USER GUIDE

applied biosystems® by life technologies™

TaqMan® Universal PCR Master Mix

Catalog Numbers 4324018, 4364341, 4364343, 4324020, 4326614, 4304437, 4364338, 4364340, 4305719, 4318157, 4326708

Publication Number 4304449

Revision E

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

life technologies™

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

The information in this guide is subject to change without notice.

Important Licensing Information:

This product may be covered by one or more Limited Use Label Licenses. By use of this product, you accept the terms and conditions of all applicable Limited Use Label Licenses.

DISCLAIMER

LIFE TECHNOLOGIES CORPORATION AND/OR ITS AFFILIATE(S) DISCLAIM ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NON-INFRINGEMENT. TO THE EXTENT ALLOWED BY LAW, IN NO EVENT SHALL LIFE TECHNOLOGIES AND/OR ITS AFFILIATE(S) BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF.

TRADEMARKS

Contents

About this guide

Revision history

Revision	Date	Description
E	March 2014	Update to genotyping content to include: • TaqMan® SNP Genotyping PCR • TaqMan® Drug Metabolism PCR Updated to current style and format.
D	July 2010	

Purpose of the guide

This user guide describes the two primary applications of the TaqMan® Universal PCR Master Mix: quantitative RT-PCR and genotyping. Although TaqMan® Universal PCR Master Mix can be used in a broad variety of PCR applications, this document primarily describes the use of the master mix with pre-optimized TaqMan® assays. General guidelines are provided for the design and optimization of custom quantitation and allelic discrimination (genotyping) assays.

Because analysis methods vary greatly between applications, this user guide provides general guidelines for the analysis of data generated from experiments that use TaqMan® Universal PCR Master Mix and TaqMan® assays. For detailed information about data analysis or the procedures outlined in this protocol, consult the appropriate documentation for your instrument.

User attention words

Two user attention words may appear in this document. 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 or accurate chemistry kit use.

1 Product Information

Product description

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

This document describes the use of the TaqMan® Universal PCR Master Mix with pre-optimized:

- TaqMan® Gene Expression Assays
- TaqMan® MicroRNA Assays
- TaqMan® Drug Metabolism Genotyping Assays
- TaqMan® SNP Genotyping Assays
- Custom TaqMan® Probes and Sequence Detection Primers

to amplify your DNA target of choice. Guidelines are also provided for the design and optimization of custom primers and TaqMan® probes.

In RNA quantitation assays the TaqMan® Universal PCR Master Mix is used in the second step of a 2-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 Life Technologies.

Kit contents and storage

The TaqMan® Universal PCR Master Mix is supplied at 2X 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 PCR Master Mix, no AmpErase® UNG, contains all the above ingredients except UNG.

TaqMan® Universal PCR Master Mix, with or no AmpErase® UNG, is supplied at 2X concentration and is available in the following volumes:

Master Mix	Catalog Number	Quantity	# of reactions		Storage
			20–µL	50-µL	
TaqMan® Universal PCR Master Mix	4304437, 1–Pack	1 × 5 mL	500	200	2°C to 8°C.
	4364338, 2–Pack	2 × 5 mL	1000	400	
	4364340, 5–Pack	5 × 5 mL	2500	1000	
	4305719, 10–Pack	10 × 5 mL	5000	2000	
	4318157, 10 Unit Pack	10 × 5 mL	5000	2000	
	4326708, 1 Bulk Pack	1 × 50 mL	5000	2000	
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	4324018, 1–Pack	1 × 5 mL	500	200	2°C to 8°C.
	4364341, 2–Pack	2 × 5 mL	1000	400	
	4364343, 5–Pack	5 × 5 mL	2500	1000	
	4324020, 10 Unit Pack	10 × 5 mL	5000	2000	
	4326614, 1 Bulk Pack	1 × 50 mL	5000	2000	

Compatible real-time instruments

The TaqMan® Universal PCR Master Mix, with or without AmpErase® UNG, 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 available from Life Technologies:

- 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® ViiA™ 7 Real-Time PCR System
- QuantStudio™ 6 Flex Real-Time PCR System
- QuantStudio™ 7 Flex Real-Time PCR System
- QuantStudio™ 12k Flex System

Fast instruments and thermal cycling conditions

IMPORTANT! Use TaqMan® Universal PCR Master Mix with Standard mode thermal cycling conditions only. TaqMan® Universal PCR Master Mix is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan® Universal PCR Master Mix on the StepOne™, StepOnePlus™, 7500 Fast, 7900HT Fast, ViiA 7 Fast 96-well, or QuantStudio Fast 96-well 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.

Materials required but not included

Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Materials		Catalog no.
High Capacity RNA-to-cDNA™ Kit, 50 rxns		4387406
SuperScript® VILO™ cDNA Synthesis Kit	50 reactions	11754050
	250 reactions	11754250
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® SNP Genotyping Assays	Small-scale (40X, 300 × 25-µL rxns)	4351379
Custom TaqMan® SNP Genotyping Assays	Small-scale (40X, 300 × 25-µL rxns)	4331349
TaqMan® Drug Metabolism Genotyping Assays	Small-scale (20X, 150 × 25-µL rxns)	4362691
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

2 Gene expression quantitation

This chapter provides a protocol for performing 2-step reverse transcription polymerase chain reaction (RT-PCR) using TaqMan® Universal PCR Master Mix with TaqMan® Gene Expression Assays, Custom TaqMan® Gene Expression Assays, or Custom TaqMan® Probes and Sequence Detection Primers.

Reverse transcription

Synthesis of single-stranded cDNA from RNA is the first step in the two-step 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 we recommend using a Life Technologies reverse transcription kit.

For additional RT guidelines and instructions, refer to the appropriate protocol. You can download the protocols for Life Technologies kits at: **www.lifetechnologies.com**.

Note: For additional documentation, see "Obtaining support" on page 70.

RNA template guidelines

The quality of your results is directly related to the purity of your RNA template. For optimal performance use RNA that is:

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

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.

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 PCR Master Mix) 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 "Custom TaqMan® assay design" on page 47 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 PCR Master Mix thoroughly by swirling the bottle.
  - Thaw frozen TaqMan® assays by placing them on ice. When thawed, resuspend the samples by vortexing, then briefly centrifuge to collect.
  - Thaw frozen samples by placing them on ice. When thawed, resuspend the samples by briefly vortexing and then centrifuge the tubes to collect.

Determine the number of required reactions

Determine the number of reactions to perform for each assay. We recommend performing four replicates of each reaction. Include 10% extra volume to compensate for the volume loss that occurs during pipetting. 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.
- 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.

Set up plate document or experiment

Refer to your instrument user guide for instructions on how to configure the plate document or experiment. See "Obtaining support" on page 70 to obtain user documentation for Life Technologies real-time PCR systems.

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

- Thermal Cycling Parameters:

Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temperature	50°C	95°C	95°C	60°C
Time (mm:ss)	02:00	10:00	00:15	01:00

† Required for optimal UNG activity. If using TaqMan® Universal PCR Master Mix, no AmpErase® UNG, this step is not necessary.

‡ Required to activate the DNA Polymerase.

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

- Run Mode: **Standard mode**
- Sample Volume:

Plate format	Reaction volume (µL)
MicroAmp® Optical 96-Well Reaction Plate	20–50
MicroAmp® Optical 384-Well Reaction Plate	10–20

- 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)

IMPORTANT! TaqMan® Universal PCR Master Mix is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan® Universal PCR Master Mix on the StepOne™, StepOnePlus™, 7500 Fast, 7900HT Fast, ViiA 7 Fast 96-well, or QuantStudio Fast 96-well instruments, use Standard mode thermal cycling conditions.

Prepare and run 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 PCR Master Mix (2X)	25.0	10.0	1X
TaqMan® Gene Expression Assay (20X)†	2.5	1.0	1X
cDNA template + H_2O‡	22.5	9.0	1–100 ng
Total Volume	**50.0**	**20.0**	**—**

† See **www.LifeTechnologies.com** for TaqMan® Gene Expression Assay information.

‡ Use 1–100 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.
   - We recommend performing four technical 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.

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

2. Cap the tube(s) and vortex briefly to mix the solutions.
3. Centrifuge the tube(s) briefly to spin down the contents and eliminate any air bubbles from the solutions.
4. Transfer the appropriate volume of each reaction mixture to each well of an optical reaction plate.
5. Cover the plate with a MicroAmp® Optical Adhesive Film. For standard 96-well plates, you may use MicroAmp® Optical Caps.
6. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.
7. Apply a compression pad to the plate if required by your real-time PCR system.
8. In the system software, open the plate document or experiment that corresponds to the reaction plate.
9. Load the reaction plate into the real-time PCR system.
10. Start the run.

3 Data Analysis

This chapter provides general guidelines for analyzing data obtained from gene expression assays.

Overview

Data analysis varies depending on the product, assay and real-time PCR system used. Refer to the appropriate PCR instrument user guide for detailed analysis instructions. See "Documentation and Support" on page 69 for more information.

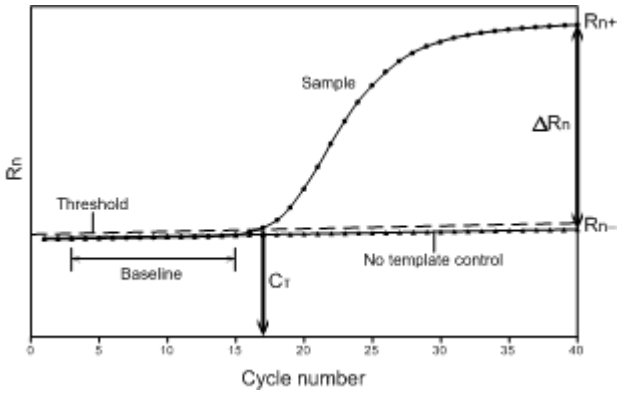
The general process for analyzing gene expression data involves:

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

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.



Normalization

The Passive Reference is a dye included in the TaqMan® Universal PCR Master Mix and 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.

R_n and ΔR_n values

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 Rn (normalized reporter) for a given reaction tube.

R_n^+ is the R_n value of a reaction containing all components including the template.

R_n^- is 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.

ΔR_n is 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:

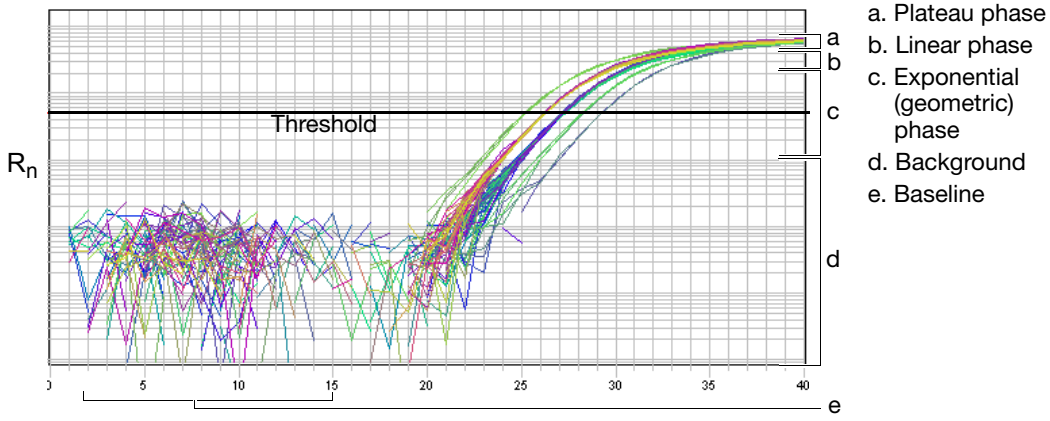
$$R_n^+ = \frac{\text{Emission Intensity of Reporter}}{\text{Emission Intensity of Passive Reference}}$$ PCR with template

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

Automatic calculation of the baseline and threshold

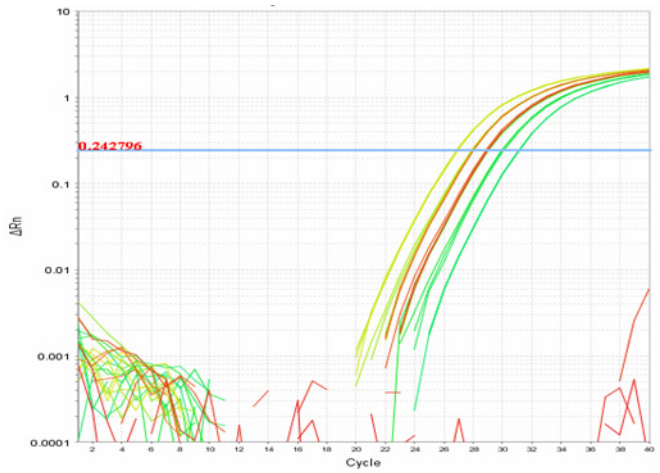
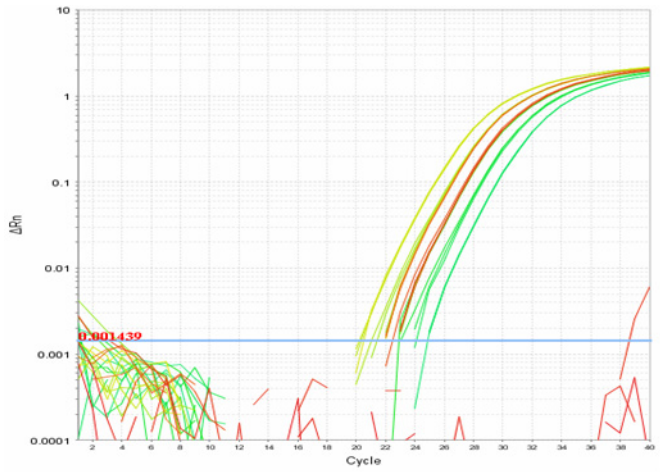
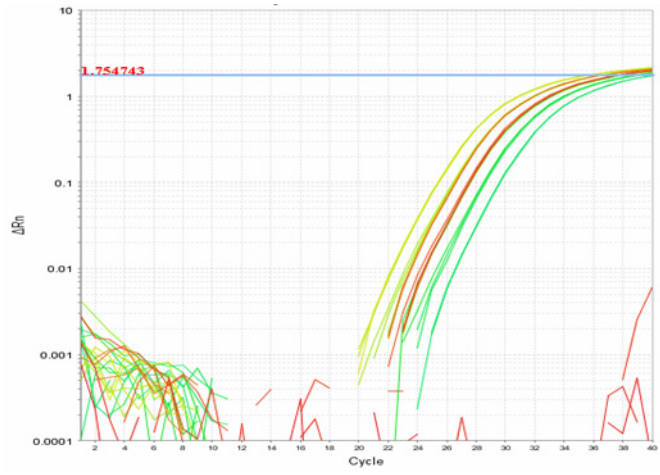
The system software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the "typical" amplification curve shown below. Experimental error (such as contamination or pipetting errors) can produce atypical data that can cause the software algorithm to generate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

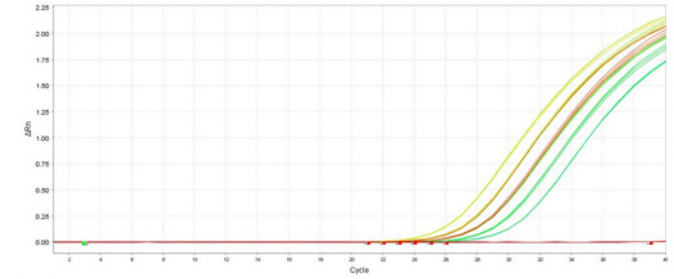
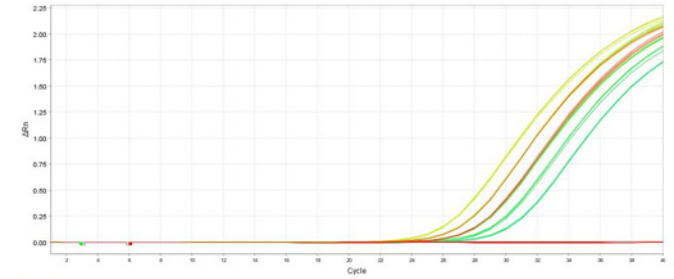
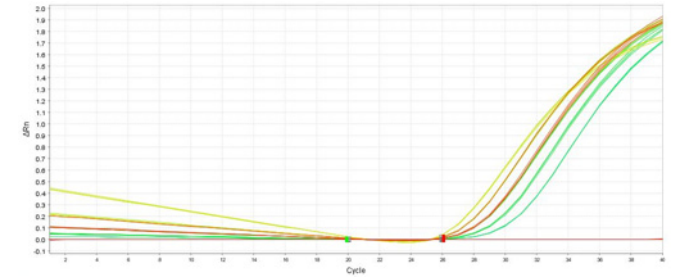
IMPORTANT! After an analysis, verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots, and adjust the values manually if necessary.



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.

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.	

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 PCR Master Mix:

- 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 Primer Express® Software amplicon design criteria.

4 Genotyping

This chapter describes how to perform genotyping of single nucleotide polymorphisms (SNPs) within genomic DNA (gDNA) using TaqMan® Universal PCR Master Mix with any TaqMan® genotyping assay.

Before you begin

Review Appendix B, "PCR Good Laboratory Practices" on page 39.

Quantitate the DNA

For a genotyping assay, add 1–10 ng of gDNA template per reaction well. To quantitate gDNA, use a reliable method such as absorbance (A_{260}/A_{280}) measurements or real-time quantification by RNase P. If using the RNase P method, generate a standard curve using the DNA template standards in the TaqMan® DNA Template Reagents Kit (Cat. no. 401970) and the RNase P gene primers and probe in the TaqMan® RNase P Detection Reagents Kit (Cat. no. 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! Multiple genotyping assays can be run on one reaction plate. Include controls for each assay run on the 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 reaction 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. Thaw frozen genomic DNA sample(s) by placing on ice. After the samples thaw, mix if needed by vortexing, then centrifuge the tubes briefly to collect.
2. Thaw frozen TaqMan® assay(s) by placing on ice. Vortex the tubes to mix, then centrifuge briefly to collect.
3. Thoroughly mix the TaqMan® Universal PCR Master Mix by swirling the bottle.
4. In an appropriate volume tube, combine the PCR reaction mix components as outlined in the following tables.

Component	Volume (µL) per reaction		
	5-µL rxn	10-µL rxn	25-µL rxn
TaqMan® Universal PCR Master Mix (2X)	2.5	5.0	12.5
TaqMan® genotyping assay mix (20X)†	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 40X and 80X Assay Mixes to 20X working solutions with 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Use DNase-free water.

Recommended volumes according to instrument		
System	**Plate well volume**	**Reaction volume per well**
StepOne™, StepOnePlus™	48 wells, 0.1 mL	10–30 µL
7300, 7500, 7900HT	96 wells, 0.2 mL	20–50 µL
7500 Fast, 7900HT Fast	96 wells, 0.1 mL	10–30 µL
7900HT (384 block)	384 wells, 0.02 mL	5–20 µL
ViiA™ 7	96 wells, 0.1 mL 96 wells, 0.2 mL 384 wells, 0.02 mL	10–30 µL 20–50 µL 5–20 µL
QuantStudio™ (6 Flex, 7 Flex & 12k Flex)	96 wells, 0.1 mL 96 wells, 0.2 mL 384 wells, 0.02 mL	10–30 µL 20–50 µL 5–20 µL

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

6. Pipet the PCR reaction mix volume (4, 8, or 20 µL) appropriate to your plate, into each well of a reaction plate.
7. Pipet the appropriate volume containing 1–10 ng of sample or control DNA for each reaction into the appropriate well(s) as outlined in the following table.

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

8. Seal the plate using MicroAmp® Optical Adhesive Film or MicroAmp® Optical Caps. Invert the plate 3–5 times to mix (alternatively vortex briefly at low speed), then briefly centrifuge the plate to collect the contents and eliminate air bubbles.
9. Apply a compression pad to the plate if required by your real-time PCR system.
10. Load the plate into a real-time PCR system.

Perform TaqMan® SNP Genotyping PCR

IMPORTANT! TaqMan® Universal PCR Master Mix is not supported for use with Fast Mode thermal cycling conditions. When using TaqMan® Universal PCR Master Mix on the StepOne™, StepOnePlus™, 7500 Fast, 7900HT Fast, ViiA 7 Fast 96-well, Veriti® 96-well Fast, or QuantStudio Fast 96-well instruments, use Standard mode thermal cycling conditions. If you use assays other than the TaqMan® assays, or use thermal cycling instruments and conditions other than those specified in this protocol, validate your assays and re-optimize your thermal cycling conditions as needed.

Note: Genotyping PCR reactions can be carried out on non real-time thermal cycling instruments (e.g., ProFlex™ or Veriti® thermal cycler) with subsequent endpoint plate read fluorescent determinations made on a real-time PCR instrument. Real-time PCR data is not necessary but may be helpful when troubleshooting.

1. Set up the following run conditions:

Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temperature(°C)	50°C	95°C	95°C	60°C
Time (mm:ss)	02:00	10:00	00:15	01:00

† Required for optimal UNG activity. If using TaqMan® Universal PCR Master Mix, no AmpErase® UNG, this step is not necessary

‡ Required to activate the DNA Polymerase.

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

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

Perform TaqMan® Drug Metabolism Genotyping PCR

TaqMan® Drug Metabolism Genotyping Assays use a 90 second extension time and 50 cycles because the average amplicon size is longer than that of most TaqMan® SNP Genotyping Assays.

1. Set up the following run conditions:

Parameter	UNG incubation†	Polymerase activation‡	PCR (50 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temperature(°C)	50°C	95°C	95°C	60°C
Time (mm:ss)	02:00	10:00	00:15	01:30

† Required for optimal UNG activity. If using TaqMan® Universal PCR Master Mix, no AmpErase® UNG, this step is not necessary.

‡ Required to activate the DNA Polymerase.

   - Run speed: Standard
   - Reaction volume:

Plate format	Reaction volume
MicroAmp® Optical 96-Well ReactionPlate	25 µL/well
MicroAmp® Optical 384-Well ReactionPlate	5 µL/well

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

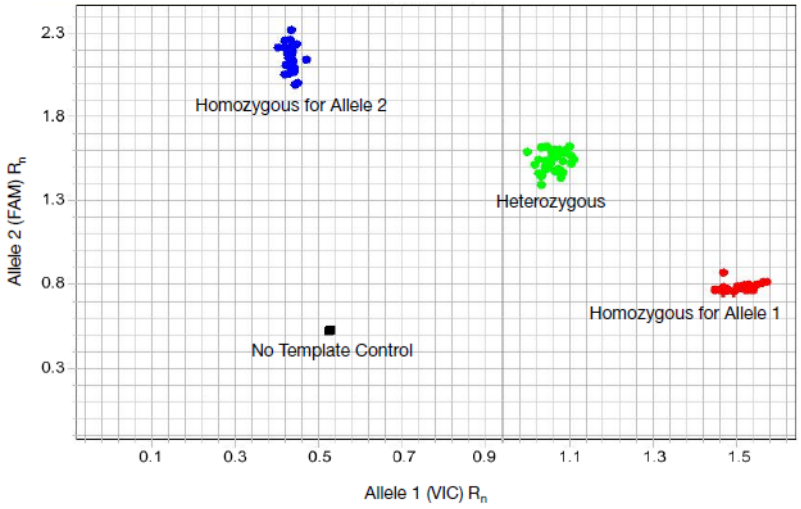
Read the plate

After PCR amplification, 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 (VIC® dye) versus Allele 2 (FAM™ dye). Each well of the 96-well or 384-well reaction plate is represented as an individual point on the plot. The clusters in the plot show the three genotypes of one SNP.



A

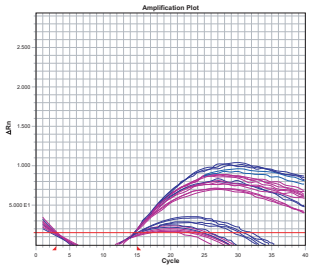
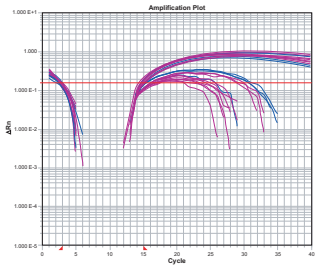
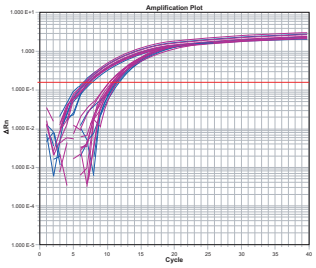
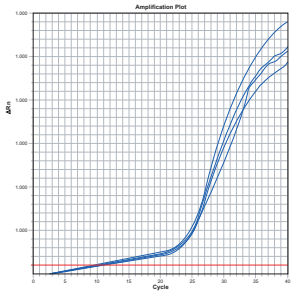
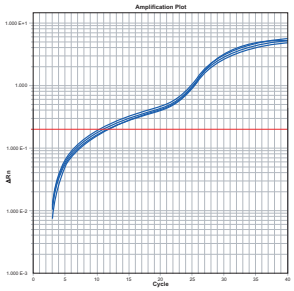
Troubleshooting

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

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.

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: • *Custom TaqMan® Assays Design and Ordering Guide* (Pub. no. 4367671) • *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays* (Pub. no. 4371002)
	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 60X TaqMan® Gene Expression Assay to a 20X working stock.)
	Degraded or contaminated template	• Improve the sample integrity (extraction methods). See "Reverse transcription" on page 13. • 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 "Reverse transcription" on page 13.
	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 pipetting: Little or no TaqMan® Universal PCR Master Mix	Follow accurate pipetting practices.

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 PCR Master Mix were added to the reaction plate. (If the master mix is missing, the passive reference fails.)
	Incorrect dye components were selected	Check the dye components settings and reanalyze the data.
	The annealing temperature on the thermal cycler was too high for the primers and/or probe	Verify that the thermal cycler is set to the correct annealing and extension temperatures. Ensure that the thermal cycler is calibrated and maintained regularly.
	Inappropriate reaction conditions were used	Troubleshoot the RT-PCR optimization.
	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.

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 PCR Master Mix. 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.

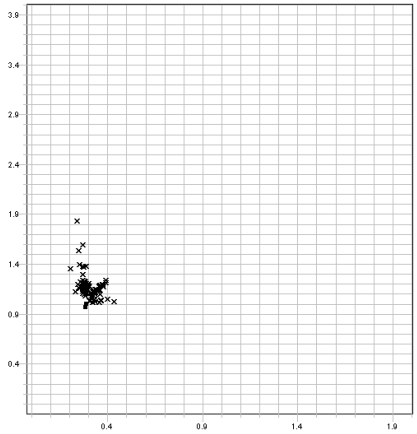
Observation	Possible cause	Recommendation
No template control (NTC) shows amplification.	Contaminated reagents (contaminated with gDNA, amplicon, or plasmid clones)	• Rerun the assay using new reagents. • Be sure your workspace and equipment are cleaned properly. • Use UNG. • Run no-reverse-transcription 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.

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 assay to span an exon-exon junction. For more information, refer to: – *Custom TaqMan® Assays Design and Ordering Guide* (Pub. no. 4367671) – *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays* (Pub. no. 4371002) • 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 assay to span an exon-exon junction. For more information, refer to: – *Custom TaqMan® Assays Design and Ordering Guide* (Pub. no. 4367671) – *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays* (Pub. no. 4371002) • 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.

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.

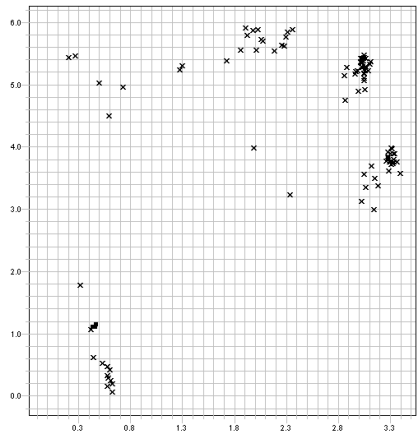
Troubleshooting genotyping experiments

Observation 1: No or low amplification



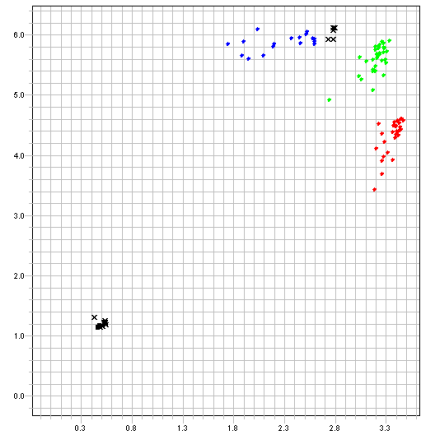
Possible cause		Recommendation
Samples	Sample degradation	Run an agarose gel to verify that DNA is degraded.
	Incorrect DNA quantitation (genomic only)	Perform concentration measurements.
	PCR inhibitors	Dilute the DNA sample.
	Too much or too little starting material	Titrate sample input for the DNA 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



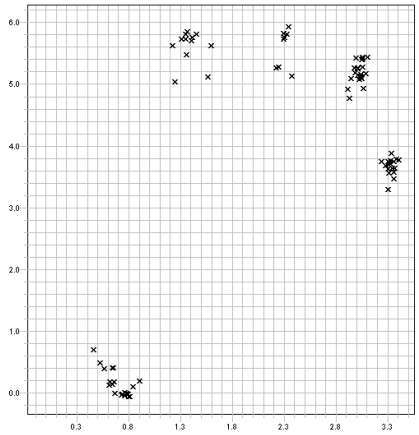
Possible cause		Recommendation
Samples	PCR inhibitors	Dilute the DNA sample.
	Too little DNA used for PCR	Perform another 10 PCR cycles, increase the DNA input for PCR, or perform preamplification reactions.
Instrument	Wrong reporter dyes chosen	Verify the dye settings and reanalyze the plate read.
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

Observation 3: Clusters too close



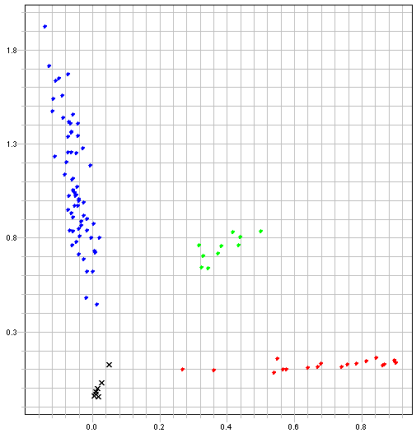
Possible cause		Recommendation
Samples	Sample degradation	Run an agarose gel to verify if DNA is degraded.
Reagents	Probe degradation	Perform the assay again with newly prepared reagents. Ensure that the reagents are stored correctly.
	Assay design	Verify that the probe designs are within good T_m range.
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 PCR Master Mix 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.

B 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 (Cat. no. 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 PCR Master Mix is a pure, nuclease-free, 26-kDa recombinant enzyme encoded by the Escherichia coli uracil-N glycosylase gene which has been inserted into an E. coli host to direct the expression of the native form of the enzyme (Kwok and Higuchi, 1989).

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

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

Prevention of PCR product carryover

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

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

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

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

Fluorescent contaminants

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

C

Chemistry Overview

About two-step RT-PCR

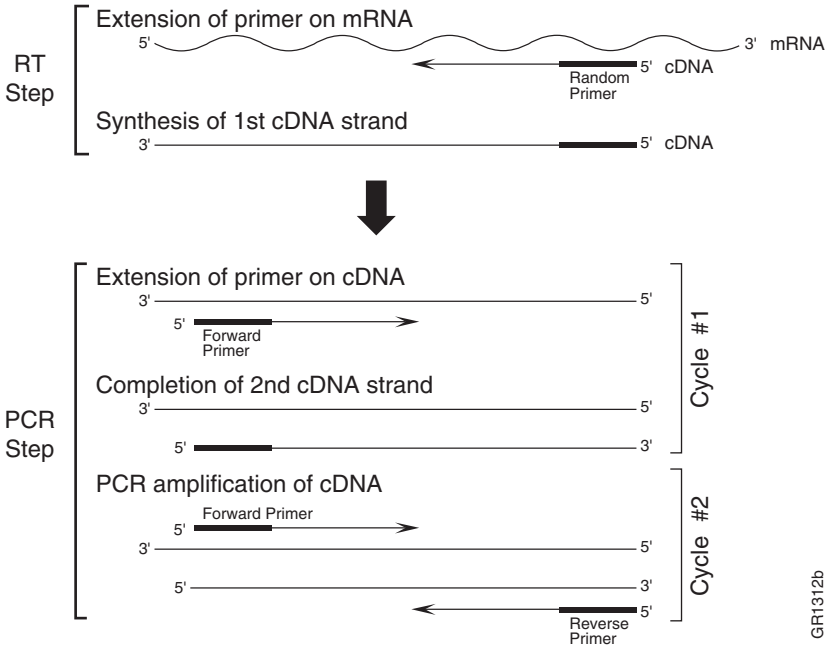
Gene quantitation assays using TaqMan® Universal PCR Master Mix and TaqMan® Gene Expression Assays are performed in a two-step RT-PCR:

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 PCR Master Mix.

The figure below illustrates two-step PCR.

Note: Figure 1 does not show hybridization of the TaqMan® MGB probe. See , "Basics of the 5′ nuclease assay" on page 44 for details on how the TaqMan® MGB probe is used in the PCR step.

Figure 1 Two-step RT-PCR



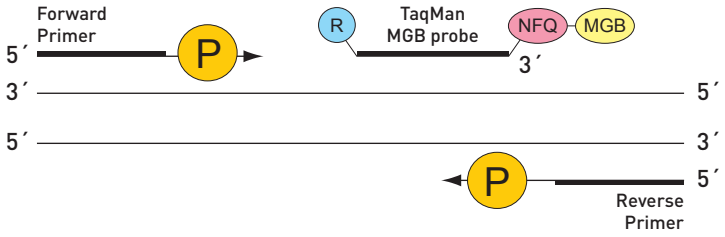
Basics of the 5’ nuclease assay

The PCR reaction exploits the 5′ nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan® probe during the PCR process. The TaqMan® probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. During the PCR, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. This process occurs in every cycle and does not interfere with the exponential accumulation of product. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.

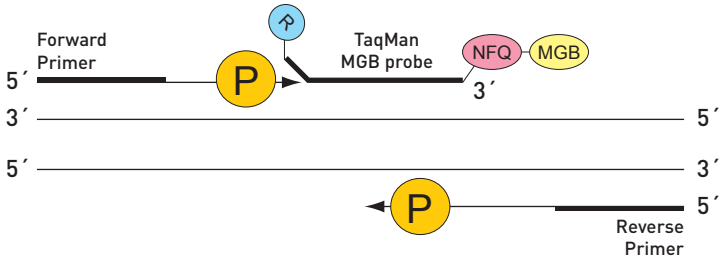
The forklike-structure-dependent, polymerization- associated 5′ to 3′ nuclease activity of AmpliTaq Gold® enzyme during PCR is graphically outlined below.

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

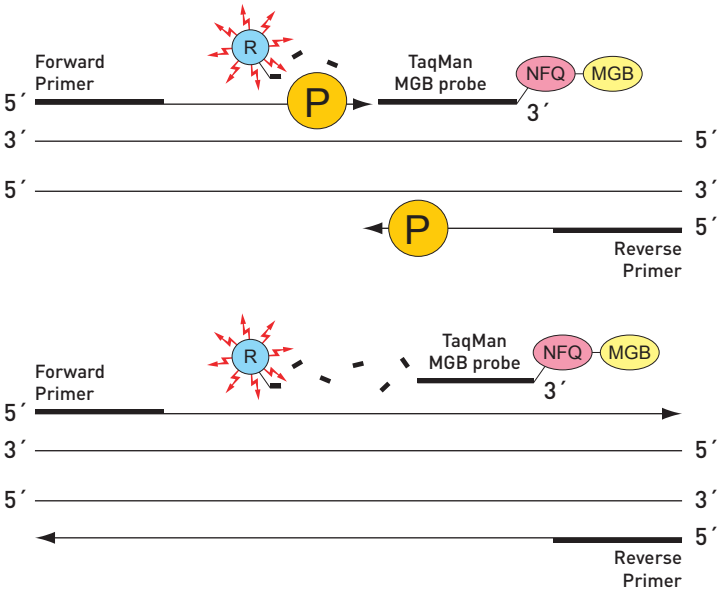
During PCR, the TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer.



The DNA polymerase cleaves only probes that are hybridized to the target. The 5′ to 3′ nucleolytic activity cleaves the probe separating the reporter dye from the quencher dye which results in increased fluorescence by the reporter.

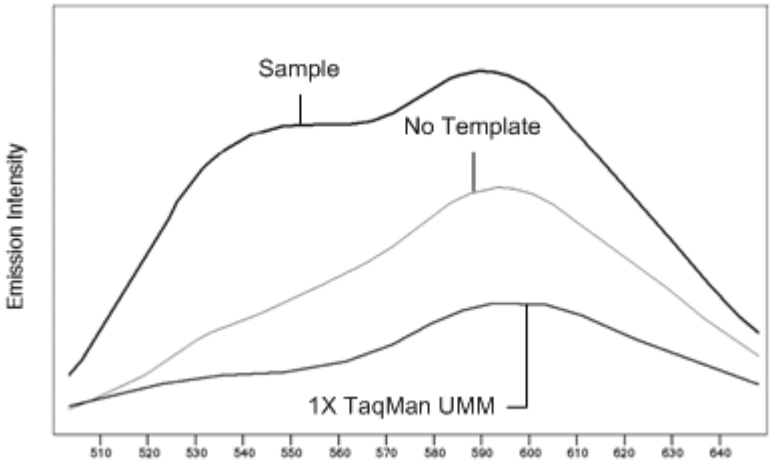


Probe fragments are displaced and polymerization of the strand continues, but because the 3′ end of the probe is blocked, no extension of the probe occurs during PCR.



The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR.

Figure 2 An overlay of three emission scans, post 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, Life Technologies 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 40 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.

D Custom TaqMan® assay design

This chapter describes how to design a custom 5′ nuclease assay (primers and TaqMan® hydrolysis probe) for gene expression quantitation assays.

Before you begin

Review Appendix B, "PCR Good Laboratory Practices" on page 39.

Identifying target sequence and amplicon size

Target template

Target template is a genomic DNA, cDNA, or plasmid nucleotide sequence.

Amplicon

A short segment of DNA within the target sequence that is the template and/or product of amplification. An amplicon's sequence and length are determined by specific forward and reverse primers.

General amplicon site selection guidelines

Using Primer Express® software, or your preferred suite of software tools for sequence analysis and design, select an *amplicon site* (short segment of cDNA) within the target sequence. Selecting a good amplicon site ensures amplification of the target cDNA without coamplification of the genomic sequence, pseudogenes, or related genes.

- The amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA.
- The shortest amplicons work the best. Consistent results are obtained for amplicon size ranges from 50–150 bp.
- Design the hydrolysis probe before determining primer pairs during assay design.
- Design hydrolysis probes and primer pairs according to the guidelines provided on page 48.
- The primer pair must be specific to the target gene; the primer pair must not amplify pseudogenes or other related genes.
- Test the primer pairs, then select the primer pair that produces the highest signal-to-noise ratio (that is, earliest C_T with total RNA or mRNA and no amplification with genomic DNA or negative controls).

If the gene does not contain introns

If the gene you are studying does not contain introns, then you cannot ensure amplification of the target cDNA sequence without coamplification of the genomic sequence. In this case, you may need to run controls in your reverse transcription reactions that do not contain reverse transcriptase (RT-controls) to determine whether your RNA sample contains DNA. Amplification in the RT-controls indicates that your RNA sample contains DNA. To remove the DNA from the RNA sample, treat the RNA sample with DNase I.

Probe and primer design

Using Primer Express® software, or your preferred suite of software tools for sequence analysis and design, design a TaqMan® probe to detect amplification of the target sequence, then design primers to amplify the target sequence. Refer to the *Primer Express Software Version 3.0 Getting Started Guide* for detailed primer and probe selection procedures.

TaqMan® Universal PCR Master Mix has been optimized for use with primers and TaqMan® probes that have been designed according to Life Technologies development guidelines. For the vast majority of TaqMan® assays developed using the following guidelines a concentration of 900 nM primers and a 250 nM fluorescent probe provides a highly reproducible and sensitive assay when using cDNA or DNA as template in a singleplex assay.

General probe design guidelines

- Keep the GC content in the 20–80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than four consecutive G bases.
- The base at the 5′ end must not be a G.
- Select the strand in which the probe contains more C bases than G bases.
- For singleplex assays, keep the T_m between 68°C to 70°C.

General primer design guidelines

- Choose the primers after the probe.
- Do not overlap primer and probe sequences. The optimal primer length is 20 bases.
- Keep the GC content in the 20–80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than four consecutive G bases.
- **Important: Keep the T_m between 58–60°C.**
- Be sure the last 5 nucleotides at the 3′ end contain no more than two G and/or C bases.
- If you cannot find acceptable primer sequences, you may need to examine the sequence and select another amplicon site or screen for more sites.

Calculation of primer and probe concentrations

After you receive your primers and probe, use a spectrophotometric method to determine the concentrations of the oligonucleotides in your assay.

Calculate oligonucleotide concentrations

1. Calculate the sum of extinction coefficient contributions for each oligonucleotide sequence:

Chromophore	Extinction Coefficient
A	15,200
C	7050
G	12,010
T	8400
FAM™ dye	20,958
TAMRA™ dye	31,980
TET™ dye	16,255
JOE™ dye	12,000
VIC® dye	30,100

2. Measure the absorbance at 260 nm (A_{260}) of each oligonucleotide diluted in TE buffer (for example, 1:100).

3. Calculate the oligonucleotide concentration using the formula:

   A_{260} = (sum of extinction coefficient contributions × cuvette pathlength × concentration) ÷ dilution factor

   Rearrange to solve for concentration:

   Concentration (C) = (dilution factor × A_{260}) ÷ (sum of extinction coefficient contributions × cuvette pathlength)

An example calculation of primer concentration

If the primer sequence is CGTACTCGTTCGTGCTGC:

- Sum of extinction coefficient contributions:
  = A × 1 + C × 6 + G × 5 + T × 6
  = 167,950 $M^{-1}cm^{-1}$
- Example A_{260} measurements:
  Dilution = 1:100
  Cuvette pathlength = 0.3 cm
  A_{260} = 0.13
- Primer concentration:
  = (100 × 0.13) ÷ (167,950 $M^{-1}cm^{-1}$ × 0.3 cm)
  = 2.58×10^{-4} M
  = 258 μM

An example calculation of probe concentration

If the probe sequence is CGTACTCGTTCGTGCTGC, FAM™ dye is attached to the 5′ end, and TAMRA™ dye is attached to the 3′ end:

- Sum of extinction coefficient contributions:
  = A × 1 + C × 6 + G × 5 + T × 6 + FAM × 1 + TAMRA × 1
  = 220,888 $M^{-1}cm^{-1}$
- Example A_{260} measurements:
  Dilution = 1:100
  Cuvette pathlength = 0.3 cm
  A_{260} = 0.13
- Probe concentration:
  = (100 × 0.13) ÷ (220,888 $M^{-1}cm^{-1}$ × 0.3 cm)
  = 1.96 × 10^{-4} M
  = 196 μM

Determine optimal primer concentrations

To determine the minimum primer concentrations that yield the minimum threshold cycle (C_T) and the maximum baseline-corrected normalized reporter (ΔR_n) values for the user-designed assay.

Primer concentrations to test

Use the TaqMan® Universal PCR Master Mix to prepare at least four technical replicates of each of the nine conditions shown below:

Forward primer final concentration (nM)	Reverse primer final concentration (nM)		
	50	300	900
50	50/50	50/300	50/900
300	300/50	300/300	300/900
900	900/50	900/300	900/900

Prepare and run the real-time qPCRs

1. Prepare the real-time qPCRs as outlined in the following table:

   Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume for one reaction			Final Concentration
TaqMan® Universal PCR Master Mix (2X)	5 µL	10 µL	25 µL	1X
Forward primer	1 µL	2 µL	5 µL	50–900 nM
Reverse primer	1 µL	2 µL	5 µL	50–900 nM
TaqMan® probe (2.5 µM)	1 µL	2 µL	5 µL	250 nM
Sample†	1 µL†	2 µL†	5 µL†	1–100 ng
Nuclease-free Water	1 µL	7 µL	17.5 µL	—
Total Volume	**10 µL**	**20.0 µL**	**50.0 µL**	

† Use 1–100 ng of DNA diluted in Nuclease-free Water.

2. In the real-time PCR system software set up a plate document or experiment using the following universal thermal cycling conditions.

Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temperature	50°C	95°C	95°C	60°C
Time (mm:ss)	02:00	10:00	00:15	01:00

† Required for optimal UNG activity. If using TaqMan® Universal PCR Master Mix, no AmpErase® UNG, this step is not necessary.

‡ Required to activate the DNA Polymerase.

3. Run the PCR plate.

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

Determine optimal probe concentration

To determine the minimum probe concentration that yields the minimum C_T for the target sequence without decreasing the ΔR_n for the user-defined assay.

Probe concentrations to test

Use the TaqMan® Universal PCR Master Mix to prepare at least four technical replicates at each 50 nM interval from 50–250 nM final probe concentration. Use the optimal primer concentrations you determined in the experiment you performed in "Determine optimal primer concentrations" on page 50.

Prepare and run the real-time qPCRs

1. Prepare the real-time qPCRs as outlined in the following table:

   Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume for one reaction			Final Concentration
TaqMan® Universal PCR Master Mix (2X)	5 µL	10 µL	25 µL	1X
Forward primer	1 µL	2 µL	5 µL	Optimal‡
Reverse primer	1 µL	2 µL	5 µL	Optimal‡
TaqMan® probe	1 µL	2 µL	5 µL	50–250 nM
Sample†	1 µL†	2 µL†	5 µL†	1–100 ng
Nuclease-free Water	1 µL	2 µL	5 µL	
Total Volume	**10 µL**	**20.0 µL**	**50.0 µL**	

† Use 1–100 ng of DNA diluted in Nuclease-free Water.

‡ Use the forward- and reverse-primer concentrations determined in "Determine optimal primer concentrations" on page 50.

2. In the real-time PCR system software set up a plate document or experiment using the following universal thermal cycling conditions.

Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temperature	50°C	95°C	95°C	60°C
Time (mm:ss)	02:00	10:00	00:15	01:00

† Required for optimal UNG activity. If using TaqMan® Universal PCR Master Mix without AmpErase® UNG, this step is not necessary.

‡ Required to activate the DNA Polymerase.

3. Run the PCR plate.

4. Tabulate the results for the C_T and ΔR_n values. Choose the minimum probe concentration to yield the maximum ΔR_n and the minimum C_T values.

Evaluate the results

Review the results from the primer optimization and probe optimization experiments to identify the optimal assay (combination of TaqMan® probe and forward- and reverse-primers) based upon ΔR_n and C_T values.

An optimal assay should have:

- maximal PCR yield (ΔR_n).
- minimum C_T values.
- no nonspecific amplification in the negative controls.
- minimum forward- and reverse-primer concentrations that yield the maximum ΔR_n values and the lowest C_T.
- minimum probe concentrations that yield the minimum C_T and the highest ΔR_n values

Performing routine analysis

For routine assays that are optimized as described here, perform analysis using optimum probe and primer concentrations and universal thermal cycling conditions.

Use optimized conditions to amplify DNA, or cDNA obtained from the first step of the two-step RT-PCR. For routine analysis the following ranges of RNA and DNA template can be used:

- RNA– 10 pg to 100 ng
- Genomic DNA– 100 pg to 1 µg

E Allelic discrimination probe/primer design

This appendix describes how to design and optimize probes and primers for allelic discrimination assays.

Before you begin

Review Appendix B, "PCR Good Laboratory Practices" on page 39.

Identifying target sequence and amplicon size

Target template defined

A target is a nucleotide sequence, two primers, and a probe. The location of the polymorphism constrains the number of possible target amplicons. For allelic discrimination, each allele associated with a target has a probe labeled with its own fluorescent reporter dye. Forward and reverse primers are common and have complete homology for both alleles.

General amplicon site selection guidelines

Using Primer Express® software, or your preferred suite of software tools for sequence analysis and design, select an *amplicon site* (short segment of cDNA) spanning the target sequence polymorphism. Selecting a good amplicon site ensures amplification of the target cDNA without coamplification of the genomic sequence, pseudogenes, or related genes.

- The amplicon must contain the SNP target.
- Design the allele specific hydrolysis probes before determining primer pairs during assay design.
- The shortest amplicons work the best, 50–150 bp for optimum PCR efficiency.
- Design hydrolysis probes and primer pairs according to the guidelines provided on page 56.
- The primer pair must be specific to the target gene; the primer pair must not amplify pseudogenes or other related genes.
- Test the primer pairs, then select the primer pair that produces the highest signal-to-noise ratio (that is, earliest C_T with total RNA or mRNA and no amplification with genomic DNA or negative controls).

Probe and primer design

The purpose of this procedure is to determine:

the probe concentrations that give the most reliable autocalls.

the primer concentrations that give the maximum R_n and the minimum C_T values.

Using Primer Express® software, or your preferred suite of software tools for sequence analysis and design, design SNP specific probes to detect the target sequence polymorphism, then design primers to amplify the target sequence. Refer to the *Primer Express Software Version 3.0 Getting Started Guide* for detailed primer and probe selection procedures.

TaqMan® Universal PCR Master Mix has been optimized for use with primers and hydrolysis probes that have been designed according to Life Technologies development guidelines. For the vast majority of TaqMan® assays developed using the following guidelines a concentration of 900 nM primers and 200 nM fluorescent probes provides a highly reproducible and sensitive assay.

General probe design guidelines

- Use VIC™ and FAM™ reporter dyes to label the allelic discrimination probes.
- For best results use TaqMan® MGB probes.
- Probe length of 13–30 bases (13–25 bases if using MGB probes).
- Locate SNP site in the middle third of the probe sequence or towards the 3′ end (but not in the last 2 bases).
- The two probes must be designed complimentary to the same strand (sense strand).
- Keep the GC content in the 30–80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than four consecutive G bases.
- Avoid six consecutive A residues anywhere in the probe sequence.
- Avoid two or more CC dinucleotides in the middle of the probe sequence.
- The base at the 5′ end must not be a G.
- Avoid the sequence motifs 5′–...GGG–MGB –3′ and 5′–...GGAG–MGB –3′ when designing MGB probes.
- Select the strand in which the probe contains more C bases than G bases.
- Keep the T_m between 65°C to 67°C.
- Keep T_m difference between probes ≤ 1°C.

General primer design guidelines

- Choose the primers after the probes.
- Optimal primer length is 20 bases. Position primers as close as possible to the probe without overlapping probe sequences.
- Keep the GC content in the 20–80% range.
- Avoid runs of identical nucleotides. If repeats cannot be avoided, there must be fewer than four consecutive G bases.
- Important: Keep the T_m between 58–60°C.
- The 3′five nucleotides should contain no more than two G and/or C bases.

Calculation of primer and probe concentrations

After you receive your primers and probe, use a spectrophotometric method to determine the concentrations of the oligonucleotides in your assay. See page 49 for detailed instructions on calculating primer and probe concentrations.

Determine optimal primer concentrations

To determine the minimum primer concentrations that yield the minimum threshold cycle (C_T) and the maximum baseline-corrected normalized reporter (ΔR_n) values for the user-designed assay.

Primer concentrations to test

Use the TaqMan® Universal PCR Master Mix to prepare at least four technical replicates of each of the nine primer conditions (shown below). Additionally run four technical replicates each of No Amplification Control (NAC) and No Template Control (NTC) using 900 nM forward and reverse primer concentrations.

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

Prepare and run the real-time qPCRs

1. Prepare the real-time qPCRs as outlined in the following table using one of the allelic discrimination probes at 100 nM.

   Note: Include 10% extra volume to compensate for the volume loss that occurs during pipetting.

Component	Volume for one reaction			Final Concentration
TaqMan® Universal PCR Master Mix (2X)	5 µL	10 µL	25 µL	1X
Forward primer	1 µL	2 µL	5 µL	50–900 nM
Reverse primer	1 µL	2 µL	5 µL	50–900 nM
Allelic Discrimination probe	1 µL	2 µL	5 µL	100 nM
Sample†‡	1 µL	2 µL	5 µL	1–20 ng
Nuclease-free Water§	1 µL	2 µL	5 µL	—
Total Volume	**10.0 µL**	**20.0 µL**	**50.0 µL**	

† Use 1–20 ng of gDNA (complementary to the allelic probe used) diluted in Nuclease-free Water.

‡ Use nuclease-free water for NTC wells.

§ Substitute 1 µL 0.5% sodium dodecyl sulfate (SDS) to each of the four NAC wells to inhibit enzyme activity in those wells.

2. In the real-time PCR system software set up a plate document or experiment using the following universal thermal cycling conditions.

Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temperature	50°C	95°C	95°C	60°C
Time (mm:ss)	02:00	10:00	00:15	01:00

† Required for optimal UNG activity. If using TaqMan® Universal PCR Master Mix without AmpErase UNG, this step is not necessary.

‡ Required to activate the DNA Polymerase.

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

Determine optimal probe concentrations

Determine the probe concentrations that give the most reliable autocalls for the user-defined assay.

Probe concentration considerations

The following points should be noted when determining optimal probe concentrations:

The initial fluorescence signals from the two probes are matched approximately.

Fluorescence is measured directly. No thermal cycling is required.

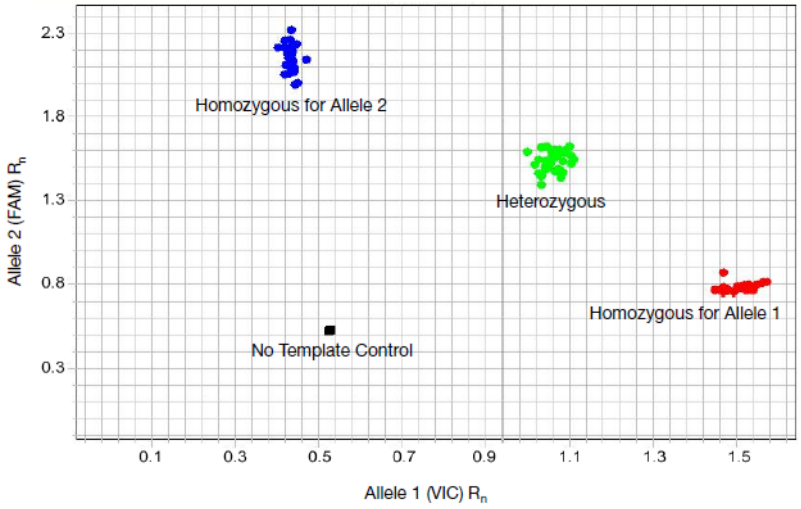
Performing routine analysis

For routine allelic discrimination assays using any TaqMan® genotyping assay or custom assays optimized as described here, perform analysis using optimum probe and primer concentrations and universal thermal cycling conditions.

Allelic discrimination assays use endpoint plate read fluorescence determinations, real-time PCR data is not necessary but may be helpful when troubleshooting.

Analyze the results

The system software records the results of the genotyping run on a scatter plot of Allele 1 (VIC® dye) versus Allele 2 (FAM™ dye). Each well of the 96-well or 384-well reaction plate is represented as an individual point on the plot. The clusters in the plot show the three genotypes of one SNP.



F

Ordering Information

How to order

The consumables and reagents in this appendix are for use with the TaqMan® Universal Master Mix and can be ordered from the Life Technologies 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 and TaqMan® assays. For a complete list of PCR systems and consumables, go to: **www.lifetechnologies.com**

System	Reaction plates and accessories
7300 system 7500 system	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad (Cat. no. 4312639) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (Cat. no. 4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (Cat. no. 4323032)
7500 Fast system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
7900HT Fast system, standard 96-well block	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad (Cat. no. 4312639) for use with one plate • MicroAmp® Snap-On Optical Film Compression Pad (Cat. no. 4333292) for use with automation accessory
7900HT Fast system, Fast 96-well block	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad (Cat. no. 4312639) for use with one plate • MicroAmp® Snap-On Optical Film Compression Pad (Cat. no. 4333292) for use with automation accessory
7900HT Fast system, 384-well block	• MicroAmp® Optical 384-Well Reaction Plate with Barcode: – 1000 plates (Cat. no. 4343814) – 500 plates (Cat. no. 4326270) – 50 plates (Cat. no. 4309849) • MicroAmp® Optical 384-Well Reaction Plate, 1000 plates (Cat. no. 4343370) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
ViiA™ 7 Real-time PCR System, standard 96-well block	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)

System	Reaction plates and accessories
ViiA™ 7 Real-time PCR System, Fast 96-well block	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
ViiA™ 7 Real-time PCR System, 384-well block	• MicroAmp® Optical 384-Well Reaction Plate with Barcode: – 1000 plates (Cat. no. 4343814) – 500 plates (Cat. no. 4326270) – 50 plates (Cat. no. 4309849) • MicroAmp® Optical 384-Well Reaction Plate, 1000 plates (Cat. no. 4343370) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
QuantStudio™ 6/7/12k Flex System, standard 96-well block	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
QuantStudio™ 6/7/12k Flex System, Fast 96-well block	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
QuantStudio™ 6/7/12k Flex System, 384-well block	• MicroAmp® Optical 384-Well Reaction Plate with Barcode: – 1000 plates (Cat. no. 4343814) – 500 plates (Cat. no. 4326270) – 50 plates (Cat. no. 4309849) • MicroAmp® Optical 384-Well Reaction Plate, 1000 plates (Cat. no. 4343370) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
9700 instrument	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • ABI PRISM® 384-Well Clear Optical Reaction Plate with Barcode: – 1000 plates (Cat. no. 4343814) – 500 plates (Cat. no. 4326270) – 50 plates (Cat. no. 4309849) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971) • MicroAmp® Clear Adhesive Films, 100 films (Cat. no. 4306311) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (Cat. no. 4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (Cat. no. 4323032)
StepOne™ system	• MicroAmp® Fast Optical 48-Well Reaction Plate, 20 plates (Cat. no. 4375816) • MicroAmp® 48-Well Optical Adhesive Film (Cat. no. 4375323)
StepOnePlus™ system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)

System	Reaction plates and accessories
Veriti® 96-well thermal cycler	• MicroAmp™ Optical 96-Well Reaction Plate: – 500 plates (Cat. no. 4316813) – 10 plates (Cat. no. N8010560) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971) • MicroAmp® Clear Adhesive Films, 100 films (Cat. no. 4306311)
Veriti® 384-well thermal cycler	• MicroAmp™ Optical 384-Well Reaction Plate with Barcode: – 1000 plates (Cat. no. 4343814) – 500 plates (Cat. no. 4326270) – 50 plates (Cat. no. 4309849) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971)
ProFlex™ PCR System	• MicroAmp™ Optical 96-Well Reaction Plate: – 500 plates (Cat. no. 4316813) – 10 plates (Cat. no. N8010560) • MicroAmp® Optical Adhesive Film (Cat. no. 4311971) • MicroAmp® Clear Adhesive Films, 100 films (Cat. no. 4306311) • MicroAmp® Optical 8-Tube Strips, 0.2-mL, 1000 tubes in strips of eight (Cat. no. 4316567) • MicroAmp® Optical 8-Cap Strips, 300 strips (Cat. no. 4323032)

Gene expression assay and array products

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

Assay or array	For more information...
TaqMan® Express Plates†	**www.lifetechnologies.com/taqmanexpress**
TaqMan® MicroRNA Assays	**www.lifetechnologies.com/miRNA**
Custom TaqMan® Small RNA Assays	Contact Life Technologies Sales
Custom TaqMan® Probes and Primers‡	**www.lifetechnologies.com/customtaqman**
TaqMan® Arrays: • TaqMan® Custom Arrays • TaqMan® Gene Signature Array • TaqMan® Gene Sets	**www.lifetechnologies.com/taqmanarrays**
Megaplex™ Pools for microRNA Expression Analysis: • Megaplex™ RT Primers • Megaplex™ PreAmp Primers • TaqMan® MicroRNA Arrays	**www.lifetechnologies.com/miRNA/megaplex**

† TaqMan® Gene Expression Assays dried in MicroAmp® Optical 96-Well Reaction Plates.

‡ Probes and primers synthesized by Life Technologies to your sequence and choice of quencher and reporter dyes.

Reverse transcription kits and reagents

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

Kit	Source	Catalog no.
High Capacity RNA-to-cDNA™ Kit, 50 rxns	Life Technologies	4387406
SuperScript® VILO™ cDNA Synthesis Kit • 50 reactions • 250 reactions	Life Technologies	 11754050 11754250
TaqMan® MicroRNA Reverse Transcription Kit • 1000 reactions • 200 reactions	Life Technologies	 4366597 4366596

Optional user-supplied reagents for gene expression quantitation

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

Materials	Cat. no.
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
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		Life Technologies
Barrier (Filter) Tips	10 µL size - Pipetman™ (Ten 8 × 12 racks)	Life Technologies
	10 µL size - Eppendorf® (Ten 8 × 12 racks)	Life Technologies
	20 µL size (Ten 8 × 12 racks)	Life Technologies
	1000 µL size (Ten 100 ct racks)	Life Technologies
	200 µL size (Ten 8 x 12 racks)	Life Technologies
Pipettors	Positive-displacement	MLS
	Air-displacement	
	Multichannel	
Vortexer		MLS
Microsoft Excel® software or equivalent spreadsheet and analysis software		Software suppliers

† Major laboratory supplier (MLS).

‡ For the MSDS of any chemical not distributed by Life Technologies, contact the chemical manufacturer. Before handling any chemicals, refer to the MSDS provided by the manufacturer, and observe all relevant precautions.

G

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

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:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **www.cdc.gov/biosafety**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **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**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at: **www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Documentation and Support

Related documentation

To obtain portable document format (PDF) versions of the documents listed in this section or additional documentation, visit **www.lifetechnologies.com**.

Note: To view PDFs, you can use Adobe® Reader® software, available as a free download from **www.adobe.com**.

Document	Part No.
Primer Express® Software Version 3.0 Getting Started Guide	4362460
Application note: *Factors Influencing Multiplex Real-Time PCR*	136AP04-01
Quick Reference: *TaqMan® Gene Expression Assays*	4401212
Real-Time PCR Handbook, 2nd edition	—
Tutorial: *Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays*	4371002
Tutorial: *Bioinformatic Evaluation of a Sequence for Custom TaqMan® SNP Genotyping Assays*	4371003
Custom TaqMan® Assays Design and Ordering Guide	4367671

Note: For additional documentation, see "Obtaining support" on page 70.

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**.

Note: For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining Certificates of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

Obtaining support

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

www.lifetechnologies.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Limited Product Warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.lifetechnologies.com/termsandconditions**. If you have any questions, please contact Life Technologies at **www.lifetechnologies.com/support**.

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in USA 800 955 6288
For support visit **lifetechnologies.com/support** or email **techsupport@lifetech.com**

lifetechnologies.com

25 February 2014