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Power SYBR® Green RNA-to-C_T™ *1-Step* Kit

Protocol

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TRADEMARKS

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Preface

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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

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

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

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

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

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 (MSDSs) 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 in the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About MSDSs

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

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

Instructions for Obtaining MSDSs

The MSDS for any chemical supplied by Applied Biosystems or Ambion is available to you free 24 hours a day.

IMPORTANT! For the MSDSs of chemicals not supplied by Applied Biosystems or Ambion, contact the chemical manufacturer.

Obtain MSDSs from Applied Biosystems

1. Go to **docs.appliedbiosystems.com/msdssearch.html**
2. In the Keyword Search field of the MSDS Search page:
   a. Enter the chemical name, part number, or other information that you expect to appear in the MSDS of interest.
   b. Click **Search**.
3. To view, download, or print the MSDS:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the MSDS
      - **Save Target As** – To download a PDF version of the MSDS
      - **Print Target** – To print the MSDS

Obtain MSDSs from Ambion

1. Go to **www.ambion.com/techlib/index.html**
2. In the Restrict by Title Words or Keywords field of the Technical Resources page:
   a. Enter the chemical name or catalog number for the MSDS of interest.
   b. Select the **MSDSs** check box.
   c. Click **Find Documents**.
3. To view, download, or print the MSDS:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the MSDS
      - **Save Target As** – To download a PDF version of the MSDS
      - **Print Target** – To print the MSDS

Chemical Waste Hazards

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

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

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

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

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

Waste Disposal

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

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

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

Biological Hazard Safety

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

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

Additional information about biohazard guidelines is available at **www.cdc.gov**

How to Obtain More Information

Related Documentation

For additional documentation, see "How to Obtain Support" on page xii.

Real-Time PCR System	Document	Part Number
All real-time PCR systems	*Power SYBR® Green RNA-to-C_T™ 1-Step Kit Quick Reference Card*	4391112
	Primer Express® Software Version 3.0 Getting Started Guide	4362460
	Real-Time PCR Systems Chemistry Guide	4348358
7000 System	*ABI PRISM® 7000 Sequence Detection System User Guide*	4330228
7300, 7500, and 7500 Fast Systems (system software v1.4)	*Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Installation and Maintenance Getting Started Guide*	4347828
	Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825
	Applied Biosystems 7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
7500 and 7500 Fast Systems (system software v2.0)	*Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Maintenance Guide*	4387777
	Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments	4387783
	Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4387779
	Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Reagent Guide	4387787

Real-Time PCR System	Document	Part Number
7900HT System	*Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide*	4347825
	Applied Biosystems *7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide*	4364014
	Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide	4364016
	Applied Biosystems 7900HT Fast Real-Time PCR System Maintenance and Troubleshooting Guide	4365542
StepOne and StepOnePlus Systems	*Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Relative Standard Curve and Comparative C_T Experiments*	4376785
	Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4376784
	Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking, and Maintenance Guide	4376782
	Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Reagent Guide	4379704

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**

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

How to Obtain Support

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

At the Support page, you can:

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

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

Power SYBR® Green RNA-to-C_T™ *1-Step* Kit Protocol

Introduction

Purpose of the Kit

Use the *Power* SYBR® Green RNA-to-C_T™ *1-Step* Kit to perform one-step RT-PCR with SYBR® Green reagents for quantitation experiments on a real-time PCR system.

About This Protocol

This protocol provides:

- A description of kit contents
- A list of equipment and materials required for using the *Power* SYBR Green RNA-to-C_T *1-Step* Kit
- Procedures and guidelines for performing a quantitation experiment using the *Power* SYBR Green RNA-to-C_T *1-Step* Kit:
  - Experiment design and setup
  - Reaction preparation
  - Analysis of results

For more information, refer to the documents shipped with your Applied Biosystems Real-Time PCR System:

- **User guide** – Procedures for using and maintaining the instrument, including performing instrument calibrations
- **Getting started guides for quantitation experiments** – Background information on real-time PCR, experiment examples, and guidelines for experiment design, setup, run, and analysis
- **Chemistry guide or reagent guide** – Information on Applied Biosystems reagents and applications that are supported on your real-time PCR system

For document titles and part numbers, see "Related Documentation" on page x.

Materials and Equipment

Kit Contents

The *Power* SYBR Green RNA-to-C_T *1-Step* Kit contains two tubes:

- *Power* SYBR® Green RT-PCR Mix (2×) contains:
  - SYBR® Green I dye
  - AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)
  - dNTPs (dATP, dCTP, dGTP, dTTP, and dUTP)
  - ROX™ passive reference
  - Optimized buffer components
- RT Enzyme Mix (125×) contains:
  - ArrayScript™ UP Reverse Transcriptase
  - RNase inhibitor

Available Quantities

Power SYBR Green RNA-to-C_T *1-Step* Kit quantities available:

Quantity	Part Number
Reagents sufficient for 40 × 50-µL reactions: • *Power* SYBR® Green RT-PCR Mix (2×), 1 mL • RT Enzyme Mix (125×), 20 µL	4391178
Reagents sufficient for 200 × 50-µL reactions: • *Power* SYBR® Green RT-PCR Mix (2×), 5 mL • RT Enzyme Mix (125×), 80 µL	4389986

Kit Storage

Store the *Power* SYBR Green RNA-to-C_T *1-Step* Kit at –15 to –25 °C.

Note: After the first use, Applied Biosystems recommends storing the *Power* SYBR® Green RT-PCR Mix (2×) at 2 to 8 °C to minimize freeze-thaw cycles.

Materials and Equipment Not Included

Reaction Plates and Accessories

Use reaction plates and accessories appropriate for your real-time PCR system.

Real-Time PCR System	Reaction Plates and Accessories
7000 System 7300 System 7500 System	• MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Film Compression Pad (PN 4312639)
7500 Fast System	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film (PN 4311971)
7900HT Fast System (96-Well Block)	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical Film Compression Pad (PN 4312639)
7900HT Fast System (384-Well Block)	• MicroAmp® Optical 384-Well Reaction Plate with Barcode: – 1000 plates (PN 4343814) – 500 plates (PN 4326270) – 50 plates (PN 4309849) • MicroAmp® Optical 384-Well Reaction Plate (1000 plates) (PN 4343370) • MicroAmp® Optical Adhesive Film (PN 4311971)
StepOne™ System	• MicroAmp® Fast Optical 48-Well Reaction Plate (20 plates) (PN 4375816) • MicroAmp® 48-Well Optical Adhesive Film (PN 4375323)
StepOnePlus™ System	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film (PN 4311971)

Other Materials and Equipment

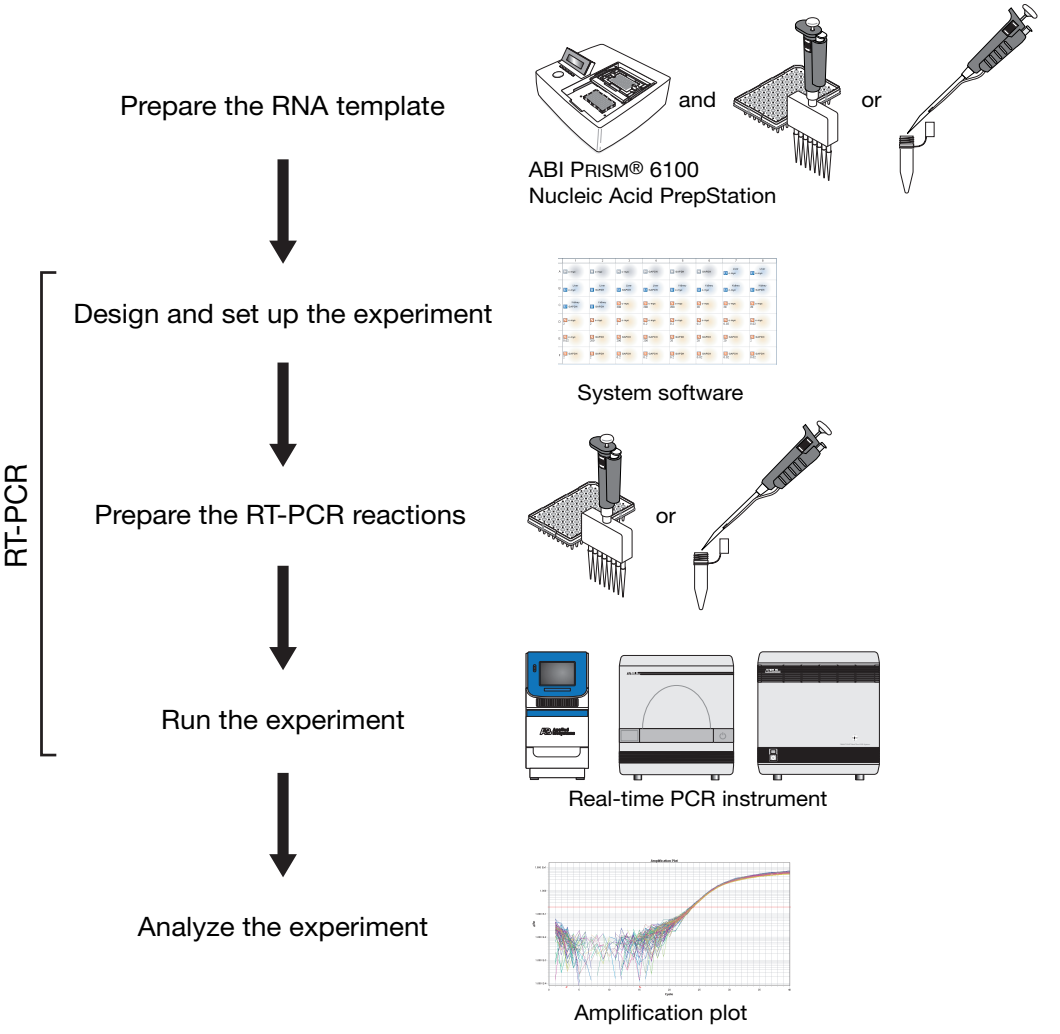
For more product recommendations, visit the real-time PCR decision tree: **www.appliedbiosystems.com/qpcrtree**

Item	Source
MicroAmp® 96-Well Tray/Retainer Set (10 sets)	Applied Biosystems (PN 403081)
MicroAmp® Cap Installing Tool (Handle)	Applied Biosystems (PN 4330015)
MicroAmp® Optical 8-Cap Strip	Applied Biosystems (PN 4323032)
MicroAmp® Optical Adhesive Film Kit (20 optical adhesive films, 1 applicator, and 1 compression pad)	Applied Biosystems (PN 4313663)
MicroAmp® Optical Tube without Cap (0.2-mL)	Applied Biosystems (PN N8010933)
Nuclease-free water (not DEPC-treated) (500 mL)	Applied Biosystems (PN AM9930)
RNase-free Barrier (Filter) Tips • 10-µL size - Eppendorf® (8 × 12 racks) • 10-µL size - Pipetman™ (8 × 12 racks) • 20-µL size (8 × 12 racks) • 100-µL size (8 × 12 racks) • 200-µL size (8 × 12 racks) • 1000-µL size (100 ct racks)	Applied Biosystems • PN AM12635 • PN AM12640 • PN AM12645 • PN AM12648 • PN AM 12655 • PN AM12665
RNase-free Tubes: • RNase-free Microfuge Tubes (1.5 mL) • Non-Stick RNase-free Microfuge Tubes (1.5 mL)	Applied Biosystems • PN AM12400 • PN AM12450
RNase*Zap*® RNase Decontamination Solution (250 mL)	Applied Biosystems (PN AM9780)
Primer Express® Software	Contact your Applied Biosystems representative
Centrifuge with plate adapter	Major laboratory supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS

Item	Source
NuSieve 4% (3:1) agarose gels, for DNA < 1 kb	FMC BioProducts (PN 54928)
Pipettors, positive-displacement or air-displacement	MLS
Tris-EDTA (TE) buffer, pH 8.0	MLS
Vortexer	MLS

Illustrated Workflow

The figure below shows the workflow for one-step RT-PCR using the *Power* SYBR Green RNA-to-C_T *1-Step* Kit.



PCR Good Laboratory Practices

Overview

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

About AmpliTaq Gold DNA Polymerase, UP

The *Power* SYBR Green RT-PCR Mix (2×) contains AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), a highly purified DNA polymerase that allows an automated and efficient hot start PCR. The enzyme is modified to be inactive until heat-activated.

The high-temperature incubation step required for activation ensures that the enzyme is activated only at temperatures where the DNA is fully denatured.

When AmpliTaq Gold DNA Polymerase, UP is added to the reaction mixture at room temperature, the inactive enzyme is not capable of primer extension. Therefore, any low-stringency mispriming events that may have occurred are not enzymatically extended and subsequently amplified.

About UDG

Uracil-DNA glycosylase (UDG), also known as uracil-N-glycosylase (UNG), hydrolyzes single- or double-stranded DNA that contains dUMP.

UDG treatment can prevent the reamplification of carryover PCR products by removing any uracil incorporated into amplicons (Longo *et al.*, 1990). UDG treatment is effective only if all previous PCR for that assay was performed using a master mix that contains dUTP.

UDG is not compatible with reverse transcription when dUTP is available because UDG will degrade the resulting cDNA. Therefore, UDG should not be used during one-step RT-PCR. However, the *Power* SYBR Green RNA-to-C_T *1-Step* Kit contains a dUTP/dTTP blend so that amplicons produced by the kit are degradable by UDG in subsequent PCR reactions.

General RNA Handling Practices

When working with RNA, prevent RNase contamination:

- Wear clean gloves throughout the experiment to prevent contamination from RNases on your hands.
- Change gloves frequently, including after touching skin, door knobs, and common surfaces.
- Maintain an RNase-free area in the lab and use dedicated RNase-free equipment and supplies:
  - Use tips and tubes that are tested and guaranteed RNase-free.
  - Use RNase-free chemicals and reagents.
  - Work in a low-traffic area that is away or shielded from air vents or open windows.
  - Clean laboratory surfaces, such as benchtops, centrifuges, and electrophoresis equipment, with Ambion's RNase*Zap*® or a mild solution of bleach or sodium hydroxide.

For more information about working with RNA, search Ambion's RNA Reference Database:
www.ambion.com/techlib/references/index.php

General PCR Practices

When preparing PCR reactions:

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Centrifuge tubes briefly before opening them. Open and close all sample tubes carefully.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution.

Design and Set Up the Experiment

Design the Experiment

1. Design your own assay with Primer Express® software.

   For more information on how to design your own assay, see Appendix A, "Guidelines for Custom-Designed Assays," on page 17.

2. Select a method for determining the quantity of target in the samples:
   - **Standard curve** – Uses a standard curve to determine the absolute quantity of a target in a sample. Typically used for quantifying viral load.
   - **Relative standard curve** – Uses standard curves to determine the change in expression of a target in a sample relative to the same target in a reference sample. Used for relative quantitation if the amplification efficiency of the target and the amplification efficiency of the endogenous control are not approximately equal.
   - **Comparative C_T** – Uses arithmetic formulas to determine the change in expression of a target in a sample relative to the same target in a reference sample. Best for high-throughput measurements of relative gene expression of many genes in many samples.

3. Determine the numbers of samples in your experiment:
   - **Samples or Unknowns** – Samples in which the quantity of the target is unknown.
   - **Standards** (standard curve and relative standard curve experiments) – Samples that contain known standard quantities. A set of standards containing a range of known quantities is a standard dilution series.
   - **Reference sample** (relative standard curve and comparative C_T experiments) – The sample used as the basis for relative quantitation results. Also called *calibrator*.

   Note: Applied Biosystems recommends performing at least 3 technical replicates of each reaction.

Set Up the Experiment

Use the software for your real-time PCR system to set up the experiment:

1. Create a file for your experiment.
2. Indicate the contents of each well in the reaction plate.
3. Set up the run method using the following conditions:
   - Ramp speed or mode: **Standard**
   - Reaction volume (µL): **10**, **20**, or **50**
   - Thermal cycling conditions:

Stage	Step	Temp	Time
Holding	Reverse transcription	48 °C	30 min
Holding	Activation of AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/Extend	60 °C	1 min
Melt curve *(Optional)*‡	Denature	95 °C	15 sec
	Anneal	60 °C	15 sec
	Denature	95 °C	15 sec

‡ To detect nonspecific amplification, perform a melt curve (dissociation curve) after the RT-PCR.

For More Information

Refer to the user guide or getting started guides for your real-time PCR system for more information about designing a quantitation experiment and using the software to set up the experiment.

Prepare the RT-PCR Reactions

About the RNA Template

With the *Power* SYBR® Green RNA-to-C_T™ *1-Step* Kit, the target template is the RNA sequence that you want to amplify and detect.

RNA Template Quality

For optimal performance, Applied Biosystems recommends using purified total RNA or mRNA that is:

- Less than 0.005% of genomic DNA by weight
- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer
- Free of RNase activity
- Nondenatured

IMPORTANT! It is not necessary to denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some gene targets.

RNA Template Quantitation

Template quantitation is critical for successful PCR reactions. Measure the absorbance (optical density or O.D.) of a sample at 260 nm (A_{260}) in a spectrophotometer.

Concentration of single-stranded RNA = $A_{260} \times 40$ µg/mL

Note: Absorbance measurements of highly concentrated (O.D.>1.0) or very dilute (O.D.<0.05) RNA samples can be inaccurate. Dilute or concentrate the RNA to obtain a reading within the acceptable range.

RNA Template Storage Conditions

Store purified RNA templates at –20 °C or –70 °C in RNase-free water.

Preparation Guidelines

- **RNA samples** – If you dilute your samples, use TE buffer or water as the diluent.
- **Template quantity** – You can use up to 100 ng of RNA template in each reaction. To determine the optimal template quantity for your assay, prepare serial dilutions of the template and set up RT-PCR reactions with different template quantities. Select the quantity that produces the earliest C_T without inhibiting PCR.
- **Primer concentrations** – Applied Biosystems recommends starting with 100 to 200 nM forward primer and 100 to 200 nM reverse primer in each reaction. With custom-designed assays, determine the optimal primer concentrations using the procedure in Appendix A, "Guidelines for Custom-Designed Assays," on page 17.
- **Standards** (for standard curve and relative standard curve experiments) – Standards are critical for accurate analysis of run data. Mistakes or inaccuracies in making the dilutions directly affect the quality of results. The quality of pipettors and tips and the care used in measuring and mixing dilutions affect accuracy. Use TE buffer or water to prepare the standard dilution series.
- **Master Mix**– Prior to use, mix thoroughly by swirling the bottle. After the first use, Applied Biosystems recommends storing the *Power* SYBR® Green RT-PCR Mix (2×) at 2 to 8 °C to minimize freeze-thaw cycles.

  IMPORTANT! Keep the *Power* SYBR Green RT-PCR Mix (2×) protected from light until you are ready to use it. Excessive exposure to light may affect the ROX™ dye and the SYBR® Green dye.

- **Replicates** – Applied Biosystems recommends performing at least 3 technical replicates of each reaction.
- **Excess reaction volume** – Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers. Applied Biosystems recommends an excess volume of at least 10%.

Prepare the RT-PCR Reactions

CAUTION CHEMICAL HAZARD. *Power* SYBR® Green RT-PCR Mix (2×) may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Calculate the volume of components needed, based on the reaction volume and the number of reactions, including excess.

   Note: Applied Biosystems recommends performing at least 3 technical replicates of each reaction. Include excess volume in your calculations to provide excess volume for the loss that occurs during reagent transfers.

Component	Volume for One Reaction		
	10 µL	**20 µL**	**50 µL**
Power SYBR® Green RT-PCR Mix (2×)	5.0 µL	10.0 µL	25.0 µL
Forward primer (100 to 200 nM final)	Variable	Variable	Variable
Reverse primer (100 to 200 nM final)	Variable	Variable	Variable
RT Enzyme Mix (125×)	0.08 µL	0.16 µL	0.4 µL
RNA template (up to 100 ng)	Variable	Variable	Variable
RNase-free H_2O	to 10 µL	to 20 µL	to 50 µL
Total Volume	**10.0 µL**	**20.0 µL**	**50.0 µL**

2. Place the tubes for the *Power* SYBR® Green RT-PCR Mix (2×), RT Enzyme Mix (125×), primers, and RNA templates on ice. After all reagents are thawed, gently invert the tubes to mix and return to the ice. Do **not** vortex the tubes.

   IMPORTANT! Keep the *Power* SYBR Green RT-PCR Mix (2×) protected from light until you are ready to use it. Excessive exposure to light may affect the fluorescent dyes.

3. Combine the required volumes of reaction components in microcentrifuge tubes on ice, then return the remaining kit components to storage.

   Note: For recommended kit storage conditions, see page 2.

4. Invert the tubes to mix, then centrifuge the tubes briefly.

5. Pipette the reactions into wells of a reaction plate appropriate for your real-time PCR system:
   - MicroAmp® Fast Optical 48-Well Reaction Plate: 20 µL
   - MicroAmp® Fast Optical 96-Well Reaction Plate: 20 µL
   - MicroAmp® Optical 96-Well Reaction Plate: 50 µL
   - MicroAmp® Optical 384-Well Reaction Plate: 10 µL
6. Seal the reaction plate with optical adhesive film or optical caps, then centrifuge the reaction plate briefly.

   For example, centrifuge at 140 x *g* for 60 seconds.

Run the Experiment

Run the RT-PCR Reaction Plate

For run conditions, see page 10.

1. Load the reaction plate into a real-time PCR instrument.
2. Perform the run.
3. Unload the reaction plate after the run is complete.

For More Information

Refer to the user guide or getting started guides for your real-time PCR system for more information about running a quantitation experiment.

Analyze the Experiment

Review the Results

- **Standard curve** (standard curve and relative standard curve experiments) – Slope, amplification efficiency, R^2 values, y-intercept, C_T values, outliers
- **Gene expression plot** (relative standard curve and comparative C_T experiments) – Differences in gene expression, standard deviation in the replicate groups
- **Amplification plots** – Baseline and threshold values, outliers
- **Well table or results table** – C_T values for each well and for each replicate group
- *(Optional)* **Melt curve** – Number of T_m peaks

Check the Purity of the PCR Product *(Optional)*

The first time you run the assay, you can confirm the absence of nonspecific amplification by analyzing the PCR amplification products by agarose gel electrophoresis. Do not perform this procedure after subsequent runs to avoid contamination.

IMPORTANT! Never bring amplified PCR products into the PCR setup area.

WARNING CHEMICAL HAZARD. Ethidium bromide causes eye, skin, and respiratory tract irritation and is a known mutagen (that is, it can change genetic material in a living cell and has the potential to cause cancer). Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Load 12 to 15 µL of each sample in an ethidium bromide-stained 4% NuSieve 3:1 agarose gel.
2. Run the gel:
   - For PCR fragments <100 bp, run the gel at 80 to 100 V for 45 to 60 minutes.
   - For PCR fragments 100 bp to 250 bp, run the gel at 100 to 115 V for 1 to 1.5 hours.
3. Run samples 1/3 to 1/2 of the length of the gel, without letting the dye run off the bottom of the gel.
4. Place the gel on a UV transilluminator, and make sure the lanes contain one distinct band.

For More Information

Refer to the getting started guides for your real-time PCR system for more information about analyzing a quantitation experiment.

Appendix A: Guidelines for Custom-Designed Assays

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Amplicon Site Selection

Using Primer Express® Software, select an *amplicon site* (segment of cDNA) within the target sequence.

Using Primer Express Software

Select the amplicon site using Primer Express Software as described in the *Primer Express® Version 3.0 Getting Started Guide* and *Software Help*.

General Amplicon Site Selection Guidelines

Selecting a good amplicon site ensures amplification of the target cDNA without coamplification of the genomic sequence, pseudogenes, and related genes.

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The primer pair must be specific to the target gene; the primer pair must not amplify pseudogenes or other related genes.
- Design primer pairs according to Primer Express Software guidelines.
- Test the primer pairs, then select the primer pair that produces the highest signal-to-noise ratio (that is, earliest C_T with total RNA or mRNA and no amplification with genomic DNA or negative controls).

If the Gene Does Not Contain Introns

If the gene you are studying does not contain introns, then you cannot ensure amplification the target cDNA sequence without coamplification of the genomic sequence. In this case, you may need to run control reactions that do not contain reverse transcriptase (RT– controls) to determine whether your RNA sample contains DNA. Amplification in the RT– controls indicates that your RNA sample contains DNA. To remove the DNA from the RNA sample, treat the RNA sample with DNase I.

Primer Design

Overview

Using Primer Express® Software, design primers to amplify the target sequence.

Using Primer Express Software

Design the primers using Primer Express Software as described in the *Primer Express® Version 3.0 Getting Started Guide* and *Software Help*.

General Primer Design Guidelines

- The optimal primer length is 20 bases.
- Keep the GC content in the 30 to 80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than 4 consecutive G bases.
- **Important: Keep the T_m between 58 to 60 °C**.
- Make sure the last 5 nucleotides at the 3′ end contain no more than two G and/or C bases.
- If you cannot find acceptable primer sequences, you may need to examine the sequence and select another amplicon site or screen for more sites.

Calculation of Oligonucleotide Concentrations

After you receive your primers, use a spectrophotometric method to determine the concentrations of the oligonucleotides in your assay.

Calculate Oligonucleotide Concentrations

1. Calculate the sum of extinction coefficient contributions for each oligonucleotide sequence:

Chromophore	Extinction Coefficient
A	15,200
C	7050
G	12,010
T	8400

2. Measure the absorbance at 260 nm (A_{260}) of each oligonucleotide diluted in TE buffer (for example, 1:100).

3. Calculate the oligonucleotide concentration using the formula:

   A_{260} = (sum of extinction coefficient contributions × cuvette pathlength × concentration) ÷ dilution factor

   Rearrange to solve for concentration:

   Concentration (C) = (dilution factor × A_{260}) ÷ (sum of extinction coefficient contributions × cuvette pathlength)

An Example Calculation of Primer Concentration

If the primer sequence is CGTACTCGTTCGTGCTGC:

- Sum of extinction coefficient contributions:
  $= A \times 1 + C \times 6 + G \times 5 + T \times 6$
  $= 167{,}950\ M^{-1}cm^{-1}$
- Example A_{260} measurements:
  Dilution = 1:100
  Cuvette pathlength = 0.3 cm
  $A_{260} = 0.13$
- Primer concentration:
  $= (100 \times 0.13) \div (167{,}950\ M^{-1}cm^{-1} \times 0.3\ cm)$
  $= 2.58 \times 10^{-4}\ M$
  $= 258\ \mu M$

Determine Optimal Primer Concentrations

With your custom-designed assay, determine the primer concentrations to use to obtain the earliest threshold cycle (C_T) and the maximum baseline-corrected normalized reporter (ΔR_n).

Primer Concentrations to Test

Use the *Power* SYBR® Green RT-PCR Mix (2×) to prepare four technical replicates of each of the nine conditions shown below:

Forward Primer Final Concentration (nM)	Reverse Primer Final Concentration (nM)		
	100	**200**	**450**
100	100/100	100/200	100/450
200	200/100	200/200	200/450
450	450/100	450/200	450/450

Prepare and Run the RT-PCR Reactions

1. Prepare the 36 RT-PCR reactions:

Component	Volume for One Reaction		
	10 µL	**20 µL**	**50 µL**
Power SYBR® Green RT-PCR Mix (2×)	5.0 µL	10.0 µL	25.0 µL
Forward primer (100, 200, or 450 nM final)	Variable	Variable	Variable
Reverse primer (100, 200, or 450 nM final)	Variable	Variable	Variable
RT Enzyme Mix (125×)	0.08 µL	0.16 µL	0.4 µL
RNA template (0.01 to 20 ng)	Variable	Variable	Variable
RNase-free H_2O	to 10 µL	to 20 µL	to 50 µL
Total Volume	**10.0 µL**	**20.0 µL**	**50.0 µL**

2. Run the RT-PCR reactions:
   - Ramp speed or mode: **Standard**
   - Reaction volume (µL): **10**, **20**, or **50**
   - Thermal cycling conditions:

Stage	Step	Temp	Time
Holding	Reverse transcription	48 °C	30 min
Holding	Activation of AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)	95 °C	10 min
Cycling (40 cycles)	Denature	95 °C	15 sec
	Anneal/Extend	60 °C	1 min
Melt curve *(Optional)*‡	Denature	95 °C	15 sec
	Anneal	60 °C	15 sec
	Denature	95 °C	15 sec

‡ To detect nonspecific amplification, perform a melt curve (dissociation curve) after the RT-PCR.

Evaluate the Results

1. Review the ΔR_n values to identify the optimal primer concentrations for PCR yield.
2. Review the C_T values to identify the optimal primer concentrations for C_T and detect any potential nonspecific amplification in the negative controls.
3. Select the forward primer and reverse primer combination that produces the earliest C_T and the highest ΔR_n.

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Glossary

amplicon

A segment of DNA amplified during PCR.

amplification efficiency

Calculation of efficiency of the PCR amplification. The amplification efficiency is calculated using the slope of the regression line in the standard curve. A slope close to −3.32 indicates optimal, 100% PCR amplification efficiency. Factors that affect amplification efficiency:

- **Range of standard quantities** – To increase the accuracy and precision of the efficiency measurement, use a broad range of standard quantities, 5 to 6 logs (10^5 to 10^6 fold).
- **Number of standard replicates** – To increase the precision of the standard quantities and decrease the effects of pipetting inaccuracies, include replicates.
- **PCR inhibitors** – PCR inhibitors in the reaction can reduce amplification and alter measurements of the efficiency.

amplification plot

Display of data collected during the cycling stage of PCR amplification. Can be viewed as:

- Baseline-corrected normalized reporter (ΔR_n) vs. cycle
- Normalized reporter (R_n) vs. cycle
- Threshold cycle (C_T) vs. well

amplification stage

Part of the instrument run in which PCR produces amplification of the target. The amplification stage, called a cycling stage in the thermal profile, consists of denaturing, primer annealing, and extension steps that are repeated.

For quantitation experiments, fluorescence data collected during the amplification stage are displayed in an amplification plot, and the data are used to calculate results.

See also cycling stage.

baseline

In the amplification plot, a line fit to the fluorescence levels during the initial stages of PCR, when there is little change in fluorescence signal.

baseline-corrected normalized reporter (ΔRn)

The magnitude of normalized fluorescence signal generated by the reporter.

In experiments that contain data from real-time PCR, the magnitude of normalized fluorescence signal generated by the reporter at each cycle during the PCR amplification. In the ΔRn vs. Cycle amplification plot, ΔRn is calculated at each cycle as:

ΔRn (cycle) = Rn (cycle) –Rn (baseline)

where Rn = normalized reporter

See also normalized reporter (Rn).

biological replicates

Reactions that contain identical components and volumes, but evaluate separate samples of the same biological source (for example, multiple samples of the same liver tissue).

See also technical replicates.

C_T

See threshold cycle (C_T).

calibrator

See reference sample.

comparative C_T ($\Delta\Delta C_T$) method

Method for determining relative target quantity in samples. With the comparative C_T ($\Delta\Delta C_T$) method, amplification of the target and of the endogenous control is measured in samples and in a reference sample. Measurements are normalized using the endogenous control. The relative quantity of target in each sample is determined by comparing normalized target quantity in each sample to normalized target quantity in the reference sample.

cycle threshold

See threshold cycle (C_T).

cycling stage

In the thermal profile, a stage that is repeated. A cycling stage is also called an amplification stage.

delta Rn (ΔRn)

See baseline-corrected normalized reporter (ΔRn).

diluent A reagent used to dilute a sample or standard before it is added to the PCR reaction. The diluent can be buffer or water.

dissociation curve *See* melt curve.

endogenous control A target or gene that should be expressed at similar levels in all samples you are testing. Endogenous controls are used in relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments to normalize fluorescence signals for the target you are quantifying. Housekeeping genes can be used as endogenous controls.

See also housekeeping gene.

forward primer Oligonucleotide that flanks the 5′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

holding stage In the thermal profile, a stage that can include one or more steps. You can add a holding stage to the thermal profile to activate enzymes, to inactivate enzymes, or to incubate a reaction.

housekeeping gene A gene that is involved in basic cellular functions and is constitutively expressed. Housekeeping genes can be used as endogenous controls.

See also endogenous control.

melt curve A plot of data collected during the melt curve stage. Peaks in the melt curve can indicate the melting temperature (Tm) of the target or can identify nonspecific PCR amplification. You can view the melt curve as normalized reporter (Rn) vs. temperature or as derivative reporter (–Rn′) vs. temperature. Also called dissociation curve.

melt curve stage In the thermal profile, a stage with a temperature increment to generate a melt curve.

melting temperature (Tm) In melt curve experiments, the temperature at which 50% of the DNA is double-stranded and 50% of the DNA is dissociated into single-stranded DNA. The Tm is displayed in the melt curve.

mode *See* ramp speed.

negative control (NC) The task for targets in wells that contain water or buffer instead of sample. No amplification of the target should occur in negative control wells. Also called no template control (NTC).

no template control (NTC) *See* negative control (NC).

normalized quantity Quantity of target divided by the quantity of endogenous control.

normalized reporter (Rn) Fluorescence signal from the reporter dye normalized to the fluorescence signal of the passive reference.

outlier For a set of data, a datapoint that is significantly smaller or larger than the others.

passive reference A dye that produces fluorescence signal. Because the passive reference signal should be consistent across all wells, it is used to normalize the reporter dye signal to account for non-PCR related fluorescence fluctuations caused by minor well-to-well differences in concentrations or volume. Normalization to the passive reference signal allows for high data precision.

quantitation method In quantitation experiments, the method used to determine the quantity of target in the samples. There are three types of quantitation methods: standard curve, relative standard curve, and comparative C_T ($\Delta\Delta C_T$).

quantity In quantitation experiments, the amount of target in the samples. Absolute quantity can refer to copy number, mass, molarity, or viral load. Relative quantity refers to the fold-difference between normalized quantity of target in the sample and normalized quantity of target in the reference sample.

R^2 value Regression coefficient calculated from the regression line in the standard curve. The R^2 value indicates the closeness of fit between the standard curve regression line and the individual C_T data points from the standard reactions. A value of 1.00 indicates a perfect fit between the regression line and the data points.

ramp The rate at which the temperature changes during the instrument run. The ramp is defined as a percentage. For some real-time PCR systems, the ramp for the melt curve step is defined as a temperature increment. In the graphical view of the thermal profile, the ramp is indicated by a diagonal line.

ramp speed Speed at which the temperature ramp occurs during the instrument run. For optimal results using the *Power* SYBR Green RNA-to-C_T *1-Step* Kit, Applied Biosystems recommends using the standard ramp speed.

reaction mix A solution that contains all components to run the PCR reaction, except for the template (sample, standard, or control).

real-time PCR Process of collecting fluorescence data during PCR. Data from the real-time PCR are used to calculate results for quantitation experiments.

reference sample In relative standard curve and comparative C_T ($\Delta\Delta C_T$) experiments, the sample used as the basis for relative quantitation results. Also called the calibrator.

regression coefficients Values calculated from the regression line in standard curves, including the R^2 value, slope, and y-intercept. You can use the regression coefficients to evaluate the quality of results from the standards.

See also standard curve.

regression line In standard curve and relative standard curve experiments, the best-fit line from the standard curve. Regression line formula:

$C_T = m\ [\log (Qty)] + b$

where m is the slope, b is the y-intercept, and Qty is the standard quantity.

See also regression coefficients.

relative standard curve method Method for determining relative target quantity in samples. With the relative standard curve method, amplification of the target and of the endogenous control is measured in samples, in a reference sample, and in a standard dilution series. Measurements are normalized using the endogenous control. Data from the standard dilution series are used to generate the standard curves. Using the standard curves, the target quantity is interpolated in the samples and in the reference sample. The relative quantity of target in each sample is determined by comparing target quantity in each sample to target quantity in the reference sample.

replicate group A set of identical reactions in an experiment.

replicates *See* biological replicates or technical replicates.

reporter Fluorescent dye used to detect amplification. With SYBR® Green reagents, the reporter dye is SYBR® Green dye.

reverse primer An oligonucleotide that flanks the 3′ end of the amplicon. The reverse primer and the forward primer are used together in PCR reactions to amplify the target.

reverse transcriptase An enzyme that converts RNA to cDNA. Reverse transcriptase is added to the PCR reaction to perform 1-step RT-PCR.

Rn *See* normalized reporter (Rn).

ROX™ dye A dye supplied by Applied Biosystems. ROX dye is used as the passive reference.

run method Definition of the reaction volume and the thermal profile for the instrument run.

sample The template that you are testing.

slope Regression coefficient calculated from the regression line in the standard curve. The slope indicates the PCR amplification efficiency for the assay. A slope close to –3.32 indicates 100% amplification efficiency.

See also amplification efficiency and regression line.

stage

In the thermal profile, a group of one or more steps. There are three types of stages: holding stage (including pre-PCR read and post-PCR read), cycling stage (also called amplification stage), and melt curve stage.

standard

Sample that contains a known quantity. Standard reactions are used in standard curve and relative standard curve experiments to generate standard curves.

See also standard curve and standard dilution series.

standard curve

In standard curve and relative standard curve experiments:

- The best-fit line in a plot of the C_T values from the standard reactions plotted against standard quantities.

  See also regression line.
- A set of standards containing a range of known quantities. Results from the standard curve reactions are used to generate the standard curve. The standard curve is defined by the number of points in the dilution series, the number of standard replicates, the starting quantity, and the serial dilution factor.

  See also standard dilution series.

standard curve method

Method for determining absolute target quantity in samples. With the standard curve method, amplification of the target is measured in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the absolute quantity of target in the samples is interpolated.

See also standard and standard curve.

standard dilution series

In standard curve and relative standard curve experiments, a set of standards containing a range of quantities. The standard dilution series is prepared by serially diluting standards. For example, the standard stock is used to prepare the first dilution point, the first dilution point is used to prepare the second dilution point, and so on.

See also standard curve.

standard quantity A known quantity in the PCR reaction.

- In standard curve experiments, the quantity of target in the standard. The units for standard quantity can be for mass, copy number, viral load, or other units for measuring the quantity of target.
- In relative standard curve experiments, a known quantity in the standard. Standard quantity can refer to the quantity of cDNA in the PCR reaction, the quantity of standard stock in the PCR reaction, or the dilution factor of the standard (for example, 1, 0.5, 0.25, 0.125, and 0.0625). The units are not relevant for relative standard curve experiments because they cancel out in the calculations.

step A component of the thermal profile. For each step in the thermal profile, you can set the ramp rate (ramp increment for melt curve steps), hold temperature, and hold time (duration); and you can turn data collection on or off for the ramp or the hold parts of the step.

SYBR® Green reagents PCR reaction components that consist of two primers designed to amplify the target and SYBR® Green dye to detect double-stranded DNA.

target The nucleic acid sequence that you want to amplify and detect.

technical replicates Identical reactions that contain identical components and volumes; and that evaluate the same sample.

See also biological replicates.

thermal profile Part of the run method that specifies the temperature, time, ramp, and data collection points for all steps and stages of the real-time PCR instrument run.

threshold In amplification plots, the level of fluorescence above the baseline and within the exponential growth region.

threshold cycle (C_T) The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.

Tm *See* melting temperature (Tm).

unknown In quantitation experiments, the task for the target in wells that contain a sample with unknown target quantities.

y-intercept In the standard curve, the value of y where the regression line crosses the y-axis. The y-intercept indicates the expected threshold cycle (C_T) for a sample with quantity equal to 1.

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