TaqMan® Gene Expression Assays —single-tube assays

USER GUIDE

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Revision Q

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ThermoFisher SCIENTIFIC

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Revision	Date	Description
Q	15 May 2018	• Updated thermal cycling conditions for TaqMan® Fast Advanced Master Mix. • Added option of cDNA preamplification. • Add option of C_{rt} algorithm for troubleshooting. • Corrected troubleshooting for inhibitors in the real-time PCR reaction.
P	22 November 2017	• Added new instruments, Master Mixes, and other products applicable for the workflows. • Removed content that is described in other resources; added references as appropriate. • Streamlined and clarified content for ease of use and reading. • Updated for general style, formatting, and branding.
N	November 2010	Baseline for this revision history.

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Contents

1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product description

TaqMan® Gene Expression Assays are a comprehensive collection of predesigned, preformulated primer and probe sets to perform quantitative gene expression studies on a variety of species. For a current list of available species and assays, use the Assay Search Tool at **thermofisher.com/taqmangeneexpression**.

- **TaqMan® Gene Expression Assays**
  - A general collection of assays that target protein-coding transcripts from a variety of species and for specific diseases, pathways, or biological processes.
  - TaqMan® Non-coding RNA Assays that target long non-coding RNA (ncRNA) in human, mouse, and rat species. These assays are designed for ncRNAs that are > 60 nt in length.
- **Endogenous control assays** (see page 25 for more information).

Note: Custom TaqMan® Gene Expression Assays can also be designed. To design a custom assay, go to **www.thermofisher.com/cadt**. For more information on the design tool, see *Custom TaqMan® Assays Design and Ordering Guide* (Pub. No. 4367671).

This document provides guidance for preparing cDNA templates (see page 12) and protocols for performing real-time PCR using a variety of compatible instruments and Master Mixes (see page 13).

For detailed information about TaqMan® Gene Expression Assays, see page 25.

Contents and storage

Item	Storage
TaqMan® Gene Expression Assay (single-tube format)	–25°C to –15°C[1]

[1] Shipped at ambient temperature. See thermofisher.com/ambientshippping.

Go to **thermofisher.com/taqmanfiles**, then enter your order number to download the following files.

- Assay information files (AIFs)
- User Instruction Documents (Protocols, User Guides, and Quick Reference Cards)
- Certificates of Analysis
- Safety Data Sheets

For detailed information about the shipment and assay information files (AIF), see *Understanding Your Shipment* (Pub. No. MAN0017153).

TaqMan® Gene Expression Assay formulations

To find and order predesigned, preformulated primer and probe sets in a variety of species, go to **thermofisher.com/taqmangeneexpression**, then use the Assay Search Tool.

Table 1 Standard formulations

Product	Dye	Size	Number of 20-µL reactions	Cat. No.	Concentration
TaqMan® Gene Expression Assays	FAM™	Extra Small	75	4453320[1] or 4448892	20X
		Small	250	4331182[1]	
		Small	360	4351372	
		Medium	750	4351370	
		Large	2900	4351368	60X
	VIC™	Small	360	4448489	20X
		Medium	750	4448490	
		Large	2900	4448491	60X
TaqMan® Gene Expression Assays, Primer-Limited (PL)	VIC™	Small	360	4448484	20X
		Medium	750	4448485	
		Large	2900	4448486	60X
TaqMan® Non-coding RNA Assay	FAM™	Small	360	4426961	20X
		Medium	750	4426962	
		Large	2900	4426963	60X

[1] This product is inventoried.

Custom TaqMan® Gene Expression Assay formulations

Use the Custom TaqMan® Assay Design Tool (**www.thermofisher.com/cadt**) to enter and submit sequences for new assay design. The tool also supports submission files created using FASTA file format. For details, see the *Custom TaqMan® Assays Design and Ordering Guide* (Pub. No. 4367671).

For a comparison of Custom and Custom Plus Assay products go to **thermofisher.com/customtaqmangex**.

Table 2 Custom formulations

Product	Dye	Size	Number of 20 µL reactions	Cat. No.	Concentration
Custom Plus TaqMan® RNA Assay	FAM™	Small	360	4441114	20X
		Medium	750	4441117	
		Large	2900	4441118	60X
	VIC™	Small	360	4448514	20X
		Medium	750	4448515	
		Large	2,900	4448516	60X
Custom PlusTaqMan® RNA Assay, Primer-Limited (PL)	VIC™	Small	360	4448511	20X
		Medium	750	4448512	
		Large	2900	4448513	60X
Custom TaqMan® Gene Expression Assays	FAM™	Small	360	4331348	20X
		Medium	750	4332078	
		Large	2900	4332079	60X
	VIC™	Small	360	4448508	20X
		Medium	750	4448509	
		Large	2900	4448510	60X
Custom TaqMan® Gene Expression Assay, Primer-Limited (PL)	VIC™	Small	360	4448487	20X
		Medium	750	4448488	
		Large	2900	4448492	60X

Assay primer and probe concentrations

Assay type	Concentration					
	Forward primer		Reverse primer		Probe	
	1X	20X	1X	20X	1X	20X
TaqMan® Gene Expression Assays (FAM™ or VIC™)	900 nM	18 µM	900 nM	18 µM	250 nM	5 µM
TaqMan® Gene Expression Assays (VIC™, Primer Limited)[1]	150 nM	3 µM	150 nM	3 µM		

[1] Recommended for multiplexing. For more information, see "Guidelines for duplex reactions using TaqMan® Gene Expression Assays" on page 30.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Table 3 Recommended products for isolation of RNA

Item	Source
Kits for RNA isolation	thermofisher.com/rnaisolation

Table 4 Recommended products for preparation of cDNA

Item	Source
cDNA kit or cDNA Master Mix, one of the following:	
SuperScript™ IV VILO™ Master Mix	11756050
SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme	11766050
High-Capacity cDNA Reverse Transcription Kit	4368813

Table 5 PCR Master Mixes

Item	Source
(Recommended) TaqMan® Fast Advanced Master Mix	4444556
TaqMan® Gene Expression Master Mix	4369016
TaqMan® Universal Master Mix II, with UNG	4440038
TaqMan® Universal Master Mix II, no UNG	4440047
TaqMan® Fast Universal PCR Master Mix, no AmpErase™ UNG	4352042

Table 6 Other materials and equipment required for the workflow

Item	Source
Real-time PCR instrument, one of the following:	
QuantStudio™ 3 or 5 Real-Time PCR System	Contact your local sales office
QuantStudio™ 6 / QuantStudio™ 7 Flex Real-Time PCR System	
QuantStudio™ 12K Flex Real-Time PCR System	
StepOne™ or StepOnePlus™ Real-Time PCR System	
ViiA™ 7 Real-Time PCR System	
7500/7500 Fast Real-Time PCR System	

Item	Source
Software	
(Optional) Relative Quantification app	Available on the Thermo Fisher Cloud
(Optional) Standard Curve app	
(Optional) ExpressionSuite™ Software	Available at **thermofisher.com/expressionsuite**
Equipment	
Thermal cycler, one of the following (or equivalent): • Veriti™ Thermal Cycler • SimpliAmp™ Thermal Cycler • ProFlex™ PCR System	Contact your local sales office
Centrifuge, with adapter for 96-well or 384-well plates	MLS
Microcentrifuge	MLS
Vortex mixer	MLS
(Optional) Eppendorf™ MixMate™ (shaker)	Fisher Scientific™ 21-379-00
Pipettes	MLS
Tubes, plates, and other consumables	
Tubes, plates, and film	**thermofisher.com/plastics**
Aerosol-resistant barrier pipette tips	MLS
Disposable gloves	MLS
Reagents	
Nuclease-free Water	AM9930
RNase Inhibitor	N8080119
RNaseOUT™ Recombinant Ribonuclease Inhibitor	10777019
TURBO DNA-*free*™ KitDNase	AM1907
TE, pH 8.0	AM9849
(Optional) TaqMan® PreAmp Master Mix	4391128
(Optional) TaqMan® PreAmp Master Mix Kit	4384267

Workflow

Start with cDNA templates prepared from RNA samples (page 12)

▼

Prepare the PCR Reaction Mix (page 14)

▼

Set up and run the real-time PCR instrument (page 15)

▼

Analyze the results (page 16)

2 Guidelines for preparation of cDNA

Guidelines for isolation of high–quality RNA

- See Table 3 on page 9 for recommended RNA isolation kits.
- *(Optional)* Use DNase to ensure minimal genomic DNA contamination of the RNA.

Guidelines for preparing cDNA templates

- See Table 4 on page 9 for recommended cDNA synthesis kits.
- Use the same reverse transcription procedure for all samples.
- For optimal reverse transcription, input RNA should be:
  - Free of inhibitors of reverse transcription (RT) and PCR
  - Dissolved in PCR-compatible buffer
  - Free of RNase activity

    Note: We recommend using RNase Inhibitor (Cat. No. N8080119) or RNaseOUT™ Recombinant Ribonuclease Inhibitor (Cat. No. 10777019).
  - Nondegraded total RNA (not applicable for double-stranded templates)

    IMPORTANT! Degradation of the RNA may reduce the yield of cDNA for some gene targets.
- For the input RNA amount, follow the recommendations provided by the cDNA kit.
- Small amounts of cDNA can be pre-amplified.
  Use TaqMan® PreAmp Master Mix (Cat. No. 4391128) or TaqMan® PreAmp Master Mix Kit (Cat. No. 4384267).
- Calculate the number of required reactions. Scale reaction components based on the single-reaction volumes, then include 10% overage, unless otherwise indicated.
- If using strip tubes to prepare cDNA templates, change to a new cap after each step or incubation.
- See your instrument user guide for detailed instructions about using plates, tubes, or strip tubes to prepare cDNA templates.

3 Perform real-time PCR

Procedural guidelines for performing real-time PCR

- Follow best-practices when preparing or performing PCR (see page 31).
- Prepare the real-time PCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Configure run documents according to the instructions provided in the real-time PCR instrument user documents.
- Protect the assays from light and store as indicated until ready for use. Excessive exposure to light can negatively affect the fluorescent probes of the assays.
- Run technical replicates in triplicate to identify outliers.

Before you begin (60X assays)

Dilute 60X assays to 20X working stocks with TE, pH 8.0, then divide the solutions into smaller aliquots to minimize freeze-thaw cycles. The size of the aliquots depends upon the number of PCR reactions you typically run. An example dilution is shown in the following table.

1. Gently vortex the tube of 60X assay, then centrifuge briefly to spin down the contents and eliminate air bubbles.

2. In a 1.5-mL microcentrifuge tube, dilute sufficient amounts of 60X assay for the required number of reactions.

Component	Volume
TaqMan® Gene Expression Assays (60X) or Custom TaqMan® Gene Expression Assays (60X)	40 µL
TE, pH 8.0 (1X)	80 µL
Total aliquot volume	**120 µL**

3. Store aliquots at –20°C until use.

Prepare the PCR Reaction Mix

Thaw the cDNA samples on ice. Resuspend the cDNA samples by inverting the tube, then gently vortexing.

1. Mix the Master Mix thoroughly but gently.

2. Combine the PCR Reaction Mix and assays in an appropriately-sized microcentrifuge tube according to the following table.

Component	Volume for 1 reaction	
	Standard 96–well or 48–well Plates	**384–well Plate or 96–well Fast Plate**
Master Mix (2X)[1,2]	10 µL	5 µL
TaqMan® Gene Expression Assay (20X) or Custom TaqMan® Gene Expression Assay (20X)	1 µL	0.5 µL
Nuclease-free water[3]	7 µL	3.5 µL
Total PCR Reaction Mix volume	**18 µL**	**9 µL**

[1] Recommended: TaqMan® Fast Advanced Master Mix

[2] (*Optional*) If you add AmpErase™ UNG (uracil-N-glycosylase), the final concentration must be 0.01U/ µL. Reduce the volume of water in the PCR reaction mix to compensate for additional volume from the UNG.

[3] Adjust the volume of nuclease-free water for a larger volume of cDNA.

3. Vortex to mix the PCR Reaction Mix thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.

4. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.

5. Add cDNA template (1 pg–100 ng in nuclease-free water), or nuclease-free water for NTC, to each well.

   - 1 µL for a 384-well plate or 96-well Fast Plate
   - 2 µL for a 96-well and 48-well Standard Plate

   Note: Be sure to adjust the volume of nuclease-free water in the PCR reaction mix for a larger volume of cDNA.

   IMPORTANT! For optimal results when using TaqMan® Fast Universal PCR Master Mix, no AmpErase™ UNG, prepare the plate on ice. Run the plate within 2 hours of preparation, or store the plate at 2–8°C for up to 24 hours.

6. Seal the plate with a MicroAmp™ Optical Adhesive Film, then vortex briefly to mix the contents.

7. Centrifuge the plate briefly to collect the contents at the bottom of the wells.

Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

Note: The instrument must be configured with the block appropriate for the plate type.

1. Select the cycling mode appropriate for the Master Mix.

   IMPORTANT! The cycling mode depends on the Master Mix that is used in the reaction. The cycling mode does not depend on a Standard or a Fast plate format.

2. Set up the thermal protocol for your instrument.

   See "Thermal protocols" on page 29 for the thermal protocols for other Master Mixes.

   Table 7 TaqMan® Fast Advanced Master Mix (StepOne™, StepOnePlus™, ViiA™ 7, and QuantStudio™ systems with fast cycling mode)

Step	Temperature	Time	Cycles
UNG incubation[1]	50°C	2 minutes	1
Enzyme activation	95°C	20 seconds[2]	1
Denature	95°C	1 second	40
Anneal / Extend	60°C	20 seconds	

   [1] Optional, for optimal UNG activity.

   [2] Enzyme activation can be up to 2 minutes. The time should not cause different results. See Enzyme activation time.

   Table 8 TaqMan® Fast Advanced Master Mix (7500 and 7500 Fast systems with fast cycling mode)

Step	Temperature	Time	Cycles
UNG incubation[1]	50°C	2 minutes	1
Enzyme activation	95°C	20 seconds[2]	1
Denature	95°C	3 seconds	40
Anneal / Extend	60°C	30 seconds	

   [1] Optional, for optimal UNG activity.

   [2] Enzyme activation can be up to 2 minutes. The time should not cause different results. See Enzyme activation time

3. Set the reaction volume appropriate for the reaction plate.
   - **96-well Standard (0.2-mL) Plate**: 20 µL
   - **96-well Fast (0.1-mL) Plate and 384–well Plate**: 10 µL

4. Load the plate into the real-time PCR instrument.

5. Start the run.

Analyze the results

For detailed information about data analysis, see the appropriate documentation for your instrument. Use the absolute or relative quantification ($\Delta\Delta C_t$) methods to analyze results.

The general guidelines for analysis include:

- View the amplification plot; then, if needed:
  - Adjust the baseline and threshold values.
  - Remove outliers from the analysis.
- In the well table or results table, view the C_t values for each well and for each replicate group.

Perform additional analysis using any of the following software:

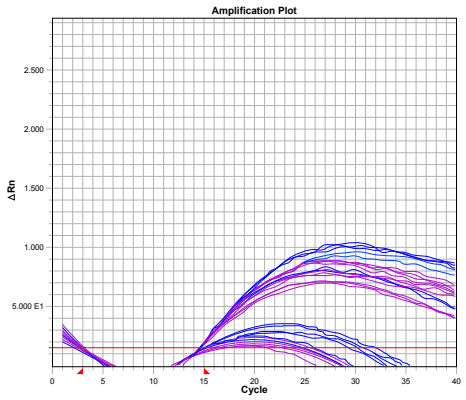
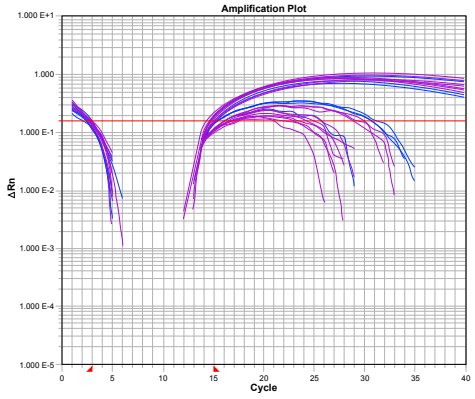
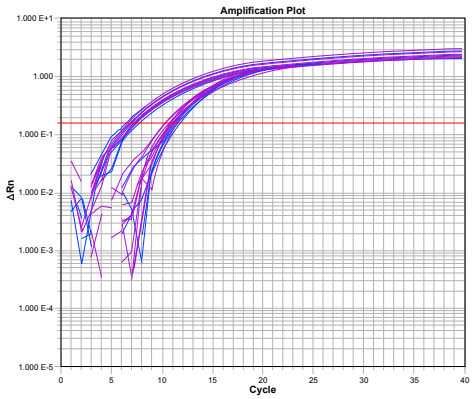
Software	Resource
Relative Quantification app	**thermofisher.com/cloud**
Standard Curve app	
ExpressionSuite™ Software[1]	**thermofisher.com/expressionsuite**

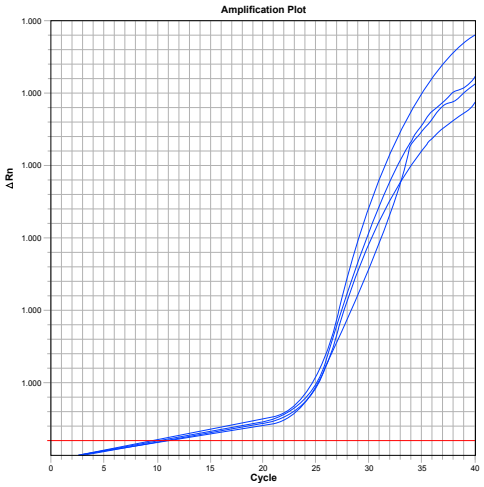
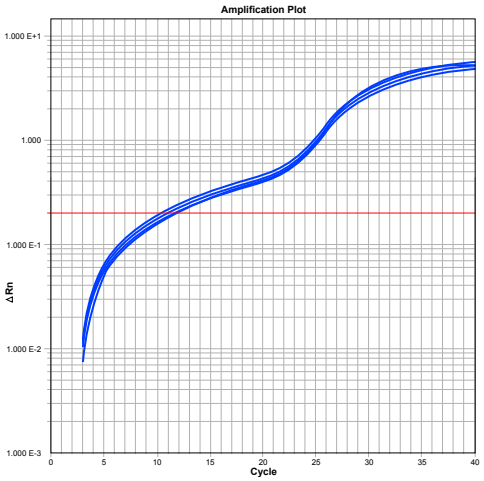
[1] Can automatically define the baseline. Files from a QuantStudio™ 3 or 5 System are not compatible.

For more information about real-time PCR, see *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239) or go to **thermofisher.com/qpcreducation**.

A

Troubleshooting

Observation	Possible cause	Recommended action
Amplification curve shows abnormal plot and/or low ΔRn values Linear view:  Log view:  	The baseline was set improperly (some samples have C_t values lower than the baseline stop value).	See 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 crosses the threshold). Corrected log view: 
	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.

Observation	Possible cause	Recommended action
Amplification curve shows a rising baseline Linear view:  Log view:  	There is interaction between the primer and probe.	• Adjust the threshold manually. • Select another assay from the same gene, if available.
Amplification curve shows weak amplification	(*Custom TaqMan® Gene Expression Assays only*) Sequence provided for the assay design contains mismatches with sample sequences.	Perform bioinformatics analysis. For more information, see *Custom TaqMan® Assays Design and Ordering Guide* (Pub. No. 4367671).
The Multicomponent Plot shows low ROX™ dye (passive reference dye)	Little or no Master Mix is present due to inaccurate pipetting.	Follow accurate pipetting practices.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_t=40) across all assays or in an unusually large number of assays	One or more of the reaction components was not added.	Ensure that the cDNA, the TaqMan® Gene Expression Assays, and the Master Mix were added to the reaction plate. The passive reference fails if the Master Mix is missing.
	Incorrect dyes were selected for each target.	Check the dyes selected for each target, then reanalyze the data.
	The annealing temperature was too high for the primers and/or probe.	Ensure that the correct annealing and extension temperatures are set.
		Ensure that the real-time PCR instrument is calibrated and maintained regularly.
	Inappropriate reaction conditions were used.	Troubleshoot the real-time PCR optimization.
		Ensure that the properties and the thermal protocol are correct.
	The template is degraded.	• Determine the quality of the template. • Rerun the assay with fresh template. • Use RNase-free reagents. • Use an RNase inhibitor.
	Inhibitors are present in the reaction.	Ensure 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, high 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.	See your real-time PCR system user guide for procedures on setting the baseline and threshold: • Switch from an automatic baseline to a manual baseline, or from a manual baseline to an automatic baseline. • Lower the threshold value to within the appropriate range.
	The reverse transcription failed.	• Check the RNA integrity and concentration. • Check for RNase activity. • Follow the recommended thermal profile. • Repeat the reverse transcription using new reagents.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_t=40) across all assays or in an unusually large number of assays	(*Custom TaqMan® Gene Expression Assays only*) Assay design or synthesis failure: The wrong sequence was submitted to Thermo Fisher Scientific.	• Ensure that the sequence that you submitted is correct. • Check for an alternative transcript or a splice variant.
	(*Custom TaqMan® Gene Expression Assays only*) Assay is designed in a variable region of the gene transcript.	Ensure that the location that is 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.
Amplification curve shows samples targeted by the same assay that have differently shaped curves	The baseline was set improperly.	See your real-time PCR system user guide for procedures on setting the baseline: • Switch from an automatic baseline to a manual baseline, or from a manual baseline to an automatic baseline. • Increase the upper or lower value of the baseline range.
	The sample quality was poor.	1. Perform a quality check on the sample. 2. If needed, re-extract the sample.
	There were different concentrations caused by imprecise pipetting.	Follow accurate pipetting practices.
	The reagents or equipment are contaminated.	Be sure that your workspace and equipment are cleaned properly.

Observation	Possible cause	Recommended action
Amplification curve shows no amplification of the sample (C_t=40) in the target assay	The gene is not expressed in the tested sample.	• Ensure that the gene is expressed in the sample type or tissue type. Go to **ncbi.nlm.nih.gov/unigene**. • Confirm the results. – Rerun the sample using the same assay. – Rerun the experiment using more sample. Avoid preparing PCR reaction mixes with more than 20% reverse transcription reaction. – Run the experiment using an alternative assay, if available, that detects a different transcript or more than one transcript from the same gene. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	The sample does not have enough copies of the target RNA.	Confirm the results. • Rerun the sample using the same assay. • Rerun the assay using more sample. Avoid PCR reaction mix with more than 20% from the reverse transcription reaction. **Note:** If the recommended actions do not resolve the problem, the result may be correct.
	One or more of the reaction components was not added.	Check your pipetting equipment and/or technique.
	Incorrect dyes were selected for each target.	Check the dyes selected for each target, then reanalyze the data.
Decrease in ROX™dye fluorescence (passive reference dye)	There was precipitation in the buffers.	Mix the Master Mix thoroughly to produce a homogenous solution.
	The reagents are degraded.	Ensure that the kits and reagents have been stored according to the instructions on the packaging and that they have not expired.
There was a simultaneous increase in fluorescence from both the passive reference dye (ROX™ dye) and the reporter dyes	The sample evaporated.	Check the seal of the adhesive film for leaks.
The multicomponent signal for ROX™dye is not flat	Incorrect dyes were selected for each target.	Check the dyes selected for each target, then reanalyze the data.

Observation	Possible cause	Recommended action
The Rn in the Rn vs Cycle plot is very high	The ROX™ dye was not set as the passive reference.	Set ROX™ dye as the passive reference, then reanalyze the data.
The no template control (NTC) shows amplification	The reagents are contaminated with gDNA, amplicon, or plasmid clones.	• Rerun the assay using new reagents. • Ensure that your workspace and equipment are cleaned properly. • Use a Master Mix containing UNG or add UNG separately, then repeat the experiment. • Run no-RT controls to rule out genomic DNA contamination. • Treat the sample with DNase. • Design an assay that spans an exon-exon boundary if genomic DNA contamination is suspected.
The endogenous control C_t values vary or they do not normalize the sample well	The endogenous control is not consistently expressed across the samples.	Ensure that the endogenous control is consistently expressed in your sample type. See "Endogenous controls" on page 25.
	The sample concentrations vary.	Quantitate and normalize the PCR samples.
	Pipetting was inaccurate.	• Check the calibration of the pipettes. • Pipet at least 5 µL of sample to prepare the reaction mix.
There is a high standard deviation in the replicates, inconsistent data, or a variable C_t	The reagents were not mixed properly.	• Increase the length of time that you mix the reagents. • Verify your mixing process by running a replicate assay.
	Pipetting was inaccurate.	• Check the calibration of the pipettes. • Pipette at least 5 µL of sample to prepare the reaction mix.
	The threshold was not set correctly.	Set the threshold above the noise level and where the replicates are tightest. See your real-time PCR system user documentation.
	There was a low concentration of the target of interest.	Rerun the assay using more cDNA template.

Observation	Possible cause	Recommended action
The C_t value is lower than expected	Contamination occurred.	• Run no-RT control to confirm that there was genomic DNA (gDNA) contamination. • Use DNase to ensure minimal gDNA contamination of the RNA. • *(Custom TaqMan® Gene Expression Assays only)* Design an assay that spans an exon–exon boundary. See *Custom TaqMan® Assays Design and Ordering Guide* (Pub. No. 4367671).
	Too much cDNA template was added.	• Reduce the amount of cDNA template. • Quantitate the RNA before the RT reaction, then adjust the concentration of cDNA from the RT reaction that is added.
	The cDNA template or the amplicon is contaminated.	Follow established PCR good laboratory practices.
Amplification occurs in the no-RT controls	Genomic DNA (gDNA) contamination occurred.	• Improve sample extraction methods to eliminate gDNA. See Chapter 2, "Guidelines for preparation of cDNA". • Use DNase to ensure minimal gDNA contamination of the RNA. • *(Custom TaqMan® Gene Expression Assays only)* Design an assay that spans an exon–exon boundary. See *Custom TaqMan® Assays Design and Ordering Guide* (Pub. No. 4367671).
	The cDNA template or amplicon is contaminated.	Follow established PCR good laboratory practices.
There was a shifting Rn 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 an automatic baseline. • Use the relative threshold algorithm (C_{rt}). See *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239).
There was a small ΔRn	The PCR efficiency was poor.	Ensure that the reagents were used at the correct concentration.
	The quantity of the cDNA is low (a low copy number of the target).	Increase the quantity of the cDNA.
There is a noisy signal above the threshold	The sample evaporated.	Check the seal of the adhesive film for leaks.

Observation	Possible cause	Recommended action
There is a noisy signal above the threshold	The well is empty because of inaccurate pipetting.	• Check the calibration of the pipettes. • Pipet at least 5 µL of sample.
	The well is assigned a sample or target in the plate document or experiment, but the well is empty.	• Be sure that your plate document or experiment is set up correctly. • Exclude the well and reanalyze the data.

B

Supplemental information

Endogenous controls

An endogenous control shows gene expression that is relatively constant and moderately abundant across tissues and cell types and treatment protocols. Normalization to endogenous control genes is currently the most accurate method to correct for potential biases that are caused by:

- Sample collection
- Variation in the amount of starting material
- Reverse transcription (RT) efficiency
- Nucleic acid (RNA/DNA) preparation and quality

No single control can act as a universal endogenous control for all experimental conditions, so we recommend verifying the chosen endogenous control or set of controls for the sample tissue, cell, or treatment. See *Using TaqMan® Endogenous Control Assays to select an endogenous control for experimental studies* (Pub. No. CO01971 0612), available from **thermofisher.com**.

To select and order endogenous control assays, go to **thermofisher.com/taqmancontrols**.

TaqMan® Gene Expression Assays chemistry overview

TaqMan® MGB probes

TaqMan® MGB probes contain:

- A reporter dye (for example, FAM™) at the 5′ end of the probe.
- A non-fluorescent quencher (NFQ) dye at the 3′ end of the probe.
  The NFQ dye does not fluoresce, which allows the real-time PCR system to measure the reporter dye contributions more accurately.
- A minor groove binder (MGB) at the 3′ end of the probe that:
  - Increases the melting temperature (T_m) without increasing the probe length.
  - Allows for the design of shorter probes.

About the 5' nuclease assay

Note: The following figures are general representations of real-time PCR with TaqMan® MGB probes and TaqMan® Gene Expression Assays. The sequence regions are not necessarily drawn to scale.

The 5' nuclease assay process takes place during PCR amplification. It occurs in every cycle and does not interfere with the exponential accumulation of cDNA synthesis product.

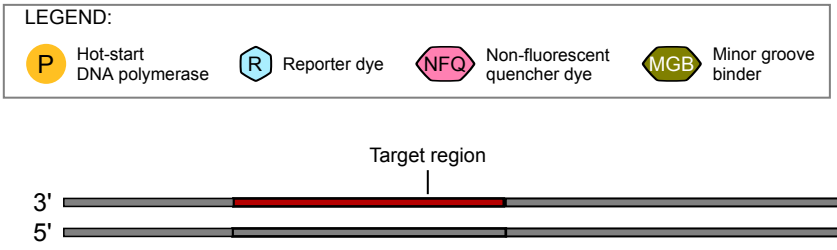


Figure 1 cDNA synthesis product

During the PCR, the forward and reverse primers anneal to complementary sequences along the denatured cDNA template strands (See Figure 2).

The TaqMan® MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites (See Figure 2). When the probe is intact, the proximity of the reporter dye and quencher dye suppresses the reporter fluorescence, primarily by Förster-type energy transfer.

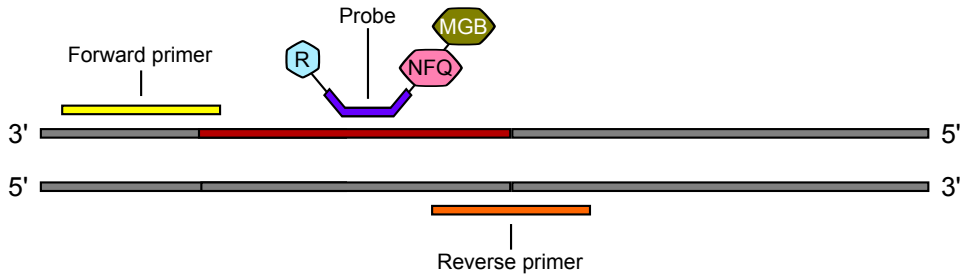


Figure 2 Annealing of probes and primers to cDNA strands

During polymerization, the DNA polymerase only cleaves probes that hybridize to the target sequence. Cleavage separates the reporter dye from the probe. The separation of the reporter dye from the quencher dye results in increased fluorescence by the reporter dye (see Figure 3).

This increase in fluorescence occurs only if the probe is complementary to the target sequence and if the target sequence is amplified during PCR. Because of these conditions, nonspecific amplification is not detected.

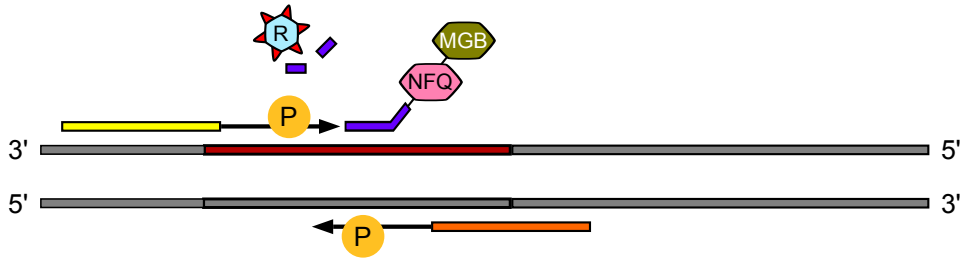


Figure 3 Initial polymerization and cleavage of reporter dye

Polymerization of the strand continues (see Figure 4), but because the 3' end of the probe is blocked, no extension of the probe occurs during PCR.

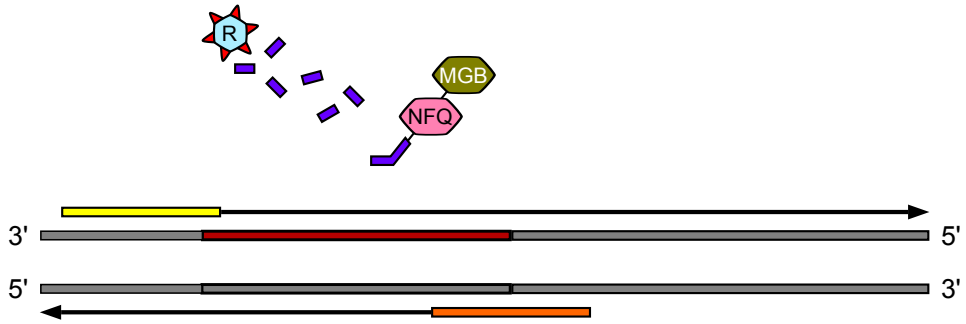


Figure 4 Completion of polymerization

Enzyme activation time

Using TaqMan® Fast Advanced Master Mix, the enzyme activation step can range from 20 seconds to 2 minutes. A 20–second enzyme activation step is sufficient when the template is cDNA. A longer enzyme activation time should not cause different results. The enzyme activation time for the default fast thermal cycling conditions on the instruments is 20 seconds. If a longer enzyme activation time is required, the thermal cycling conditions need to be changed before the run is started. A longer enzyme activation time can help to denature double-stranded genomic DNA when genomic DNA is used.

Algorithms for data analysis

Table 9 Algorithm recommendations for single–tube assays

Algorithm	Recommendation
Threshold (C_t)	Recommended.
Relative threshold (C_{rt})	*(Optional)* Use for troubleshooting abnormal or unexpected results.

The relative threshold algorithm is available in the Relative Quantification app on the Thermo Fisher Cloud, **thermofisher.com/cloud**

Thermal protocols

The thermal protocol settings depend on:

- The real-time PCR instrument
- Whether the Master Mix requires fast or standard cycling mode based on its chemistry
- Whether the Master Mix contains UNG

The thermal protocols in "Set up and run the real-time PCR instrument" on page 15 are optimized for the TaqMan® Fast Advanced Master Mix.

The following tables provide thermal protocols for other Master Mixes that are compatible with TaqMan® Gene Expression Assays.

IMPORTANT! The cycling mode depends on the Master Mix that is used in the reaction. The cycling mode does not depend on a Standard or a Fast plate format.

Table 10 TaqMan® Gene Expression Master Mix or TaqMan® Universal Master Mix II, with UNG (any compatible instrument)

Step	Temperature	Time (standard cycling mode)	Cycles
UNG incubation[1]	50°C	2 minutes	1
Enzyme activation	95°C	10 minutes	1
Denature	95°C	15 seconds	40
Anneal / Extend	60°C	1 minute	

[1] For optimal UNG activity.

Table 11 TaqMan® Universal Master Mix II, no UNG (any compatible instrument)

Step	Temperature	Time (standard cycling mode)	Cycles
Enzyme activation	95°C	10 minutes	1
Denature	95°C	15 seconds	40
Anneal / Extend	60°C	1 minute	

Table 12 TaqMan® Fast Universal PCR Master Mix, no AmpErase™ UNG (StepOne™, StepOnePlus™, ViiA™ 7, or QuantStudio™ system)

Step	Temperature	Time (fast cycling mode)	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	1 second	40
Anneal / Extend	60°C	20 seconds	

Table 13 TaqMan® Fast Universal PCR Master Mix, no AmpErase™ UNG (7500 or 7500 Fast system)

Step	Temperature	Time (fast cycling mode)	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	3 seconds	40
Anneal / Extend	60°C	30 seconds	

Guidelines for duplex reactions using TaqMan® Gene Expression Assays

Duplex real-time PCR is the simultaneous amplification and measurement of two target sequences in one reaction. TaqMan® Gene Expression Assays can be used in duplex real-time PCR when using a FAM™ dye-labeled assay in combination with a primer-limited, VIC™ dye-labeled assay. When setting up a duplex reaction:

- Validate that your duplex assay combinations provide similar results to your singleplex reactions.
- Consider the relative expression levels of each target.
- Perform serial dilutions of your sample in both singleplex and duplex reactions, and compare the results for relative expression.
- Select the higher-expressing target as the primer-limited, VIC™ dye-labeled assay.
- Use TaqMan® Fast Advanced Master Mix, which has been optimized for duplexing reactions.

For more details on how to validate your duplex assay reactions and interpret the results, see *TaqMan® Multiplex PCR Optimization User Guide* (Pub. No. MAN0010189) or go to **thermofisher.com/multiplexqpcr**.

Best practices for PCR and RT-PCR experiments

Good laboratory practices for PCR and RT-PCR

When preparing samples for PCR or RT-PCR amplification:

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

Use UNG to prevent false-positive amplification

Carryover amplicons can result in false-positive amplification during PCR. Use a Master Mix that contains uracil-N-glycosylase (UNG; also known as uracil-DNA glycosylase (UDG)) to degrade many contaminating carryover amplicons.

UNG enzymatic activity occurs during an initial incubation at 50°C. UNG is partially inactivated during the 95°C incubation step for template denaturation and polymerase activation. 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°C to 8°C in order to prevent amplicon degradation.

To ensure the desired UNG activity:

- Use PCR components and thermal cycling conditions as specified.

  UNG-containing Master Mixes incorporate the optimal concentration of UNG to prevent cross-contamination while not affecting real-time PCR performance.
- Do not attempt to use UNG-containing Master Mixes in subsequent amplification of dU-containing PCR products, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR products, preventing further amplification.

Although treatment with UNG can degrade or eliminate large numbers of carryover PCR products, use good laboratory practices to minimize cross-contamination from non-dU-containing PCR products or other samples.

Detect fluorescent contaminants

Fluorescent contaminants can generate false positive results. To help detect these contaminants, we recommend including a no-amplification control reaction that contains sample, but no Master Mix.

After PCR, if the absolute fluorescence of the no-amplification control is greater than the fluorescence of the no template control (NTC), fluorescent contaminants may be present in the sample or in the heat block of the real-time PCR instrument.

C

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. 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).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- 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. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Related documentation

Document	Pub. No.
TaqMan® Gene Expression Assays Quick Reference—single-tube assays	4401212
Introduction to Gene Expression Getting Started Guide	4454239
Understanding Your Shipment	MAN0017153
Custom TaqMan® Assays Design and Ordering Guide	4367671
TaqMan® Multiplex PCR Optimization User Guide	MAN0010189
TaqMan® PreAmp Master Mix User Guide	4384557
TaqMan® PreAmp Master Mix Quick Reference	4384556
QuantStudio™ 3 or 5 Real-Time PCR System	
QuantStudio™ 3 and 5 Real-Time PCR Systems Installation, Use, and Maintenance Guide	MAN0010407
QuantStudio™ Design and Analysis desktop Software User Guide	MAN0010408
QuantStudio™ 6 / QuantStudio™ 7 Flex Real-Time PCR System	
QuantStudio™ 6 and 7 Flex Real-Time PCR Systems Maintenance and Administration Guide	4489821
QuantStudio™ 6 and 7 Flex Real-Time PCR System Software Getting Started Guide	4489822
QuantStudio™ 12K Flex Real-Time PCR System	
QuantStudio™ 12K Flex Real-Time PCR System Maintenance and Administration Guide	4470689
QuantStudio™ 12K Flex Real-Time PCR System: Multi-Well Plates and Array Card Experiments User Guide	4470050
StepOne™ or StepOnePlus™ Real-Time PCR System	
StepOne™ and StepOnePlus™ Real-Time PCR Systems Installation, Networking and Maintenance User Guide	4376782
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR Systems Relative Standard Curve and Comparative C_t Experiments Getting Started Guide	4376785
ViiA™ 7 Real-Time PCR System	
Applied Biosystems™ ViiA™ 7 Real-Time PCR System User Guide: Calibration, Maintenance, Networking, and Security	4442661
Applied Biosystems™ ViiA™ 7 Real-Time PCR System Getting Started Guide	4441434

Document	Pub. No.
7500/7500 Fast Real-Time PCR System	
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Installation and Maintenance Guide	4347828
Applied Biosystems™ 7500/7500 Fast Real-Time PCR System Getting Started Guide: Relative Standard Curve and Comparative C_t Experiments	4387783
Data analysis	
Real-Time PCR Systems Chemistry Guide: Applied Biosystems™ 7900HT Fast Real-Time PCR System and 7300/7500 Real-Time PCR Systems	4348358
Applied Biosystems™ 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide	4364016
Applied Biosystems™ 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide	4364014
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Absolute Quantitation using Standard Curve	4347825
Applied Biosystems™ 7300/7500/7500 Fast Real-Time PCR System Getting Started Guide: Relative Quantitation using Comparative C_t	4347824
Applied Biosystems™ StepOne™ and StepOnePlus™ Real-Time PCR Systems Relative Standard Curve and Comparative C_t Experiments Getting Started Guide	4376785
Applied Biosystems™ Relative Quantitation Analysis Module User Guide	MAN0014820
Applied Biosystems™ Standard Curve Analysis Module User Guide	MAN0014819

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  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.