amplification Plot or the Standard Curve plot.

Removing Outliers on the Amplification Plot

1. Select the **Amplification Plot** tab.
2. In the Data drop-down list, select **Ct vs Well Position**.
3. Select wells to examine, then verify the uniformity of each replicate population by comparing the groupings of C_T values.
4. If you identify an outlier, locate the associated well:
   a. Determine the approximate well position of the outlier from the x-axis of the plot.

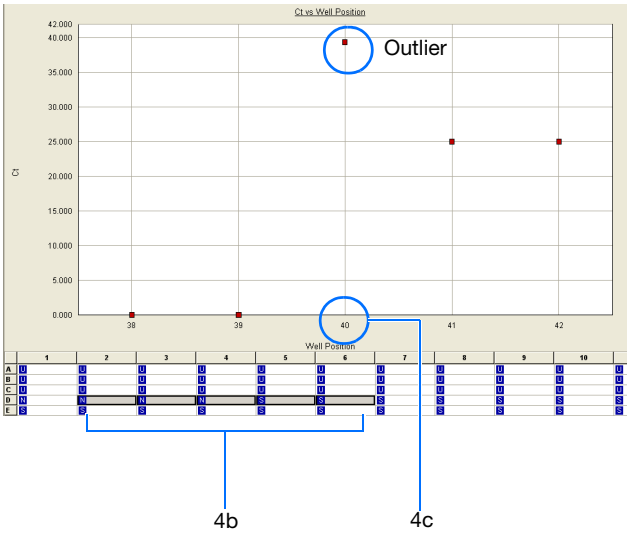


Notes

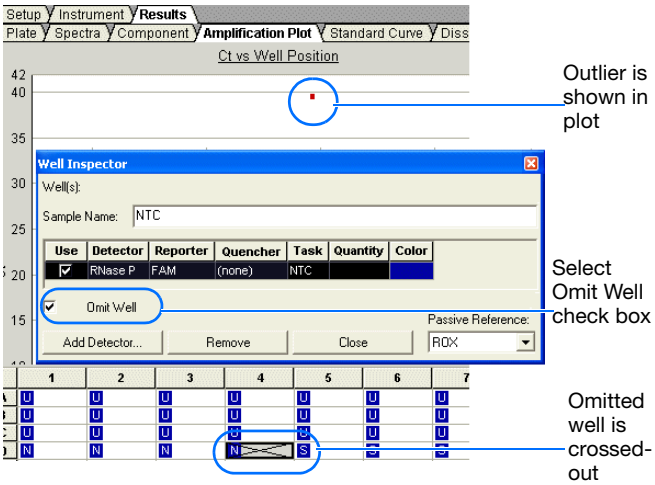
b. In the plate grid, select a range of wells that includes the approximate well position of the outlier.

The plot displays only the selected wells.

c. From the plot, determine the exact well position of the outlier.



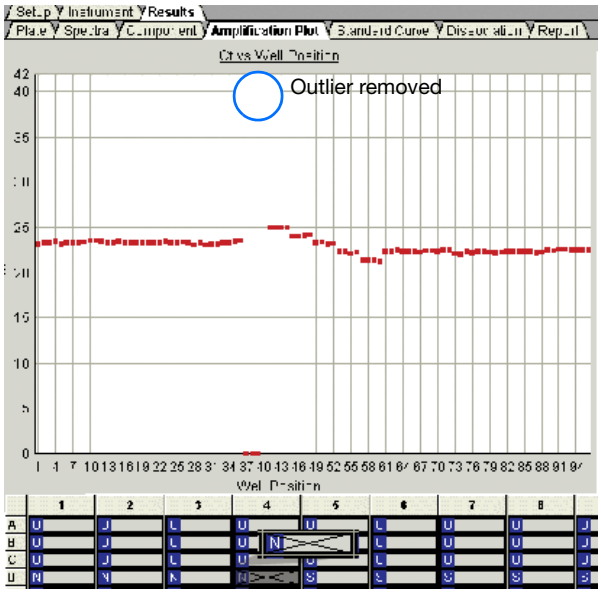
d. Select **View > Well Inspector**, then select the **Omit** check box for the appropriate well.



Notes

5. Click ▶ or select **Analysis > Analyze** to reanalyze the run without the outlier data.

6. Repeat steps 4 and 5 for other wells you want to screen.



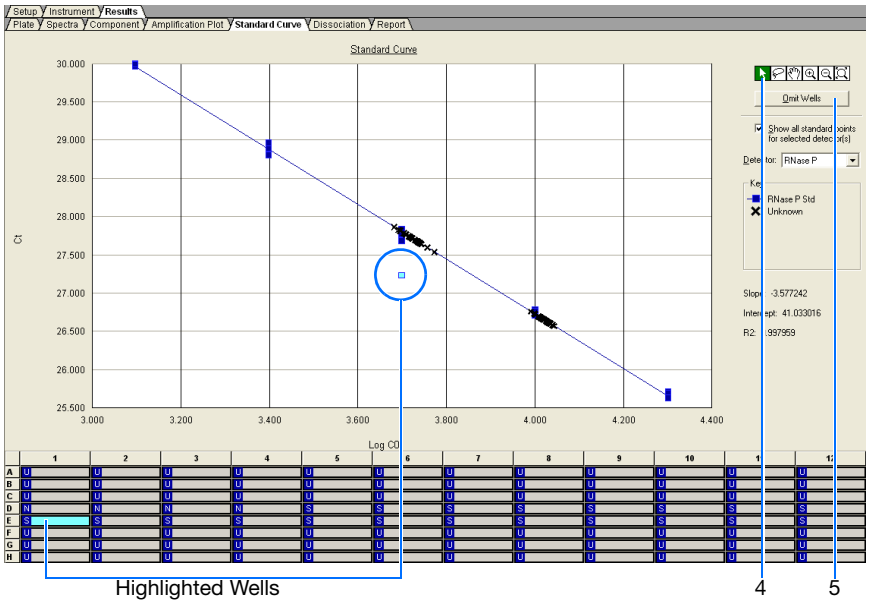
Notes

Removing Outliers on the Standard Curve

1. Select the **Standard Curve** tab.
2. Select all wells in the plate grid.
3. Examine the curve for outliers.

Note: To zoom in, click (Zoom In), then click the standard curve plot (or click-drag to zoom in on a group of wells).

4. If an outlier is present, click (Select), then click the outlier on the standard curve plot to highlight the well (or click-drag to highlight a group of wells).
5. Click **Omit Wells** (or right-click, then select **Omit Wells**).
6. Click or select **Analysis > Analyze** to reanalyze the run without the outlier data.
7. Repeat steps 4 through 6 for other outliers you want to remove.



Notes

Exporting AQ Plate Data

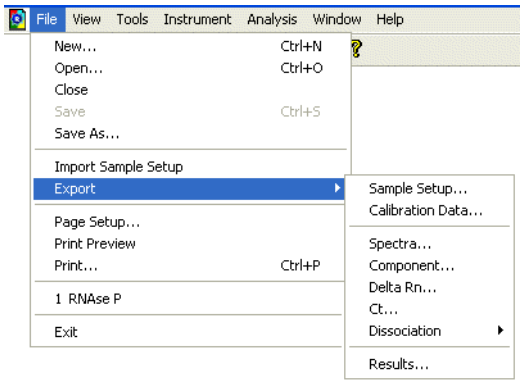
You can export numeric data from AQ plates into text files, which can then be imported into spreadsheet applications such as Microsoft® Excel® software. You can export graphs as a Microsoft® PowerPoint® software presentation or as JPEG files.

Note: You must have PowerPoint installed for the export graphs to PowerPoint feature to work.

To export data to a spreadsheet application:

1. Select **File** > **Export**, then select the data type to export.
   - **Sample Setup** (*.txt)
   - **Calibration Data** (*.csv)
   - **Spectra** (*.csv)
   - **Component** (*.csv)
   - **Delta Rn** (*.csv)
   - **Ct** (*.csv)
   - **Dissociation** (*.csv)
   - **Results**

   Refer to the Online Help for information about the export file types.



2. Enter a file name for the export file.
3. Click **Save**.

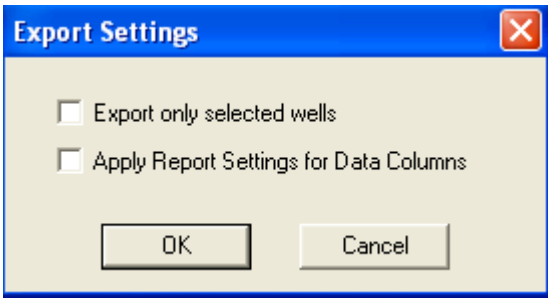
To export data for selected wells and/or report columns to a spreadsheet application:

1. Select **File** > **Export** > **Results**.
2. Enter a file name for the export file.
3. Click **Save**. The Export Settings dialog box opens.

Notes

4. (Optional) Select export settings:
   - **Export only selected wells**
   - **Apply Report Settings for Data Columns** to export the columns selected in the Report Settings dialog box (see "Report" on page 65).

5. Click **OK**.

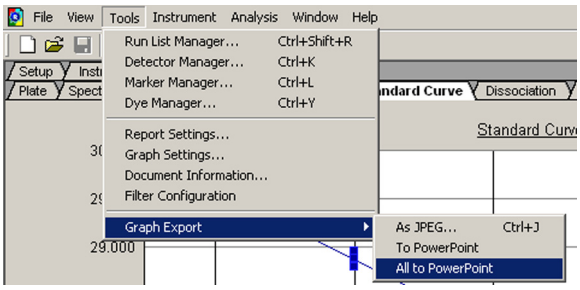


To export graphs to PowerPoint:

1. Select **Tools** > **Graph Export** > **All to PowerPoint** (or right-click any graph or plate, then select **Export All To PowerPoint**).

   The All to PowerPoint option exports screenshots from all tabs (except the Results > Report tab) of the active file.

   Note: To export only the current view, select **Tools > Graph Export > To PowerPoint** in any view (or right-click any graph or plate, then select **Export To PowerPoint).**



2. When prompted, click **OK** to export to PowerPoint. PowerPoint opens and displays your presentation.

   Note: Title and document information slides are automatically added to your presentation.

3. (Optional) In PowerPoint, modify your presentation.

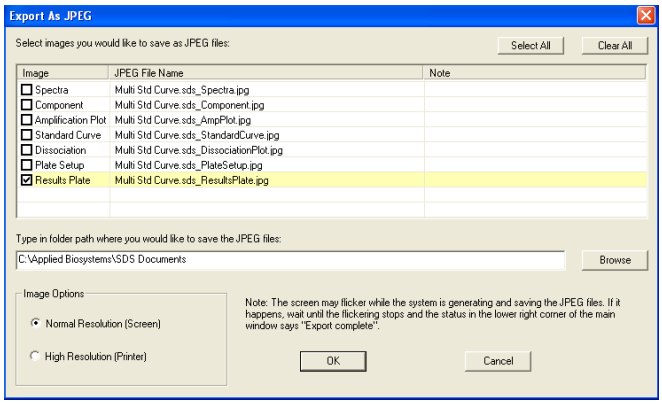
4. In PowerPoint, click (Save) to save your presentation.

To export plate views or graphs as JPEG files:

1. Select **Tools** > **Graph Export** > **As JPEG** (alternately, right-click any graph or plate, then select **Export as JPEG**).

Notes

The Export as JPEG dialog box opens.



Note: In the Export as JPEG dialog box, you can change default file names, select image resolution, and select which plate views or graphs to export, and where the file(s) are saved. Refer to Online Help for more information about this dialog box.

2. Click **OK**.

Notes

Creating Detectors

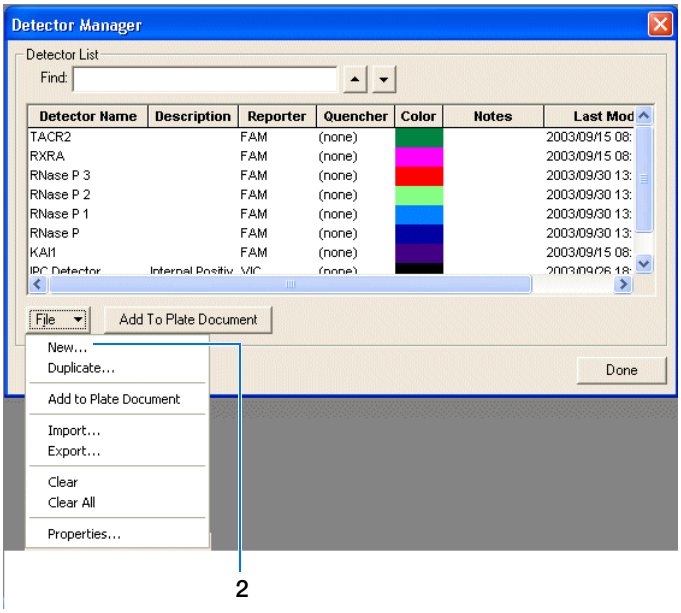
Before you can use a plate document to run a plate, you need to create and apply detectors for all samples on the plate. A detector is a virtual representation of a gene- or allele-specific nucleic acid probe reagent used for analyses performed on instruments.

To create a detector:

1. Select **Tools > Detector Manager**.

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

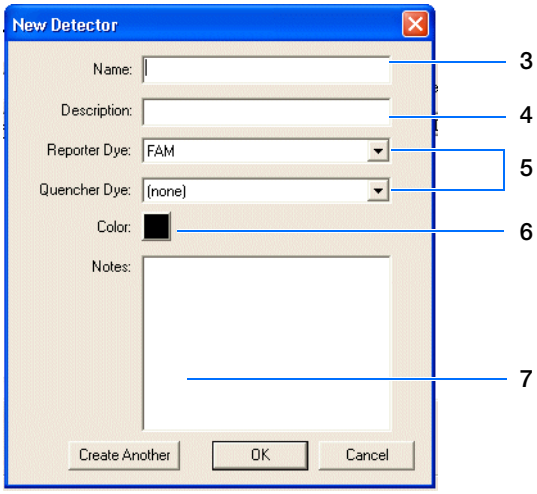
2. In the Detector Manager, select **File > New**.



3. In the New Detector dialog box, enter a name for the detector.

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

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



Notes

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

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

   Note: Select **TAMRA** as the dye quencher for TaqMan® probes and **None** for TaqMan MGB probes.

6. Click the **Color** box, select a color to represent the detector using the Color dialog box, then click **OK**.

7. Optionally, click the **Notes** field, then enter any additional comments for the detector.

8. Click **OK** to save the detector and return to the Detector Manager.

9. Repeat steps 2 through 8 for the remaining detectors.

10. In the Detector Manager, click **Done** when you finish adding detectors.

Note: TaqMan® Gene Expression Assays are shipped with an assay information file (AIF). This text-based file contains information about the assays that you ordered, including the Applied Biosystems Assay ID number, well-location of each assay, primer concentration, and primer sequence. The file also indicates the reporter dyes and quenchers (if applicable) that are used for each assay. When creating detectors, you use the reporter dye and quencher information (and optionally, the gene name or symbol for the sample name). You can view the contents of AIFs in a spreadsheet program, such as Microsoft® Excel® software.

Sample Experiment

In the example AQ experiment, a single detector was created for the single target being quantitated in the assay. The detector was named RNase P and assigned a blue color. Following conventions, the probe was a TaqMan MGB probe labeled with FAM™ dye. TaqMan MGB probes possess a nonfluorescent quencher.

In AQ experiments where two or more targets are being quantitated, a detector is created for each target.

Notes

Guidelines for Generating Standard Curves

Absolute quantitation using the 7300/7500/7500 Fast system requires that the absolute quantities of the standards be determined by independent means. Plasmid DNA or *in vitro* transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A_{260} and converted to the number of copies using the molecular weight of the DNA or RNA.

The following critical points must be considered for the proper use of absolute standard curves:

- The standard DNA or RNA must be a single, pure species. For example, plasmid DNA prepared from *E. coli* is often contaminated with RNA, increasing the A_{260} measurement and inflating the copy number determined for the plasmid.
- Accurate pipetting is required because the standard must be diluted over several orders of magnitude. Plasmid DNA or *in vitro* transcribed RNA must be concentrated to measure an accurate A_{260} value. This concentrated DNA or RNA must be diluted 10^6 to 10^{12}-fold to be at a concentration similar to the target in biological samples.
- The stability of the diluted standards must be considered, especially for RNA. Divide diluted standards into small aliquots, store at –80 °C, and thaw only once before use. An example of the effort required to generate trustworthy standards is provided by Collins, *et al*. (1995), who report on the steps they used in developing an absolute RNA standard for viral quantitation.
- Generally, it is not possible to use DNA as a standard for absolute quantitation of RNA because there is no control for the efficiency of the reverse transcription step.

Notes

Notes

Dissociation-curve Analysis

Overview

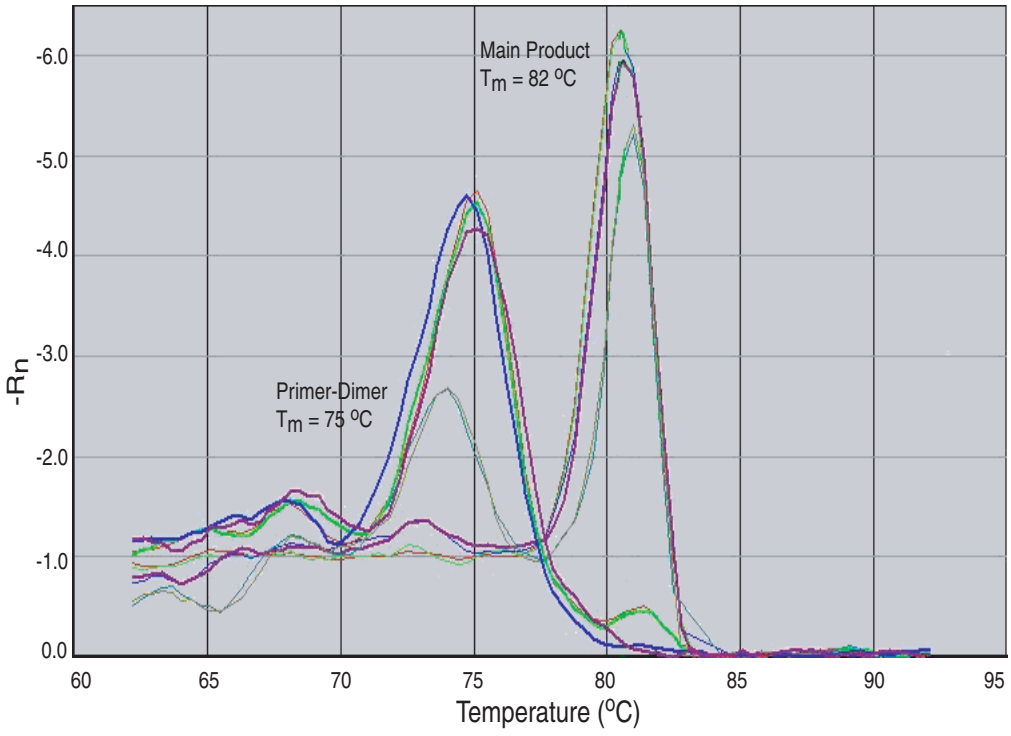
The 7300/7500/7500 Fast system supports dissociation-curve analysis of nucleic acids using SYBR® Green I dye. The objective of dissociation-curve analysis is to determine the melting temperature (T_m) of a single target nucleic acid sequence within an unknown sample. Typical uses of dissociation curves include detection of nonspecific products and primer concentration optimization.

The process begins by loading a plate with PCR samples and the SYBR Green I dye. The plate is loaded into an instrument that has been programmed to slowly elevate the temperature of the plate over several minutes.

The binding characteristic of the SYBR Green I dye allows the instrument to monitor the hybridization activity of nucleic acids. During the run, the instrument records the decrease in SYBR Green dye fluorescence resulting from the dissociation of double-stranded DNA.

Results

The following figure illustrates a typical dissociation curve from a run to detect nonspecific amplification in cDNA samples.



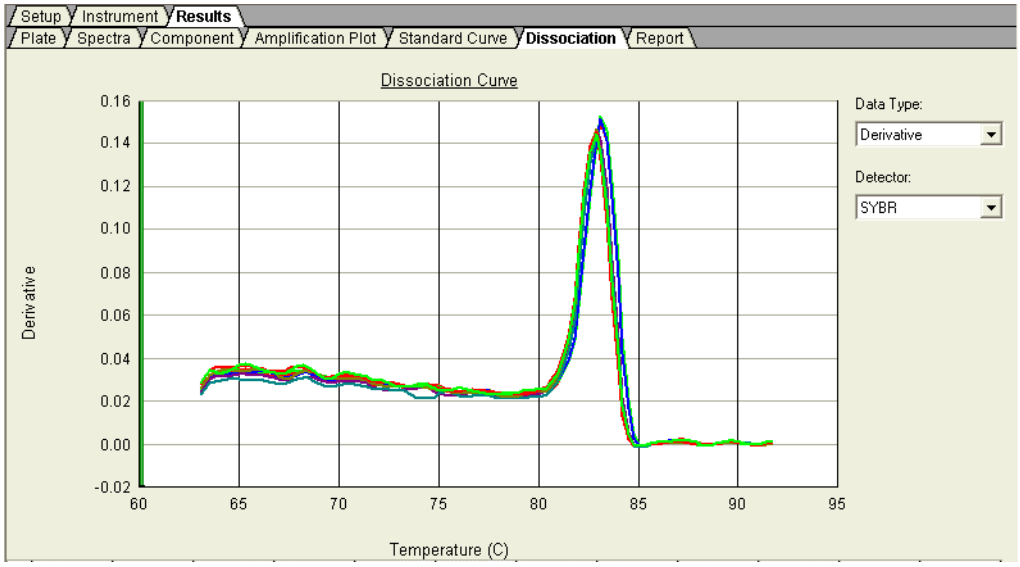
Notes

The dissociation curve plot displays the dual amplification peaks typical of primer-dimer formation. The amplification from the specific product is displayed with a T_m of 82 °C, while the primer-dimer product has a characteristically lower T_m of 75 °C.

Viewing Dissociation-curve Data

To view dissociation-curve data, select the **Dissociation** tab, then in the Data Type field, select:

- **Derivative** – Displays a plot of the first derivative of the rate of change in fluorescence as a function of temperature.
- **Raw** – Displays a plot of fluorescence as a function of temperature.



The Online Help provides information about using the 7300/7500/7500 Fast system to perform dissociation-curve analysis.

Designing Dissociation-curve Analysis Experiments

For a detailed explanation of the SYBR Green I double-stranded DNA binding dye chemistry, refer to:

- *SYBR® Green PCR and RT-PCR Reagents Protocol* (PN 4304965)
- *SYBR® Green PCR Master Mix Protocol* (PN 4310251)

Chemistry Kits for Dissociation-curve Analysis

The following Applied Biosystems kits are available:

Kit	Part Number
SYBR® Green RT-PCR Reagents	4310179
SYBR® Green PCR Core Reagents	4304886
SYBR® Green PCR Master Mix	4309155
Power SYBR® Green PCR Master Mix	4367659

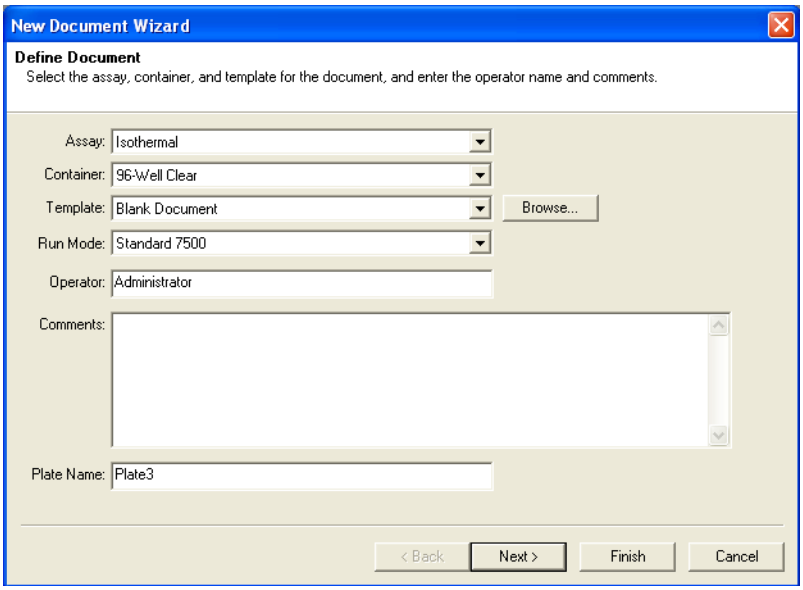
Notes

Isothermal Assays

The 7300/7500/7500 Fast System supports isothermal assays.

Creating an Isothermal Assay

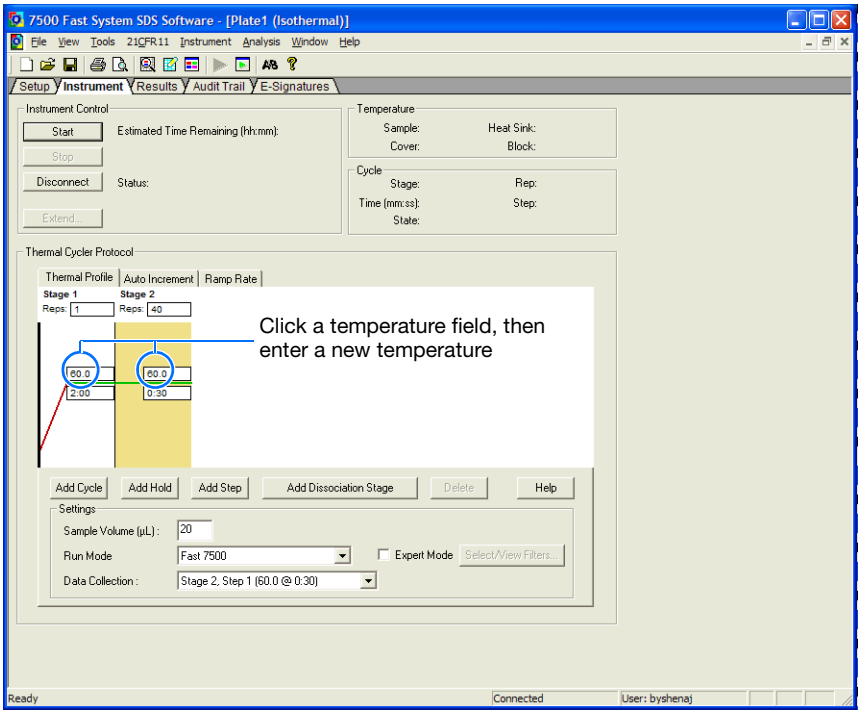
1. Select **Start > All Programs > Applied Biosystems 7300/7500/7500 Fast System > 7300/7500/7500 Fast System Software** ( ) to start the SDS Software.
2. In the Quick Startup document dialog box, select **Create New Document**.
3. In the assay drop-down list of the New Document Wizard, select **Isothermal**.



4. Complete your plate set up as directed in "Creating an AQ Plate Document" on page 25 (7300 or Standard 7500 System) or page 39 (7500 Fast System).
5. Click the **Instrument** tab to view the Thermal Profile of the new plate document.
6. (Optional) Click **Add Cycle**, **Add Hold**, or **Add Step** to add additional isothermal stages or steps.

Notes

7. (Optional) To change the default 60 °C temperature setting, click a temperature field, then enter the new temperature.



8. (Optional) Click **Add Dissociation Stage**.

Note: The dissociation stage uses the standard dissociation stage temperatures, not the isothermal temperature setting.

9. Complete your plate run as directed in "Specifying Thermal Cycling Conditions and Starting the Run" on page 29 (7300 or Standard 7500 System) or page 43 (7500 Fast System).

Notes

Example AQ Experiment

Description

The objective of the example AQ experiment is to determine the copy number of the RNase P gene in individuals from two populations.

The experiment is designed for singleplex PCR, and primers and probes are designed using Primer Express® Software.

A set of standards is generated by making serial dilutions from a sample of known quantity.

Data are generated by running a single AQ plate containing both the standard curve and the samples, then analyzed using software for the 7300/7500/7500 Fast system.

Notes

Example AQ Experiment Procedure

1. Design the experiment. (See Chapter 2 on page 10).
   a. Designate the unknowns, prepare the standard curve, and determine the number of replicates.
   b. Order the reagents for TaqMan® probe-based chemistry or design primers and probes using Primer Express® Software.
2. Isolate total RNA. (See Chapter 3 on page 16.)
3. Use the High-Capacity cDNA Reverse Transcription Kit to generate cDNA from total RNA. (See Chapter 3 on page 17.)
   a. Prepare the reverse transcription (RT) master mix, as indicated in the table to the right. Additional guidelines are provided in the *High-Capacity cDNA Reverse Transcription Kits Protocol* (PN 4375575).

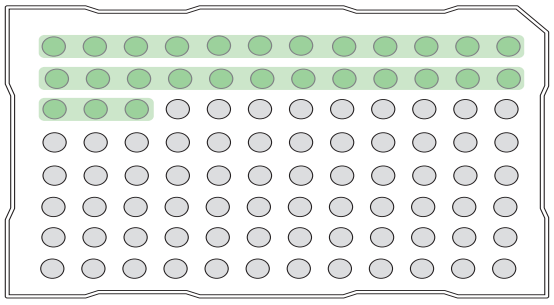
WARNING **CHEMICAL HAZARD. 10 × Reverse Transcription Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

RT Master Mix - Standard Plate		
Component	**µL/Reaction**	**µL/27 Reactions‡**
10× Reverse Transcription Buffer	2.0	54
25× dNTPs	0.8	21.6
10× random primers	2.0	54
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.0	27
Nuclease-free water	4.2	113.4
Total	10	270

‡ Each RT reaction is 20 µL (see step 3b). If you need 5 µL of cDNA at 50-µL total volume for each of 104 PCR reactions per plate (see step 4), you need 27 RT reactions. Extra volume is included to account for pipetting losses, as well as extra cDNA for archiving.

   b. Prepare the cDNA plate by pipetting into each well of the plate:
      - 10 µL RT master mix
      - 10 µL RNA sample

      Convert up to 2 µg of total RNA to cDNA per 20 µL reaction.



Notes

c. Program the thermal cycler using the indicated parameter values for the RT step of the two-step RT-PCR method.

Step Type	Time	Temperature
HOLD	10 min	25 °C
HOLD	120 min	37 °C
HOLD	5 sec	85 °C

Note: You have the option to use one-step RT-PCR, as explained in "Selecting One- or Two-Step RT-PCR" on page 12. RT reactions are performed with Standard Universal Master Mix for one-step RT-PCR or EZ-RT core reagents.

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

4. Prepare the PCR master mix as indicated in the table to the right.

   See Chapter 4 on page 20 for more information.

Note: The reaction volumes for TaqMan® Gene Expression Assays and TaqMan® Custom Gene Expression Assays are specified in the product insert that accompanies these products.

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

PCR Master Mix‡			
Reaction Component	**Standard µL/ Sample**	**Fast µL/ Sample**	**Final Concen-tration**
TaqMan Universal PCR Master Mix (2×) or TaqMan Fast Universal PCR Master Mix	25.0	10.0	1×
Forward primer	5.0	2.0	50 to 900 nM
Reverse primer	5.0	2.0	50 to 900 nM
TaqMan probe	5.0	2.0	50 to 250 nM
cDNA sample	5.0	2.0	10 to 100 ng
Nuclease-free water	5.0	2.0	—
Total	50.0	20.0	—

‡ For the example experiment, eight PCR master mixes were prepared, one for each of the two sample populations (for 37 reactions), and one for each of the six standards (for 5 reactions). Include extra volume to account for pipetting losses. cDNA is added directly into each master mix.

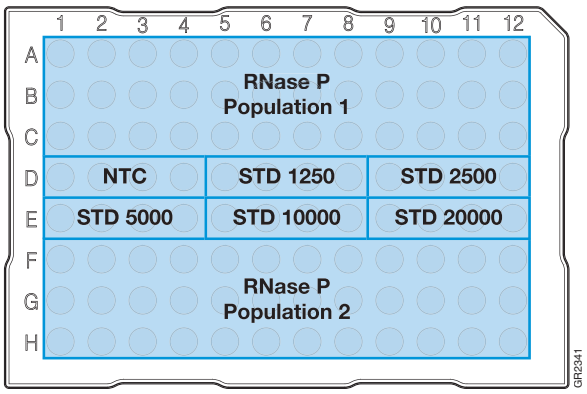
Notes

5. Prepare the reaction plate.

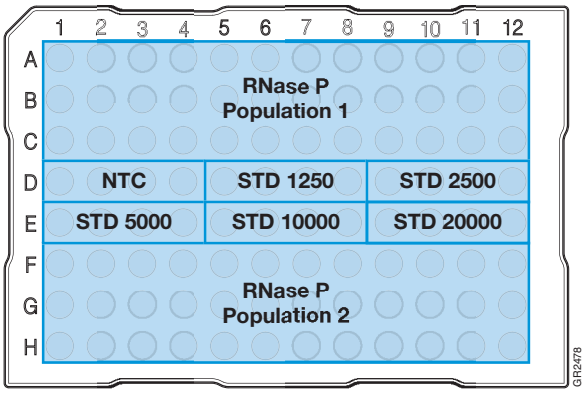
   a. Label the reaction plate, ensuring that you include a set of standards for every target sequence. The standards must be on the same plate as the target sequence.

   b. Pipette 50 µL of the appropriate PCR master mix (containing cDNA) into each well of a standard plate or pipette 20 µL into a Fast plate.

   c. Keep the reaction plate on ice until you are ready to load it into the 7300/7500/7500 Fast system.



Standard Plate

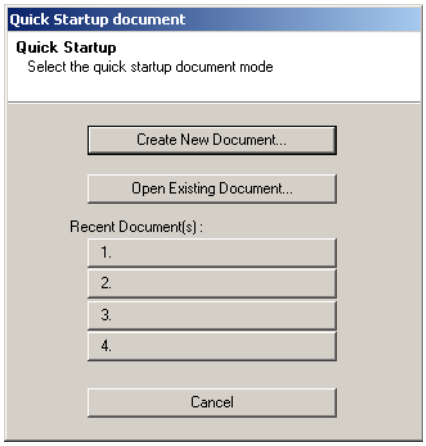


Fast Plate

6. Create an AQ plate document. (See "Creating an AQ Plate Document" on page 24.)

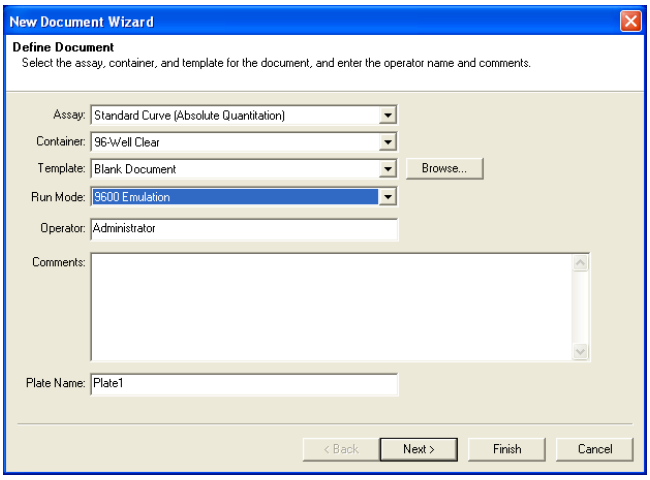
   a. Select **Start > All Programs > Applied Biosystems > 7300/7500/7500 Fast System > 7300/7500/7500 Fast System Software** ( ) to start the SDS software.

   b. In the Quick Startup document dialog box, select **Create New Document**.



Notes

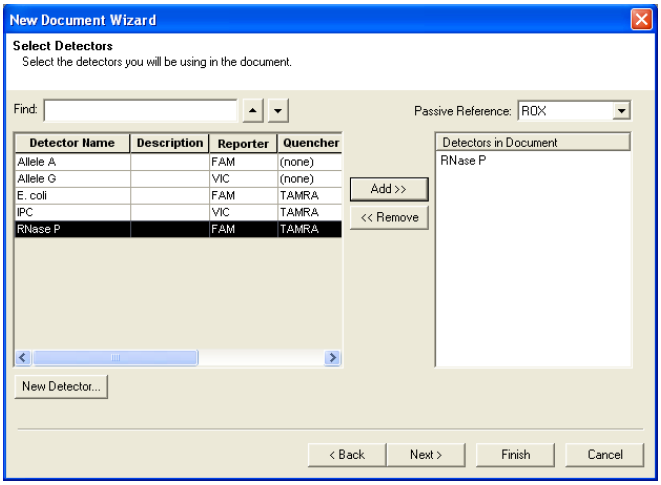
c. Select **Standard Curve (Absolute Quantitation)** in the Assay drop-down list, then click **Next >**.



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

d. Add detectors to the plate document, then click **Next >**.

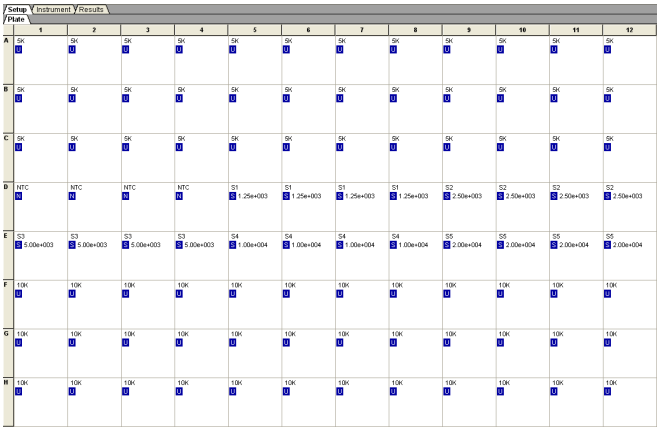
e. Specify the detectors and tasks for each well, then click **Finish**.



7. Enter the sample names in the Well Inspector (**View > Well Inspector**).

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

The figure on the right shows a completed plate setup with detectors, tasks, quantities, and sample names.



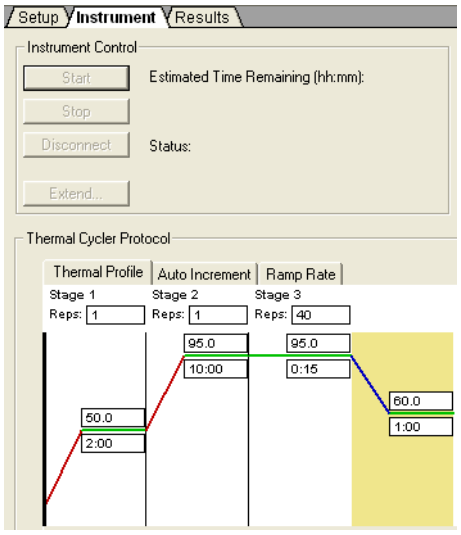
E

Notes

8. Start the AQ run.

   a. Select the **Instrument** tab. By default, the standard PCR conditions for the PCR step of the two-step RT-PCR method are displayed. See the figure to the right.

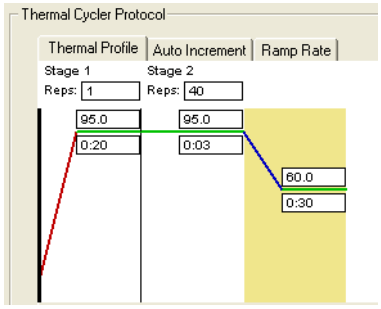
   The figure on page 86 shows the default PCR conditions for the 7500 Fast system.



   b. Select **File > Save**, enter a name for the AQ plate document, then click **Save**.

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

   d. Click **Start**.

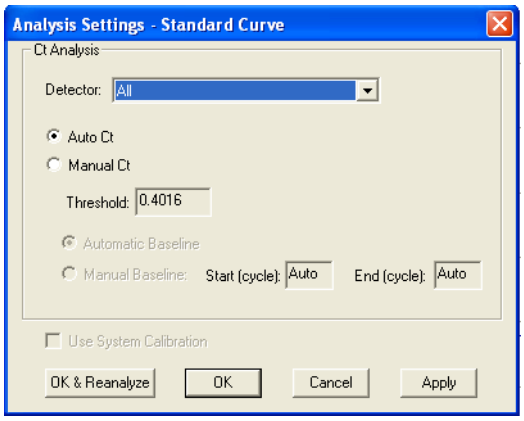


   After the run, a message indicates if the run is successful or if errors were encountered.

9. Analyze the AQ data, as explained in Chapter 6.

   a. Click (**Analysis > Analysis Settings**) to configure analysis settings. Use the **Auto Ct** option. (See "Configuring Analysis Settings" on page 52.)

   b. Click **OK & Reanalyze**, or select **Analysis > Analyze** to reanalyze the data.

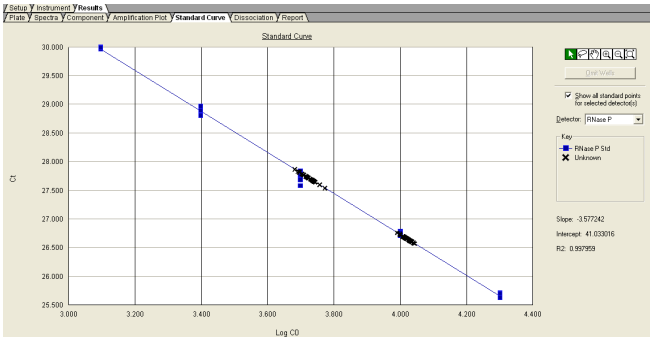


   c. If necessary, manually adjust the baseline and threshold. (See "Adjusting the Baseline and Threshold" on page 54.)

Notes

d. Click **OK & Reanalyze**, or select **Analysis > Analyze** to reanalyze the data.

e. View analysis results.



Conclusion

From the standard curve, derive the number of copies of the RNase P gene in population 1 and population 2.

E

Notes

Notes

References

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

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.

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Headquarters
850 Lincoln Centre Drive
Foster City, CA 94404 USA
Phone: +1 650.638.5800
Toll Free (In North America): +1 800.345.5224
Fax: +1 650.638.5884

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