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TaqMan® Pri-miRNA Assays

Protocol

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Contents

Safety information

Note: For general safety information, see this Preface and Appendix D, "Safety" on page 29. When a hazard symbol and hazard type appear by a chemical name or 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 signal word is to be limited to the most extreme situations.

MSDSs

The MSDSs for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining MSDSs, see "Obtaining MSDSs" on page 29.

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

How to use this guide

Text conventions

This guide uses the following conventions:

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

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

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

  Select **File ▸ Open ▸ Spot Set**.

  Right-click the sample row, then select **View Filter ▸ View All Runs**.

User attention words

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

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

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

How to obtain support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

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

TaqMan® Pri-miRNA Assays

Product information

Purpose of the product

TaqMan® Pri-miRNA Assays enable quantitative expression studies of primary microRNA (pri-miRNA) transcripts in human, mouse, and rat species using reverse transcription and real-time PCR (real-time RT-PCR). These predesigned, preformulated primer and probe sets work with the same optimized reverse transcriptions kits, master mixes, reagents, and instruments that are used with TaqMan® Gene Expression Assays.

Note: For information about TaqMan® assay reactions or TaqMan Pri-miRNA Assay design, see "Background Information" on page 25.

Kit contents

Size‡	No. of 20-µL reactions	Part number	Availability
Small	360	4427012	Made-to-Order (20×)
Medium	750	4427013	Made-to-Order (20×)
Large	2900	4427014	Made-to-Order (60×)

‡ See Appendix A on page 17 for details on selecting and ordering assays.

TaqMan Pri-miRNA Assays include:

- One tube for each assay that is ordered, containing:
  - Two unlabeled primers (1× final concentration is 900 nM per primer; 20× stock concentration is 18 µM per primer)
  - One 6-FAM™ dye-labeled, TaqMan MGB probe (1× final concentration is 250 nM; 20× stock concentration is 5 µM)

  Note: Each TaqMan Pri-miRNA Assay is identified by its assay ID, a unique, alphanumeric string followed by "_pri." See "About the assay name and assay ID" on page 17.

- A data sheet containing assay and order details.
- An Information CD that includes the following files:
  - Assay information file (AIF)
  - *Understanding Your Shipment*, which outlines the contents of your shipment and provides information on each of the included items.
  - *TaqMan® Pri-miRNA Assays Protocol* (PN 4427719)
  - *TaqMan® Pri-miRNA Assays Quick Reference Card* (PN 4427720)

Storage

Store TaqMan Pri-miRNA Assays at –15 to –25 °C and keep them protected from light.

Materials and equipment not included

Endogenous control assay(s)

TaqMan® Endogenous Controls are a collection of predesigned assays for candidate control genes, used to normalize for differences in sample RNA added to a reaction. They are compatible with both TaqMan Pri-miRNA Assays and TaqMan Gene Expression Assays. For more information about selecting endogenous controls, see the Application Note: *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies* (search for Stock Number 127AP08-01 at **www.appliedbiosystems.com**).

Materials for reverse transcription and PCR

See "Step 4: Order materials and equipment not included" on page 19 for a complete list of materials.

Table 1 Required materials and equipment

✔	Material	Source
	Reverse transcription reagents	Applied Biosystems (see Table 6 on page 19)
	PCR reagents	
	Thermal cycler (or real-time PCR instrument)	Applied Biosystems
	Real-Time PCR Instrument	
	Reaction plates and accessories for your real-time PCR instrument	Applied Biosystems (see Table 7 on page 20)
	Centrifuge (with plate adapter)	MLS‡
	Disposable gloves	MLS
	Microcentrifuge	MLS
	Pipette tips, aerosol-resistant	MLS
	Pipettors (positive/air-displacement or multichannel)	MLS
	Polypropylene tubes (various sizes)	MLS
	Vortexer	MLS
	Nuclease-free water (no diethyl pyrocarbonate [DEPC])	MLS

‡ Major laboratory supplier

Compatible real-time instruments

TaqMan Pri-miRNA Assays can be used with the Applied Biosystems:

- 7300 Real-Time PCR System
- 7500 Real-Time PCR System
- 7500 Fast Real-Time PCR System
- 7900HT Real-Time PCR System
- StepOne™ Real-Time PCR System
- StepOnePlus™ Real-Time PCR System

Workflow

Prepare the cDNA sample

Prepare the total RNA

↓

Perform reverse transcription

↓

Evaluate the cDNA

↓

High Capacity RNA-to-cDNA Kit

Thermal cycler or
real-time PCR instrument

Prepare the reaction mix and load the plate

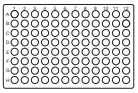
Prepare the PCR reaction mix

↓

Load the plate

↓

TaqMan® Gene Expression
Master Mix



48-, 96-, or 384-well
reaction plate

Run the real-time PCR reaction

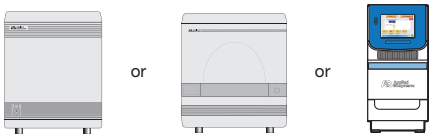
Create the plate document/ experiment

↓

Run the plate

↓

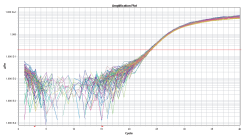

System software and information CD



Real-time PCR instrument

Analyze the results

Refer to the user guide for your real-time PCR instrument



Amplification plot

Prepare the cDNA sample

Isolate total RNA

Before running the TaqMan Pri-miRNA Assays, isolate total RNA to use as a template for synthesis of single-stranded cDNA. For optimal performance, Applied Biosystems recommends using an Ambion® RNA isolation kit. Go to **www4.appliedbiosystems.com**, select **RNA Isolation ▸ Which RNA Isolation Kit to Choose?** to view a list of kits.

Applied Biosystems recommends using total RNA that is:

- Between 0.002 and 0.2 µg/µL
- Less than 0.005% of genomic DNA by weight

IMPORTANT! TaqMan® Pri-miRNA Assays will detect genomic DNA. If your RNA purification method does not include DNase treatment, treat the purified RNA with the Ambion TURBO DNA-*free*™ Kit (recommended; PN AM1907) using the standard protocol.

- Dissolved in a PCR-compatible buffer
- Free of RNase activity
- Free of inhibitors of reverse transcription and PCR
- Nondenatured

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

Perform reverse transcription

Applied Biosystems recommends using one of the following kits to obtain cDNA from RNA samples.

- High Capacity RNA-to-cDNA Kit (PN 4387406)
- High Capacity cDNA Reverse Transcription Kit (PN 4368813, 4374966)

Note: Use the same reverse transcription procedure for all samples in an experimental study. See Table 6 on page 19 for a list of compatible reverse transcription kits.

Evaluate the cDNA

Applied Biosystems recommends that you use:

- 1 to 100 ng of cDNA per 20-µL amplification reaction (PCR)
- The same amount of cDNA in each reaction

DNA quantitation methods

- TaqMan® RNase P Detection Reagents (recommended; PN 4316831). These reagents enable quantitation of cDNA that is able to function as a template in PCR. Refer to *Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR* (search for PN 4371090 at **www.appliedbiosystems.com**).

  or

- UV absorbance (A_{260}/A_{280}) measurements.

(Optional) Store the cDNA

If you do not proceed immediately to PCR amplification, store all cDNA samples at –15 to –25 °C. To minimize freeze-thaw cycles, store the cDNA in smaller aliquots.

Prepare the reaction mix and load the plate

Thaw and mix the reagents

1. Thaw on ice, resuspend completely by vortexing gently, then centrifuge briefly:
   - TaqMan® Pri-miRNA Assays (20×)
   - cDNA samples
2. Mix the master mix reagent by gently swirling the bottle. (See Table 6 on page 19 for a list of compatible master mixes available from Applied Biosystems.)

Calculate the number of reactions

Calculate the number of reactions that you need for each assay. Applied Biosystems recommends performing four replicates of each reaction. Be sure to include on each plate:

- A TaqMan Pri-miRNA Assay for each cDNA sample
- Endogenous control assays
- (Optional) No template controls (NTCs) for each gene expression assay on the plate

Prepare the PCR reaction mix

For the following hazard, see the complete safety alert description in Appendix D, "Safety" on page 29.

CAUTION! CHEMICAL HAZARD. TaqMan® Gene Expression Master Mix, TaqMan® Universal PCR Master Mix (2×; with or without AmpErase® UNG), TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG

1. For each sample (to be run in quadruplicate), pipette the following into a nuclease-free 1.5-mL microcentrifuge tube:

PCR reaction mix component	Volume per 20-µL reaction (µL)	
	Single reaction	Four replicates‡
20× TaqMan® Pri-miRNA Assays	1.0	5.0
2× TaqMan® Gene Expression Master Mix§	10.0	50.0
cDNA template (1 to 100 ng)#	4.0	20.0
RNase-free water	5.0	25.0

‡ Replicate volumes include 20% excess for volume loss from pipetting.
§ (Optional) Use TaqMan® Fast Universal Master Mix (2×), No AmpErase® UNG or TaqMan® Universal Master Mix. If you add AmpErase® UNG (uracil-N-glycosylase), the final concentration must be 0.01 U/µL. Reduce the volume of water in the PCR reaction mix to compensate for additional volume from the UNG.
# Applied Biosystems recommends that no more than 20% of the PCR be composed of the reverse transcription reaction.

2. Cap the tube and invert it several times to mix the reaction components.

3. Centrifuge the tube briefly.

Load the plate

1. Transfer 20 µL of PCR reaction mix into each well of a 48-, 96-, or 384-well reaction plate.

   See Table 7 on page 20 for a a list of compatible reaction plates and accessories.

2. Seal the plate with the appropriate cover.

3. Centrifuge the plate briefly.

IMPORTANT! If you use TaqMan® Fast Universal PCR Master Mix (2✕), run the reaction plate within 2 hours of completing the reaction setup. Otherwise, refrigerate or freeze the plate until you can load it into the instrument.

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4. Load the plate into the instrument.

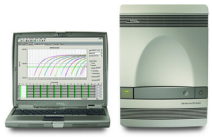
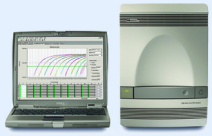
Run the real-time PCR reaction

1. Create a plate document/experiment for the run using the parameter values shown in Table 2 on page 8.

2. Run the plate.

   For instructions on how to create and run a plate document/experiment, see "Related documentation" on page 33 for a list of resource documents for your instrument.

Table 2 Plate document/experiment parameters for TaqMan® Pri-miRNA Assays

System	Run	Reaction plate	Plate document/experiment parameters	Thermal cycling conditions		
				Stage	Temp (°C)	Time (mm:ss)
Applied Biosystems 7300/7500 Real-Time PCR System 	Standard	96-well standard	• Rxn. Volume: 20 µL • Ramp Rate: Standard‡	Hold§	50	2:00
				Hold	95	10:00
				Cycle (40 Cycles)	95	0:15
					60	1:00
Applied Biosystems 7500 Fast Real-Time PCR System 	Standard	96-well Fast	• Rxn. Volume: 20 µL • Ramp Rate: Standard	Hold§	50	2:00
				Hold	95	10:00
				Cycle (40 Cycles)	95	0:15
					60	1:00
	Fast	96-well Fast	• Rxn. Volume: 20 µL • Ramp Rate: Fast	Hold§	50	2:00
				Hold	95	0:20
				Cycle (40 Cycles)	95	0:03
					60	0:30
Applied Biosystems 7900HT Real-Time PCR System 	Standard	96-well standard	• Rxn. Volume: 20 µL • Ramp Rate: Standard	Hold§	50	2:00
				Hold	95	10:00
		384-well standard	• Rxn. Volume: 20 µL • Ramp Rate: Standard	Cycle (40 Cycles)	95	0:15
					60	1:00
	Fast	96-well Fast	• Rxn. Volume: 20 µL • Ramp Rate: Fast	Hold§	50	2:00
				Hold	95	0:20
		384-well standard	• Rxn. Volume: 20 µL • Ramp Rate: Standard	Cycle (40 Cycles)	95	0:01
					60	0:20
Applied Biosystems StepOne™/StepOnePlus™ Real-Time PCR System 	Standard	48/96-well Fast	• Rxn. Volume: 20 µL • Ramp Speed: Standard	Hold§	50	2:00
				Hold	95	10:00
				Cycle (40 Cycles)	95	0:15
					60	1:00
	Fast	48/96-well Fast	• Rxn. Volume: 20 µL • Ramp Speed: Fast	Hold§	50	2:00
				Hold	95	0:20
				Cycle (40 Cycles)	95	0:01
					60	0:20

‡ The 7300 system has only one run mode (Standard 7300).
§ Required for optimal AmpErase® UNG activity; not needed when UNG is not in the reaction.

Analyze the results

Analyzing the data from TaqMan® Pri-miRNA Assays requires you to:

- View the amplification plots for the entire plate
- Set the baseline and threshold values
- Use the relative standard curve or the comparative C_T method to analyze your data

Resources for data analysis

The details of data analysis depend on the real-time PCR instrument that you use; refer to its user guide for instructions on how to analyze your data.

Table 3 Applied Biosystems real-time PCR systems: data analysis guides

Real-time PCR system	Document	Part number
7900HT Fast system	*Relative Quantitation Using Comparative C_T: Getting Started Guide*	4364016
	Performing Fast Gene Quantification: Quick Reference Card	4351892
	Performing Fast Gene Quantitation with 384-Well Plates: User Bulletin	4369584
7300/7500/ 7500 Fast system	*Relative Quantification: Getting Started Guide*	4347824
	Relative Standard Curve and Comparative C_T Experiments: Getting Started Guide	4387783
StepOne™/ StepOnePlus™ system	*Comparative C_T/Relative Standard Curve and Comparative C_T Experiments: Getting Started Guide*	4376785
All	*Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems: Chemistry Guide*	4348358

Troubleshooting

Table 4 Troubleshooting

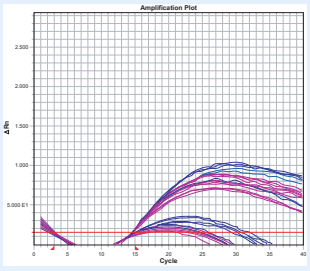
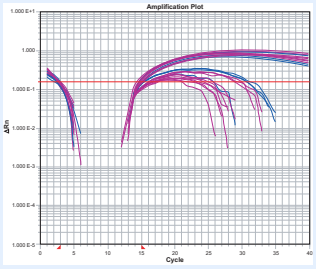
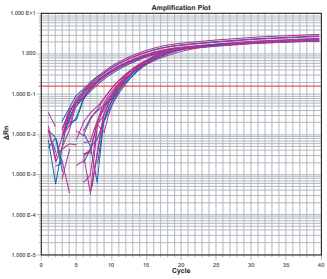
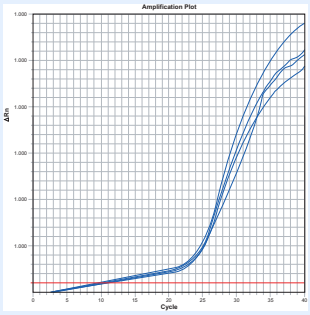
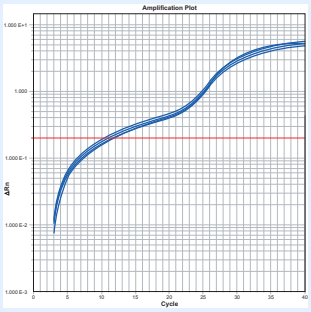
Observation	Possible cause	Recommended action
Amplification curve shows abnormal plot and/or low ΔR_n values. Linear view:  Log view: 	The baseline was set improperly (some samples have C_T values lower than the baseline stop value).	Refer to your real-time PCR system user guide for procedures on setting the baseline. Switch from manual to automatic baselining, or move the baseline stop value to a lower C_T (2 cycles before the amplification curve for the sample crosses the threshold). Log view corrected: 
	An amplification signal is detected in the early cycles (no baseline can be set because the signal is detected too early).	Dilute the sample to increase the C_T value.
Amplification curve shows a rising baseline. Linear view:  Log view: 	Primer and probe interaction.	• Adjust the threshold manually. • Select another assay from the same gene, if available.

Table 4 Troubleshooting *(continued)*

Observation	Possible cause	Recommended action
Amplification curve shows weak amplification.	Degraded reagents and/or probe.	• Check the expiration date of the reagents. • Verify that you follow the correct handling and storage conditions. • Avoid excessive freeze-thaw cycles.
	Degraded or contaminated template.	• Improve the sample integrity (extraction methods). See "Prepare the cDNA sample" on page 4. • Check each template preparation by agarose gel electrophoresis or bioanalyzer to determine the: – Purity (only one product should be formed). – Level of degradation. • Use RNase-free, sterile, filtered water.
	Inhibitors are present in the reaction.	• Verify the presence of an inhibitor: a. Create a serial dilution of your sample. b. Run the serial dilution with an assay for an expressed gene (for example, an endogenous control). If an inhibitor is present, low concentrations yield higher-than-expected C_T values. (High concentration means more inhibition because the sample is not diluted.) c. Rerun the assay with purified template. • Improve the sample integrity (extraction methods). See "Prepare the cDNA sample" on page 4.
	Poor reverse transcription (RT) conversion to cDNA.	• Check the RNA sample for degradation. • Input RNA could be too concentrated or too dilute. Verify the concentration by optical density (OD), make new serial dilutions of template RNA from original stock, then repeat the RT-PCR. • Ensure that the RT-PCR setup is performed under the appropriate conditions to avoid premature cDNA synthesis. • Check the RT reagents for contamination and/or degradation.
	Primer-dimer formation and residual polymerase activity.	(*Fast chemistry only*) For optimal results, run the reaction plate as soon as possible after completing the reaction setup. If you cannot run a reaction plate within 2 hours after completing the reaction setup, refrigerate or freeze the reaction plate until you can run it.
Amplification curve shows low ROX™ dye (passive reference dye).	Inaccurate pipetting: Little or no TaqMan® Universal PCR Master Mix.	Follow accurate pipetting practices.

Table 4 Troubleshooting *(continued)*

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_T = 40) across all assays or in an unusually large number of assays.	One or more of the reaction components was not added.	Verify that the cDNA, TaqMan Pri-miRNA Assays, and TaqMan Gene Expression Master Mix were added to the reaction plate. (If the master mix is missing, the passive reference fails.)
	Incorrect dye components were selected.	Check the dye components settings and reanalyze the data.
	The annealing temperature on the thermal cycler was too high for the primers and/or probe.	Verify that the thermal cycler is set to the correct annealing and extension temperatures. Ensure that the thermal cycler is calibrated and maintained regularly.
	Inappropriate reaction conditions were used.	Troubleshoot the RT-PCR optimization.
	The template is degraded.	• Determine the quality of the template. • Rerun the assay with fresh template. • Use RNase-free reagents. • Use an RNase inhibitor.
	Inhibitors are present in the reaction.	Verify the presence of an inhibitor: 1. Create a serial dilution of your sample. 2. Run the serial dilution with an expressing assay (for example, an endogenous control). If an inhibitor is present, low concentrations yield higher-than-expected C_T values. (High concentration means more inhibition because the sample is not diluted.) 3. Rerun the assay with purified template.
	The baseline and/or threshold was improperly set.	Refer to your real-time PCR system user guide for procedures on setting the baseline and threshold: • Switch from automatic to manual baselining, or from manual to automatic. • Lower the threshold value to within the appropriate range.
	cDNA conversion failed.	• Check the RNA integrity and concentration. • Check for RNase activity. • Follow Applied Biosystems recommended thermal profile. • Repeat the RT step using new reagents.
Amplification curve shows samples targeted by the same assay that have differently shaped curves.	The baseline was set improperly.	Refer to your real-time PCR system user guide for procedures on setting the baseline: • Switch from automatic to manual baselining, or from manual to automatic. • Increase the upper or lower value of the baseline range.
	Sample quality is poor.	1. Perform a quality check on the sample. 2. If necessary, reextract the sample.
	Imprecise pipetting: different concentrations.	Follow accurate pipetting practices.
	Contamination of reagents or equipment.	Be sure that your workspace and equipment are properly cleaned.

Table 4 Troubleshooting *(continued)*

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_T = 40) in the target assay.	The gene is not expressed in the tested sample.	• Verify the known expression of the gene in the sample type. • Verify by: – Rerunning the sample using the same assay. – Rerunning the assay using more sample. Avoid preparing the PCR reaction mix with more than 20% from the reverse transcription reaction. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	The sample may not have enough copies of the target RNA.	Verify by: • Rerunning the sample using the same assay. • Rerunning the assay using more sample. Avoid preparing the PCR reaction mix with more than 20% from the reverse transcription reaction. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	One or more of the reaction components was not added.	Check your pipetting equipment and/or technique.
	Incorrect dye components were selected.	Check the settings of the dye components before data analysis.
Decrease in ROX™ dye fluorescence (passive reference dye).	Precipitation in the TaqMan® buffers.	• When using the TaqMan® PCR Core Reagents Kit, be sure to mix the tubes well. • Use TaqMan® Gene Expression Master Mix (2X). Be sure to mix thoroughly to produce a homogenous solution.
	Degraded reagents.	Verify that kits and reagents have been stored according to the instructions on the packaging and have not expired.
Simultaneous increase in fluorescence from both the: • Passive reference (ROX™) dye. • Reporter dye(s).	Evaporation.	Check the seal of the optical adhesive cover for leaks.
Multicomponent signal for ROX™ dye is not linear.	Pure dye components spectra are incorrect.	Rerun the pure dye spectra.
	Incorrect dye components were selected.	Select the correct dyes for the data analysis.
R_n on R_n-vs.-Cycle plot is very high.	ROX™ dye was not selected as the passive reference when the plate document/ experiment was set up.	Select the ROX™ dye as the passive reference, then reanalyze the data.
No template control (NTC) shows amplification.	Contaminated reagents (contaminated with gDNA, amplicon, or plasmid clones).	• Rerun the assay using new reagents. • Be sure your workspace and equipment are cleaned properly. • Use AmpErase® UNG. • Run no-reverse-transcription controls to rule out genomic DNA contamination. • Treat the sample with the TURBO DNA-*free*™ Kit (PN AM1907).

Table 4 Troubleshooting *(continued)*

Observation	Possible cause	Recommended action
The endogenous control C_Ts vary, or do not normalize the sample well.	Endogenous control is not consistently expressed across the samples.	See the Application Note: *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies* (127AP08-01) for information on selecting an endogenous control.
	Sample concentrations vary widely.	If desired, quantitate and normalize samples before running them.
	Inaccurate pipetting.	• Check the calibration of the pipettes. • Pipette more than 5 µL of sample.
High standard deviation of replicates (inconsistent data, C_T varies).	Inefficient mixing of reagents.	• Increase the length of time that you mix the reagents. • Validate your mixing process by running a replicate plate.
	Inaccurate pipetting.	• Check the calibration of the pipettes. • Pipette more than 5 µL of sample.
	Threshold was set improperly.	Set the threshold above the noise and where the replicates are tightest. Refer to your real-time PCR system user documentation for procedures on setting the threshold.
	Low concentration of target.	Rerun the assay using more template.
	Template absorption (adhering to the tube).	Add a carrier (for example, yeast tRNA).
C_T value is lower than expected.	gDNA contamination.	• Verify contamination by running an RT-minus reaction (without the reverse transcriptase). • Treat the sample with the TURBO DNA-*free*™ Kit (PN AM1907).
	More sample added than expected.	• Reduce the amount of sample. • Quantitate and adjust the concentration of the sample.
	Template or amplicon contamination.	Follow established PCR good laboratory practices.
Amplification occurs in the no-RT controls.	gDNA contamination.	• Improve sample extraction methods to eliminate gDNA. See "Isolate total RNA" on page 4. • Treat the sample with the TURBO DNA-*free*™ Kit (PN AM1907).
	Template or amplicon contamination.	Follow established PCR good laboratory practices.
Shifting R_n value during the early cycles of the PCR (cycles 0 to 5).	Fluorescence did not stabilize to the buffer conditions of the reaction mix. **Note:** This condition does not affect PCR or the final results.	• Reset the lower value of the baseline range. • Use automatic baselining.
Small ΔR_n.	PCR efficiency is poor.	Recheck the concentration of the reagents.
	Quantity of starting target is low (low copy number of target).	Increase the quantity of the starting target.

Table 4 Troubleshooting *(continued)*

Observation	Possible cause	Recommended action
Noisy signal above the threshold.	Evaporation.	Check the seal of the optical adhesive cover for leaks.
	Empty well due to inaccurate pipetting.	• Check the calibration of the pipettes. • Pipette more than 5 µL of sample.
	The well is labeled with a detector in the plate document/ experiment, but the well is empty.	• Be sure that your plate document/experiment is set up correctly. • Exclude the well and reanalyze the data.

Step 1: Search for and order a TaqMan® Pri-miRNA Assay

TaqMan® Pri-miRNA Assay searches use the same online tool as for TaqMan® MicroRNA Assays.

1. At **www.appliedbiosystems.com**, place the cursor over **Products**, then select **TaqMan® MicroRNA Assays** under Assay Searches.
2. At the Assay Search page, select **TaqMan Pri-miRNA Assays**, **TaqMan MicroRNA Assays (mature)**, or **All MicroRNA Assays** in the pull-down menu of the keyword search tab.
3. Follow the directions within the tool to search for and order assays designed to the pri-miRNA of interest. TaqMan Pri-miRNA Assays are available in the following formulations:

Size	No. of 20-µL reactions	Part number	Availability
Small	360	4427012	Made-to-Order (20X)
Medium	750	4427013	Made-to-Order (20X)
Large	2900	4427014	Made-to-Order (60X)

About the assay name and assay ID

The assay name corresponds to the Sanger miRBase stem-loop ID.

The assay ID consists of a prefix (indicating the species to which the assay is designed), a unique numeric string, and the suffix "_pri."

Assay ID Prefix	Species
Hs	Homo sapiens
Mm	Mus musculus
Rn	Rattus norvegicus

Step 2: Order a candidate endogenous control assay

Select and order endogenous control assays from Applied Biosystems:

1. At **www.appliedbiosystems.com**, place the cursor over **Products**, then select **TaqMan® Gene Expression Assays** under Assay Searches.

2. At the Assay Search page, check the box for your species of interest.
3. Under Choose Set Membership select **Assay Attributes ▸ Endogenous Controls**.

Refer to the *TaqMan® Gene Expression Assays Protocol* (PN 4333458) for a comprehensive list of candidate endogenous control assays.

A valid normalization or endogenous control is needed to correct for differences in RNA sampling and sample variation. The ideal control is expressed consistently under experimental conditions and is sufficiently abundant across all tissues and cell types studied.

Note: Applied Biosystems recommends that you experimentally validate all candidate genes to be used as endogenous controls. For more information, see the Application Note: *Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies* (Stock Number 127AP08-01), available at **www.appliedbiosystems.com**.

Step 3: Select a chemistry

Select standard or Fast chemistry

StepOne™, StepOnePlus™, 7500 Fast, and 7900HT Fast Real-Time PCR Systems contain Fast thermal cycling blocks that can perform Fast quantitative PCR. Applied Biosystems Fast PCR systems use high-speed thermal cycling blocks, TaqMan® Fast Universal PCR Master Mix, and optical Fast thermal cycling plates and tubes to reduce quantitative PCR run times to less than 40 minutes. For more information on Fast chemistries available from Applied Biosystems, refer to the Data Sheet: *Comparing Fast and Standard Data on Applied Biosystems 7500 and 7500 Fast Real-Time PCR Systems* (SN 117MI08-01).

Table 5 Chemistry and plates for a standard or Fast run

Component	Standard chemistry per run	Fast chemistry per run
cDNA quantity	1–100 ng	1–100 ng
TaqMan® Master Mix	• TaqMan® Gene Expression Master Mix • TaqMan® Universal PCR Master Mix (2×), with or without AmpErase® UNG	TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG
96-well plate	MicroAmp® Optical 96-Well Reaction Plate with Barcode	MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode
384-well plate	MicroAmp® Optical 384-Well Reaction Plate with Barcode	
48-well plate	MicroAmp® Fast Optical 48-Well Reaction Plate	

Select 1- or 2-step RT-PCR

Applied Biosystems offers several chemistries that you can use to perform RT-PCR in one or two steps. See Table 6 for a list of kits.

Step 4: Order materials and equipment not included

See "Materials for reverse transcription and PCR" on page 2 for a list of required materials.

Related reagents

Table 6 Reagents for reverse transcription and PCR

Reagent	Description and part number
TaqMan® Gene Expression Master Mix (2X)	• One 1-mL tube (PN 4370048) • One 5-mL bottle (PN 4369016) • One 6-mL bottle (PN 4393469) • Two 5-mL bottles (PN 4369514) • Five 5-mL bottles (PN 4369510) • Ten 5-mL bottles (PN 4369542) • One 50-mL bottle (PN 4370074)
TaqMan® Universal PCR Master Mix (2X)	• One 5-mL bottle (PN 4304437) • Two 5-mL bottles (PN 4364338) • Five 5-mL bottles (PN 4364340) • Ten 5-mL bottles (PN 4305719) • One 50-mL bottle (PN 4326708)
TaqMan® Universal Master Mix (2X) No AmpErase® UNG	• One 5-mL bottles (PN 4324018) • Two 5-mL bottles (PN 4364341) • Five 5-mL bottles (PN 4364343) • Ten 5-mL bottles (PN 4324020) • One 50-mL bottle (PN 4326614)
TaqMan® Fast Universal Master Mix (2X) No AmpErase® UNG	• 250 × 20-µL reactions (PN 4352042) • 500 × 20-µL reactions (PN 4366072) • 1250 × 20-µL reactions (PN 4366073) • 2500 × 20-µL reactions (PN 4364103) • 5000 × 20-µL reactions (PN 4367846)
High Capacity RNA-to-cDNA Kit	50 reactions (PN 4387406)
High Capacity cDNA Reverse Transcription Kit	• 200 reactions (PN 4368814) • 200 reactions with RNase Inhibitor (PN 4374966) • 1000 reactions (PN 4368813) • 1000 reactions with RNase Inhibitor (PN 4374967)
TaqMan® RNA-to-C_T™ *1-Step* Kit	• 40 × 50-µL reactions (PN 4392653) • 200 × 50-µL reactions (PN 4392938) • 2000 × 50-µL reactions (PN 4392656)
Nuclease-free water (no diethyl pyrocarbonate [DEPC])	500 mL (PN AM9930)

Reaction plates and accessories

Table 7 Reaction plates and accessories for Applied Biosystems thermal cyclers and real-time PCR systems

Instrument	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 (PN 4311971) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (PN 4316567) • MicroAmp® Optical 8-Cap Strips, 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 (PN 4311971)
7900HT Fast system, standard 96-well block	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical Film Compression Pad (PN 4312639) for use with one plate
7900HT Fast system, Fast 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) for use with one plate
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)

Table 7 Reaction plates and accessories for Applied Biosystems thermal cyclers and real-time PCR systems *(continued)*

Instrument	Reaction plates and accessories
9700 instrument	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical 384-Well Clear Optical Reaction Plate with Barcode: – 1000 plates (PN 4343814) – 500 plates (PN 4326270) – 50 plates (PN 4309849) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Clear Adhesive Films, 100 films (PN 4306311) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (PN 4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (PN 4323032)
Veriti® 96-well thermal cycler	• MicroAmp® Optical 96-Well Reaction Plate: – 500 plates (PN 4316813) – 10 plates (PN N8010560) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Clear Adhesive Films, 100 films (PN 4306311)
Veriti® 384-well thermal cycler	• MicroAmp® Optical 384-Well Reaction Plate with Barcode: – 1000 plates (PN 4343814) – 500 plates (PN 4326270) – 50 plates (PN 4309849) • MicroAmp® Optical Adhesive Film (PN 4311971)

Related gene expression assays and arrays products

Table 8 Related gene expression assays and arrays products

Assay or array		For more information...
TaqMan® Assays	TaqMan® MicroRNA Assays	**miRNA.appliedbiosystems.com**
	Custom TaqMan® Small RNA Assays	Contact an Applied Biosystems Sales Representative
	TaqMan® Gene Expression Assays	**www.allgenes.com**
	TaqMan® Non-coding RNA Assays	**www.allgenes.com**
	Custom TaqMan® Gene Expression Assays	**www.allgenes.com**
	Custom TaqMan® Probes and Primers‡	**www.appliedbiosystems.com**
TaqMan® Arrays	TaqMan® Array Cards: • TaqMan® Custom Arrays • TaqMan® Gene Signature Array • TaqMan® Gene Sets	**taqmanarray.appliedbiosystems.com**
	TaqMan® Array Plates§	**www.allgenes.com**
	• TaqMan® MicroRNA Arrays – Human – Rodent • Megaplex™ Primer Pools – Megaplex™ RT Primers – Megaplex™ PreAmp Primers	**miRNA.appliedbiosystems.com**

‡ Probes and primers that are synthesized by Applied Biosystems to your exact sequence and choice of quencher and reporter dyes.
§ TaqMan® Gene Expression Assays dried in MicroAmp® Optical 96-Well Reaction Plates.

Prevent contamination and nonspecific amplification

PCR assays require special laboratory practices to avoid false positive amplifications. The high throughput and repetition of these assays can lead to amplification of one DNA molecule.

AmpErase® UNG

AmpErase Uracil-N-glycosylase (UNG) prevents reamplification of carryover-PCR products in an assay if all previous PCR for that assay is performed using a dUTP-containing master mix. 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, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA (Longo *et al.*, 1990).

PCR good laboratory practices

When preparing samples for PCR amplification:

- 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. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution.

About TaqMan® chemistry

About the probes

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 the 5′ nuclease assay

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

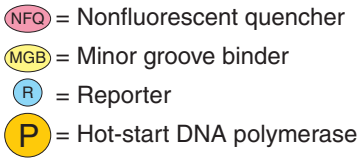


Figure 1 Legend for Figures 2 through 5

During PCR, the TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 2).

When the probe is intact (Figures 2 and 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).

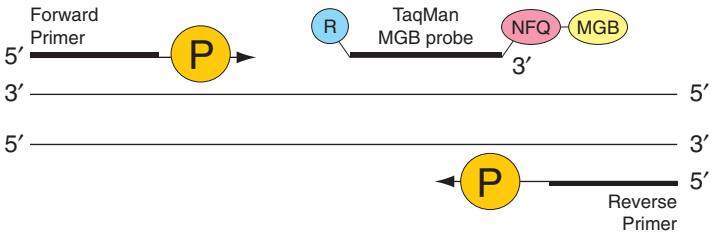


Figure 2 Polymerization

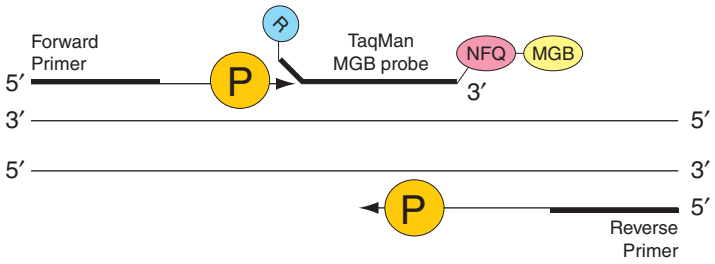


Figure 3 Strand displacement

The DNA polymerase cleaves only probes that are hybridized to the target (Figure 4). 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.

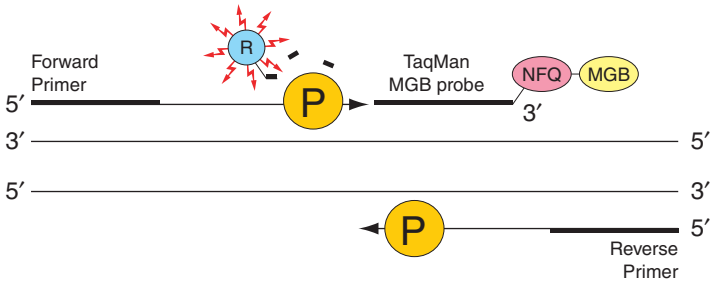


Figure 4 Cleavage

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).

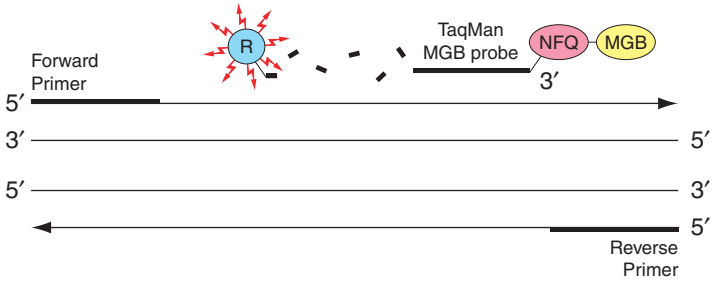


Figure 5 Completion of polymerization

About TaqMan® Pri-miRNA Assays

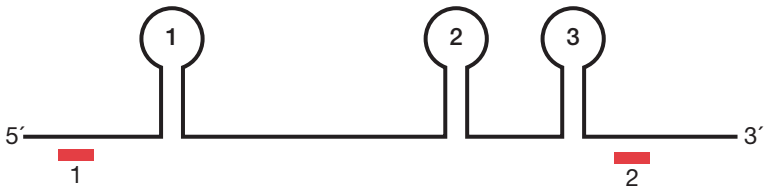
About pri-miRNAs

The miRNA maturation pathway begins with synthesis of long primary miRNA transcripts (pri-miRNAs) by RNA polymerase II. Pri-miRNAs contain single or, in many cases, multiple ~65-nt stem-loop structures that each encode one or more mature miRNA molecules. Pri-miRNAs are processed in the nucleus by Drosha and DGCR8/Pasha, releasing the stem-loops as intermediary pre-miRNAs. Pre-miRNAs are exported from the nucleus to the cytoplasm and then cleaved by Dicer into ~22 nt-long miRNAs. These short RNA molecules are finally incorporated into mature miRNA-containing RNA-protein complexes, referred to as miRISC. miRISC complexes regulate gene expression by binding to messenger RNA targets, resulting either in translational inhibition or, less commonly, degradation of the message.

Recent findings suggest that the miRNA maturation pathway and ultimately the level of the active mature species are regulated by developmental and tissue-specific factors (Heo et al, 2008; Viswanathan et al, 2008). An added complication to understanding mature miRNA expression arises from the observation that two or more pri-miRNA transcripts may encode identical mature miRNA sequences. As a consequence, gene-level expression changes at any one of these multiple genetic loci can result in changes to mature miRNA levels. Quantitation of the pri-miRNA, reflecting the transcriptional activity of the miRNA "gene," along with quantitation of the mature miRNA, reflecting the number of functionally active molecules, is important to understanding this layer of gene regulation.

About TaqMan® Pri-miRNA Assay design

TaqMan Pri-miRNA Assays are designed using the same design algorithms as TaqMan Gene Expression Assays. TaqMan Pri-miRNA Assays have been designed in close proximity to each stem-loop sequence identified in the Sanger miRBase sequence repository. In each case, the assay is located within 500 nucleotides on either side of the stem-loop sequence. In a small portion of cases, stem-loop sequences may be sufficiently close so as to prevent design to the intervening sequence. In these cases, Applied Biosystems recommends using the closest available assay (see Figure 6).



Stem-loop 1: detected by assay 1
Stem-loops 2 and 3: detected by assay 2

Figure 6 TaqMan® Pri-miRNA Assay alignments

RT-PCR with TaqMan Pri-miRNA Assays

RT-PCR targeting pri-miRNAs is performed using TaqMan Gene Expression Assay-compatible master mixes, instruments, and reaction conditions.

About the assay information file (AIF)

The assay information file (AIF) contains reference information about your order and technical details of all assays in the shipment. The AIF is included on the Information CD accompanying your order, in a folder labeled with the Rack or Plate ID.

AIF formats

The AIF may be provided in TXT format and/or in both XML and HTML formats, depending on the product line and order date. You can use the:

- HTML-format AIFs as a reference; open them in a Web browser.
- XML- and TXT-format AIFs for electronic data importation and manipulation.

Table 9 Assay information file formats and naming conventions

File format	Filename convention
HTML	Assay_Info_Pri_miRNA_SalesOrder_*XXXX*_RackID_*YYYY*
XML	Assay_Info_Pri_miRNA_SalesOrder_*XXXX*_RackID_*YYYY*
TXT	Assay_Info_Pri_miRNA_SalesOrder_*XXXX*_RackID_*YYYY* *-or-* *ProdNum_LotNum*_AIF

AIF field descriptions

Select assay information field descriptions are found in *Understanding Your Shipment*, which is included in the Information CD.

Chemical safety

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.

Obtaining MSDSs

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

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **MSDS**.
2. In the Keyword Search field, enter the chemical name, product name, MSDS part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document

- **Save Target As** – To download a PDF version of the document to a destination that you select

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

Chemical alerts

General alerts for all chemicals

Avoid contact with skin, eyes, and/or clothing. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Specific chemical alerts

CAUTION! CHEMICAL HAZARD. TaqMan® Gene Expression Master Mix, TaqMan® Universal PCR Master Mix (2×) with or without AmpErase® UNG, TaqMan® Fast Universal PCR Master Mix (2×), No AmpErase® UNG may cause eye and skin irritation.

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Related documentation

For additional documentation, see "How to obtain support" on page vi.

Real-time PCR system	Document	PN/SN
All real-time PCR systems	*Custom TaqMan® Assays: Design and Ordering Guide*	4367671
	Online Ordering Guide for TaqMan® Gene Expression Assays	127MI07-05
	Online Selection Guide for TaqMan® Gene Expression Assays	127GU08-01
	TaqMan® Gene Expression Assays Application Note: Amplification Efficiency of TaqMan® Gene Expression Assays	127AP05-03
	TaqMan® Gene Expression Assays Application Note: Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies	127AP08-01
	Real-Time PCR Systems Chemistry Guide	4348358
	High-Capacity cDNA Reverse Transcription Protocol	4375575
	TaqMan® Gene Expression Master Mix Protocol	4371135
	TaqMan® Universal PCR Master Mix (2X) Protocol	4304449
	TaqMan® Fast Universal PCR Master Mix (2X) Protocol	4351891
	TaqMan® RNA-to-C_T™ 1-Step Kit Protocol	4393463
	White Paper: The Design Process for a New Generation of Quantitative Gene Expression Analysis Tools: TaqMan® Probe-Based Assays for Human, Mouse, and Rat Genes	127WP02-02
	White Paper: Product Stability Study: TaqMan® Gene Expression Assays	127WP03-01
	White Paper: TaqMan® Gene Expression Assays for Validating Hits from Fluorescent Microarrays	127WP01-02
7900HT Fast system Fast or standard sample blocks	*Performing Fast Gene Quantification: Quick Reference Card*	4351892
	Relative Quantitation Using Comparative C_T: Getting Started Guide	4364016
	Performing Fast Gene Quantitation with 384-Well Plates: User Bulletin	4369584
7300, 7500, and 7500 Fast systems	*Relative Quantification: Getting Started Guide*	4347828
StepOne™ and StepOnePlus™ systems	*Reagent Guide*	4379704
	Relative Standard Curve and Comparative C_T Experiments: Getting Started Guide	4376785

Send us your comments

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

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is for submitting comments and suggestions relating *only* to documentation. To order documents, download PDF files, or for help with a technical question, see "How to obtain support" on page vi.

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Technical Resources and Support
For the latest technical resources and support information for all locations, please refer to our Web site at
www.appliedbiosystems.com/support