applied biosystems
by life technologies

TaqMan® Small RNA Assays

TaqMan® MicroRNA Assays
TaqMan® siRNA Assays
Custom TaqMan® Small RNA Assays

Protocol

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Contents

About This Guide

Purpose

This protocol provides:

- Background information about the TaqMan® MicroRNA Assays, TaqMan® siRNA Assays, and Custom TaqMan® Small RNA Assays.
- A list of equipment and materials needed for the protocol.
- Procedures for using TaqMan® MicroRNA Assays, TaqMan® siRNA Assays, or Custom TaqMan® Small RNA Assays, including:
  - Specialized procedures for performing reverse transcription (RT) of single-stranded or double-stranded RNA templates.
  - A unified procedure for performing PCR amplification of the RT product and subsequent data analysis.

Safety information

Note: For general safety information, see this section and Appendix D, "Safety" on page 31. 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.

SDSs

The 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 33.

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

TaqMan® Small RNA Assays

Product overview

Purpose

TaqMan® Small RNA Assays are preformulated primer and probe sets designed to detect and quantify mature microRNAs (miRNAs), small interfering RNAs (siRNAs), and other small RNAs using Applied Biosystems real-time PCR instruments. The assays can detect and quantify small RNA in 1 to 10 ng of total RNA with a dynamic range of greater than six logs. When used for microRNA analysis, the assays can discriminate mature miRNA sequences from their precursors.

Note: In this protocol, "small RNA" refers to miRNA, siRNA, or other small RNA that are less than 200 bases in length.

Both predesigned and custom TaqMan® Small RNA Assays are available for a variety of small RNA classes:

- **TaqMan® MicroRNA Assays** – Predesigned assays that are available for the majority of content found in the miRBase miRNA sequence repository. These assays are ideal for targeted quantification, screening, and validation of miRNA profiling results.
- **TaqMan® siRNA Assays for Ambion *Silencer*® Select siRNAs** – Predesigned assays for the quantification of Ambion *Silencer*® Select siRNAs. These assays are ideal for assessing siRNA transfection efficiency, half-life, and bio-distribution. (For novel, proprietary, or other classes of small, double-stranded RNA, see Custom TaqMan® Small RNA Assays below.)
- **TaqMan® Small RNA Controls** – Predesigned assays for small, non-coding RNAs unrelated to miRNAs, that can be used to normalize for differences in sample RNA. TaqMan® Small RNA Controls are available for a number of species. For more information on using TaqMan® control assays, see "Select a TaqMan® Small RNA Control" on page 10.
- **Custom TaqMan® Small RNA Assays** – Custom assays designed for any small RNA sequence between 17 to 200 nucleotides in length. Submit a target sequence for any organism and Applied Biosystems sends you a ready-to-use TaqMan® Small RNA Assay with optimized primers and probe.

Note: For information about the mechanics of the TaqMan® assays, see Appendix C, "Chemistry Overview" on page 27.

About this product

TaqMan® MicroRNA Assays, TaqMan® siRNA Assays, and Custom TaqMan® Small RNA Assays use a stem-looped primer for reverse transcription and a sequence-specific TaqMan® assay to accurately detect mature miRNAs, siRNAs, and other small RNAs respectively.

Each TaqMan® Assay includes:

- One tube containing small RNA-specific RT primer
- One tube containing a mix of:
  - Small RNA-specific forward PCR primer
  - Specific reverse PCR primer
  - Small RNA-specific TaqMan® MGB probe

For a current list of assays, refer to the Applied Biosystems website:

appliedbiosystems.com/TaqManMicroRNAAssays

To access the Custom TaqMan Small RNA Design Tool, refer to:

appliedbiosystems.com/smallrna

Materials and equipment

Storage

Store all TaqMan® Small RNA Assays at – 15 to – 25 °C.

Available products

Each kit provides enough material for the number of reactions in the following table.

Product	Scale	Part no.	20-µL qPCR reactions	Reagent volume (concentration)	
				RT primer	TaqMan® Assay
Custom TaqMan® Small RNA Assays	Large	4398989	2900	1 × 725µL (60X)‡	1 × 967µL (60X)§
	Medium	4398988	750	1 × 575 µL (20X)‡	1 × 750 µL (20X)
	Small	4398987	50/150	1 × 150 µL (5X)	1 × 150 µL (20X)
	Extra small	4440418	25/75	1 × 75 µL (5X)	1 × 75 µL (20X)
TaqMan® siRNA Assays (Predesigned*)	Large	4440880	2900	1 × 725 µL (60X)‡	1 × 967 µL (60X)§
	Medium	4440879	750	1 × 575 µL (20X)‡	1 × 750 µL (20X)
	Small	4440878	50/150	1 × 150 µL (5X)	1 × 150 µL (20X)
	Extra small	4440877	25/75	1 × 75 µL (5X)	1 × 75 µL (20X)
TaqMan® MicroRNA Assays (Made-to-order*)	Large	4440888	2900	1 × 725 µL (60X)‡	1 × 967 µL (60X)§
	Medium	4440887	750	1 × 575 µL (20X)‡	1 × 750 µL (20X)
	Small	4440886	50/150	1 × 150 µL (5X)	1 × 150 µL (20X)
	Extra small	4440885	25/75	1 × 75 µL (5X)	1 × 75 µL (20X)
TaqMan® MicroRNA Assays (Inventoried*)	Small	4427975	50/150	1 × 150 µL (5X)	1 × 150 µL (20X)

* Predesigned and inventoried assays are manufactured and stocked ahead of time. Made-to-order assays are manufactured when the order is placed.
‡ Dilute to a 5X working stock solution before use.
§ Dilute to a 20X working stock solution before use.

Preparing the TaqMan® assays

- For medium- and large-scale orders, the RT primer is supplied in 20X and 60X concentrations that must be diluted to a 5X working stock solution using 0.1X TE buffer.
- For large-scale orders, the TaqMan® assay mix is supplied in a 60X concentration that must be diluted to a 20X working stock using 0.1X TE buffer.
- Before use, thaw the RT primer or TaqMan® assay mix on ice, resuspend the solution completely by gently vortexing, then briefly centrifuge the tube.

Select a reverse transcription kit

For optimal performance of TaqMan® assays, we recommend using the TaqMan® MicroRNA Reverse Transcription Kit. For more information on ordering reverse transcription reagents, see "Recommended kits and reagents" on page 23.

Select a PCR master mix

We recommend that you use TaqMan® Small RNA Assays with the following master mixes:

- TaqMan® Universal PCR Master Mix II, No UNG
- TaqMan® Universal PCR Master Mix II, With UNG
- TaqMan® Universal PCR Master Mix 2×, No AmpErase® UNG
- TaqMan® Universal PCR Master Mix 2×, With AmpErase® UNG

For more information on ordering master mix, see "Recommended kits and reagents" on page 23.

Select a TaqMan® Small RNA Control

We recommend using TaqMan® Small RNA Controls with TaqMan® Small RNA Assays. When quantifying small RNA gene expression levels, variation in the amount of starting material, sample collection, RNA preparation and quality, and reverse transcription (RT) efficiency can contribute to quantification errors. Normalization to endogenous control genes is currently the most accurate method to correct for potential RNA input or RT efficiency biases. An ideal endogenous control generally shows gene expression that is relatively constant and highly abundant across tissues and cell types. You need to validate the chosen endogenous control or set of controls for the target cell, tissue, or treatment because no single control can act as a universal endogenous control for all experimental conditions.

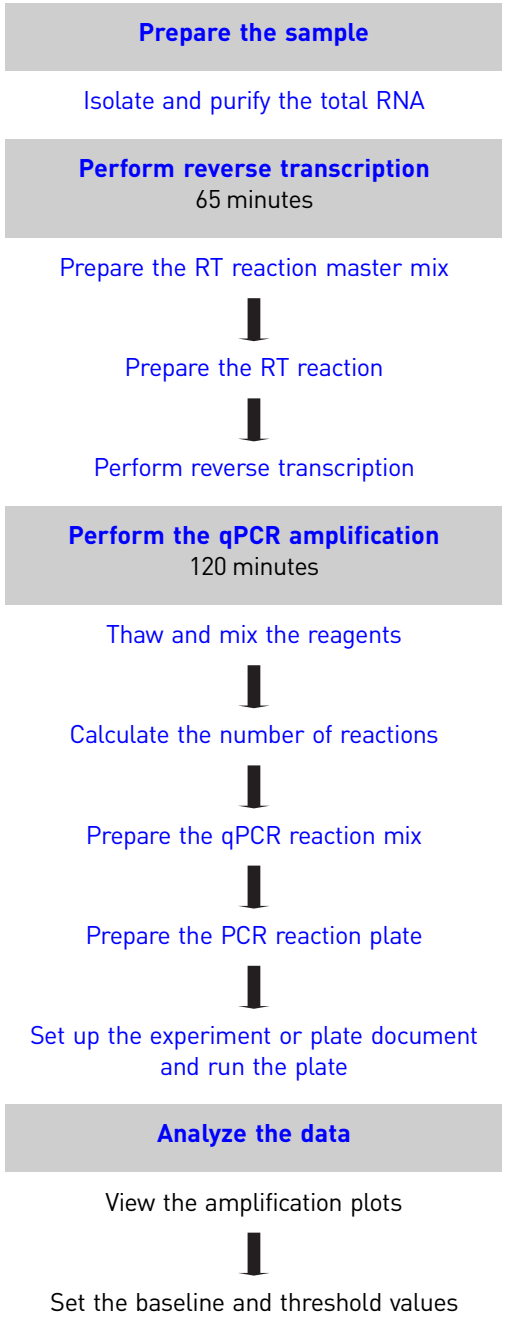
For more information on selecting an endogenous control, refer to the *Application Note: Using TaqMan® Endogenous Control Assays to Select an Endogenous Control for Experimental Studies* (Stock Number 127AP08-01) available from:
www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042279.pdf

Note: To view a complete list of TaqMan® Small RNA controls available from Applied Biosystems, visit **appliedbiosystems.com/TaqManMicroRNAAssays**.

Materials and equipment not included

See "Materials and equipment not included" on page 22 for a list of required and optional equipment and materials for use with TaqMan® Small RNA Assays.

Procedural overview



Prepare the sample

Isolate and purify the total RNA

Prepare samples using a method that preserves small RNAs. To prevent the loss of the longer control transcripts (such as snoRNAs), We recommend that you do *not* perform size fractionation.

Note: See "Materials and equipment not included" on page 22 for a list of recommended RNA isolation kits.

Perform reverse transcription

RNA template guidelines

For optimal performance of TaqMan® Small RNA Assays, we recommend using RNA with the following characteristics:

- Free of inhibitors of reverse transcription (RT) and PCR
- Dissolved in PCR-compatible buffer
- Free of RNase activity
- Nondenatured total RNA (not applicable for double-stranded templates)

IMPORTANT! Do not denature the total RNA.

IMPORTANT! TaqMan® MicroRNA and Small RNA Assays are specifically optimized to work with the MuLV Reverse Transcriptase contained in the TaqMan® MicroRNA Reverse Transcription Kit. We cannot guarantee assay performance if you use a different reverse transcriptase enzyme.

Input quantity

Use 1 to 10 ng of total RNA per 15-µL RT reaction.

Prepare the RT reaction master mix

IMPORTANT! When working with double-stranded template, such as siRNAs, denature your siRNA template with sequence-specific RT primer before performing the reverse transcription.

IMPORTANT! Prepare the RT reactions in an area free of artificial templates, amplified material, and siRNA transfections. High-copy-number templates can easily contaminate the reactions.

To prepare the RT master mix using the TaqMan® MicroRNA Reverse Transcription Kit components:

1. Allow the kit components to thaw on ice.
2. (Medium- or large-scale orders only) Dilute the 20× or 60× RT primer to a 5× working stock solution using 0.1× TE buffer.
3. In a polypropylene tube, prepare the RT master mix on ice by scaling the volumes listed below to the desired number of RT reactions. We recommend adding 10 to 20% excess volume to compensate for losses that occur during pipetting.

Component	Master mix volume per 15-µL reaction*
100mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µL
10× Reverse Transcription Buffer	1.50 µL
RNase Inhibitor, 20 U/µL	0.19 µL
Nuclease-free water	4.16 µL
Total volume	**7.00 µL**

* Each 15-µL RT reaction consists of 7 µL master mix, 3 µL of 5X RT primer, and 5 µL RNA sample.

4. Mix gently. Centrifuge to bring the solution to the bottom of the tube.
5. Place the RT master mix on ice until you prepare the RNA reaction.

Prepare the RT reaction

1. Thaw the 5× RT primer and RNA template on ice. Before use, vortex the RT primer tubes to mix, then centrifuge briefly.

2. If you are performing quantitation of:
   - Ambion *Silencer*® Select siRNAs, go to step 3.
   - All other templates, go to step 4.

3. If you are performing Ambion *Silencer*® Select siRNAs quantitation, denature and prepare the double-stranded template:
   a. For each 15-µL RT reaction, combine 3 µL of 5× RT primer and 5 µL of double-stranded template in a 0.2-mL polypropylene reaction tube (the RT reaction tube) or in a well of a 96-well reaction plate.
   b. Incubate the tube or plate at 85 °C for 5 minutes, then at 60 °C for 5 minutes.
   c. Place the denatured template on ice.
   d. For each 15-µL RT reaction, combine the RT master mix (from step 3 on page 13) to the tube or well containing denatured RNA and RT primer (from step 3c) in the ratio of:

      7 µL RT master mix : 8 µL denatured RNA and RT primer (1 to 10 ng of RNA per reaction)

      Note: The amount of input total RNA for optimal detection depends on the transfection protocol used. We recommend using total RNA from non-transfected cells as a control in both *in-vitro* and *in-vivo* studies.

   e. Go to step 5.

4. If you are preparing single-stranded RNA, prepare the total RNA template:
   a. For each 15-µL RT reaction, combine RT master mix (from step 3 on page 13) with total RNA in the ratio of:

      7 µL RT master mix : 5 µL total RNA (1 to 10 ng per reaction)

   b. Mix gently. Centrifuge to bring the solution to the bottom of the tube.
   c. Before opening the RT primer tubes, thaw the tubes on ice and mix by vortexing, then centrifuge them.
   d. For each 15-µL RT reaction, add 12.0 µL of RT master mix containing total RNA (from step 4b) into a 0.2-mL polypropylene reaction tube (the RT reaction tube) or into a well of a 96-well reaction plate.
   e. Add 3 µL of 5× RT primer from each assay set into the corresponding RT reaction tube or plate well.
   f. Go to step 5.

5. Seal the tube or reaction plate and mix thoroughly by inverting the solution. Centrifuge to bring the solution to the bottom of the tube or well.

6. Incubate the tube on ice for 5 minutes and keep it on ice until you are ready to load the thermal cycler.

Perform reverse transcription

Note: If applicable to your thermal cycler or real-time PCR system, perform the reverse transcription in Standard mode.

1. Use the following parameter values to program the thermal cycler:

Step	Time	Temperature
Hold	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	4

2. Set the reaction volume to 15.0 µL.
3. Load the reaction tube or plate into the thermal cycler.
4. Start the RT run.

STOPPING POINT If you do not immediately continue to PCR amplification after the RT run, store the RT reaction at – 15 to – 25 °C.

Perform the qPCR amplification

Reagent preparation guidelines

For optimal quantitative PCR (qPCR) performance:

- Prepare the qPCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Keep all TaqMan® Small RNA Assays protected from light, in the freezer, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prepare the qPCR reaction mix before transferring it to the reaction plate for thermal cycling and fluorescence analysis.

Thaw and mix the reagents

1. Thaw on ice, resuspend completely by gently vortexing, then centrifuge briefly:
   - TaqMan® Assay (20×)
   - Complementary DNA (cDNA) samples

   IMPORTANT! If using large-scale product, you need to dilute the 60× TaqMan® assay mix to a 20× working stock solution before use.

2. Mix the master mix reagent by gently swirling the bottle. (See "Recommended kits and reagents" on page 23 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. We recommend performing three replicates of each reaction. Be sure to include on each plate:

- A small RNA assay for each cDNA sample
- Endogenous control assay(s)
- No template controls (NTCs) for each assay on the plate

Note: We strongly recommend the use of NTC reactions to evaluate background signal.

Prepare the qPCR reaction mix

The recommended reaction volume is 20 µL. Prepare the plate so that each qPCR reaction contains the components as listed below.

To prepare the qPCR reaction mix:

1. Obtain a sterile 1.5-mL microcentrifuge tube for each sample (to be run in triplicate).
2. Pipet the following components into each tube:

Component	Volume per 20-µL Reaction	
	Single reaction	**Three replicates§**
TaqMan® Small RNA Assay (20X)	1.00 µL	3.60 µL
Product from RT reaction*	1.33 µL	4.80 µL
TaqMan® Universal PCR Master Mix II (2X), no UNG‡	10.00 µL	36.00 µL
Nuclease-free water	7.67 µL	27.61 µL
Total volume	**20.00 µL**	**72.01 µL**

* The maximum amount of RT product that can be added to a reaction. (The RT primer must be diluted a minimum of 1:15 in the final qPCR reaction.)
‡ For optimal performance of TaqMan® Small RNA Assays, we recommend that you use the master mixes listed in "Select a PCR master mix" on page 10.
§ Replicate volumes include 20% excess to compensate for volume loss during pipetting.

3. Cap the tube and invert several times to mix.
4. Centrifuge the tube briefly.

Prepare the PCR reaction plate

1. Transfer 20 µL of the complete qPCR reaction mix (including assay and RT product) into each of three wells on a 48-, 96-, or 384-well plate.
2. Seal the plate with the appropriate cover.
3. Centrifuge the plate briefly.
4. Load the plate into the instrument.

Set up the experiment or plate document and run the plate

Note: Refer to your real-time PCR system documentation for instructions on how to configure the experiment or plate document and run the PCR plates (see "Related documentation" on page 39).

1. In the real-time PCR system software, create an experiment or plate document on your real-time PCR system using the following parameters:
   - Run Mode: Standard
   - Sample Volume: 20 µL
   - Thermal Cycling Conditions:

Step	Optional AmpErase® UNG activity*	Enzyme Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 minutes	10 minutes	15 seconds	60 seconds

* Not needed when UNG is not in the reaction.

2. If the reaction plate is not already loaded, load the plate into the instrument.
3. Start the run.

Analyze the data

Refer to the appropriate instrument user guide for instructions on how to analyze your data.

General process

The general process for analyzing the data from gene expression assays involves the following procedures:

1. View the amplification plots.
2. Set the baseline and threshold values.

Resources for data analysis

For more information about analyzing your data, refer to "Related documentation" on page 39.

Tools for data analysis

We recommend the following software for analyzing data generated using TaqMan® Small RNA Assays.

DataAssist™ Software

DataAssist™ Software is a simple, powerful data analysis tool for sample comparison when using the comparative C_T ($\Delta\Delta C_T$) method for calculating relative quantitation of gene expression. The software uses a filtering procedure for outlier removal and various normalization methods based on lists of single or multiple genes. It provides relative quantification analysis of gene expression through a combination of statistical analysis and interactive visualization. DataAssist™ Software provides a function-rich graphic user interface (GUI) for easy data importation, experimental design setup, and interactive, high-throughput data analysis.

DataAssist™ Software is free and can be downloaded from the Applied Biosystems website at: **www.appliedbiosystems.com/dataassist**

RealTime StatMiner® Software

RealTime StatMiner® Software from Integromics is a software analysis package for qPCR experiments that is compatible with all Applied Biosystems instruments. RealTime StatMiner® Software uses a step-by-step analysis workflow guide that includes parametric, non-parametric, and paired tests for relative quantification of gene expression, as well as 2-way ANOVA for two-factor differential expression analysis.

For more information, visit: **www.integromics.com/StatMiner**

Ordering Information

How to order TaqMan® Small RNA Assays

You can order predesigned and custom TaqMan® Small RNA Assays for miRNAs, siRNAs, and other small RNAs directly from the Applied Biosystems website. The website features tools that can aid you in selecting from inventoried assays or for designing a custom assay for an unlisted small RNA.

For more information on ordering Predesigned TaqMan® MicroRNA Assays, visit:

appliedbiosystems.com/TaqManMicroRNAAssays

For more information on ordering Custom TaqMan® Small RNA Assays, or Predesigned TaqMan® siRNA Assays for Ambion *Silencer*® Select siRNAs, visit:

appliedbiosystems.com/smallrna

For more information on designing and ordering Custom TaqMan® Small RNA Assays, see the *Custom TaqMan® Small RNA Assays Design and Ordering Guide* (PN 4412550) available on the Applied Biosystems website.

Materials and equipment not included

This section covers the following user-supplied equipment and materials:

Required laboratory materials and equipment

This table includes materials and equipment required for using TaqMan® MicroRNA and Small RNA Assays. All of the items listed are available from major laboratory suppliers (MLS).

Materials and Equipment	Part No.
Centrifuge with plate holders	MLS
Disposable gloves	MLS
Microcentrifuge	MLS
Pipettors (positive-displacement, air-displacement, or multi-channel) and tips: 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range	MLS
Polypropylene tubes	MLS
RNase-free, sterile-filtered water	MLS
Vortexer	MLS
Lab equipment	MLS

Recommended kits and reagents

Note: We strongly recommend that you use these Applied Biosystems reagents with all TaqMan® Small RNA Assays.

Materials and Equipment			Part no.
RNA Isolation Kits	mirVana™ miRNA Isolation Kit	Kit, 40 purifications	AM1560
		Kit without Phenol, 40 purifications	AM1561
	mirVana™ PARIS™ Kit, 40 purifications		AM1556
	TRI Reagent®		AM9738
	TaqMan® MicroRNA Cells-to-CT™ Kit	Kit, 100 rxns	4391848
		Kit, 400 rxns	4391996
		Cells-to-CT™ Stop Solution, 1 mL	4402960
		Cells-to-CT Bulk Lysis Reagents, 2500 rxns	4391851
RT Kits	TaqMan® MicroRNA Reverse Transcription Kit	Kit, 200 rxns*	4366596
		Kit, 1000 rxns*	4366597
PCR Master Mixes	TaqMan® Universal PCR Master Mix II, No UNG	Kit, Mini-Pack, 1 × 1-mL tube	4440043
		Kit, 1-Pack, 1 × 5-mL bottle	4440040
		Kit, 2-Pack, 2 × 5-mL bottles	4440047
		Kit, 5-Pack, 5 × 5-mL bottles	4440048
		Kit, 10-Pack, 10 × 5-mL bottles	4440049
	TaqMan® Universal PCR Master Mix II, with UNG	Kit, Mini-Pack, 1 × 1-mL tube	4440042
		Kit, 1-Pack, 1 × 5-mL bottle	4440038
		Kit, 2-Pack, 2 × 5-mL bottles	4440044
		Kit, 5-Pack, 5 × 5-mL bottles	4440045
		Kit, 10-Pack, 10 × 5-mL bottles	4440046
	TaqMan® 2X Universal PCR Master Mix, No AmpErase® UNG	Kit, 1-Pack, 1 × 5-mL bottle	4324018
		Kit, 2-Pack, 2 × 5-mL bottles	4364341
		Kit, 5-Pack, 5 × 5-mL bottles	4364343
		Kit, 10-Pack, 10 × 5-mL bottles	4324020
		Kit, Bulk, 1 × 50-mL bottle	4326614
	TaqMan® 2X Universal PCR Master Mix, with AmpErase® UNG	Kit, 1-Pack, 1 × 5-mL bottle	4304437
		Kit, 2-Pack, 2 × 5-mL bottles	4364338
		Kit, 5-Pack, 5 × 5-mL bottles	4364340
		Kit, 10-Pack, 10 × 5-mL bottles	4305719
		Kit, Bulk, 1 × 50-mL bottle	4326708

* TaqMan® Small RNA Assays are specifically optimized to work with the MuLV Reverse Transcriptase contained in the TaqMan® MicroRNA Reverse Transcription Kit. Applied Biosystems cannot guarantee assay performance if you use other RT enzymes.

Good Laboratory Practices

Preventing contamination

Overview

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).

General PCR practices

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).

IMPORTANT! Prepare the PCR reactions with TaqMan® MicroRNA and Small RNA Assays in an area free of artificial templates or siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.

Chemistry Overview

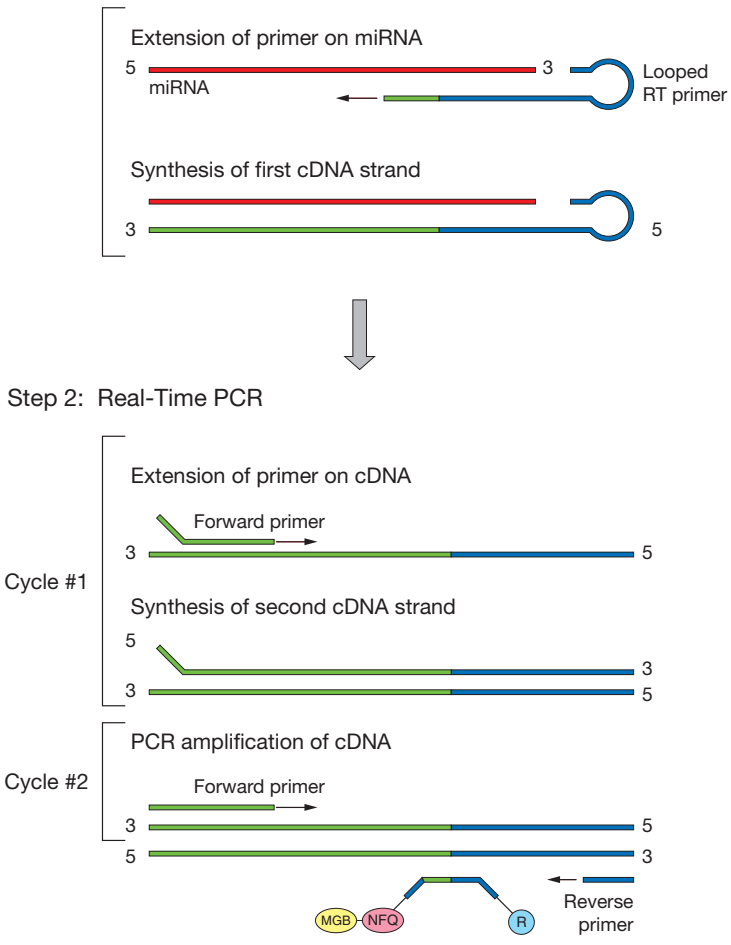
Two-step RT-PCR

Quantification using the TaqMan® Small RNA Assays is done using two-step RT-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using a small RNA-specific, stem-loop RT primer from the TaqMan® Small RNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit.

2. In the PCR step, PCR products are amplified from cDNA samples using the TaqMan® Small RNA Assay together with the TaqMan® Universal PCR Master Mix II.

Figure 1 Two-step RT-PCR



About the probes

The TaqMan® MGB probes contain:

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

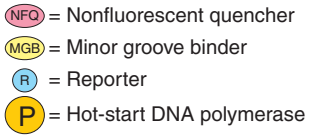
  This modification increases the melting temperature (T_m) without increasing probe length (Afonina et al., 1997; Kutyavin et al., 1997), which allows the design of shorter probes.
- A nonfluorescent quencher (NFQ) at the 3′end of the probe.

  Because the quencher does not fluoresce, Applied Biosystems sequence detection systems can measure reporter dye contributions more accurately.

5´nuclease assay process

The 5′nuclease assay process (Figure 2 through Figure 6) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

Figure 2 Legend for 5′nuclease assay process figures



During PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 3).

When the probe is intact (Figure 3 and Figure 4), 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 3 Polymerization

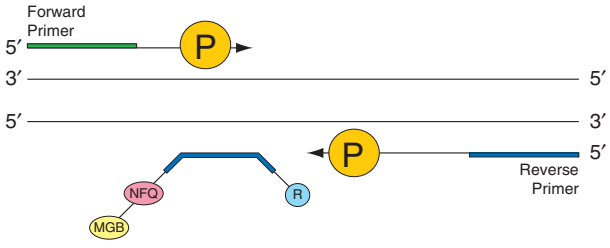
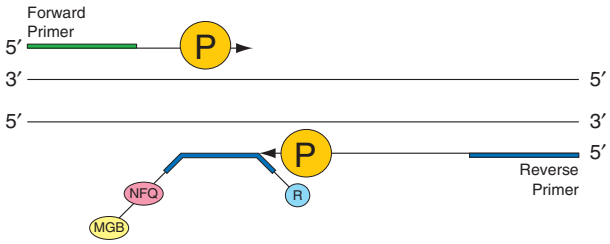
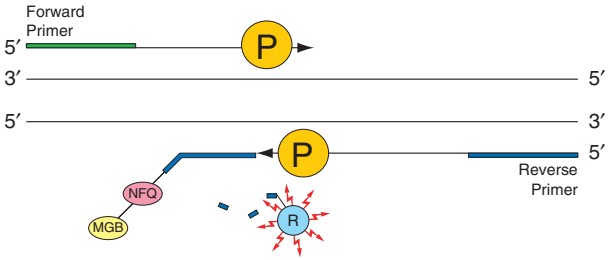


Figure 4 Strand displacement



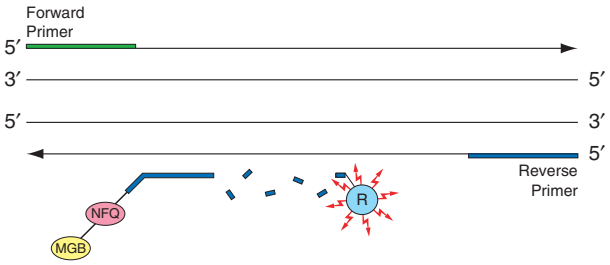
The DNA polymerase cleaves only probes that are hybridized to the target (Figure 5). Cleavage separates the reporter dye from the quencher dye; the separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter. The increase in fluorescence signal occurs only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, nonspecific amplification is not detected.

Figure 5 Cleavage



Polymerization of the strand continues, but because the 3′ end of the probe is blocked, there is no extension of the probe during PCR (Figure 6).

Figure 6 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 HAZARD. All chemicals in the instrument, including liquid in the lines, are potentially hazardous. Always determine what chemicals have been used in the instrument before changing reagents or instrument components. Wear appropriate eyewear, protective clothing, and gloves when working on the instrument.

WARNING! CHEMICAL HAZARD. Four-liter reagent and waste bottles can crack and leak. Each 4-liter 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.

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 33.)
- 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 safely dispose of the chemicals.

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

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

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the 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 when you operate the instrument, you need to:

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

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

Biological hazard safety

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/biosafety/publications/index.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

General safety alerts for all chemicals

For the definitions of the alert words **IMPORTANT**, **CAUTION**, **WARNING**, and **DANGER**, see "Safety alert words" on page 5.

General alerts for all chemicals

Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

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Documentation and Support

Related documentation

The following related documents contain supporting information:

Documents	Part No.
Applied Biosystems 7900HT Fast Real-Time PCR System	
Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4364014
Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantification Getting Started Guide	4364016
Applied Biosystems ViiA 7™ Real-Time PCR System	
Applied Biosystems ViiA™ 7 Real-Time PCR System Getting Started Guide	4441434
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems	
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments	4387779
Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Comparative CT/Relative Standard Curve Experiments	4387783
Applied Biosystems StepOne™/StepOnePlus™ Real-Time PCR Systems	
Applied Biosystems StepOne™/StepOnePlus™ Real-Time PCR System Getting Started Guide for Standard Curve Experiments	4376784
Applied Biosystems StepOne™/StepOnePlus™ Real-Time PCR System Getting Started Guide for Comparative CT/Relative Standard Curve Experiments	4387783
All Real-Time PCR Systems	
Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems	4348358
Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408.*	—
Livak, K.J. and Schmittgen, T.D. 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols* 3:1101–1108.	—
Application Note: *Endogenous Controls for Real-Time Quantitation of miRNA Using TaqMan® MicroRNA Assays*, Publication 127AP11-0	127AP11-0

* Provides the derivation, assumptions, and applications of the $2^{-\Delta\Delta Ct}$ method and variations for analyzing the relative changes in gene expression from real-time quantitative PCR experiments.

Note: Portable document format (PDF) versions of this and other protocols, and the quick reference cards are available from **www.appliedbiosystems.com** and on the CD shipped with the kit.

Note: To open the user documentation included on the CD, use the Adobe® Acrobat® Reader® software available from **www.adobe.com**

Note: For additional documentation, see "Obtaining support" below.

Obtaining 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, 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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