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by life technologies

TaqMan® Fast Advanced Master Mix

Protocol

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TRADEMARKS

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About This Guide

Safety information

Note: For general safety information, see this section and Appendix D, "Safety" on page 67. When a hazard symbol and hazard type appear by an instrument hazard, see the "Safety" Appendix for the complete alert on the chemical or instrument.

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:

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

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

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

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

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 68.

IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

TaqMan® Fast Advanced Master Mix

Product information

Purpose of the product

Use the TaqMan® Fast Advanced Master Mix to perform the PCR step in any gene expression experiment or other quantitative analysis, such as:

- Pathogen detection
- Differential gene expression analysis
- Viral load quantitation
- MicroRNA quantitation
- Microarray validation

Starting template

You can use the TaqMan Fast Advanced Master Mix with the DNA target of your choice, including cDNA or gDNA.

For RNA quantitation experiments, the TaqMan Fast Advanced Master Mix is used in the second step of a two-step reverse transcription–polymerase chain reaction (RT-PCR) protocol. You can use Applied Biosystems reverse transcription kits to generate the cDNA template from RNA.

About this protocol

TaqMan Fast Advanced Master Mix has been optimized for use with primers and TaqMan® probes that have been designed according to Applied Biosystems guidelines. This protocol describes how to perform 2-step quantitative RT-PCR, using the TaqMan Fast Advanced Master Mix in the PCR step with:

- TaqMan® Gene Expression Assays, Custom TaqMan® Gene Expression Assays, and custom TaqMan® primer and probe sets
- TaqMan® Array Plates (Fast and Standard plates)
- TaqMan® MicroRNA Assays
- TaqMan® Array Micro Fluidic Cards

Because analysis methods vary greatly between applications, this protocol provides general guidelines for analyzing data generated from experiments that use TaqMan Fast Advanced Master Mix and TaqMan® assays. For more detailed information about data analysis or the procedures outlined in this protocol, refer to the appropriate documentation for your instrument (see "Product documentation" on page 75).

About the kit

Storage and stability

Upon receipt, store the TaqMan® Fast Advanced Master Mix at –20 °C. After opening, store the TaqMan Fast Advanced Master Mix at 4 °C.

Performance of the TaqMan Fast Advanced Master Mix is guaranteed until the expiration date printed on the package and bottle labels.

Kit components

The TaqMan Fast Advanced Master Mix contains:

- AmpliTaq® Fast DNA Polymerase
- Uracil-N glycosylase (UNG)
- dNTPs with dUTP
- ROX™ dye (passive reference)
- Optimized buffer components

The TaqMan Fast Advanced Master Mix is supplied as a 2× concentration and is available in the following volumes:

Kit	Part number	Volume	No. of 20-µL reactions
TaqMan® Fast Advanced Master Mix Kit	4444556	1 × 1-mL	100
	4444557	1 × 5-mL	500
	4444558	1 × 50-mL	5000
	4444963	2 × 5-mL	1000
	4444964	5 × 5-mL	2500
	4444965	10 × 5-mL	5000

Recommended real-time PCR systems

You must perform the PCR step on a real-time PCR system. Traditional thermal cyclers cannot be used because they cannot detect and record the fluorescent signals generated by the cleavage of TaqMan® probes. Applied Biosystems recommends the following real-time PCR systems:

- ViiA™ 7 Real-Time PCR System
- StepOne™ and StepOnePlus™ Systems
- 7900HT/7900HT Fast System
- 7500/7500 Fast System
- 7300 System

Note: For a list of the reaction plates and accessories available for each real-time PCR system, see page 53.

Thermal-cycling conditions for your real-time PCR system

The TaqMan Fast Advanced Master Mix can be used with Fast and Standard real-time PCR systems, provided the thermal-cycling profile and run mode are correctly set.

- **Thermal-cycling profile** – The thermal-cycling profile defines the temperature and time for each step. Be sure to use the appropriate thermal-cycling profile for your system:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

- **Run mode** – The run mode defines the ramp rate that is used to heat or cool the sample block between temperature changes.

Applied Biosystems Real-Time PCR System	Default run mode
7900HT system	Standard
7900HT Fast system (384-Well and Standard 96-Well Block Modules)	
7500 system	
7300 system	
ViiA™ 7 system	Fast
StepOne™ system	
StepOnePlus™ system	
7900HT Fast system (Fast 96-Well Block Module)	
7500 Fast system	

Before you begin

Prevent contamination

Review Appendix B, "PCR Good Laboratory Practices" on page 57.

Other assays or thermal-cycling conditions

If you use assays other than the TaqMan® assays, or use thermal-cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal-cycling conditions as needed.

Section 1 TaqMan® and Custom TaqMan® Gene Expression Assays

This section provides a general protocol for performing two-step RT-PCR. In the PCR step, you use the TaqMan® Fast Advanced Master Mix with one of the following assays:

- TaqMan® Gene Expression Assays
- Custom TaqMan® Gene Expression Assays
- Custom TaqMan® primer and probe sets

This section covers:

For more information

For more detailed information about the procedures outlined in this protocol, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

User-supplied materials

Reagents for reverse transcription

Reagents		Applied Biosystems part number
High Capacity cDNA Reverse Transcription Kit	200 reactions	4368814
	200 reactions with RNase Inhibitor	4374966
	1000 reactions	4368813
	1000 reactions with RNase Inhibitor	4374967
High Capacity RNA-to-cDNA™ Kit	50 reactions	4387406
High Capacity RNA-to-cDNA Master Mix	500 reactions	4390779
	200 reactions	4390778
	50 reactions	4390777
	15 reactions	4390776
High Capacity RNA-to-cDNA Master Mix with No RT Control	500 reactions	4390713
	200 reactions	4390712
	50 reactions	4390711
	15 reactions	4390710
SuperScript® VILO™ cDNA Synthesis Kit	50 reactions	4453650
	250 reactions	4453651
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free, sterile-filtered water)		AM9849
RNase inhibitor		N8080119

Reagents for PCR

Reagents		Applied Biosystems part number
TaqMan® Gene Expression Assays, inventoried		4331182
TaqMan® Gene Expression Assays, made-to-order		4351372
Custom TaqMan® Gene Expression Assays	Small-scale, 20X (144 × 50-µL reactions)	4331348
	Medium-scale, 20X (300 × 50-µL reactions)	4332078
	Large-scale, 60X (1160 × 50-µL reactions)	4332079
Custom TaqMan® probes and primers		Various†
Nuclease-free Water (not DEPC-treated)		AM9930

† Probes and primers synthesized by Applied Biosystems to your sequence and choice of quencher and reporter dyes. See Applied Biosystems web site: **www.appliedbiosystems.com**.

Optional reagents

See "Optional reagents" on page 55 for a list of optional user-supplied reagents.

Reaction plates, consumables, and equipment

See:

- "Reaction plates and accessories" on page 53 for a list of reaction plates that are compatible with your real-time PCR system.
- "General laboratory equipment" on page 56 for a list of required and optional laboratory consumables and equipment.

Workflow

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Follow the protocol for your reverse transcription kit

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Run the PCR reaction plate (page 20)

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Analyze the data (page 21)

Perform reverse transcription

Synthesis of single-stranded cDNA from total RNA samples is the first step in the two-step RT-PCR.

Recommended kits and reagents

To obtain cDNA from RNA samples, Applied Biosystems recommends the reverse transcription kits and reagents listed in "Reagents for reverse transcription" on page 14.

Perform reverse transcription

For detailed RT guidelines and instructions, refer to the protocol for your reverse transcription kit. To download the protocols for Applied Biosystems kits, go to:

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

Perform real-time PCR amplification

Target amplification using cDNA as the template is the second step in the two-step RT-PCR. In this step, the DNA polymerase amplifies target cDNA using sequence-specific primers and cleaves the TaqMan® probe to generate a fluorescent signal that is measured by the real-time PCR system. (For more information on TaqMan® chemistry, see Appendix C on page 61.)

Note: If you choose to use custom TaqMan® probes and sequence detection primers, rather than a TaqMan® Gene Expression Assay or a Custom TaqMan® Gene Expression Assay, see "Using custom TaqMan® primer and probe sets" on page 21.

PCR reagent handling and preparation

Follow these guidelines to ensure optimal PCR performance:

- Keep the TaqMan® assays in the freezer, away from light, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Before use:
  - Thoroughly mix the TaqMan® Fast Advanced Master Mix by swirling the bottle.
  - Thaw frozen TaqMan® assays by placing them on ice. When thawed, resuspend the assays by vortexing, then briefly centrifuge.
  - Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by briefly vortexing, then briefly centrifuge.

Prepare the PCR reaction mix

1. Determine the total number of PCR reactions to perform. On each reaction plate, include:
   - A gene expression assay for each cDNA sample
   - No template controls (NTCs) for each assay on the plate

   IMPORTANT! You can run multiple assays on one reaction plate. Include controls for each assay that you run on a plate.

   Applied Biosystems recommends that you perform 3 replicates of each reaction.

2. Per the table below, calculate the total volume required for each reaction component.

   Note: Include extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume (µL) for 1 reaction		Final concentration
	384-well plate	96-well and 48-well plates (both Standard and Fast)	
TaqMan® Fast Advanced Master Mix (2X)	5.0	10.0	1X
TaqMan® Gene Expression Assay (20X) or Custom TaqMan® Gene Expression Assay (20X)†	0.5	1.0	1X
Nuclease-free Water	3.5	7.0	—
Total volume per reaction	**9.0**	**18.0**	**—**

† See **allgenes.com** for assay information.

3. Label a 1.5-mL microcentrifuge tube, add all components to the labeled tube, then cap the tube.
4. Vortex the tube briefly to mix the components.
5. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.

Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate:

Reaction plate format	Reaction volume (µL)
384-well plate	9.0
96-well and 48-well plates (both Standard and Fast)	18.0

2. Cover the reaction plate with an optical adhesive film.
3. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

4. Remove the optical adhesive film.

5. Per the table below, add cDNA template or water to each well.

Component	Volume (µL) for 1 reaction	
	384-well plate	96-well and 48-well plates (both Standard and Fast)
cDNA template + Nuclease-free Water†	1.0	2.0
Nuclease-free Water (for the NTC reactions)	1.0	2.0
Total volume per reaction	**10.0**	**20.0**

† Use 100 ng to 1 pg of cDNA diluted in Nuclease-free Water.

6. Cover the reaction plate with a new optical adhesive film.

7. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

8. If required by your real-time PCR system, apply a compression pad to the plate.

Set up a plate document or experiment

When you set up a plate document or experiment, use the following thermal-cycling conditions:

- Thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

- Run mode:

Applied Biosystems Real-Time PCR System	Default run mode
7900HT system	Standard
7900HT Fast system (384-Well and Standard 96-Well Block Modules)	
7500 system	
7300 system	
ViiA™ 7 system	Fast
StepOne™ system	
StepOnePlus™ system	
7900HT Fast system (Fast 96-Well Block Module)	
7500 Fast system	

- Sample volume:

Reaction plate format	Reaction volume (µL)
384-well plate	10.0
96-well and 48-well plates (both Standard and Fast)	20.0

Run the PCR reaction plate

1. In the system software, open the plate document or experiment that corresponds to the reaction plate.
2. Load the reaction plate into the real-time PCR system.
3. Start the run.

Analyze the data

Data analysis varies depending on the real-time PCR system that you use. The general process for analyzing gene expression quantitation data involves:

1. Viewing the amplification plots for the entire reaction plate.
2. Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
3. Using the relative standard curve method or the comparative C_T method to analyze the data.

For detailed analysis information, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

Using custom TaqMan® primer and probe sets

To design custom probes and primers for a quantitation experiment:

- Determine the target template and amplicon (this page).
- Design the custom sequence detection primers and TaqMan® probe(s) (this page).
- Determine the optimal concentrations of the sequence detection primers (this page).
- Determine the optimal concentration of the custom TaqMan probe(s) (page 24).
- Perform real-time quantitative PCR (page 26).

Determine the target template and amplicon

A target template is a DNA sequence, including a cDNA, gDNA, or plasmid nucleotide sequence.

Design primers to amplify amplicons (short segments of DNA) within the target sequence. The shortest amplicons work the best. Consistent results are obtained for amplicon size ranges from 50 to 150 bp.

Design the custom TaqMan® probes and primers

You can design primers and probes with the Primer Express® Software. Refer to the *Primer Express® Software Version 3.0 Getting Started Guide*.

Determine the optimal primer concentration

The purpose of this procedure is to determine the minimum primer concentrations that yield the minimum threshold cycle (C_T) for each probe target without a decrease in the ΔR_n.

Note: Applied Biosystems real-time PCR systems can provide additional data for optimization using the minimum C_T. Refer to your instrument documentation for more information.

To determine the optimal primer concentration:

1. Per the table below, prepare a PCR reaction mix.

Reaction component	Volume (µL) for 1 reaction		Final concentration
	384-well plate	96-well and 48-well plates (both Standard and Fast)	
TaqMan® Fast Advanced Master Mix	5.0	10.0	1×
Forward primer	1.0	2.0	50 to 900 nM
Reverse primer	1.0	2.0	50 to 900 nM
TaqMan® probe (2.5-µM)	1.0	2.0	250 nM
Nuclease-free Water	1.0	2.0	—
Total volume per reaction	**9.0**	**18.0**	**—**

2. In an optical reaction plate, load at least 3 replicates of each of the following conditions:

Condition	Forward primer (nM)	Reverse primer (nM)
1	50	50
2	300	300
3	900	900

3. Run the reaction plate on your real-time PCR system using the following thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

4. At the end of run, tabulate the results for the C_T and ΔR_n Choose the minimum forward- and reverse-primer concentrations that yield the maximum ΔR_n and the lowest C_T.

Determine the optimal probe concentration

The purpose of this procedure is to determine the minimum probe concentrations that yield the minimum C_T for each probe target without decreasing the ΔR_n.

Most TaqMan® assays are designed and run following Applied Biosystems assay development guidelines. A concentration of 900-nM primers and a 250-nM probe provides a highly reproducible and sensitive assay.

To determine the optimal probe concentration for single-probe assays:

1. Per the table below, prepare a PCR reaction mix.

Reaction component	Volume (µL) for 1 reaction		Final concentration
	384-well plate	96-well and 48-well plates (both Fast and Standard)	
TaqMan® Fast Advanced Master Mix	5.0	10.0	1×
Forward primer	1.0	2.0	Optimal†
Reverse primer	1.0	2.0	Optimal†
TaqMan® probe (2.5-µM)	1.0	2.0	50 to 250 nM
Nuclease-free Water	1.0	2.0	—
Total volume per reaction	**9.0**	**18.0**	**—**

† Use the forward- and reverse-primer concentrations determined in "Determine the optimal primer concentration" on page 22.

2. In an optical reaction plate, load at least 3 replicates at each 50-nM interval from 50 to 250 nM

3. Run the plate on your real-time PCR system using the following thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

4. Tabulate the results for the C_T and ΔR_n. Choose the minimum probe concentrations that yield the minimum C_T and the highest ΔR_n.

Perform real-time PCR

For routine assays that are optimized as described here, perform real-time quantitative PCR as follows:

1. Per the table below, prepare a PCR reaction mix, then transfer the appropriate volume to each well of an optical reaction plate.

Reaction component	Volume (µL) for 1 reaction		Final concentration
	384-well plate	96-well and 48-well plates (both Fast and Standard)	
TaqMan® Fast Advanced Master Mix	5.0	10.0	1×
Forward primer	1.0	2.0	Optimal†
Reverse primer	1.0	2.0	Optimal†
TaqMan® probe (2.5-µM)	1.0	2.0	Optimal‡
Nuclease-free Water	1.0	2.0	—
Total volume per reaction	**9.0**	**18.0**	**—**

† Use the forward- and reverse-primer concentrations determined in "Determine the optimal primer concentration" on page 22.
‡ Use the probe concentration determined in "Determine the optimal probe concentration" on page 24.

2. Per the table below, add cDNA template or water to each well.

Component	Volume (µL) for 1 reaction	
	384-well plate	96-well and 48-well plates (both Standard and Fast)
cDNA template + Nuclease-free Water†	1.0	2.0
Nuclease-free Water (for the NTC reactions)	1.0	2.0
Total volume per reaction	**10.0**	**20.0**

† Use 100 ng to 1 pg of cDNA diluted in Nuclease-free Water.

3. Run the plate on your real-time PCR system using the following thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

Section 2 TaqMan® Array Plates

This section provides a general protocol for performing two-step RT-PCR. In the PCR step, you use the TaqMan® Fast Advanced Master Mix with the TaqMan® Array Plates. The TaqMan Array Plates are TaqMan® Gene Expression Assays that are dried in MicroAmp® Optical 96-Well Reaction Plates and MicroAmp® Fast 96-Well Optical Reaction Plates.

This section covers:

For more information

For more detailed information about the procedures outlined in this protocol, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

User-supplied materials

Reagents for reverse transcription

Reagents		Applied Biosystems part number
High Capacity cDNA Reverse Transcription Kit	200 reactions	4368814
	200 reactions with RNase Inhibitor	4374966
	1000 reactions	4368813
	1000 reactions with RNase Inhibitor	4374967
High Capacity RNA-to-cDNA™ Kit	50 reactions	4387406
High Capacity RNA-to-cDNA Master Mix	500 reactions	4390779
	200 reactions	4390778
	50 reactions	4390777
	15 reactions	4390776
High Capacity RNA-to-cDNA Master Mix with No RT Control	500 reactions	4390713
	200 reactions	4390712
	50 reactions	4390711
	15 reactions	4390710
SuperScript® VILO™ cDNA Synthesis Kit	50 reactions	4453650
	250 reactions	4453651

Reagents	Applied Biosystems part number
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free, sterile-filtered water)	AM9849
RNase inhibitor	N8080119

Reagents for PCR

Reagents	Applied Biosystems part number
TaqMan® Array Standard 96 well Plates	Std_96
TaqMan® Array Fast 96 well Plates	Fast_96
TaqMan® Array Gene Signature Plates	Various†
Nuclease-free Water (not DEPC-treated)	AM9930

† See the Applied Biosystems web site: **www.appliedbiosystems.com**.

Optional reagents

See "Optional reagents" on page 55 for a list of optional user-supplied reagents.

Reaction plates, consumables, and equipment

See:

- "Reaction plates and accessories" on page 53 for a list of reaction plates that are compatible with your real-time PCR system.
- "General laboratory equipment" on page 56 for a list of required and optional laboratory consumables and equipment.

Workflow

Perform reverse transcription (page 32)

Follow the protocol for your reverse transcription kit

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Analyze the data (page 35)

Perform reverse transcription

Synthesis of single-stranded cDNA from total RNA samples is the first step in the two-step RT-PCR.

Recommended kits and reagents

To obtain cDNA from RNA samples, Applied Biosystems recommends the reverse transcription kits and reagents listed in "Reagents for reverse transcription" on page 29.

Perform reverse transcription

For detailed RT guidelines and instructions, refer to the protocol for your reverse transcription kit. To download the protocols for Applied Biosystems kits, go to:

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

Perform real-time PCR amplification

Target amplification using cDNA as the template is the second step in the two-step RT-PCR. In this step, the DNA polymerase amplifies target cDNA using sequence-specific primers and cleaves the TaqMan® probe to generate a fluorescent signal that is measured by the real-time PCR system. (For more information on TaqMan® chemistry, see Appendix C on page 61.)

PCR reagent handling and preparation

Follow these guidelines to ensure optimal PCR performance:

- Keep the TaqMan® Array Plates away from light until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Before use:
  - Thoroughly mix the TaqMan® Fast Advanced Master Mix by swirling the bottle.
  - Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by briefly vortexing, then briefly centrifuge.

Prepare the PCR reaction mix

1. For each cDNA sample, determine the total number of PCR reactions to perform (1 reaction = 1 well in the TaqMan Array Plate).
2. Per the table below, calculate the total volume required for each reaction component.

   Note: Include extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume (µL) for 1 reaction	
	Fast 96-well plate	**Standard 96-well plate**
cDNA template + Nuclease-free Water†	10.0	10.0
TaqMan® Fast Advanced Master Mix (2×)	10.0	10.0
Total volume per reaction	**20.0**	**20.0**

† Use 5 to 50 ng of cDNA diluted in Nuclease-free Water.

Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate:

Reaction plate format	Reaction volume (µL)
Fast 96-well plate	20.0
Standard 96-well plate	20.0

2. Cover the reaction plate with an optical adhesive film.
3. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.
4. If required by your real-time PCR system, apply a compression pad to the plate.

Set up a plate document or experiment

When you set up a plate document or experiment, use the following thermal-cycling conditions:

- Thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well or Standard 96-Well)					
ViiA™ 7 system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

- Run mode:

Applied Biosystems Real-Time PCR System	Default run mode
7900HT system	Standard
7900HT Fast system (Standard 96-Well Block Modules)	
7500 system	
7300 system	
ViiA™ 7 system	Fast
StepOnePlus™ system	
7900HT Fast system (Fast 96-Well Block Module)	
7500 Fast system	

- Sample volume: 20 µL

Run the PCR reaction plate

1. In the system software, open the plate document or experiment that corresponds to the reaction plate.
2. Load the reaction plate into the real-time PCR system.
3. Start the run.

Analyze the data

Data analysis varies depending on the real-time PCR system that you use. The general process for analyzing gene expression quantitation data involves:

1. Viewing the amplification plots for the entire reaction plate.
2. Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
3. Using the relative standard curve method or the comparative C_T method to analyze the data.

For detailed analysis information, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

Section 3 TaqMan® MicroRNA Assays

This section provides a general protocol for performing two-step RT-PCR. In the PCR step, you use the TaqMan® Fast Advanced Master Mix with TaqMan® MicroRNA Assays. The TaqMan MicroRNA Assays are designed to detect and accurately quantitate mature microRNAs (miRNAs) using Applied Biosystems real-time PCR systems.

This section covers:

For more information

For more detailed information about the procedures outlined in this protocol, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

User-supplied materials

Reagents for reverse-transcription

Reagents		Applied Biosystems part number
TaqMan® MicroRNA Reverse Transcription Kit†	200 reactions	4366596
	1000 reactions	4366597
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free, sterile-filtered water)		AM9849
RNase inhibitor		N8080119
Nuclease-free Water (not DEPC-treated)		AM9930

† TaqMan® MicroRNA Assays are specifically optimized to work with the TaqMan® MicroRNA Reverse Transcription Kit. Applied Biosystems cannot guarantee assay performance if you use other reverse transcription kits.

Reagents for PCR

The TaqMan MicroRNA Assays are available for a range of species. Because many mature miRNA sequences are identical across related species, many human assays are also valid for mouse and rat assays.

Reagents	Applied Biosystems part number
TaqMan® MicroRNA Assays	Various†
Custom Small RNA Assays (Early-Access)	4398987, 4398988, 4398989

† See the Applied Biosystems web site: **miRNA.appliedbiosystems.com**.

Optional reagents

See "Optional reagents" on page 55 for a list of optional user-supplied reagents.

Reaction plates, consumables, and equipment

See:

- "Reaction plates and accessories" on page 53 for a list of reaction plates and that are compatible with your real-time PCR system.
- "General laboratory equipment" on page 56 for a list of required and optional laboratory consumables and equipment.

Workflow

Perform reverse transcription (page 40)

Follow the protocol for your reverse transcription kit

⬇

Perform real-time PCR amplification (page 40)

Prepare the PCR reaction mix (page 41)

⬇

Prepare the PCR reaction plate (page 42)

⬇

Set up a plate document or experiment (page 42)

⬇

Run the PCR reaction plate (page 43)

⬇

Analyze the data (page 43)

Perform reverse transcription

Synthesis of single-stranded cDNA from total RNA samples is the first step in the two-step RT-PCR.

Recommended kit

To obtain cDNA from RNA samples, Applied Biosystems recommends the TaqMan® MicroRNA Reverse Transcription Kit. TaqMan® MicroRNA Assays are specifically optimized to work with the TaqMan MicroRNA Reverse Transcription Kit. Applied Biosystems cannot guarantee assay performance if you use other reverse transcription kits. (See "Reagents for reverse-transcription" on page 37 for the kit part numbers.)

Perform reverse transcription

For detailed RT guidelines and instructions, refer to the protocol provided with the TaqMan MicroRNA Reverse Transcription Kit.

Perform real-time PCR amplification

Target amplification using cDNA as the template is the second step in the two-step RT-PCR. In this step, the DNA polymerase amplifies target cDNA using sequence-specific primers and cleaves the TaqMan® probe to generate a fluorescent signal that is measured by the real-time PCR system. (For more information on TaqMan® chemistry, see Appendix C on page 61.)

Reagent preparation guidelines

- Keep all TaqMan® MicroRNA Assays in the freezer, protected from light, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Before use, mix the TaqMan Fast Advanced Master Mix thoroughly by swirling the bottle.
- Prepare the PCR reaction mix before transferring to the reaction plate for thermal cycling.

Prepare the PCR reaction mix

1. Determine the total number of PCR reactions to perform. On each reaction plate include:
   - A mircoRNA assay for each cDNA sample
   - Endogenous control assays
   - No template controls (NTCs) for each assay on the plate

   IMPORTANT! You can run multiple assays on one reaction plate. Include controls for each assay that you run on a plate.

   Applied Biosystems recommends that you perform 3 replicates of each reaction.

2. Per the table below, calculate the total volume required for each reaction component.

   Note: Include extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume (µL) for 1 reaction	
	384-well plate	96-well and 48-well plates (both Fast and Standard)
TaqMan® Fast Advanced Master Mix (2X)	5.00	10.00
Nuclease-free Water	3.83	7.67
TaqMan® MicroRNA Assay (20X)	0.50	1.00
Product from RT reaction†	0.67	1.33
Total volume per reaction	**10.00**	**20.00**

† The minimum final dilution of the RT reaction in the PCR reaction is 1:15.

3. Combine the TaqMan Fast Advanced Master Mix and water:
   a. Label a 1.5-mL microcentrifuge tube.
   b. Add the TaqMan Fast Advanced Master Mix and water to the labeled tube, then cap the tube.
   c. Mix gently, then centrifuge to bring the solution to the bottom of the tube.
   d. Transfer the solution to a polypropylene tube (the PCR reaction tube). (Necessary to transfer to a different tube? Nathalie to check this.)

4. Add the TaqMan® MicroRNA Assay to the PCR reaction tube.

5. Add the RT reaction product to the PCR reaction tube.

6. Mix gently, then centrifuge to bring the solution to the bottom of the plate.

Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate:

Reaction plate format	Reaction volume (µL)
384-well plate	10.0
96-well and 48-well plates (both Fast and Standard)	20.0

2. Cover the reaction plate with an optical adhesive film.
3. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.
4. If required by your real-time PCR system, apply a compression pad to the plate.

Set up a plate document or experiment

When you set up a plate document or experiment, use the following thermal-cycling conditions:

- Thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

- Run mode:

Applied Biosystems Real-Time PCR System	Default run mode
7900HT system	Standard
7900HT Fast system (384-Well and Standard 96-Well Block Modules)	
7500 system	
7300 system	
ViiA™ 7 system	Fast
StepOne™ system	
StepOnePlus™ system	
7900HT Fast system (Fast 96-Well Block Module)	
7500 Fast system	

- Sample volume:

Reaction plate format	Reaction volume (µL)
384-well plate	10.0
96-well and 48-well plates (both Standard and Fast)	20.0

Run the PCR reaction plate

1. In the system software, open the plate document or experiment that corresponds to the reaction plate.
2. Load the reaction plate into the real-time PCR system.
3. Start the run.

Analyze the data

Data analysis varies depending on the real-time PCR system that you use. The general process for analyzing gene expression quantitation data involves:

1. Viewing the amplification plots for the entire reaction plate.
2. Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
3. Using the relative standard curve method or the comparative C_T method to analyze the data.

For detailed analysis information, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

Section 4 TaqMan® Array Micro Fluidic Cards

This section provides a general protocol for performing two-step RT-PCR. In the PCR step, you use the TaqMan® Fast Advanced Master Mix with TaqMan® Array Micro Fluidic Cards. The TaqMan Array cards are 384-well cards preloaded with TaqMan® Gene Expression Assays. The TaqMan Array cards allow you to measure gene expression using the comparative C_T ($\Delta\Delta C_T$) method of relative quantitation. You can run 1 to 8 samples per card, against 12 to 384 TaqMan Gene Expression Assay targets (including controls).

This section covers:

For more information

For more detailed information about the procedures outlined in this protocol, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

User-supplied materials

Reagents for reverse transcription

Reagents		Applied Biosystems part number
High Capacity cDNA Reverse Transcription Kit	200 reactions	4368814
	200 reactions with RNase Inhibitor	4374966
	1000 reactions	4368813
	1000 reactions with RNase Inhibitor	4374967
High Capacity RNA-to-cDNA™ Kit	50 reactions	4387406
High Capacity RNA-to-cDNA Master Mix	500 reactions	4390779
	200 reactions	4390778
	50 reactions	4390777
	15 reactions	4390776

Reagents		Applied Biosystems part number
High Capacity RNA-to-cDNA Master Mix with No RT Control	500 reactions	4390713
	200 reactions	4390712
	50 reactions	4390711
	15 reactions	4390710
SuperScript® VILO™ cDNA Synthesis Kit	50 reactions	4453650
	250 reactions	4453651
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free, sterile-filtered water)		AM9849
RNase inhibitor		N8080119

Reagents for PCR

Reagents		Applied Biosystems part number
TaqMan® Array Custom Micro Fluidic Card	One TaqMan Array card; 384 wells per card	Various†
TaqMan® Gene Set	One TaqMan Array card; 384 wells per card	
TaqMan® Gene Signature Array	The endogenous control Gene Signature Arrays come in sets of two cards; the remaining Gene Signature Arrays come in sets of four cards. 384 wells per card.	
Nuclease-free Water (not DEPC-treated)		AM9930

† See the Applied Biosystems web site: **taqmanarray.appliedbiosystems.com**.

Optional reagents

See "Optional reagents" on page 55 for a list of optional user-supplied reagents.

Reaction plates, consumables, and equipment

See:

- "Reaction plates and accessories" on page 53 for a list of reaction plates and that are compatible with your real-time PCR system.
- "General laboratory equipment" on page 56 for a list of required and optional laboratory consumables and equipment.

Workflow

Perform reverse transcription (page 48)

Follow the protocol for your reverse transcription kit

⬇

Perform real-time PCR amplification (page 49)

Prepare the PCR reaction mix (page 50)

⬇

Prepare the TaqMan Array card (page 50)

⬇

Set up a plate document or experiment (page 51)

⬇

Run the TaqMan Array card (page 51)

⬇

Analyze the data (page 51)

Perform reverse transcription

Synthesis of single-stranded cDNA from total RNA samples is the first step in the two-step RT-PCR.

Recommended kits and reagents

To obtain cDNA from RNA samples, Applied Biosystems recommends the reverse transcription kits and reagents listed in "Reagents for reverse transcription" on page 45.

Perform reverse transcription

For detailed RT guidelines and instructions, refer to the:

- *TaqMan® Array Micro Fluidic Card User Guide*

  and
- Protocol for your reverse transcription kit. To download the protocols for Applied Biosystems kits, go to:

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

Perform real-time PCR amplification

Target amplification using cDNA as the template is the second step in the two-step RT-PCR. In this step, the DNA polymerase amplifies target cDNA using sequence-specific primers and cleaves the TaqMan® probe to generate a fluorescent signal that is measured by the real-time PCR system. (For more information on TaqMan® chemistry, see Appendix C on page 61.)

Card handling and preparation

Follow these guidelines to ensure optimal PCR performance:

- Do not remove a TaqMan Array card from its packaging until the packaging has reached room temperature and you are ready to fill it with sample-specific PCR mix. Prolonged exposure to indoor lighting can photo-degrade the fluorescent probes contained within the card. Do not expose the card to sunlight.
- Fill each fill reservoir with sample-specific PCR mix made from a single cDNA sample.
- Add 100 µL of the sample-specific PCR mix per fill reservoir to ensure adequate filling. Volumes smaller than 100 µL will result in insufficiently filled cards.
- Do not add sample after centrifuging the cards. When you centrifuge the card, the sample-specific PCR mix resuspends the dried TaqMan® probes and primers within the wells of the card. Adding sample after centrifuging disrupts the resuspended assay positions.
- To ensure a high degree of reproducibility, Applied Biosystems recommends scheduling your runs so that each card is run as soon as possible. After sealing, there is no measurable well-to-well contamination for up to 64 hours.

Recommended amounts of cDNA

For the PCR reactions, Applied Biosystems recommends that you use:

- 30 to 1000 ng (0.3 to 10 ng/mL) of cDNA (converted from total RNA) per fill reservoir. The amount to use depends on the expression level of your target genes and the number of target copies per well that you need to detect. For example, you can use:
  - 1000 ng (10 ng/µL) per fill reservoir to detect genes with low expression. However, the cDNA concentration will be high (10 ng/µL); be sure to use high-quality cDNA without inhibitors.
  - 100 to 200 ng per fill reservoir to detect genes with moderate expression.
  - 30 to 50 ng per fill reservoir to detect genes with moderate to high expression.
- The same amount of cDNA sample for all reactions.

Prepare the PCR reaction mix

1. For each cDNA sample, determine the total number of fill reservoirs in the TaqMan Array card that you will use.

2. Per the table below, calculate the total volume required for each reaction component.

   Note: Include extra volume to compensate for the volume loss that occurs during pipetting.

Reaction Component	Volume (µL) for 1 fill reservoir
cDNA template + Nuclease-free Water†	50.0
TaqMan® Fast Advanced Master Mix (2X)	50.0
Total volume	**100.0**

† Each sample-specific PCR reaction mix should contain 30 to 1000 ng (0.3 to 10 ng/µL) of total RNA converted to cDNA. The amount of cDNA to add depends upon the abundance of the specific gene transcript.

3. If frozen, thaw the cDNA samples on ice. Resuspend the cDNA samples by inverting the tube, then gently vortexing.

4. Mix the master mix thoroughly by swirling the bottle.

5. For each sample, label a 1.5-mL microcentrifuge tube, then add the required components to the labeled tube.

6. Cap the microcentrifuge tubes, then gently vortex the tubes to thoroughly mix the solution.

7. Briefly centrifuge the tubes to spin down the contents and eliminate air bubbles.

Prepare the TaqMan Array card

Fill, centrifuge, and seal the TaqMan Array card according to the *TaqMan® Array Micro Fluidic Card User Guide*.

Set up a plate document or experiment

When you set up a plate document or experiment, use the following thermal-cycling conditions:

- Thermal-cycling profile:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	92	97	62
7900HT Fast system (TaqMan® Array Micro Fluidic Card Thermal Cycling Block)	Time (mm:ss)	02:00	10:00	00:01	00:20

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

- Default run mode: Standard
- Ramp rate: 100%
- Sample volume: 1.0 µL

Run the TaqMan Array card

1. In the system software, open the plate document or experiment that corresponds to the TaqMan Array card.
2. Load the card into the real-time PCR system.
3. Start the run.

Analyze the data

Data analysis varies depending on the real-time PCR system that you use. The general process for analyzing gene expression quantitation data involves:

1. Viewing the amplification plots for the entire reaction plate.
2. Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.
3. Using the relative standard curve method or the comparative C_T method to analyze the data.

For detailed analysis information, refer to the appropriate documentation for your instrument. See "Product documentation" on page 75.

Additional User-Supplied Materials

This appendix covers:

Reaction plates and accessories

The table below lists the reaction plates and accessories available for Applied Biosystems real-time PCR systems.

Real-Time PCR System	Reaction plates and accessories
7300 system 7500 system	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (PN 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (PN 4323032)
7500 Fast system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Optical 8-Cap Strip, 300 strips (PN 4323032)
ViiA™ 7 system, 384-Well Block Module 7900HT/7900HT Fast system, 384-Well Block Module	• 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, 100 films (PN 4311971)
7900HT/7900HT Fast system, Standard 96-Well Block Module	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (PN 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (PN 4323032) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (PN 4333292)

Real-Time PCR System	Reaction plates and accessories
7900HT/7900HT Fast system, Fast 96-well Block Module	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (PN 4333292)
7900HT/7900HT Fast system, TaqMan® Array Micro Fluidic Card Thermal Cycling Block	TaqMan® Array Micro Fluidic Card
ViiA™ 7 system, TaqMan® Array Micro Fluidic Card Thermal Cycling Block	
StepOne™ system	• MicroAmp® Fast Optical 48-Well Reaction Plate, 20 plates (PN 4375816) • MicroAmp® 48-Well Optical Adhesive Film, 100 films (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, 100 films (PN 4311971)

Optional reagents

Item	Applied Biosystems part number
DNA*Zap*™ Solution (2 bottles, 250 mL each)	AM9890
MagMAX™ AI/ND Viral RNA Isolation Kit, 50 purifications	AM1929
MagMAX™ Viral RNA Isolation Kit, 50 purifications	AM1939
mirVana™ miRNA Isolation Kit, 40 purifications	AM1560
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE, 40 purifications	AM1975
RiboPure™ Bacterial Kit	AM1925
RiboPure™ Blood Kit, 40 purifications	AM1928
RiboPure™ RNA Isolation Kit, 50 purifications	AM1924
RiboPure™ Yeast Kit	AM1926
RNA*later*® ICE Solution, 25-mL	AM7030
RNA*later*® Solution, 100 mL	AM7020
RNAqueous® -4PCR Kit, 30 purifications	AM1914
RNAqueous® Kit, 50 purifications	AM1912
RNase*Zap*® RNase Decontamination Solution, 250 mL	AM9780
RT-PCR Grade Water, 10, 1.75-mL bottles	AM9935
TRI Reagent®, 100-mL	AM9738
Turbo DNA-*free*™, 50 reactions	AM1907

General laboratory equipment

The following table lists required and optional laboratory equipment and consumables.

Item		Source	Part number
Accessories for tubes of assay mixes: • Decapper for single caps • Decapper for eight caps • TPE cap cluster for simultaneously capping 96 individual polypropylene tubes, 50 capmats/bag		Micronic BV‡	---
Centrifuge with plate adapter		Major laboratory suppliers (MLS)	---
Disposable gloves		MLS	---
Microcentrifuge		MLS	---
Heat block or water bath or thermal cycler to 95 °C		MLS	---
Microcentrifuge tubes, 1.5-mL		Applied Biosystems	AM12400
Barrier (Filter) Tips	10 µL size - Pipetman™ (Ten 8 × 12 racks)	Applied Biosystems	AM12640
	10 µL size - Eppendorf® (Ten 8 × 12 racks)	Applied Biosystems	AM12635
	20 µL size (Ten 8 × 12 racks)	Applied Biosystems	AM12645
	1000 µL size (Ten 100 ct racks)	Applied Biosystems	AM12665
	200 µL size (Ten 8 x 12 racks)	Applied Biosystems	AM12655
Pipettes	Positive-displacement	MLS	---
	Air-displacement		
	Multichannel		
Vortexer		MLS	---
Microsoft® Excel® software or equivalent spreadsheet and analysis software		Software suppliers	---

‡ See the Micronic BV web site: **www.micronic.com**. Other vendors supply similar products.

PCR Good Laboratory Practices

This appendix covers:

Sample preparation

When preparing samples for PCR amplification:

- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation).
- 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.
- Open and close all sample tubes carefully. Centrifuge tubes before opening. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution. Use DNA*Zap*™ Solution (PN AM9890).

Preventing contamination

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).

False positives

Special laboratory practices are necessary in order to avoid false positive amplifications (Higuchi, et al., 1989). This is because of the capability for single DNA molecule amplification provided by the PCR process (Saiki et al., 1985; Mullis et al., 1987; Saiki et al., 1988). Because of the enormous amplification possible with PCR, amplicon carryover can result in sample contamination. Other sources of contamination could be from samples with high DNA levels or from positive control templates.

When dUTP replaces dTTP as a dNTP substrate in PCR and the method described below is used, UNG treatment can prevent the reamplification of carryover PCR products in subsequent experiments (Sninsky and Gelfand, pers. comm.) This method uses enzymatic and chemical reactions analogous to the restriction-modification and excision-repair systems of cells to degrade specifically PCR products from previous PCR amplifications or to degrade mis-primed, non-specific products produced prior to specific amplifications, but not degrade native nucleic acid templates.

The method used to make PCR products susceptible to degradation involves substituting dUTP for dTTP in the PCR mix and treating subsequent PCR mixes with the enzyme uracil-N glycosylase (UNG, EC 3.2.2-) prior to amplification (Longo et al., 1990).

Although the protocol and reagents described here are capable of degrading or eliminating large numbers of carried over PCR products, we encourage users to continue using the specific devices and suggestions described in this protocol booklet and in Kwok (1990) and Higuchi (1989) to minimize cross-contamination from non-dU-containing PCR products or other samples.

Uracil-N glycosylase (UNG)

The UNG provided in the TaqMan® Fast Advanced Master Mix is a pure, nuclease-free, 26-kDa recombinant enzyme encoded by the Escherichia coli uracil-N glycosylase gene which has been inserted into an E. coli host to direct the expression of the native form of the enzyme (Kwok and Higuchi, 1989).

UNG acts on single- and double-stranded dU-containing DNA by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, and creates an alkali-sensitive apyrimidic site in the DNA. Apyrimidic sites block replication by DNA polymerases. The enzyme has no activity on RNA or dT-containing DNA.

UNG incubation at 50 °C is necessary to cleave any dU-containing PCR carryover products. Ten-minute incubation at 95 °C is necessary to substantially reduce UNG activity, and to denature the native DNA in the experimental sample. Because UNG is not completely deactivated during the 95 °C incubation, it is important to keep the annealing temperatures greater than 55 °C and to refrigerate PCR products at 2 to 8 °C in order to prevent amplicon degradation.

Prevention of PCR product carryover

Use primers that contain dA nucleotides near the 3′ ends so that any primer-dimer generated is efficiently degraded by UNG at least as well as any dU-containing PCR products. The further a dA nucleotide is from the 3′ end, the more likely that partially degraded primer-dimer molecules may serve as templates for a subsequent PCR amplification.

Production of primer dimer could lower the amplification yield of the desired target region. If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3' terminal dU-nucleotides should be considered. Single-stranded DNA with terminal dU nucleotides are not substrates for UNG (Delort et al., 1985) and thus the primers will not be degraded. Biotin-dUMP derivatives are not substrates for UNG.

The concentration of UNG and the time of the incubation step necessary to prevent amplification of contaminating dU-containing PCR product depends on the PCR conditions necessary to amplify your particular DNA sequence and the level of contamination expected. In most cases, using UNG at 1 U/l00 mL reaction and incubation at 50 °C for two minutes is sufficient.

Do not attempt to use UNG in subsequent amplification of dU-containing PCR template, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR product, preventing further amplification.

Fluorescent contaminants

Since fluorescent contaminants may interfere with this assay and give false-positive results, it may be necessary to include a No-Amplification Control tube that contains sample, but no enzyme. If the absolute fluorescence of the No-Amplification Control is greater than that of the no template control (NTC) after PCR, fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler.

About Two-Step RT-PCR

This appendix covers:

Overview

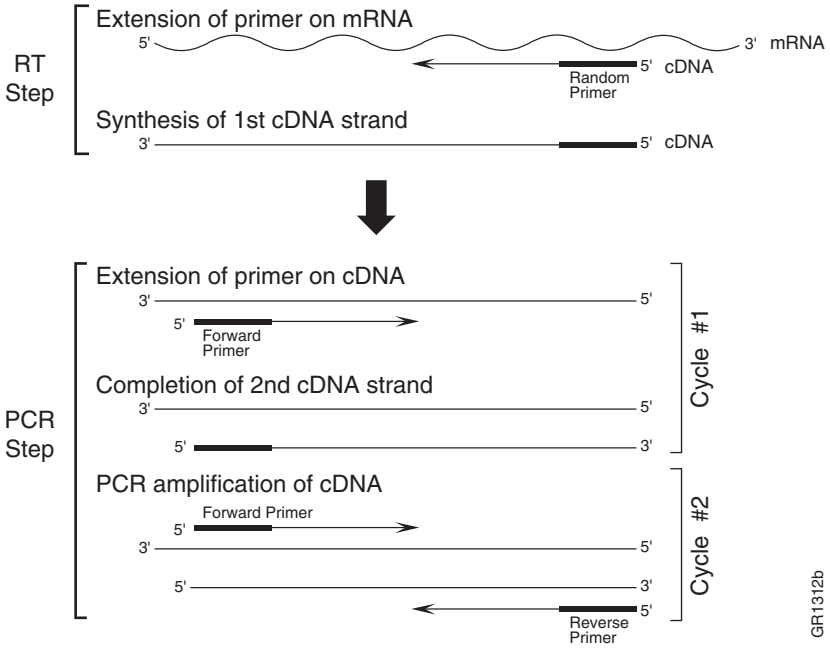
Gene quantitation assays using TaqMan® Fast Advanced Master Mix and TaqMan® assays are performed in a two-step RT-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse-transcribed from RNA.
2. In the PCR step, PCR products are quantitatively synthesized from cDNA samples using the TaqMan® Fast Advanced Master Mix.

The figure below illustrates two-step PCR.

Note: Figure 1 does not show hybridization of the TaqMan® MGB probe. See Figure 2 on page 64 for details on how the TaqMan MGB probe is used in the PCR step.

Figure 1 Two-step RT-PCR



About TaqMan® MGB Probes

The TaqMan® MGB probes contain:

- A reporter dye (for example, FAM™ dye) linked to the 5′ end of the probe.
- A minor groove binder (MGB) at the 3′ end of the probe.

  MGBs increase the melting temperature (T_m) without increasing probe length (Afonina *et al.*, 1997; Kutyavin *et al.*, 1997); they also allow for the design of shorter probes.
- A nonfluorescent quencher (NFQ) at the 3′ end of the probe.

  Because the quencher does not fluoresce, Applied Biosystems real-time PCR systems can measure reporter dye contributions more accurately.

About AmpliTaq® Fast DNA Polymerase

The AmpliTaq® Fast DNA Polymerase enzyme is purified through a proprietary process to reduce bacterial DNA introduced from the host organism. The purification process ensures that non-specific, false-positive DNA products due to bacterial DNA contamination are minimized during PCR.

When AmpliTaq® Fast DNA Polymerase is added to the reaction mixture at room temperature, the inactive enzyme is not capable of primer extension. Any low-stringency mispriming events that may have occurred will not be enzymatically extended and subsequently amplified. A thermal incubation step is required for activation to ensure that active enzyme is generated only at temperatures where the DNA is fully denatured.

About uracil-N glycosylase

Uracil-N glycosylase (UNG) treatment can prevent the reamplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded amplicons. (Longo et al., 1990). UNG prevents reamplification of carryover-PCR products in an assay if all previous PCR for that assay was performed using a dUTP-containing master mix. See "Preventing contamination" on page 58 for more information about UNG.

About ROX™ Passive Reference dye

The ROX™ Passive Reference dye provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

About the 5′ nuclease assay

The 5′ nuclease assay process (Figure 2 through Figure 5) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

Legend for Figures 2 to 5:

NFQ = Nonfluorescent quencher
MGB = Minor groove binder
R = Reporter
P = Hot-start DNA polymerase

During PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites.

When the probe is intact (Figure 2 and Figure 3), the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983).

Figure 2 Polymerization

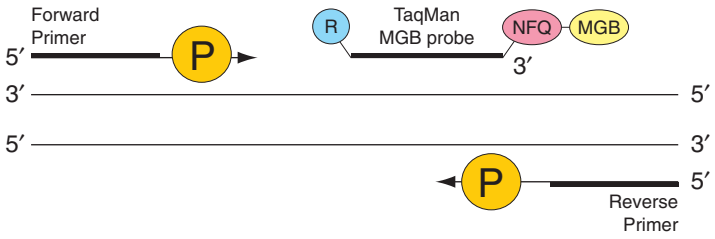
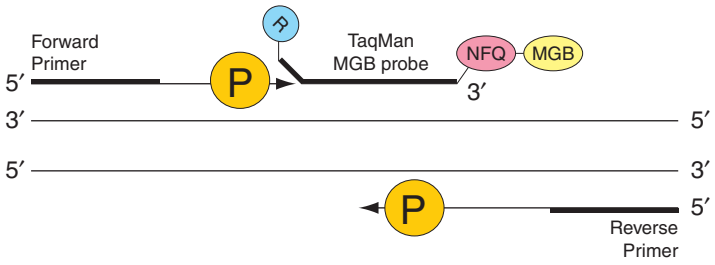
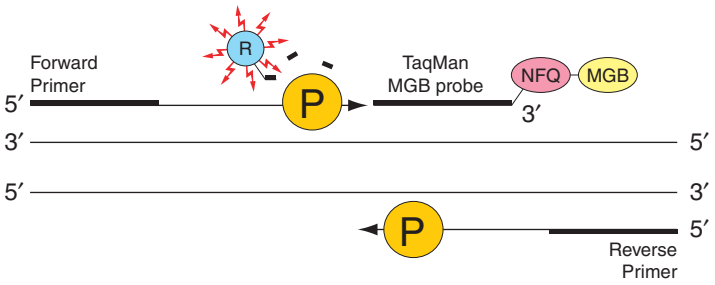


Figure 3 Strand displacement



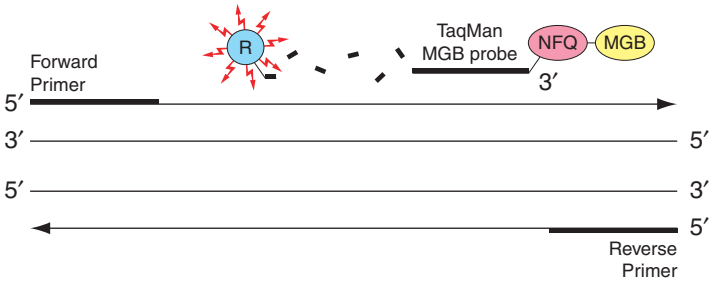
The DNA polymerase cleaves only probes that are hybridized to the target (Figure 4 on page 65). Cleavage separates the reporter dye from the quencher dye; the separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter. The increase in fluorescence occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Figure 4 Cleavage



Polymerization of the strand continues, but because the 3′ end of the probe is blocked, no extension of the probe occurs during PCR (Figure 5).

Figure 5 Completion of polymerization



Safety

This appendix covers:

Chemical safety

General chemical safety

Chemical hazard warning

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

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

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page 68.)
- 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 SDS.
- 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 SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

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

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

Obtaining SDSs

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

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.

3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

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

Chemical waste safety

Chemical waste hazards

CAUTION! HAZARDOUS WASTE. Refer to 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.

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) 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 SDS.
- 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 SDS.
- Handle chemical wastes in a fume hood.
- After emptying a waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste disposal

If potentially hazardous waste is generated, 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 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

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* (**www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**).
- 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

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Glossary

ΔR_n

The difference between the R_n+ value and the R_n– value. It reliably indicates the magnitude of the signal generated by the given set of PCR conditions.

The following equation expresses the relationship of these terms:

$\Delta R_n = (R_n+) - (R_n-)$

where:

$R_n+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$ PCR with template

$R_n- = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$ PCR without template or early cycles of a real-time reaction

C_T

See threshold cycle (C_T).

fold difference

The measured ratio of the quantity of template in Sample A over the quantity of template in Sample B, where quantity A > quantity B, so that the ratio is > 1.

full replicate

Repeated wells of the same sample with the same assay, where the contents of each well go through all experimental steps (sample preparation, reverse transcription, and PCR) separately.

minimum fold difference

The smallest fold difference that is statistically significant.

multicomponenting

The term used to distinguish the contribution each individual dye makes to the fluorescent spectra. The overlapping spectra from the dye components generate the composite spectrum, which represents one reading from one well.

normalization

The Passive Reference 1, a dye included in the 10× TaqMan® Buffer A, does not participate in the 5′ nuclease PCR. The Passive Reference provides an internal reference to which the reporter-dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescent fluctuations due to changes in concentration or volume.

PCR/technical replicate

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

R_n+	The R_n value of a reaction containing all components including the template. Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube.
R_n–	The R_n value of an unreacted sample. This value may be obtained from the early cycles of a real-time run (those cycles prior to a detectable increase in fluorescence). This value may also be obtained from a reaction not containing template.
statistically significant (to a 99.7% confidence level)	A result with a low probability (0.3%) of resulting from chance.
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.

Documentation and Support

Product documentation

A portable document format (PDF) version of the document listed below is available at **www.appliedbiosystems.com**

Note: To open the PDF version, use the Adobe Acrobat Reader software available from **www.adobe.com**

Document	Part number
TaqMan® Fast Advanced Master Mix Protocol	4444605

Related documentation

To obtain the documents listed in this section or additional documentation, see "Obtaining support" on page 77.

Chemistry/reagent documentation

Document	Part number
Applied Biosystems Real-Time PCR Systems Reagent Guide	4387787
Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial	–
Custom TaqMan® Assays: Design and Ordering Guide	4367671
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
Real-Time PCR Systems Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems	4348358
TaqMan® Array Micro Fluidic Card User Guide	4400263
TaqMan® Array Plates Protocol	4391016
TaqMan® Gene Expression Assays Protocol	4333458
TaqMan® MicroRNA Reverse Transcription Kit Protocol	4367038
TaqMan® Small RNA Assays Protocol (For TaqMan® MicroRNA Assays and Custom TaqMan® Small RNA Assays)	4364031

Instrument documentation

7300/7500/7500 Fast system

Document	Part number
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide	4347825
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 Getting Started Guide for Comparative C_T/Relative Standard Curve Experiments	4387783

7900HT/7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)

Document	Part number
Applied Biosystems 7900HT Fast Real-Time PCR System Quick Reference Card: Performing Fast Gene Quantification	4351892
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 Absolute Quantitation Using Standard Curve Getting Started Guide	4364014
Applied Biosystems 7900HT Fast Real-Time PCR System User Bulletin: Performing Fast Gene Quantitation with 394-Well Plates	4369584
Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide	4351684

StepOne™ and StepOnePlus™ systems

Document	Part number
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Reagent Guide	4379704
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Relative Standard Curve and Comparative C_T Experiments Getting Started Guide	4376785
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4376784

Obtaining support

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www.appliedbiosystems.com

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

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