Applied Biosystems

TaqMan® Universal Master Mix II

Protocol

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Contents

Safety information

Note: For general safety information, see this Preface and Appendix E, "Safety" on page 61. 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 "MSDSs" on page 63.

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® Universal Master Mix II

Product information

Purpose of the product

TaqMan® Universal Master Mix II is a convenient mix of components (except primers, probes, template, and water) necessary to perform a real-time polymerase chain reaction (PCR). You can use TaqMan® Universal Master Mix II to amplify complementary DNA (cDNA) and DNA targets for a variety of applications, including quantitation and genotyping. The mix is available with or without UNG.

About this protocol

This protocol describes the two primary applications of the TaqMan® Universal Master Mix II: quantitative RT-PCR and genotyping. Although TaqMan® Universal Master Mix II can be used in a broad variety of PCR applications, this document describes the use of the master mix with pre-optimized TaqMan® assays.

Because analysis methods vary greatly between applications, this protocol provides general guidelines for the analysis of data generated from experiments that use TaqMan® Universal Master Mix II and TaqMan® assays. For detailed information about data analysis or the procedures outlined in this protocol, consult your the appropriate documentation for your instrument (see "Related documentation" on page 69).

About the kit

TaqMan® Universal Master Mix II has been optimized for use with primers and TaqMan® probes that have been designed according to Applied Biosystems guidelines. The master mix can be used with custom TaqMan® assays available from the Applied Biosystems custom assay service, or with pre-optimized assays such as:

- TaqMan® Gene Expression Assays
- TaqMan® MicroRNA Assays
- TaqMan® Drug Metabolism Genotyping Assays
- TaqMan® SNP Genotyping Assays

For RNA quantitation experiments, the TaqMan® Universal Master Mix II is used in the second step of a two-step reverse transcription–polymerase chain reaction (RT-PCR) protocol. The cDNA template used with the master mix can be generated in a reverse transcription reaction using kits available from Applied Biosystems. (See "Reverse transcription kits and reagents" on page 50 for a list of recommended products.)

Materials and equipment

Storage and stability

Upon receipt, store the TaqMan® Universal Master Mix II at 2 to 8 °C.

Applied Biosystems does not recommend storing TaqMan® Universal Master Mix II at temperatures other than 2 to 8 °C or using TaqMan® Universal Master Mix II after the date printed on the package and bottle label.

Before use, thoroughly mix the TaqMan® Universal Master Mix II.

Kit components

The TaqMan® Universal Master Mix II, with or no UNG, is supplied in a 2× concentration and contains:

- AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)
- Uracil-N glycosylase (UNG)
- dNTPs with dUTP
- ROX™ Passive Reference
- Optimized buffer components

Note: TaqMan® Universal Master Mix II, no UNG, contains all the above ingredients except UNG.

TaqMan® Universal Master Mix II, with or no UNG, is supplied in a 2× concentration and is available in the following volumes:

Master mix	Item	Volume	50-µL reactions	Part number
TaqMan® Universal Master Mix II, no UNG	Mini-Pack	1 × 1-mL tube	40	4440043
	1-Pack	1 × 5-mL bottle	200	4440040
	2-Pack	2 × 5-mL bottles	400	4440047
	5-Pack	5 × 5-mL bottles	1000	4440048
	10-Pack	10 × 5-mL bottles	2000	4440049
	Bulk-Pack	1 × 50-mL bottle	2000	4440041
TaqMan® Universal Master Mix II, with UNG	Mini-Pack	1 × 1-mL tube	40	4440042
	1-Pack	1 × 5-mL bottle	200	4440038
	2-Pack	2 × 5-mL bottles	400	4440044
	5-Pack	5 × 5-mL bottles	1000	4440045
	10-Pack	10 × 5-mL bottles	2000	4440046
	Bulk-Pack	1 × 50-mL bottle	2000	4440039

Compatible real-time instruments

The TaqMan® Universal Master Mix II may be used for real-time or plate read (endpoint) detection of DNA or cDNA. Analysis is performed using any of the following real-time PCR systems:

- StepOne™ or StepOnePlus™ Real-Time PCR System
- Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System
- Applied Biosystems 7900HT/7900HT Fast Real-Time PCR System
- Applied Biosystems 7700 Sequence Detection System
- Applied Biosystems 7000 Sequence Detection System

Before you begin

Prevent contamination

Review Appendix C, "PCR Good Laboratory Practices" on page 53.

Select an instrument and reaction plate

IMPORTANT! Use TaqMan® Universal Master Mix II with *Standard* mode thermal cycling conditions only.

You can perform PCR amplification with any of the instruments and compatible plates listed in Appendix B, "Ordering Information" on page 47.

Fast reagents and thermal cycling conditions

IMPORTANT! TaqMan® Universal Master Mix II is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan® Universal Master Mix II on the StepOne™, StepOnePlus™, 7500 Fast, or 7900HT Fast instruments, use Standard mode thermal cycling conditions. If you use assays other than the TaqMan® assays, or use thermal cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal cycling conditions as needed. Refer to *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for more information on selecting thermal cycling conditions.

Section 1 Gene expression quantitation

Purpose

Use TaqMan® Universal Master Mix II with the DNA target of your choice, including cDNA or plasmid DNA. You can use TaqMan® Universal Master Mix II with any TaqMan® assay or quantitative PCR application, such as:

- Pathogen detection
- Copy number analysis
- Microarray validation
- Differential gene expression analysis
- Viral load quantitation
- MicroRNA quantitation (see Section 2 on page 19)

About this section

This section provides a protocol for performing PCR using TaqMan® Universal Master Mix II with TaqMan® Gene Expression Assays, Custom TaqMan® Gene Expression Assays, or Custom TaqMan® Probes and Sequence Detection Primers. For detailed information about specific procedures outlined in this protocol, consult the appropriate instrument user guide. A procedural overview is also provided in the TaqMan® Universal Master Mix II Quick Reference Card (PN 4428174).

Materials and equipment

Reagents not supplied

The reagents below are not supplied with the TaqMan® Universal Master Mix II.

Materials		Part number
High Capacity RNA-to-cDNA™ Kit, 50 rxns		4387406
High Capacity RNA-to-cDNA Master Mix	500 reactions	4390779
	200 reactions	4390778
	50 reactions	4390777
	15 reactions	4390776
High Capacity RNA-to-cDNA Master Mix with No RT Control	500 reactions	4390713
	200 reactions	4390712
	50 reactions	4390711
	15 reactions	4390710
RNase inhibitor		N8080119
TaqMan® Gene Expression Assays, inventoried		4331182
TaqMan® Gene Expression Assays, made-to-order		4351372
Custom TaqMan® Gene Expression Assays	Small-scale (20X, 144 × 50-µL rxns)	4331348
	Medium-scale (20X, 300 × 50-µL rxns)	4332078
	Large-scale (60X, 1160 × 50-µL rxns)	4332079
TaqMan® PreAmp Master Mix Kit, 40 rxns		4384267
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free sterile-filtered water)		AM9849
DNAZap™ Solution, two, 250-mL bottles		AM9890
RT-PCR Grade Water, ten, 1.75-mL bottles		AM9935
DNase-free water		AM9914G

Optional user-supplied reagents

See "Optional user-supplied reagents for gene expression quantitation" on page 51 for a list of optional user-supplied reagents.

Plastics not supplied

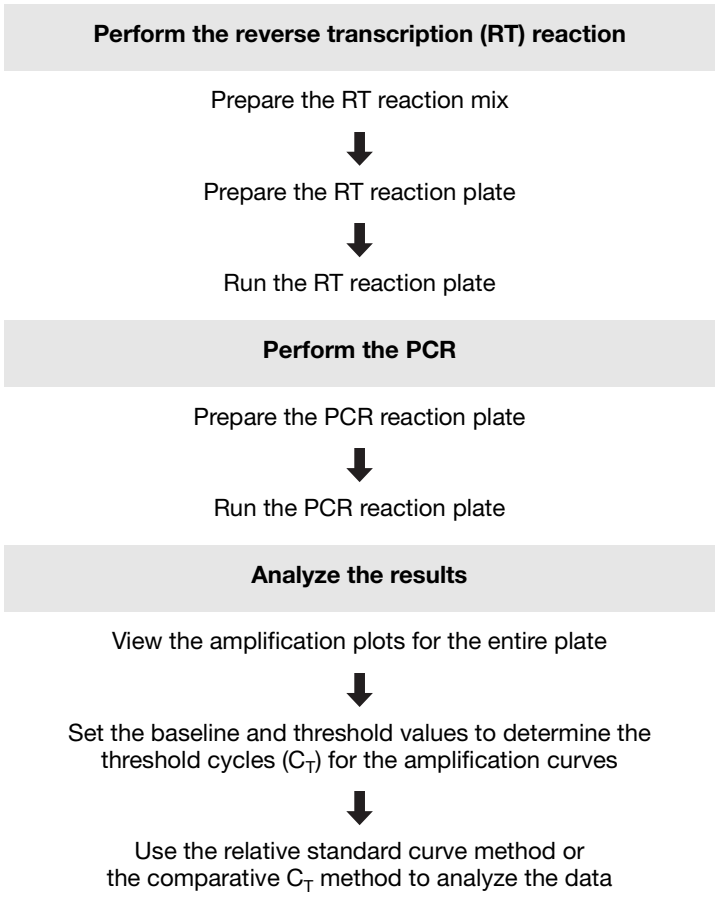
See "Real-time PCR systems, PCR systems, and consumables" on page 48 for a list of compatible real-time PCR system consumables.

Consumables and equipment not supplied

See "Consumables and equipment" on page 52 for a list of required laboratory consumables and equipment.

Workflow

The following figure shows the process for performing gene expression experiments.



Perform reverse transcription

Synthesis of single-stranded cDNA from RNA is the first step in the RT-PCR process, which requires you to:

1. Prepare the reverse transcription (RT) reaction mix.
2. Prepare the RT reaction plate.
3. Perform reverse transcription.

To obtain cDNA from RNA samples, Applied Biosystems recommends using a Applied Biosystems reverse transcription kit. See to "Reverse transcription kits and reagents" on page 50 for a list of recommended products.

For additional RT guidelines and instructions, refer to the appropriate protocol. You can download the protocols for Applied Biosystems kits at:
http://docs.appliedbiosystems.com/search.taf

RNA template guidelines

For optimal performance, Applied Biosystems recommends using RNA that is:

- Free of inhibitors of reverse transcription and PCR.
- Dissolved in TE buffer or PCR-compatible buffer.
- Free of RNase activity.

Reagent and sample preparation guidelines

To ensure optimal performance:

- Use nuclease-free pipet tips and reagents to minimize degradation of the RNA.
- Observe standard laboratory practices when handling RNA.

Perform real-time PCR amplification

Target amplification using cDNA as the template is the second step in the RT-PCR process. In this step, the DNA polymerase (from the TaqMan® Universal Master Mix II) amplifies target cDNA synthesized from the RNA sample, using sequence-specific primers and a TaqMan® probe (for example, a probe from the TaqMan® Gene Expression Assay mix).

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.

Note: If you choose to use Custom TaqMan® Probes and Sequence Detection Primers, rather than a TaqMan® Gene Expression Assay or a Custom TaqMan® Gene Expression Assay, see "Using TaqMan® Universal Master Mix II with custom TaqMan® probes and primers" on page 16 for more information.

PCR reagent handling and preparation

Following these guidelines ensures optimal PCR performance:

- Keep the TaqMan® assays in the freezer, away from light, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prior to use:
  - Mix the TaqMan® Universal Master Mix II thoroughly by swirling the bottle.
  - Thaw frozen TaqMan® assays by placing them on ice. When thawed, resuspend the samples by vortexing, then centrifuge the tubes briefly.
  - Resuspend the TaqMan® reagents (for example, the TaqMan® Gene Expression Assay mix) by vortexing and then centrifuge the tube briefly.
  - Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by briefly vortexing and then centrifuge the tubes.

Determine the number of required reactions

Determine the number of reactions to perform for each assay. Applied Biosystems recommends performing four replicates of each reaction. Include extra reactions (approximately 110% of the required volume) to compensate for the volume loss that occurs during reagent transfers. For example, if using a 96-well plate, prepare enough reaction mix for approximately 106 reactions.

Be sure to include on each plate:

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

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

Prepare the PCR reaction plate

1. Prepare the reaction mix for each sample using the components listed below.

Component	Volume (µL) per reaction		Final conc.
	50-µL rxns.	20-µL rxns.	
TaqMan® Universal Master Mix II (2×)	25.0	10.0	1×
TaqMan® Gene Expression Assay (20×)‡	2.5	1.0	1×
cDNA template + H_2O§	22.5	9.0	1 to 100 ng
Total Volume	50.0	20.0	–

‡ See **www.allgenes.com** for TaqMan® Gene Expression Assay information.
§ Use 10 to 100 ng of cDNA plus RNase-free water.

- Calculate the volume of each component of the PCR reaction mix by multiplying the volume of each component by the number of replicates for each sample.
- Applied Biosystems recommends performing four replicates of each reaction. Select the reaction size depending on the reaction plate used. Prepare 110% of the required volume to account for pipetting error.
- Use 1 to 100 ng of cDNA per replicate.

IMPORTANT! For optimal performance of TaqMan® Gene Expression Assays, use 1 to 100 ng of cDNA per 20- or 50-µL reaction.

2. Cap the tube(s).
3. Vortex the tube(s) briefly to mix the solutions.
4. Centrifuge the tube(s) briefly to spin down the contents and eliminate any air bubbles from the solutions.
5. Transfer the appropriate volume of each reaction mixture to each well of an optical plate, as specified in the following table.

Plate format	Reaction volume
MicroAmp® Optical 96-Well Reaction Plate	50 µL
• MicroAmp® Fast Optical 96-Well Reaction Plate • MicroAmp® Optical 384-Well Reaction Plate • MicroAmp® Fast Optical 48-Well Reaction Plate	20 µL

6. Cover the plate with a MicroAmp® Optical Adhesive Film. For standard 96-well plates, you may use MicroAmp® Optical Caps.
7. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.
8. Apply a compression pad to the plate if required by your real-time PCR system.

Run the PCR reaction plate

Refer to your instrument user guide for instructions on how to configure the plate document or experiment. See "Related documentation" on page 69 for a list of user documentation for Applied Biosystems real-time PCR systems.

When creating plate documents/experiments, use the following parameters:

- Thermal Cycling Parameters:

System	UNG incubation‡	Polymerase activation§	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/extend
Temp. (°C)	50	95	95	60
Time (mm:ss)	2:00	10:00	00:15	1:00
Volume (µL)	20 or 50			

‡ Required for optimal UNG activity; omit if UNG is not present in the reaction.
§ The 10-minute, 95 °C step is required to activate the AmpliTaq Gold®, UP enzyme.

IMPORTANT! Omit the 2-minute, 50 °C step if you are using TaqMan® Universal Master Mix II, no UNG.

- Run Mode: **9600 emulation** (default)
- Sample Volume:

Plate format	Reaction volume
MicroAmp® Optical 96-Well Reaction Plate	50 µL
• MicroAmp® Fast Optical 96-Well Reaction Plate • MicroAmp® Optical 384-Well Reaction Plate • MicroAmp® Fast Optical 48-Well Reaction Plate	20 µL

- Auto Increment Settings: Accept default values (default is 0)
- Data Collection: Accept default values (default is 60 °C)
- Ramp Rate Settings: Accept default values (default is Standard)

  TaqMan® Universal Master Mix II is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan® Universal Master Mix II on the StepOne™, StepOnePlus™, 7500 Fast, or 7900HT Fast instruments use Standard mode thermal cycling conditions. If you use assays other than the TaqMan® Gene Expression assays, or use thermal cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal cycling conditions as needed. Refer to *Real-Time PCR Systems Chemistry Guide* (PN 4348358) for more information on selecting thermal cycling conditions.

Run the plate

Refer to the appropriate instrument user guide for detailed instructions on loading and running the PCR plates. See "Related documentation" on page 69 for a list of user documentation for Applied Biosystems real-time PCR systems.

To run the plate:

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

Analyze the results

The general process for analyzing gene expression data involves:

a. Viewing the amplification plots for the entire plate.

b. Setting the baseline and threshold values to determine the threshold cycles (C_T) for the amplification curves.

c. Using the relative standard curve method or the comparative C_T method to analyze the data.

Data analysis varies depending on the real-time PCR system that you use. See "Related documentation" on page 69 for a list of applicable documents.

Baseline and threshold values

When using a real-time PCR system, you can use the software to set the baseline and threshold for the amplification curves either automatically or manually.

- The *baseline* refers to the initial cycles of PCR in which there is slight change in fluorescence signal.
- The intersection of the *threshold* with the amplification plot defines the C_T in real-time PCR assays. The threshold is set above the background and within the exponential growth phase of the amplification curve.

Automatic calculation of the baseline and threshold

The system software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the "typical" amplification curve shown in Figure 1. Experimental error (such as contamination or pipetting errors) can produce atypical data that can cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

IMPORTANT! After an analysis, verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots, and adjust the values manually if necessary.

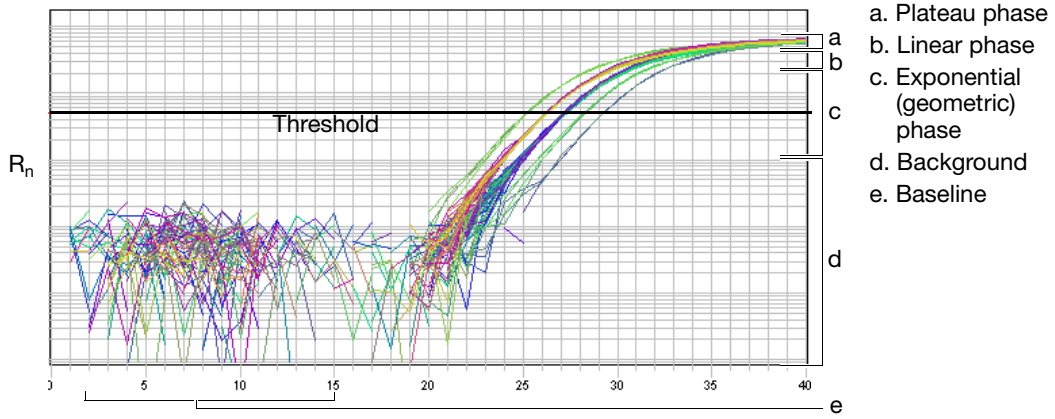


Figure 1 Typical amplification curve

Manual setting of the baseline and threshold

If you use the system software to set the baseline and threshold values manually for any detector/assay in the study, perform an adjustment procedure for each detector/assay. Refer to your real-time PCR system documentation for guidance on manually setting and adjusting your threshold and baseline.

Table 1 Correct and incorrect threshold settings

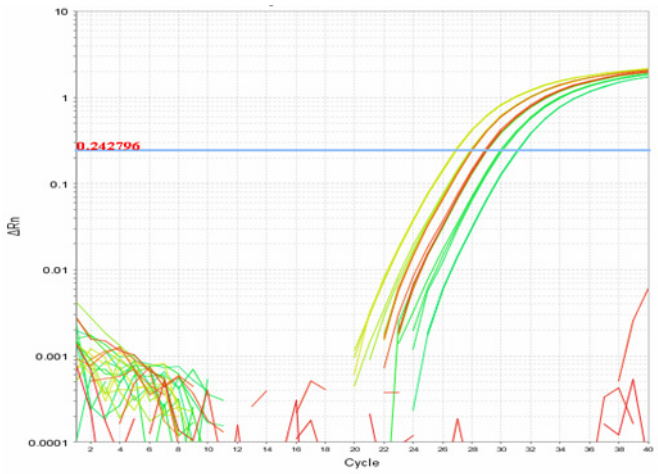
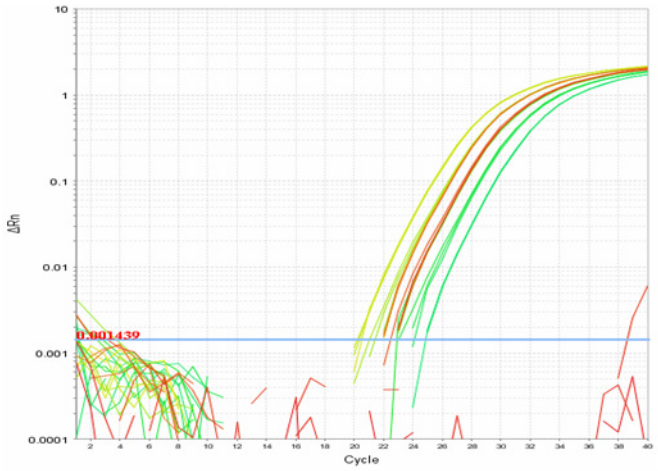
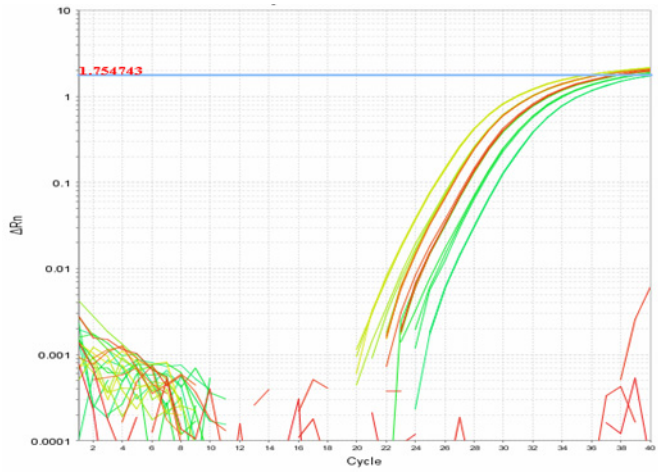
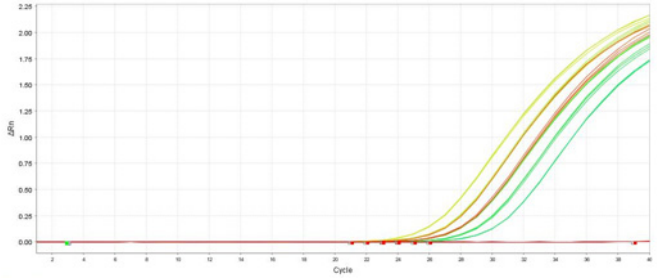
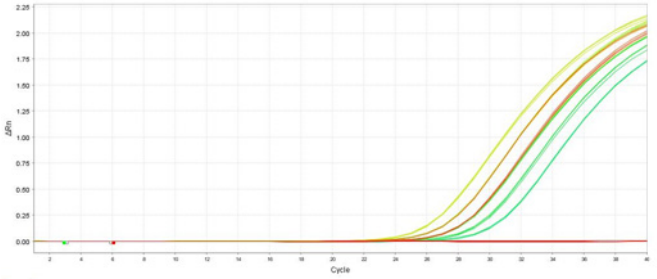
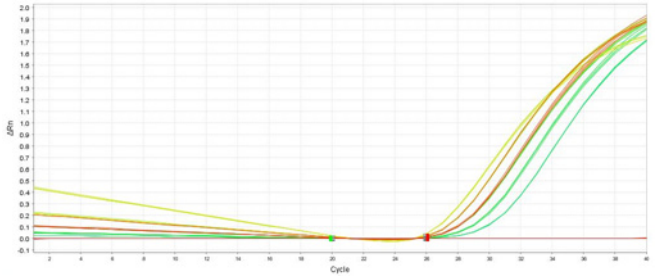
Threshold set correctly The threshold is set in the exponential phase of the amplification curve. Threshold settings above or below the optimum increase the standard deviation of the replicate groups.	
Threshold set too low The threshold is set below the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Set the threshold up into the exponential phase of the curve.	
Threshold set too high The threshold is set above the exponential phase of the amplification curve. The standard deviation is significantly higher than that for a plot where the threshold is set correctly. Set the threshold down into the exponential phase of the curve.	

Table 2 Correct and incorrect baseline settings

Baseline set correctly The amplification curve begins after the maximum baseline.	
Baseline set too low The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.	
Baseline set too high The amplification curve begins before the maximum baseline. Decrease the End Cycle value.	

Analyze the data

You can perform two types of quantitation using the TaqMan® Universal Master Mix II:

- Relative quantitation compares the relative expression of a target gene between an unknown and a reference sample. You can perform relative quantitation using the standard curve method or the comparative C_T method.
- Absolute quantitation compares the C_T of an unknown sample against a standard curve with known copy numbers.

Quantitation of cDNA relative to a calibrator sample

Gene expression can be measured by comparing the relative expression of a target gene in a unknown sample and in a physiological reference sample. In a typical experiment, gene expression levels are studied as a function of either a treatment of cells in culture, of patients, or of tissue type. The calibrator sample in each case is the cDNA from either the untreated cells or patients, or a specific tissue type.

All quantitations are also normalized to an endogenous control such as GAPDH to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. All amplicons in these determinations should follow the amplicon design criteria around the Primer Express® Software. Refer to the Real-Time PCR Systems Chemistry Guide (PN 4348358) for additional information about relative quantitation.

Using TaqMan® Universal Master Mix II with custom TaqMan® probes and primers

To design custom probes and primers for a real-time quantitative PCR assay:

- Determine your target template and amplicon.
- Design your custom sequence detection primers and TaqMan® probe(s).
- Determine the optimal concentrations of the sequence detection primer and custom TaqMan® probe(s).
- Perform real-time quantitative PCR.

Determining target template and amplicon

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

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

Designing custom TaqMan® probes and primers

Primers and probes can be designed using Primer Express® Software as described in the Primer Express® Software Version 3.0 Getting Started Guide (PN 4362460).

Determining optimal primer concentration

The purpose of this procedure is to determine the minimum primer concentrations giving the maximum ΔR_n. The Applied Biosystems Real-Time PCR Systems can provide additional data for optimization using the minimum threshold cycle (C_T). Refer to your instrument user manual for more information.

To determine the optimal primer concentration:

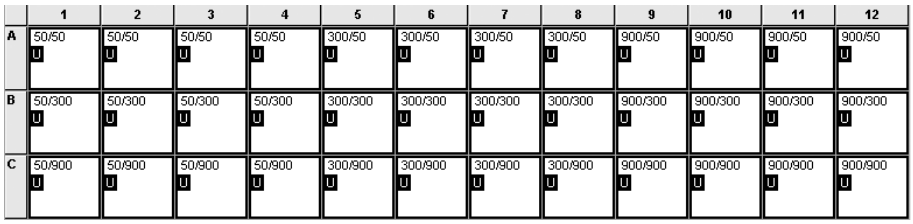
1. Prepare a PCR reaction mix for primer optimization:

Reaction component	Volume (µL) per sample		Final conc.
	50-µL rxns.	20-µL rxns.	
TaqMan® Universal Master Mix II	25.0	10.0	1×
Forward primer	5.0	2.0	50 to 900 nM
Reverse primer	5.0	2.0	50 to 900 nM
TaqMan probe (2.5-µM)	5.0	2.0	250 nM
DNA sample	5.0	2.0	10 to 100 ng
Water	5.0	2.0	—
Total	50.0	20.0	—

2. Run at least four replicates of each of the nine conditions as shown:

		Forward primer (nM)		
		50	**300**	**900**
Reverse primer (nM)	**50**	50/50	300/50	900/50
	300	50/300	300/300	900/300
	900	50/900	300/900	900/900

3. Load the plate with four replicates of each condition as shown:



4. Place the plate in the Applied Biosystems Real-Time PCR System and follow the thermal cycling conditions:

Step	**UDG incubation**	**Enzyme activation**	**PCR**	
	HOLD	**HOLD**	**Cycle (40 cycles)**	
			Denature	**Anneal/extend**
Temp. (°C)	50	95	95	60
Time (mm:ss)	2:00	10:00	0:15	1:00
Volume (µL)	20 or 50‡			

‡ Select appropriate volume for reaction plate.

IMPORTANT! The 2-minute, 50 °C step is required for optimal UNG enzyme activity. The 10-minute, 95 °C step is required to activate the AmpliTaq Gold®, UP enzyme.

5. At the end of runs, tabulate the results for ΔR_n. Choose the minimum forward- and reverse-primer concentrations that yield the maximum ΔR_n.

Determining optimal probe concentration

The purpose of this procedure is to determine the minimum probe concentrations that give the minimum C_T for each probe target.

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

To determine the optimal probe concentration:

1. Prepare a PCR reaction mix:

Reaction component	Volume (µL) per sample		Final conc.
	50-µL rxns.	20-µL rxns.	
TaqMan® Universal Master Mix II	25.0	10.0	1×
Forward primer	5.0	2.0	Optimal
Reverse primer	5.0	2.0	Optimal
TaqMan probe	5.0	2.0	50 to 250 nM
DNA sample	5.0	2.0	10 to 100 ng
Water	5.0	2.0	—
Total	50.0	20.0	—

2. For single-probe assays, determine the optimal probe concentration by running four replicates at each 50-nM interval from 50 to 250 nM.

   Note: Use the forward- and reverse-primer concentrations (determined in "Determining optimal primer concentration" on page 16).

3. Run the plate on the real-time PCR system using the following conditions:

Step	UDG incubation‡	Enzyme activation§	PCR	
	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/extend
Temp. (°C)	50 °C	95 °C	95 °C	60 °C
Time (mm:ss)	2:00	10:00	00:15	1:00
Volume (µL)	20 or 50#			

‡ The 2-minute, 50 °C step is required for optimal UNG enzyme activity.
§ The 10-minute, 95 °C step is required to activate the AmpliTaq Gold®, UP enzyme.
# Select appropriate volume for reaction plate.

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

Recommended sample input for real-time quantitative PCR

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

- 0.1 ng to 1 µg of DNA.
- The determined optimum probe and primer concentrations.
- The appropriate volume of TaqMan® Universal Master Mix II as described in "Prepare the PCR reaction plate" on page 10.
- The thermal cycling conditions specified in your instrument user guide.

Section 2 MicroRNA quantitation

Purpose

Use TaqMan® Universal Master Mix II to perform microRNA quantitation using TaqMan® MicroRNA Assays. The TaqMan® MicroRNA Assays are designed to detect and accurately quantify mature microRNAs (miRNAs) using Applied Biosystems real-time PCR instruments.

About this section

This section provides a protocol for performing PCR using TaqMan® Universal Master Mix II with TaqMan® MicroRNA assays. For detailed information about specific procedures outlined in this protocol, consult the appropriate instrument documentation. A procedural overview is also provided in the TaqMan® Universal Master Mix II Quick Reference Card (PN 4428174).

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 TaqMan® MicroRNA Assays

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. For the most current list of assays, visit the Applied Biosystems website at: **www.appliedbiosystems.com**

Materials and equipment

Reagents not supplied

The reagents below are not supplied with the TaqMan® Universal Master Mix II.

Materials and equipment		Source
TaqMan® MicroRNA Reverse Transcription Kit‡	200 reactions	4366596
	1000 reactions	4366597

‡ 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.

Plastics not supplied

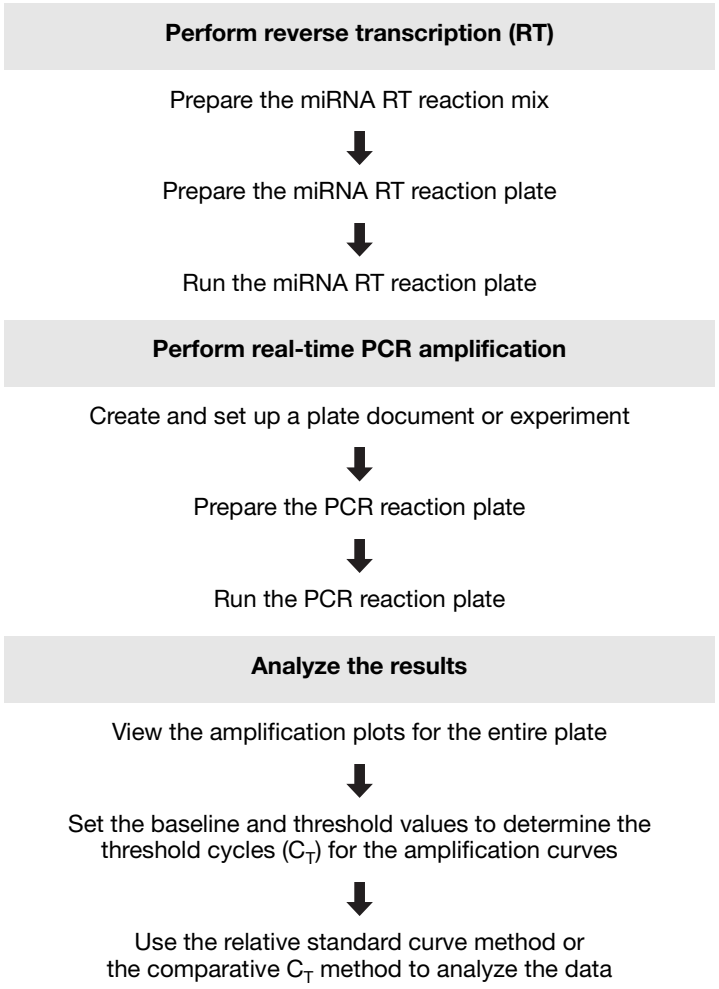
See "Real-time PCR systems, PCR systems, and consumables" on page 48 for a list of compatible real-time PCR system consumables.

Consumables and equipment not supplied

See "Consumables and equipment" on page 52 for a list of required laboratory consumables and equipment.

Workflow

The following figure shows the process for performing miRNA quantitation.



Perform reverse transcription

Synthesize single-stranded cDNA from total RNA samples using the TaqMan® MicroRNA Reverse Transcription Kit. 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 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

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.

Prepare the microRNA RT reaction master mix

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

1. Allow the kit components to thaw on ice.
2. In a polypropylene tube, prepare the RT master mix by scaling the volumes below to the desired number of reactions. Applied Biosystems recommends preparing 110% of the required volume to account for pipetting error. This procedure assumes that you are quantifying miRNA from a single total RNA sample.

Component	Volume (µL) per 15-µL reaction‡
100 mM 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

‡ 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.

Prepare the microRNA RT reaction

1. For each 15-µL RT reaction, combine RT master mix (from step 2 on page 22) with total RNA in the ratio of:

   7 µL RT master mix: 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.

   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.

   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) into a 0.2-mL polypropylene reaction tube. (This is the RT reaction tube.) Alternatively, you can 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 minutes and keep on ice until you are ready to load the thermal cycler.

Run the microRNA RT reaction plate

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 transcription run.

Perform real-time PCR amplification

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.

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.

Reagent preparation guidelines

- 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.

Component	Volume (µL) per 20-µL reaction
TaqMan® MicroRNA Assay (20X)	1.00
Product from RT reaction (Minimum 1:15 Dilution)	1.33
TaqMan® Universal Master Mix II, no UNG‡	10.00
Nuclease-free water	7.67
Total Volume	20

‡ For optimal performance of TaqMan® MicroRNA Assays, Applied Biosystems strongly recommends that you use Applied Biosystems TaqMan® Universal Master Mix II, No UNG.

Prepare the PCR reaction plate

Note: The following procedure assumes that you are testing one individual assay.

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

Reagent	Volume (µL) per 20-µL reaction
TaqMan® Universal Master Mix II, no 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).
4. Transfer 1.0 µL of 20× TaqMan® MicroRNA Assay mix (labeled Real Time) into the PCR Reaction tube.
5. Transfer 1.33 µL of the RT product from the RT reaction tube into the PCR reaction tube.
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.
9. Apply a compression pad to the plate if required by your real-time PCR system.

Set up the experiment or plate document

Refer to your instrument documentation for instructions on how to configure the plate document/experiment. See "Related documentation" on page 69 for a list of documentation for Applied Biosystems real-time PCR systems.

When creating plate documents/experiments, use the following parameters:

- Thermal Cycling Parameters:

System	UNG incubation‡	Polymerase activation§	PCR	
	Hold	Hold	Cycle (40 cycles)	
			Denature	Anneal/extend
Temp. (°C)	50	95	95	60
Time (mm:ss)	2:00	10:00	00:15	1:00

‡ Required for optimal UNG activity; not needed when UNG is not present in the reaction.
§ The 10-minute, 95 °C step is required to activate the AmpliTaq Gold®, UP enzyme.

- Run Mode: **Standard** (Default)
- Sample Volume: **20** µL
- Auto Increment Settings: Accept default values (default is 0)
- Ramp Rate Settings: Accept default values (default is Standard)
- Data Collection: Accept default values (default is 60 °C)

Run the plate

Refer to the appropriate instrument user guide for detailed instructions on loading and running the PCR plates (see "Related documentation" on page 69).

To run the plate:

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

Analyze the results

Refer to the appropriate instrument documentation for instructions on how to analyze your data. The general process for analyzing the data from gene expression assays involves:

1. Viewing the amplification plots.
2. Setting the baseline and threshold values.

Baseline and threshold values

When using a real-time PCR system, you can use the software to set the baseline and threshold for the amplification curves either automatically or manually.

- The *baseline* refers to the initial cycles of PCR in which there is slight change in fluorescence signal.
- The intersection of the *threshold* with the amplification plot defines the C_T in real-time PCR assays. The threshold is set above the background and within the exponential growth phase of the amplification curve.

See pages 13 and 15 for information on setting the threshold and baseline.

Tools for analyzing TaqMan® MicroRNA Assay results

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 $2^{-\Delta\Delta Ct}$ 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).

Section 3 Genotyping

Purpose

Use the TaqMan® Universal Master Mix II to perform genotyping of single nucleotide polymorphisms (SNPs). The master mix can be used with a genomic DNA and any TaqMan® genotyping assay, including:

- TaqMan® SNP Genotyping Assays
- Custom TaqMan® SNP Genotyping Assays
- TaqMan® Drug Metabolism Genotyping Assays
- TaqMan® Pre-Designed Assay Reagents for Genotyping

About this section

This section provides information on performing PCR using TaqMan® Universal Master Mix II with TaqMan® SNP Genotyping Assays, TaqMan® Drug Metabolism SNP Genotyping Assays, or Custom TaqMan® Probes and Sequence Detection Primers. For detailed information about specific procedures outlined in this protocol, consult the appropriate instrument user guide. A procedural overview is also provided in the TaqMan® Universal Master Mix II Quick Reference Card (PN 4428174).

Materials and equipment

Reagents not supplied

The reagents below are not supplied with TaqMan® Universal Master Mix II.

Item		Part number
Custom TaqMan® SNP Genotyping Assays	Small-scale, human, 40X (1000 × 5-µL rxns)	4331349
	Small-scale, non-human, 40X (1000 × 5-µL rxns)	4332077
	Medium-scale, human, 40X (3000 × 5-µL rxns)	4332072
	Medium-scale, non-human, 40X (3000 × 5-µL rxns)	4332075
	Large-scale, human, 80X (12,000 × 5-µL rxns)	4332073
	Large-scale, non-human, 80X (12,000 × 5-µL rxns)	4332076
TaqMan® Pre-Designed SNP Genotyping Assays	Small-scale, 40X (1500 × 5-µL rxns)	4351379
	Medium-scale, 40X (5000 × 5-µL rxns)	4351376
	Large-scale, 80X (12,000 × 5-µL rxns)	4351374
TaqMan® Validated and Coding Genotyping Assays, Small-scale, 20X concentration (750 × 5-µL rxns)		4331183
TaqMan® Pre-Developed Assay Reagents for Allelic Discrimination	CYP2C19*2, (400 rxns)	4312561
	CYP2C9*2, (400 rxns)	4312559
	CYP2C9*3, (400 rxns)	4312560
	CYP2D6*3, (400 rxns)	4312554
	CYP2D6*4, (400 rxns	4312555
	CYP2D6*6, (400 rxns)	4312556
	CYP2D6*7, (400 rxns)	4312557
	CYP2D6*8, (400 rxns)	4312558
TaqMan® Drug Metabolism Genotyping Assays‡		§
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free sterile-filtered water)		AM9849
DNAZap™ Solution, 2 × 250-mL bottles		AM9890
RT-PCR Grade Water, 10 × 1.75-mL bottles		AM9935
DNase-free water		AM9914G

‡ Includes CD with protocol, Assay Information File (AIF), DME Assay Index, and Troubleshooting Guide.
§ Go to **www.appliedbiosystems.com**, then search: **TaqMan® Drug Metabolism Assay**

Plastics not supplied

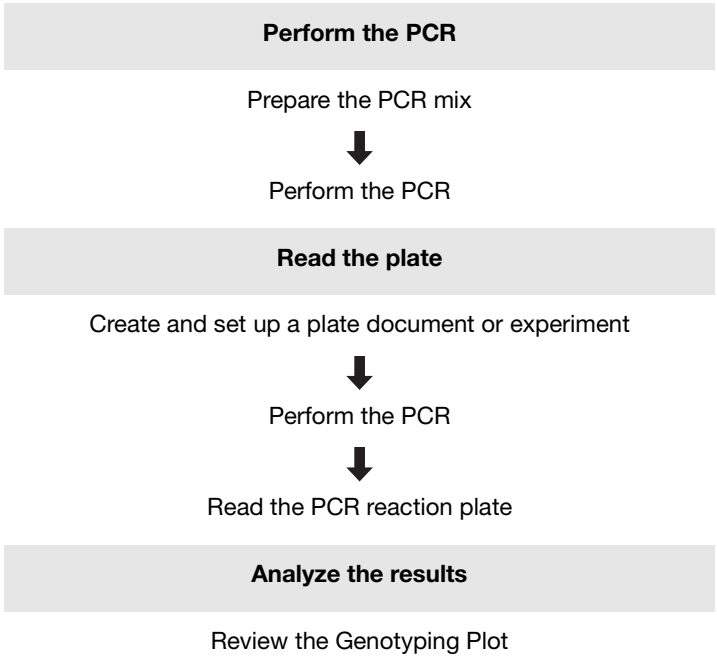
See "Real-time PCR systems, PCR systems, and consumables" on page 48 for a list of compatible real-time PCR system real-time PCR system consumables.

Consumables and equipment not supplied

See "Consumables and equipment" on page 52 for a list of required laboratory consumables and equipment.

Workflow

The following figure shows the process for performing genotyping experiments.



Before you begin

Quantitate the DNA

For a genotyping assay, add 1 to 10 ng of DNA template per reaction well. To quantitate genomic DNA, use a reliable method such as A260 measurements or real-time quantification by RNase P. If you use the RNase P method, you generate a standard curve using the DNA template standards in the TaqMan® DNA Template Reagents Kit (PN 401970) and the RNase P gene primers and probe in the TaqMan® RNase P Detection Reagents Kit (PN 4316831). For details on generating a standard curve, refer to *Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR* at:
http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf

Determine the number of required reactions

Determine the number of reactions to perform for each assay. Prepare 110% of the required volume to account for pipetting error. For example, for a 96-well plate, prepare enough volume for approximately 106 reactions.

Be sure to include on each plate at least:

- Two no-template controls (NTCs)
- (Optional) one genomic DNA control of known genotype

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

Perform genotyping

The first step in a genotyping assay is PCR amplification, which requires you to prepare the PCR mix, perform the PCR, read the plate, and analyze the results.

Prepare the PCR mix

IMPORTANT! Keep all TaqMan® reagents protected from light until you are ready to use them. Excessive exposure to light may affect the fluorescent probes. Minimize freeze-thaw cycles. Prepare the PCR reaction mix for each assay before transferring it to the reaction plate for thermal cycling and fluorescence analysis.

1. Thoroughly mix the TaqMan® Universal Master Mix II by swirling the bottle.
2. Thaw the frozen TaqMan® assays by placing them on ice. Vortex then centrifuge the tubes briefly.
3. Thaw any frozen genomic DNA by placing them on ice. After the samples thaw, mix them if needed by vortexing, then centrifuge the tubes briefly.
4. In an appropriate tube, combine the components shown in Table 3:
   a. Determine the reaction volume appropriate to the instrument and plate (see Table 4).
   b. Multiply the volume for one reaction component (see Table 4) by the total number of reactions.
   c. Add the volume calculated from step 4b for each component to the tube.

Table 3 PCR reaction mix volume (µL/well)

Component	Volume (µL) per reaction		
	5-µL rxn	10-µL rxn	25-µL rxn
TaqMan® Universal Master Mix II (2×)	2.50	5.0	12.50
TaqMan® genotyping assay mix (20×)‡	0.25	0.5	1.25
DNase-free water	1.25	2.5	6.25
Total	4.0	8.0	20.0

‡ For ease of use, dilute 40× and 80× Assay Mixes to 20× working solutions with 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Use DNase-free water.

Table 4 Recommended volumes according to instrument

System	Plate well volume	Reaction volume per well
7900HT (384 block)	384 wells, 0.02 mL	5 to 20 µL
7300, 7500, 7900HT	96 wells, 0.2 mL	20 to 50 µL
7500 Fast, 7900HT Fast	96 wells, 0.1 mL	10 to 30 µL
StepOne™, StepOnePlus™	48 wells, 0.1 mL	10 to 30 µL

5. Cap the tube(s), briefly vortex to mix the solutions, then briefly centrifuge them to spin down the contents and to eliminate air bubbles.

6. Into each well of a reaction plate, pipette the PCR reaction mix volume (4, 8, or 20 µL) appropriate to your plate, seal the plate with a MicroAmp® clear adhesive film, then centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.

7. Remove the clear adhesive film from the plate, then pipette one control or diluted DNA sample into the appropriate well(s).

8. If you use purified genomic DNA, use 1 to 10 ng of genomic DNA or control DNA for each reaction in the appropriate volume (see table below).

Volume of genomic DNA or DNA control (µL/PCR reaction)		
5-µL reaction	**10-µL reaction**	**25-µL reaction**
1.0	2.0	5.0

9. Seal the plate using MicroAmp® Optical Adhesive Film or MicroAmp® Optical Caps, then centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

10. Apply a compression pad to the plate if required by your real-time PCR system.

11. Load the plate into a real-time PCR system.

Perform the PCR

1. Set up the following run conditions:

IMPORTANT! These conditions are optimized for use only with TaqMan® genotyping assays on the instruments specified in Appendix B.

System	Polymerase activation‡	PCR	
	Hold	Cycle (40 cycles)	
		Denature	Anneal/extend
Temp. (°C)	95	95	60
Time (mm:ss)	10:00	00:15	1:00

‡ The 10-minute, 95 °C step is required to activate the AmpliTaq Gold®, UP enzyme.

- Run speed: **Standard**
- Reaction volume: **5**, **10**, or **25** µL

2. Load the reaction plate into the thermal cycler, then start the run.

Read the plate

After PCR amplification, you perform an endpoint plate read on a real-time PCR instrument.

The system software uses the fluorescence measurements from each well made during the plate read, then plots R_n (signal) values. The software determines which alleles are in each sample for later genotyping analysis. Refer to the genotyping section of the appropriate instrument documentation for instructions on how to use the system software to perform the plate read and analysis.

Analyze the results

The system software records the results of the genotyping run on a scatter plot of Allele 1 versus Allele 2. Each well of the 96-well or 384-well reaction plate is represented as an individual point on the plot (for example, see Figure 2).

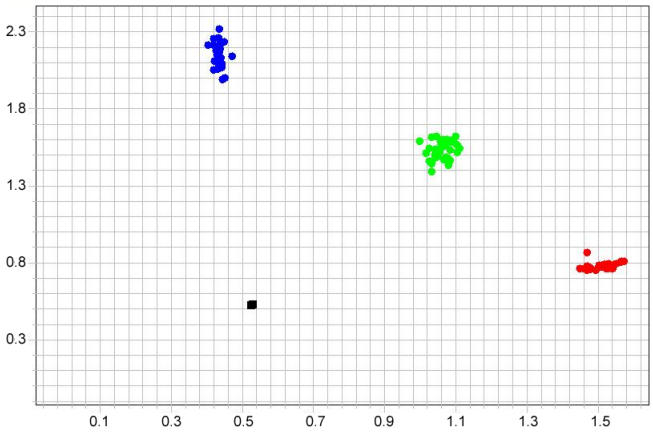


Figure 2 The clusters in the plot show the three genotypes of one SNP

Appendix A

Troubleshooting

This appendix divides the troubleshooting information according to application. Match your symptom with one of the observations below. Find the "Possible cause," then follow the "Recommended action."

Gene expression quantitation experiments:

Genotyping experiments:

Troubleshooting gene expression experiments

Table 5 Troubleshooting gene expression experiments

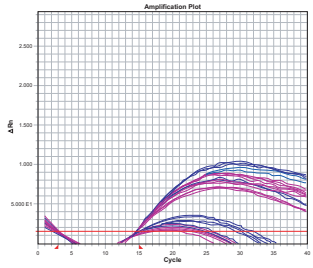
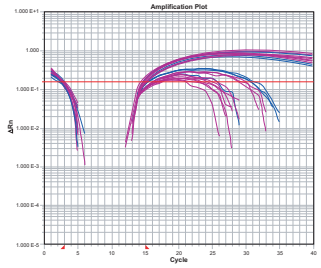
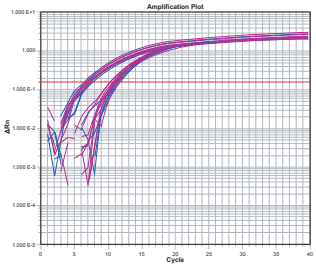
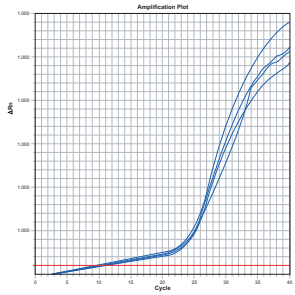
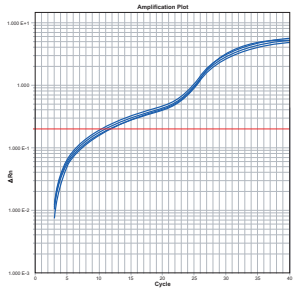
Observation	Possible cause	Recommendation
Amplification curve shows abnormal plot and/or low ΔR_n values. Linear view:  Log view: 	The baseline was set improperly (some samples have C_T values lower than the baseline stop value)	Refer to your real-time PCR system user guide for procedures on setting 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.

Table 5 Troubleshooting gene expression experiments *(continued)*

Observation	Possible cause	Recommendation
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.
Amplification curve shows weak amplification.	Sequence mismatches between target and assay sequences	Perform bioinformatics. For more information, refer to the: • *Custom TaqMan® Genomics Assays Protocol: Submission Guidelines* (PN 4367671) • *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial* (from **www.appliedbiosystems.com**)
	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. (Consider diluting the 60× TaqMan® Gene Expression Assay to a 20× working stock.)
	Degraded or contaminated template	• Improve the sample integrity (extraction methods). See "Perform reverse transcription" on page 8. • 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 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 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.) c. Rerun the assay with purified template. • Improve sample integrity (extraction methods). See "Perform reverse transcription" on page 8.
	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.
Amplification curve shows low ROX™ dye (passive reference dye).	Inaccurate pippetting: Little or no TaqMan® Universal Master Mix II	Follow accurate pipetting practices.

Table 5 Troubleshooting gene expression experiments *(continued)*

Observation	Possible cause	Recommendation
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® Gene Expression Assay, and TaqMan® Universal Master Mix II 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.
	Degraded template	• Determine the quality of the template. • Rerun the assay with fresh template. • Use RNase-free reagents. • Use an RNase inhibitor.
	Inhibitors 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.
	Assay design or synthesis failure: The wrong sequence was submitted to Applied Biosystems	• Verify that the sequence that you submitted is correct. • Check for an alternative transcript or a splice variant.
	Assay is designed in a variable region of the gene transcript	Verify that the location targeted by the assay is not within the 5′ untranslated region (UTR), which can be highly variable between transcripts. If the assay is designed within the 5′ UTR, select a different assay that is within the coding region of the transcript. Otherwise, select an assay for an alternative transcript or splice variant.
	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.

Table 5 Troubleshooting gene expression experiments *(continued)*

Observation	Possible cause	Recommendation
Amplification curve shows samples within 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 pippetting practices.
	Contamination	Be sure your workspace and equipment are properly cleaned.
Amplification curve shows no amplification of the sample (C_T = 40) in the target assay.	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.
	The gene is not expressed in the tested sample	• Verify by: – Rerunning the sample using the same assay – Running the sample using an alternative assay for the same gene • Verify the known expression of the gene in the sample type. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	The reaction may not have enough copies of the target gene	Verify by: • Rerunning the sample using the same assay • Rerunning the assay using more sample • Running the sample using an alternative assay for the same gene **Note:** If the recommended actions do not resolve the problem, the result may be correct.
Decrease in ROX™ dye fluorescence (passive reference dye).	Precipitation in the TaqMan® buffers	• Be sure to mix the tubes well. • Use TaqMan® Universal Master Mix II. Be sure to mix thoroughly to produce a homogenous solution.
	Degraded TaqMan® buffers	Verify that the kits have been stored according to the instructions on the packaging and have not expired.
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.
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 5 Troubleshooting gene expression experiments *(continued)*

Observation	Possible cause	Recommendation
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 UNG. • Run no-reverse-transcription controls to rule out genomic DNA contamination. • (*gDNA contamination only*) Design an assay that spans an exon-exon boundary.
Standard curve has a poor slope *or* poor correlation coefficient. Where: • Poor slope (a slope value of –3.32 equals approximately 100% efficiency) *or* • Poor correlation coefficient (the best correlation coefficient is 1.0).	Incorrect dilutions	• Redilute the samples. Ensure pipettes are calibrated. • Pipette more than 5 µL of sample.
	Inaccurate pipetting	• Check the calibration of the pipettes. • Pipette more than 5 µL of sample.
	Inhibitors 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.
	Improper reaction conditions	Follow the Applied Biosystems recommended thermal cycling profile.
	Inconsistent replicates (high standard deviation)	Make a master mix for each dilution point on the curve, then transfer to the reaction plate.
	Range of dilution points is too narrow	Increase the number of points and the logarithmic range.
	Incorrect baseline and threshold settings	Verify settings according to your real-time PCR system user documentation.
	(*Bad correlation coefficient only*) Improper mixing	• Increase the length of time that you mix the reagents. • Make a master mix for each dilution point on the curve, then transfer to the reaction plate.
Endogenous control C_Ts vary, or do not normalize the sample well.	Endogenous control is not consistently expressed across the samples	Selecting another 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.
Simultaneous increase in fluorescence from both the: passive reference ROX™ dye and the reporter dye(s).	Evaporation	Check the seal of the optical adhesive cover for leaks.

Table 5 Troubleshooting gene expression experiments *(continued)*

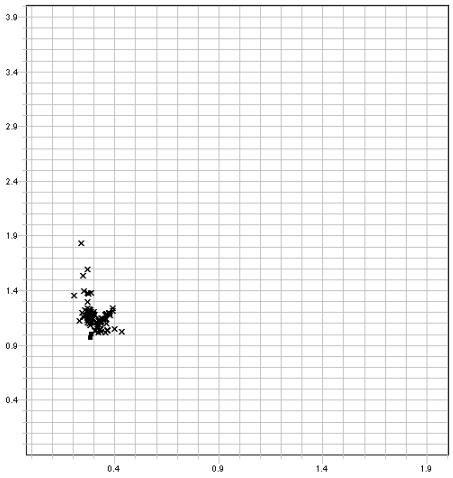
Observation	Possible cause	Recommendation
High standard deviation of replicates (inconsistent data, C_T varies).	Inefficient mixing of reagents	• Increase the length of time that you mix the reagents. • Make a master mix for each dilution point on the curve, then transfer to the reaction plate. • Validate 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 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	• Perform bioinformatics: Design the assay to span an exon-exon junction. For more information, refer to the: – *Custom TaqMan® Genomics Assays Protocol: Submission Guidelines* (PN 4367671) – *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial* (from **www.appliedbiosystems.com**) • Verify contamination by running an RT-minus reaction (without the reverse transcriptase). • Treat the sample with DNase.
	More sample added than expected	• Reduce the amount of sample. • Quantitate and normalize the sample.
	Template or amplicon contamination	Follow established PCR good laboratory practices.
Amplification occurs in the no RT controls.	gDNA contamination	• Perform bioinformatics: Design the assay to span an exon-exon junction. For more information, refer to the: – *Custom TaqMan® Genomics Assays Protocol: Submission Guidelines* (PN 4367671) – *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial* (from **www.appliedbiosystems.com**) • Improve sample extraction methods to eliminate gDNA. • Treat the sample with DNase.
	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.

Table 5 Troubleshooting gene expression experiments *(continued)*

Observation	Possible cause	Recommendation
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.
	Well is labeled with a detector/assay in the plate document/ experiment, but the well is empty	• Be sure your plate document/experiment is set up correctly. • Exclude the well and reanalyze the data.

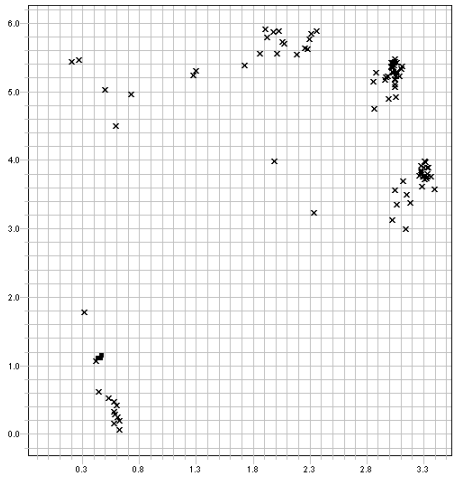
Troubleshooting genotyping experiments

Observation 1: No or low amplification



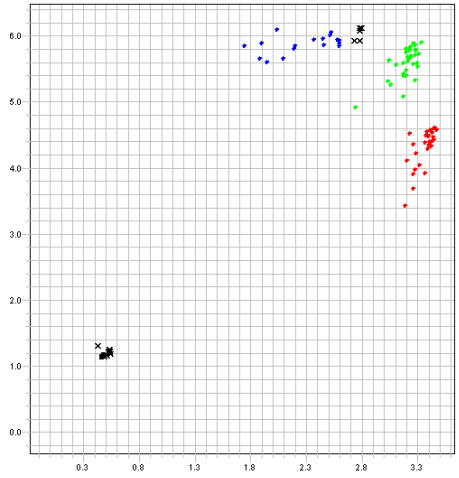
Possible cause		Recommendation
Samples	Sample degradation	Run an agarose gel to verify that DNA is degraded.
	Incorrect DNA quantitation (genomic only)	Perform concentration measurements.
	PCR inhibitors	Dilute the DNA sample.
	Too much or too little starting material	Titrate sample input for the DNA extraction step.
	Too little DNA was used for PCR	Perform another 10 PCR cycles, increase the DNA input for PCR, or perform preamplification reactions.
Reagents	Reagents expired or mishandled	Perform the assay again with newly prepared reagents. Ensure that storage conditions are correct.
	Reagents not added to a well	Visually inspect the well.
	Evaporation	Ensure that the reaction plate is sealed properly. Use a compression pad if recommended.
	Bubbles in the wells	Ensure that the reaction plate is centrifuged before thermal cycling.
	SNP is embedded in primer designs	Perform BLAST to verify that no SNP is in the primer region. If necessary, redesign the primer to avoid the SNP region.
Instrument	Wrong reporter dyes were chosen	Verify the dye settings and reanalyze the plate read.
	Thermal cycler is poorly calibrated	Check thermal-cycling conditions and make sure the thermal cycler is correctly calibrated.

Observation 2: No clusters



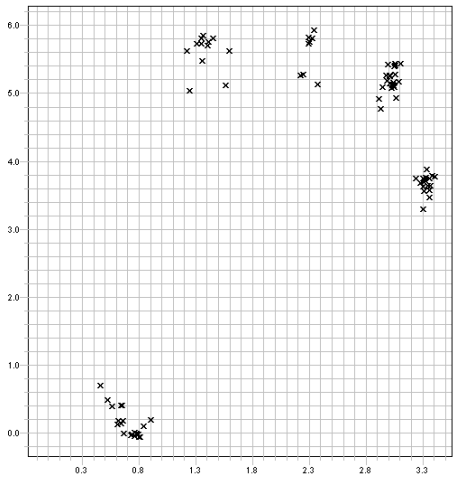
Possible cause		Recommendation
Samples	PCR inhibitors	Dilute the DNA sample.
	Too little DNA used for PCR	Perform another 10 PCR cycles, increase the DNA input for PCR, or perform preamplification reactions.
Instrument	Wrong reporter dyes chosen	Verify the dye settings and reanalyze the plate read.
	ROX™ dye is not selected	Ensure that the proper passive reference is selected.

Observation 3: Clusters too close



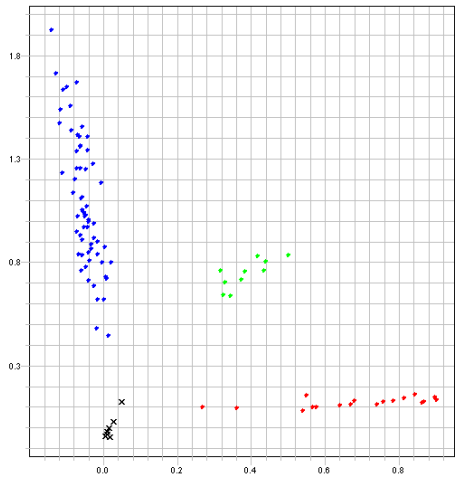
Possible cause		Recommendation
Samples	Sample degradation	Run an agarose gel to verify if DNA is degraded.
Reagents	Probe degradation	Perform the assay again with newly prepared reagents. Ensure that the reagents are stored correctly.
	Assay design	Verify that the probe designs are within good T_m range.
Instrument	Too many cycles run	If the reaction has been thermal cycling for more than 40 cycles, rerun the PCR with fewer cycles.

Observation 4: Too many clusters



Possible cause		Recommendation
Genetics	The probe sequence may contain a second SNP	Check the SNP database to see if an additional SNP has been discovered.
	Copy number: There are more than two copies of the SNP	Perform a copy number assay to determine the copy number. Perform comparative sequencing.
	SNP is multi-allelic	Perform comparative sequencing to verify the presence of more than two alleles. Repeat the experiment to verify that the performance is consistent.
Samples	Sample contamination	Check the performance of the samples in other assays to rule out problems caused by contamination or degradation.
Instrument	One marker is assigned to multiple assays	Ensure that you use only one marker per assay.

Observation 5: “Chicken-feet” clusters



Possible cause		Recommendation
Samples	Incorrect DNA quantitation	Perform concentration measurements.
	PCR inhibitors	Dilute the DNA sample.
	Variable sample input	Check the performance of the samples in other assays. Requantitate the DNA if applicable, or ensure that the sample input for DNA extraction is within the recommended range.
Reagents	Reagents expired or mishandled	Perform the assay again with newly prepared reagents. Ensure that the reagents are stored correctly.
	Reagents not added to the well	Visually inspect the well.
	Evaporation	Ensure that the reaction plate is sealed properly. If recommended, use a compression pad.
	ROX™ dye is not in the master mix	Use TaqMan® Universal Master Mix II or TaqMan® Genotyping Master Mix.
	Insufficient mixing of reagents	Ensure that the reagents are mixed properly, then rerun the reaction.
Instrument	Thermal cycler is poorly calibrated	Check the thermal-cycling conditions and make sure that the thermal cycler is correctly calibrated.
	ROX™ dye is not selected	Ensure that the proper passive reference is selected.

Ordering Information

How to order

The consumables and reagents in this appendix are for use with the TaqMan® Universal Master Mix II and can be ordered from the Applied Biosystems website.

This appendix contains ordering information for the following:

Real-time PCR systems, PCR systems, and consumables

The following table lists real-time PCR systems, thermal cyclers and consumables that can be used with TaqMan® Universal Master Mix II and TaqMan® assays. For a complete list of PCR systems and consumables, go to:
www.appliedbiosystems.com

System	Reaction plates and accessories
7300 system 7500 system	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film (PN 4311971) • MicroAmp® Optical Film Compression Pad (PN 4312639) • 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 • MicroAmp® Snap-On Optical Film Compression Pad (PN 4333292) for use with automation accessory
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 • MicroAmp® Snap-On Optical Film Compression Pad (PN 4333292) for use with automation accessory
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)

System	Reaction plates and accessories
9700 instrument	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • ABI PRISM® 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)
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)
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)

Gene expression assays and arrays products

The following gene expression products are available from Applied Biosystems. For a complete list of assays and arrays, go to: **www.appliedbiosystems.com**

Assay or array	For more information...
TaqMan® Express Plates‡	**www.allgenes.com**
TaqMan® MicroRNA Assays	**miRNA.appliedbiosystems.com**
Custom TaqMan® Small RNA Assays	Contact Applied Biosystems Sales
Custom TaqMan® Probes and Primers§	**www.appliedbiosystems.com**
TaqMan® Arrays: • TaqMan® Custom Arrays • TaqMan® Gene Signature Array • TaqMan® Gene Sets	**taqmanarray.appliedbiosystems.com**
Megaplex™ Pools for microRNA Expression Analysis: • Megaplex™ RT Primers • Megaplex™ PreAmp Primers • TaqMan® MicroRNA Arrays	**miRNA.appliedbiosystems.com**

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

Reverse transcription kits and reagents

To obtain cDNA from RNA samples, Applied Biosystems recommends the reverse transcription kits listed in the following table. For a complete list of kits and reagents, go to: **www.appliedbiosystems.com**

Kit	Source
High Capacity RNA-to-cDNA™ Kit, 50 rxns (PN 4387406)	Applied Biosystems
TaqMan® RNA-to-C_T™ 2-Step Kit • TaqMan® RNA-to-C_T™ 2-Step Kit, Mini Pack (PN 4399902) • TaqMan® RNA-to-C_T™ 2-Step Kit, 1-Pack (PN 4399367)	Applied Biosystems
TaqMan® MicroRNA Reverse Transcription Kit • 1000 reactions (PN 4366597) • 200 reactions (PN 4366596)	Applied Biosystems

Optional user-supplied reagents for gene expression quantitation

For a description of these reagents, go to: **www.ambion.com/techlib/index**

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

Consumables and equipment

The following includes required and optional laboratory equipment and materials. Unless otherwise noted, many items listed are available from major laboratory suppliers.

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

‡ Other vendors supply similar products.
§ Micronic BV, PO Box 604 8200, AP Lelystad, Netherlands: Telephone: 0031.320.277.090, Fax: 0031.320.277.088; United States: Telephone: 724.941.6411, Fax: 724.941.8662; Website: **www.micronic.com**
# Major laboratory supplier (MLS).
‡‡ For the MSDS of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

PCR Good Laboratory Practices

Sample preparation

When preparing samples for PCR amplification:

- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Centrifuge tubes before opening. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution. Use DNA*Zap*™ Solution (PN AM9890).

Preventing contamination

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

False positives

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

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

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

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

Uracil-N glycosylase (UNG)

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

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

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

Prevention of PCR product carryover

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

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

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

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

Fluorescent contaminants

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

About two-step RT-PCR

Gene quantitation assays using TaqMan® Universal Master Mix II and TaqMan Gene Expression Assays are performed in a two-step RT-PCR:

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

The figure below illustrates two-step PCR.

Note: Figure 3 does not show hybridization of the TaqMan® MGB probe. See Figure 5 on page 59 for details on how the TaqMan MGB probe is used in the PCR step.

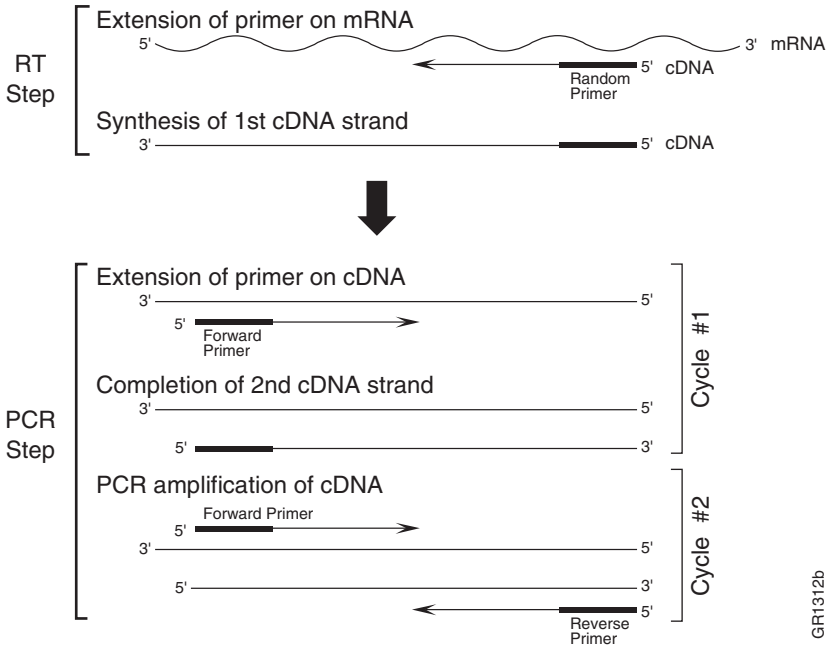


Figure 3 Two-step RT-PCR

About TaqMan® MGB Probes

The TaqMan® MGB probes contain:

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

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

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

About AmpliTaq Gold® DNA Polymerase, (UP) Ultra Pure

The AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure) enzyme is identical to AmpliTaq Gold® DNA Polymerase, but the enzyme is further purified through a proprietary process to reduce bacterial DNA introduced from the host organism. The purification process ensures that non-specific, false-positive DNA products due to bacterial DNA contamination are minimized during PCR.

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

About uracil-N glycosylase

Uracil-N glycosylase (UNG) treatment can prevent the reamplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded amplicons. (Longo et al., 1990). UNG prevents reamplification of carryover-PCR products in an assay if all previous PCR for that assay was performed using a dUTP-containing master mix. See "Preventing contamination" on page 54 for more information about UNG.

About ROX passive reference

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

About the 5′ nuclease assay

The 5′ nuclease assay process (Figure 5 through Figure 8) takes place during PCR amplification. This process occurs in every cycle and does not interfere with the exponential accumulation of product.

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

Figure 4 Legend for Figure 5 through Figure 8

During PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (Figure 5).

When the probe is intact (Figure 5 and Figure 6), 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).

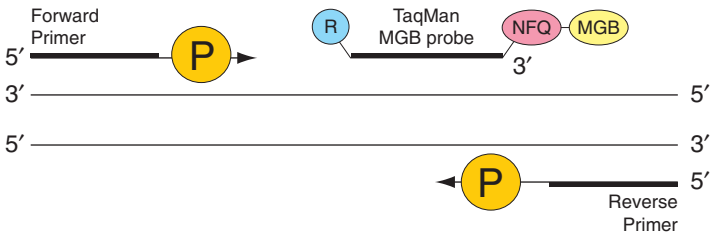


Figure 5 Polymerization

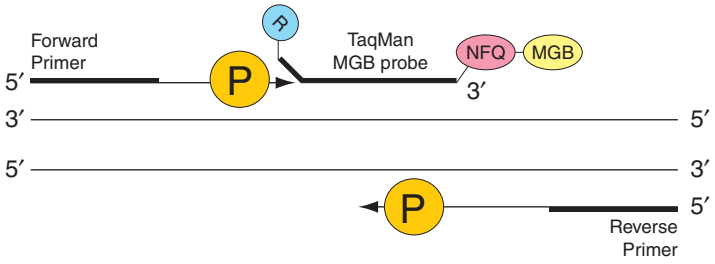


Figure 6 Strand displacement

The DNA polymerase cleaves only probes that are hybridized to the target (Figure 7). 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.

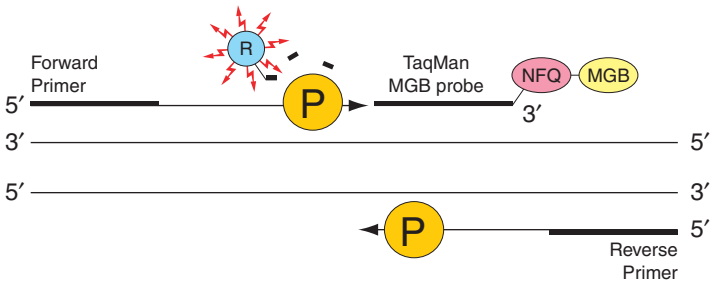


Figure 7 Cleavage

Polymerization of the strand continues, but because the 3′ end of the probe is blocked, no extension of the probe occurs during PCR (Figure 8).

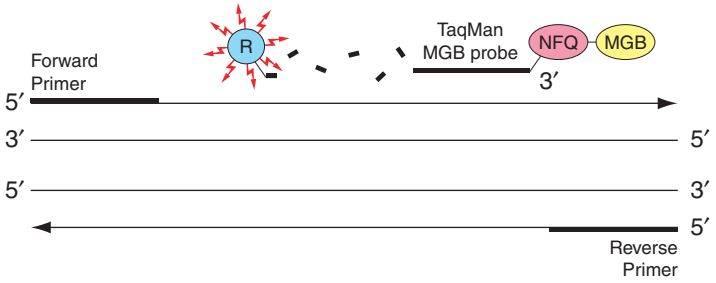


Figure 8 Completion of polymerization

Appendix E

Safety

This appendix covers:

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) 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 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" on page 63.)
- 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.

MSDSs

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 choose

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

Chemical waste safety

Chemical waste hazards

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

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

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

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

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

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

Additional information about biohazard guidelines is available at:

www.cdc.gov

Chemical alerts

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

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.

Förster, V.T. 1948. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Annals of Physics (Leipzig)* 2:55–75.

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339:237–238.

Lakowicz, J.R. 1983. *Principles of Fluorescence Spectroscopy*, ed. New York: Plenum Press. xiv, 496 pp.

Longo, M.C., Berninger, M.S., and Hartley, J.L. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 93:125–128.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.

Saiki, R.K., Scharf, S., Faloona, F., et al. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230:1350–1354.

Related documentation

Applied Biosystems documents

You can download the documents in Tables 6, 7, 8, and 9 from the Applied Biosystems Web site at: **http://docs.appliedbiosystems.com/search.taf**

Table 6 TaqMan® Universal Master Mix II documentation

Document	Part number
TaqMan® Universal Master Mix II Protocol	4428173
TaqMan® Universal Master Mix II Quick Reference Card	4428174

Table 7 Resources for gene expression quantitation experiments

System	Document	Part number
Applied Biosystems 7900HT Fast Real-Time PCR System (and SDS Enterprise Database)	*Absolute Quantification Getting Started Guide*	4364014
	Relative Quantification Getting Started Guide	4364016
	User Bulletin: Performing Fast Gene Quantification	4352533
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System	*Absolute Quantification Getting Started Guide*	4347825
	Relative Quantification Getting Started Guide	4347824
	Getting Started Guide for Standard Curve Experiments	4387779
	Getting Started Guide for Comparative C_T/Relative Standard Curve Experiments	4387783
Applied Biosystems StepOne™ or StepOnePlus™ Real-Time PCR System	*Getting Started Guide for Standard Curve Experiments*	4376784
	Getting Started Guide for Comparative C_T/Relative Standard Curve Experiments	4376785
All	*Real-Time PCR Systems Chemistry Guide*	4348358
	TaqMan® Gene Expression Assays Protocol	4333458
	TaqMan® Gene Expression Assays Quick Reference Card	4401212

Table 8 Resources for microRNA quantitation experiments

System	Document	Part number
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	*Absolute Quantification Getting Started Guide*	4347825
	Getting Started Guide for Standard Curve Experiments	4387779
Applied Biosystems StepOne™ or StepOnePlus™ Real-Time PCR System	*Getting Started Guide for Standard Curve Experiments*	4376784
All	*Real-Time PCR Systems Chemistry Guide*	4348358
	TaqMan® MicroRNA Reverse Transcription Kit Protocol	4367038

Table 9 Resources for genotyping experiments

System	Document	Part number
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System	*Allelic Discrimination Getting Started Guide*	4347822
	Getting Started Guide for Genotyping Experiments	4387784
Applied Biosystems 7900HT Fast Real-Time PCR System (and SDS Enterprise Database)	*User Guide*	4351684
GeneAmp® PCR System 9700	GeneAmp® PCR System 9700 *Base Module* User Guide	4303481
	GeneAmp® PCR System 9700 *96-Well Sample Block Module* User Guide	4316011
	GeneAmp® PCR System 9700 *Dual 384-Well Sample Block Module* User Guide	4304215
	GeneAmp® PCR System 9700 *0.5-mL Sample Block Module* User Guide	4307808
	GeneAmp® PCR System 9700 *Auto-Lid Dual 96 Sample Block Module and Dual 96-Well Sample Block Module* User Guide	4343363
	GeneAmp® PCR System 9700 *Auto-Lid Dual 384 Sample Block Module* User Guide	4310838
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems	*Getting Started Guide for Genotyping Experiments*	4376786
Applied Biosystems Veriti® Thermal Cycler	*User Guide*	4375799
All	*Applied Biosystems TaqMan® Sample-to-SNP™ Kit Protocol*	4402136
	Applied Biosystems TaqMan® Sample-to-SNP™ Quick Reference Card	4402745
	Real-Time PCR Systems Chemistry Guide	4348358
	TaqMan® SNP Genotyping Assays Protocol	4332856

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

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.

ΔR_n value

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

The following equation expresses the relationship of these terms:

$\Delta R_n = (R_n+) - (R_n–)$

where:

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

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

C_T

See threshold cycle (C_T).

fold difference

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

full replicate

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

minimum fold difference

The smallest fold difference that is statistically significant.

multicomponenting

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

normalization

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

replicate, technical or PCR

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

R_n+

The R_n value of a reaction containing all components including the template.

Normalization is accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the R_n (normalized reporter) for a given reaction tube.

R_n–	The R_n value of an unreacted sample. This value may be obtained from the early cycles of a real-time run (those cycles prior to a detectable increase in fluorescence). This value may also be obtained from a reaction not containing template.
statistically significant (to a 99.7% confidence level)	A result with a low probability (0.3%) of resulting from chance.
threshold cycle (C_T)	The PCR cycle number at which the fluorescence meets the threshold in the amplification plot.

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