TaqMan® MicroRNA Assays

Protocol

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

NOTICE TO PURCHASER: LIMITED LICENSE

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Notice to Purchaser: Disclaimer of License

The TaqManÆ microRNA Assay is optimized for use in the polymerase chain reaction (PCR) covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd. No license under these patents to use the PCR Process is conveyed expressly or by implication to the purchaser by the purchase of these products. A license to use the PCR Process for the purchaser's own internal research accompanies the purchase of certain Applied Biosystems reagents when used in conjunction with an authorized thermal cycler, or is available from Applied Biosystems.

Trademarks

Part Number 4364031 Rev. B
01/2006

Contents

Preface

This preface contains:

Safety

Safety Alert Words

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

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

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

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

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

Chemical Hazard Warning

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

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About MSDSs" below.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

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 **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field of the MSDS Search page:
    a. Type in the chemical name, part number, or other information that you expect to appear in the MSDS of interest.

b. Select the language of your choice.

c. Click **Search**.

3. To view, download, or print the document of interest:
   a. Right-click the document title.
   b. Select:
      - **Open** – To view the document
      - **Save Target As** – To download a PDF version of the document to a destination that you choose
      - **Print Target** – To print the document
4. To have a copy of an MSDS sent by fax or e-mail, in the Search Results page:
   a. Select **Fax** or **Email** below the document title.
   b. Click **RETRIEVE DOCUMENTS** at the end of the document list.
   c. Enter the required information.
   d. Click **View/Deliver Selected Documents Now**.

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

Chemical Waste Hazard

CHEMICAL WASTE HAZARD. Some wastes produced by the operation of the instrument or system are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)

- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

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

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

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

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment

devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

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

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

How to Obtain Support

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

At the Support page, you can:

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

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

Section 1 Introduction

Overview

Purpose

The TaqMan®MicroRNA Assays are designed to detect and accurately quantify mature microRNAs (miRNAs) using Applied Biosystems real-time PCR instruments.

TaqMan MicroRNA Assays offer several distinct advantages over conventional miRNA-detection methods, including:

- High-quality quantitative data – The assays can detect and quantify miRNA over more than six logs of dynamic range.
- Sensitivity – The assays can detect miRNAs in as little as 1 to 10 ng of total RNA, allowing you to conserve limited samples.
- High Specificity – The assays detect only mature miRNA, not its precursor, with single-base discrimination.
- Fast and simple methodology– The two-step protocol takes less than four hours and can be used with any Applied Biosystems Real-Time PCR instrument.

About MicroRNAs

MicroRNAs are endogenous RNAs, about 22 nucleotides long, that play important regulatory roles in animals and plants by targeting mRNA transcripts for cleavage or translational repression (Bartel, 2004). To date, hundreds of unique, mature miRNAs have been identified across species, with more continuing to be discovered. Their expression levels vary greatly among species and tissues (Kim *et al.*, 2004).

Low abundant miRNAs have been difficult to detect based on current technologies, such as cloning, Northern hybridization (Lim *et al.*, 2003), microarrays, and other techniques.

About this Product

The TaqMan MicroRNA Assays use looped-primer RT-PCR, a new real-time quantification method, to accurately detect mature miRNAs.

Each TaqMan MicroRNA assay includes:

- One tube containing miRNA-specific RT primer
- One tube containing a mix of:
  - miRNA-specific forward PCR primer
  - specific reverse PCR primer
  - miRNA-specific TaqMan MGB probe

Available TaqMan MicroRNA Assays

The TaqMan MicroRNA Assays are available for a range of species. Because many mature miRNA sequences are identical across related species, many assays for human are also valid for mouse and rat. Contact technical support or visit www.appliedbiosystems.com for details.

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

http://www.appliedbiosystems.com

About This Protocol

This protocol provides:

- Background information about the TaqMan MicroRNA Assays
- A list of equipment and materials needed for the protocol
- Procedures for using TaqMan MicroRNA assays

Chemistry Overview

Two-Step RT-PCR

Quantification using the TaqMan MicroRNA Assays is done using two-step RT-PCR:

1. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan® MicroRNA Reverse Transcription Kit.
2. In the PCR step, PCR products are amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix.

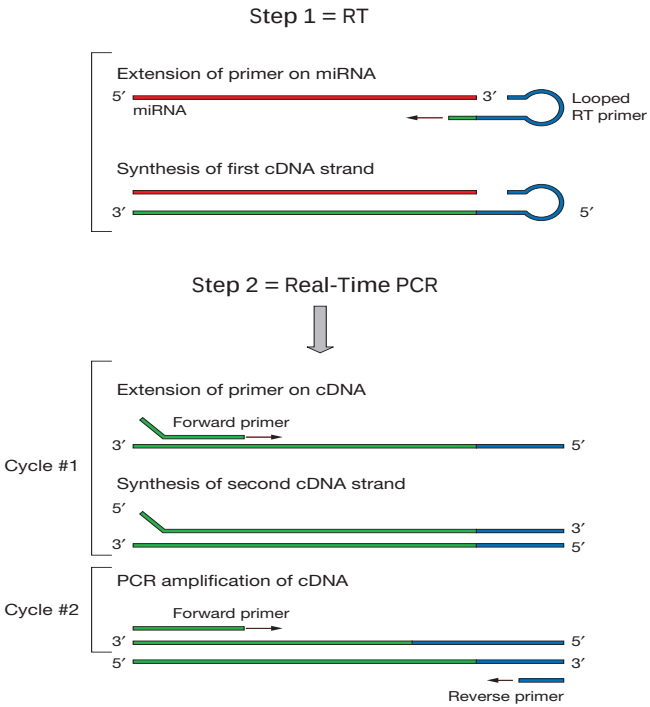


Figure 1 Two-step RT-PCR

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 (Figures 2 through 6) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

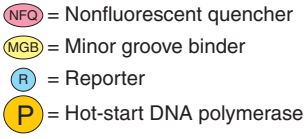


Figure 2 Legend for 5′ nuclease assay process figures

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 (Figures 3 and 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).

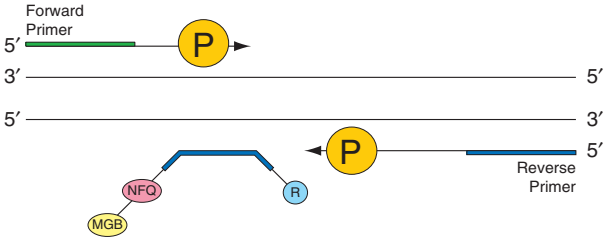


Figure 3 Polymerization

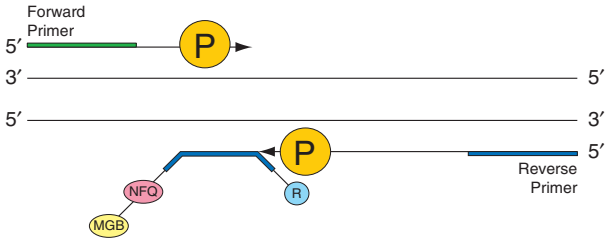


Figure 4 Strand displacement

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.

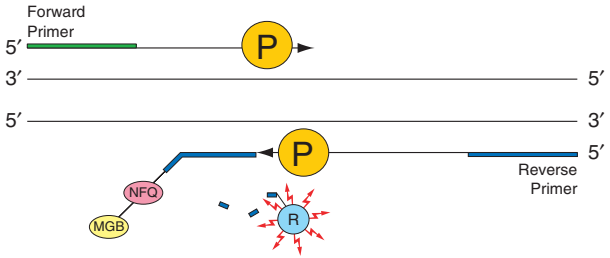


Figure 5 Cleavage

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).

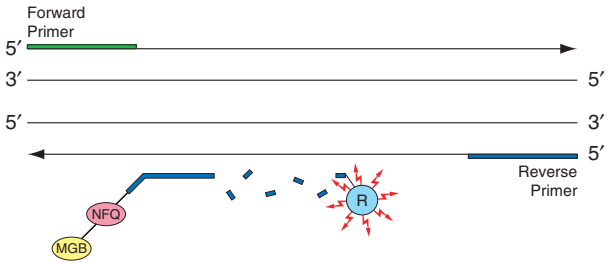


Figure 6 Completion of polymerization

Materials and Equipment

Available Products

The TaqMan MicroRNA Assays are available for a range of species. For the most current list of assays, refer to the Applied Biosystems website:

http://www.appliedbiosystems.com

Each assay consists of:

- One tube of RT primer
- One tube of TaqMan Assay (preformulated forward/reverse primer and MGB probe)
- Enough material for 150 Real-Time PCR reactions (at a total reaction volume of 20 µL)

IMPORTANT! TaqMan MicroRNA 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 reverse transcriptase enzymes.

Additionally, Applied Biosystems recommends that you use TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG with TaqMan MicroRNA Assays.

Storage

Store TaqMan MicroRNA Assays at –15 to –25 °C.

Materials and Equipment Not Included

Table 1 includes required and optional equipment and materials for using TaqMan MicroRNA Assays. Unless otherwise noted, many items listed are available from major laboratory suppliers.

Table 1 User-supplied materials and equipment

Materials and Equipment	Source
Required	
TaqMan® MicroRNA Reverse Transcription Kit[a] • 200 reactions • 1000 reactions	Applied Biosystems • PN 4366596 • PN 4366597
TaqMan® 2× Universal PCR Master Mix, No AmpErase® UNG[b]	Applied Biosystems PN 4324018

Table 1 User-supplied materials and equipment *(continued)*

Materials and Equipment	Source
Additional Materials and Equipment	
Applied Biosystems 7900HT Fast Real-Time PCR System	Contact your local Applied Biosystems sales office.
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System	
GeneAmp® PCR System 9700 thermal cycler	
ABI PRISM® 96-Well Optical Reaction Plate with Barcode (code 128)	Applied Biosystems PN 4326659
ABI PRISM® 384-Well Clear Optical Reaction Plate with Barcode (code 128)	Applied Biosystems PN 4309849
ABI PRISM® Optical Adhesive Covers (quantity 100)	Applied Biosystems PN 4311971
Optical Adhesive Covers (quantity 25)	Applied Biosystems PN 4360954
ABI PRISM® Optical Caps, 8 caps/strip	Applied Biosystems PN 4323032
ABI PRISM® Cap Installing Tool	Applied Biosystems PN 4330015
Adhesive Seal Applicator Kit	Applied Biosystems PN 4333183
MicroAmp™ Clear Adhesive Films	Applied Biosystems PN 4306311
Optical Compression Pads (quantity 5)	Applied Biosystems PN 4312639
Applied Biosystems Reagent Tubes With Caps, 10-mL	Applied Biosystems PN 4305932
Centrifuge with plate holders	Major Laboratory Supplier (MLS)
Disposable gloves	MLS
Microcentrifuge	MLS
Pipette tips, aerosol resistant, nuclease-free: 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range	MLS
Pipettors (positive-displacement, air-displacement, or multichannel): 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range	MLS
Polypropylene tubes	MLS

Table 1 User-supplied materials and equipment *(continued)*

Materials and Equipment	Source
RNase-free, sterile-filtered water	MLS
Vortexer	MLS
Microsoft® Excel or equivalent spreadsheet and analysis software	Software suppliers

a. TaqMan MicroRNA 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 reverse transcriptase enzymes.

b. Applied Biosystems strongly recommends that you use these Applied Biosystems reagents with the TaqMan MicroRNA Assays.

Table 2 Applied Biosystems documents

Documents	Part Number
TaqMan® MicroRNA Reverse Transcription Kit Product Insert	4367038
Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide	4351684
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Getting Started Guide	4347828
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4347825
Real-Time PCR Systems Chemistry Guide	4348358

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).

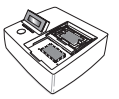
General PCR Practices

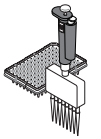
General PCR practices to prevent contamination:

- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Do not bring amplified PCR products into the PCR setup area.
- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Open and close all sample tubes and reaction plates carefully. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Use positive-displacement or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.

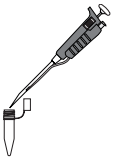
Procedural Overview

Prepare total RNA





or



↓

Perform reverse transcription

Thermal cycler

Real-Time PCR Instruments

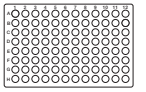
↓ 65 min

Create and set up a plate document

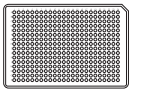
SDS software

↓ 15 min

Prepare the PCR reaction plate



Optical 96-Well
Thermal Cycling Plate



Optical 384-Well
Thermal Cycling Plate

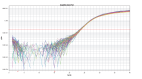
↓ 15 min

Run the PCR reaction plate

Applied Biosystems
7900HT Fast / 7300 / 7500 / 7500 Fast
Real-Time PCR System

↓ 90 min

Analyze results
(2 min)



Section 2 Using Individual Assays

Reverse Transcription

Overview

Synthesize single-stranded cDNA from total RNA samples using the TaqMan® MicroRNA Reverse Transcription Kit. The process involves the following procedures:

1. Preparing the RT master mix
2. Preparing the RT reaction plate
3. Performing reverse transcription

IMPORTANT! TaqMan MicroRNA 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 reverse transcriptase enzymes.

RNA Template Guidelines

For the optimal performance of the TaqMan MicroRNA Reverse Transcription Kit and of TaqMan MicroRNA Assays, Applied Biosystems recommends using RNA with the following characteristics:

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

IMPORTANT! Do not denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some miRNA targets.

Per Reaction Input Amount of Total RNA

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

Preparing the RT Reaction Master Mix

Prepare RT master mix using the TaqMan MicroRNA Reverse Transcription Kit components before preparing the reaction.

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

To prepare RT master mix:

1. Allow the kit components to thaw on ice.
2. In a polypropylene tube, prepare the RT master mix by scaling the volumes listed below to the desired number of RT reactions. Applied Biosystems recommends adding 10 to 20% overage to account for pipetting losses.

   IMPORTANT! This procedure assumes that you are quantifying miRNA from a single total RNA sample.

Component	Master Mix Volume/ 15-µL Reaction[a]
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00
10× Reverse Transcription Buffer	1.50
RNase Inhibitor, 20U/µL	0.19
Nuclease-free water	4.16
Total	7.00

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

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

Preparing the RT Reaction

To prepare the RT reaction:

1.	For each 15-µL RT reaction, combine RT master mix (from step 2 on page 12) with total RNA in the ratio of: 7 µL RT master mix to 5 µL total RNA For example, combine 7.7 µL of RT master mix with 5.5 µL of total RNA. Remember to include the same proportion of excess volume of total RNA that you did for the RT master mix. In this example, a 10% excess volume was included for both RT master mix and total RNA. **Note:** Applied Biosystems recommends that you use 1 to 10 ng of total RNA per reaction.
2.	Mix gently. Centrifuge to bring the solution to the bottom of the tube. **IMPORTANT!** Do not exceed 2000 RPM or 5 minutes when centrifuging.
3.	Before opening the RT Primer tubes, thaw the tubes on ice and mix by vortexing, then centrifuge them.
4.	For each 15-µL RT reaction, dispense 12.0 µL of RT master mix containing total RNA (from step 1 on page 13) into a 0.2-mL polypropylene reaction tube. (This is the RT reaction tube.) **Note:** Alternatively, you may dispense into a single well of a 96-well reaction plate.
5.	Transfer 3 µL of RT primer (tube labeled RT Primer) from each assay set into the corresponding RT reaction tube or plate well.
6.	Seal the tube and mix gently. Centrifuge to bring solution to the bottom of the tube.
7.	Incubate the tube on ice for 5 min and keep on ice until you are ready to load the thermal cycler.

Performing Reverse Transcription

To perform reverse transcription:

1. Leaving the thermal cycler in the 9600 Emulation mode (default), use the following parameter values to program the thermal cycler:

Step Type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
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 reverse transciption run.

PCR Amplification

Overview

During the target amplification step, the AmpliTaq® Gold DNA polymerase amplifies target cDNA synthesized from the RNA sample, using sequence-specific primers from the TaqMan Assay Plates.

IMPORTANT! You must perform the PCR step on a Real-Time PCR system. Traditional thermal cyclers cannot be used because they cannot detect and record the fluorescent signals generated by the cleavage of TaqMan probes.

PCR Process

Performing the PCR step requires the following procedures:

1. Preparing the reaction plate
2. Setting up the plate document
3. Running the plate

Reagent Preparation Guidelines

Following these guidelines ensures optimal PCR performance:

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

PCR Reaction Components

Applied Biosystems recommends performing four PCR replicates per RT reaction. The recommended reaction volume is 20 µL. Prepare the plate so that each PCR reaction contains the components as listed in the following table.

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

Component	Volume (µL) / 20-µL Reaction
TaqMan MicroRNA Assay (20×)	1.00
Product from RT reaction (Minimum 1:15 Dilution)	1.33
TaqMan 2× Universal PCR Master Mix, No AmpErase UNG[a]	10.00
Nuclease-free water	7.67
Total Volume	**20**

a. For optimal performance of TaqMan MicroRNA Assays, Applied Biosystems strongly recommends that you use Applied Biosystems TaqMan 2× Universal PCR Master Mix, No AmpErase UNG.

Preparing the PCR Reaction Plate

Applied Biosystems recommends performing four replicates of each 20-µL reaction.

IMPORTANT! The following procedure assumes that you are testing one individual assay.

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

To prepare the PCR reaction:

1. Scale the volumes listed below to the appropriate number of RT reactions. Applied Biosystems recommends including four replicates per RT reaction. Prepare on ice.

Reagent	Master Mix Volume for One 20-µL Reaction
TaqMan 2× Universal PCR Master Mix, No AmpErase UNG	10.00
Nuclease-free water	7.67
Total Volume	**17.67**

2. Mix gently. Centrifuge to bring solution to the bottom of the tube.

3. Add 17.67 µL of the PCR master mix/water mixture per 20-µL PCR reaction into a polypropylene tube (the PCR reaction tube), as shown in the following example.

Volume for One 20-µL Reaction	Example: Volume for 4 replicates[a]
17.67	17.67 µL x 4 replicates = 70.68 µL + 8.8 µL excess = 79.48 µL

a. Calculation includes 12.5% extra volume to account for pipetting losses. Keep this extra volume proportional with the extra volume in the next two steps.

To prepare the PCR reaction: ***(continued)***

4. Transfer 1.0 µL of 20× TaqMan MicroRNA Assay mix (labeled Real Time) into the PCR Reaction tube, as shown in the following example.

Volume for One 20-µL Reaction	Example: Volume for 4 replicates[a]
1.0	1.0 µL x 4 replicates = 4 µL + 0.5 µL excess = 4.5 µL

a. Calculation includes 12.5% extra volume to account for pipetting losses.

5. Transfer 1.33 µL of the RT product from the RT reaction tube into the PCR reaction tube, as shown in the following example.

Volume for One 20-µL Reaction	Example: Volume for 4 replicates[a]
1.33	1.33 µL x 4 replicates = 5.32 µL + 0.68 µL excess = 6.0 µL

a. Calculation includes 12.5% extra volume to account for pipetting losses.

6. Mix gently. Centrifuge to bring solution to the bottom of the plate.

7. Prepare the PCR reaction plate by dispensing 20 µL of the complete PCR master mix (including primer and RT product) into each of four wells.

8. Seal the plate with an optical adhesive cover, then centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.

Setting Up the Plate Document

Refer to the appropriate instrument user guide for instructions on how to configure the plate document.

- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)

- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (PN 4347825)

When creating plate documents, use the following parameters:

<table>
<tr><th>Parameter</th><th>Value</th></tr>
<tr><td>Run Mode</td><td>9600 emulation (Default)</td></tr>
<tr><td>Sample Volume</td><td>20 µL</td></tr>
<tr><td>Thermal Cycling Parameters</td><td>
<table>
<tr><th rowspan="2">Step</th><th rowspan="2">AmpliTaq Gold® Enzyme Activation</th><th colspan="2">PCR</th></tr>
<tr><td colspan="2">CYCLE (40 cycles)</td></tr>
<tr><td>HOLD</td><td>Denature</td><td>Anneal/ Extend</td></tr>
<tr><td>Time</td><td>10 min</td><td>15 sec</td><td>60 sec</td></tr>
<tr><td>Temp (°C)</td><td>95</td><td>95</td><td>60</td></tr>
</table>
</td></tr>
<tr><td>Auto Increment Settings</td><td>Accept default values. (Default is 0.)</td></tr>
<tr><td>Ramp Rate Settings</td><td>Accept default values. (Default is Standard.)</td></tr>
<tr><td>Data Collection</td><td>Accept default values. (Default is 60 °C.)</td></tr>
</table>

Running the Plate

Refer to the following documents for detailed instructions on loading and running the PCR plates:

- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)

- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantification Getting Started Guide* (PN 4347825)

To run the plate:

1.	In the SDS software, open the plate document that corresponds to the reaction plate.
2.	Load the reaction plate into the instrument.
3.	Start the run.

Section 3 Analyzing 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.

Tools for Analyzing TaqMan MicroRNA Assay Results

Assay Normalization with TaqMan® Endogenous Controls

Using the comparative C_T method, you can use endogenous controls to normalize the expression levels of target genes by correcting differences in the amount of cDNA loaded into PCR reactions.

To normalize human total RNA samples, an appropriate constitutively expressed endogenous control must be selected. Common mRNA control transcripts are available as TaqMan® Endogenous Controls, but must be validated for the individual researcher's samples. More information about TaqMan Endogenous controls is available on the Applied Biosystems Web site.

Resources for Data Analysis

Refer to the following documents for more information about analyzing your data:

- *Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide* (PN 4351684)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide* (PN 4347825)
- Livak and Schmittgen, 2001 – Provides the derivation, assumptions, and applications of the $2^{-\Delta\Delta C_T}$ method and variations for analyzing the relative changes in gene expression from Real-Time quantitative PCR experiments.
- Real-Time PCR Systems Chemistry Guide (Chapter 3) (PN 4348358)

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