USER GUIDE

applied biosystems® by life technologies™

TaqMan® Fast Virus 1-Step Master Mix

Catalog Numbers 4444432, 4444434, 4444436

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Contents

TaqMan® Fast Virus 1-Step Master Mix

Product information

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

Purpose of the product

Use the TaqMan® Fast Virus 1-Step Master Mix with any TaqMan® primer/probe set for RNA or DNA virus research. This master mix and assay combination allows you to perform one-step RT-PCR for the following types of experiments:

- **Presence/absence** – An endpoint experiment that indicates the presence or absence of a specific nucleic acid sequence (target) in a sample. The actual quantity of target is not determined. Presence/absence experiments are commonly used to detect the presence or absence of a pathogen, such as a viral or bacterial pathogen. (Presence/absence experiments are also referred to as *plus/minus experiments*.)
- **Standard curve** – A type of quantitation experiment that determines the absolute target quantity in samples. With the standard curve method, the real-time PCR system software measures amplification of the target in samples and in a standard dilution series. Data from the standard dilution series are used to generate the standard curve. Using the standard curve, the software interpolates the absolute quantity of target in the samples. Standard curve experiments are commonly used for quantifying viral load. (Standard curve experiments are also referred to as *absolute quantitation* or *AQ experiments*.)

  You can also perform a standard curve experiment without running standards, if you only want to collect the C_T values.

  Note: A quantitation experiment is a real-time experiment that measures the quantity of a target nucleic acid sequence (target) during each amplification cycle of the polymerase chain reaction (PCR).

Starting template

You can use this protocol for both RNA and DNA targets. During thermal cycling, the reverse transcription step will not affect performance with DNA targets.

About this protocol

This protocol provides:

- A description of TaqMan® Fast Virus 1-Step Master Mix contents
- A list of equipment and materials required for using the TaqMan® Fast Virus 1-Step Master Mix
- Procedures and guidelines for performing gene expression experiments with the TaqMan® Fast Virus 1-Step Master Mix:
  - Designing the experiment
  - Preparing and running the reactions
  - Analyzing results

For more information

For more information, refer to the appropriate documentation for your instrument:

Document type	Description
User guides	Procedures for using and maintaining the real-time PCR system, including performing instrument calibrations
Getting Started Guides	Guidelines for experiment design, setup, run, and analysis
Protocols	Information on Life Technologies reagents

For document titles and part numbers, see "Documentation and Support" on page 35.

Master mix contents and storage conditions

Contents

The TaqMan® Fast Virus 1-Step Master Mix contains:

- AmpliTaq® Fast DNA Polymerase
- Thermostable MMLV enzyme
- dNTPs including dATP, dGTP, dCTP, and dTTP
- RNaseOUT™ Recombinant Ribonuclease Inhibitor
- ROX™ dye (passive reference)
- Buffer components optimized for maximum sensitivity and tolerance to several common RT-PCR inhibitors
- (Optional) Uracil-DNA Glycosylase (UDG)

Note: The catalog numbers listed in the table below do not contain UDG. If you are interested in the TaqMan® Fast Virus 1-Step Master Mix that is premixed with UDG, contact your local Life Technologies representative.

The master mix is supplied in a 4× concentration and is available in the following quantities:

Product	Quantity/Cat. no.		
	1 × 1 mL	5 × 1 mL	1 × 10 mL
TaqMan® Fast Virus 1-Step Master Mix	4444432	4444434	4444436

Storage and stability

Store the TaqMan® Fast Virus 1-Step Master Mix at –10°C to –30°C. The master mix will not freeze at –10°C to –30°C; gelling may occur.

Do not use the master mix after the date printed on the package and bottle labels.

Recommended real-time PCR systems

Life Technologies recommends using the TaqMan® Fast Virus 1-Step Master Mix with the following real-time PCR systems:

Applied Biosystems® Real-Time PCR System	Block Module
7500 Fast system	NA
7500 system	NA
ViiA™ 7 system	384-Well Block Module
7900HT/7900HT Fast system	Fast 96-well Block Module
	Standard 96-Well Block Module
	384-Well Block Module
StepOne™ and StepOnePlus™ systems	NA

Considerations

- You must perform thermal cycling 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.
- You can use the TaqMan® Fast Virus 1-Step Master Mix with Fast or standard thermal-cycling protocols.
- If you use assays other than TaqMan® or Custom TaqMan® Gene Expression Assays, or use thermal-cycling conditions other than those specified in this protocol, validate your assays and re-optimize your thermal-cycling conditions as needed.
- For a list of the reaction plates available for each real-time PCR system, see "Reaction plates and accessories" on page 10.

User-supplied materials

Reagents

Reagent	Source	Cat. no.
MagMAX™-96 Viral RNA Isolation Kit (for preparing swab, serum, plasma, and blood research samples)	Life Technologies	AM1836
MagMAX™-96 Total RNA Isolation Kit (for viral RNA isolation from research tissue samples)	Life Technologies	AM1830
MagMAX™-96 DNA Multi-Sample Kit (for viral DNA isolation from research tissue samples)	Life Technologies	4413021
PureLink™ Viral RNA/DNA Mini Kit (50 preps)	Life Technologies	12280-050
TaqMan® Exogenous Internal Positive Control Reagents	Life Technologies	4308323
TaqMan® Gene Expression Assays	Life Technologies	Various part numbers†
TURBO DNA-*free*™ solution (for digesting trace amounts of unwanted DNA)	Life Technologies	AM1907
RNA*later*® solution (an aqueous, non-toxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA)	Life Technologies	AM7020
RNase*Zap*® solution (a surface decontamination spray that destroys RNases on contact)	Life Technologies	AM9780
DNA*Zap*™ solution (a surface spray that degrades high levels of contaminating DNA and RNA)	Life Technologies	AM9890
RT-PCR Grade Water	Life Technologies	AM9935
Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, made using DNase-free, RNase-free sterile-filtered water)	Life Technologies	AM9849

† For information on ordering TaqMan® Gene Expression Assays from the Life Technologies website, see "Design the experiment" on page 12.

General laboratory equipment

All items are available from major laboratory suppliers (MLS).

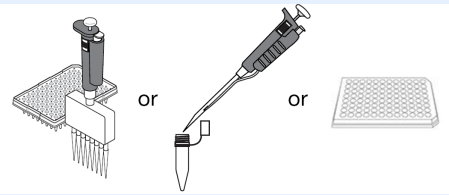
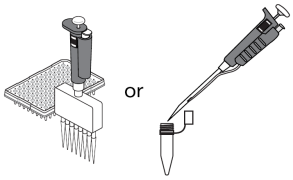
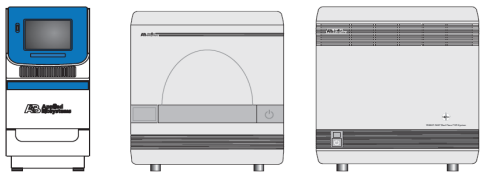
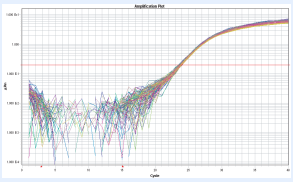
Item	Source
Disposable gloves	MLS
Pipette tips (aerosol-resistant, nuclease-free)	MLS
Pipettes (positive/air-displacement or multichannel)	MLS
Liquid reservoirs (RNase-free)	MLS
Vortexer	MLS
Centrifuge	MLS

Reaction plates and accessories

The table below lists the reaction plates and accessories available for Applied Biosystems® real-time PCR systems.

Real-Time PCR System	Reaction plates and accessories
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, 100 films (Cat. no. 4311971) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032)
7500 system	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (Cat. no. 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032)
ViiA™ 7 system, 384-Well Block Module 7900HT/7900HT Fast system, 384-Well Block Module	• 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, 100 films (Cat. no. 4311971)
7900/7900HT Fast system, Standard 96-Well Block Module	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (Cat. no. 4326659) – 20 plates (Cat. no. 4306737) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (Cat. no. 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (Cat. no. 4323032) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (Cat. no. 4333292)
7900HT/7900HT Fast system, Fast 96-well Block Module	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (Cat. no. 4366932) – 20 plates (Cat. no. 4346906) • MicroAmp® Optical Adhesive Film, 100 films (Cat. no. 4311971) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (Cat. no. 4333292)
StepOne™ system	• MicroAmp® Fast Optical 48-Well Reaction Plate, 20 plates (Cat. no. 4375816) • MicroAmp® 48-Well Optical Adhesive Film, 100 films (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, 100 films (Cat. no. 4311971)

Workflow

Design the experiment (page 12)	
Select a gene expression assay (page 12) ↓ Select your experiment type (page 14) ↓ Determine the number of reactions (page 15) ↓	
Prepare the samples (page 16)	
↓	
Prepare and run the RT-PCRs (page 17)	
Prepare the RT-PCR mix in a reaction plate (page 18) ↓	
Set up a plate document or experiment (page 20) ↓	
Run the RT-PCR plate (page 21) ↓	
Analyze the data (page 21)	

Design the experiment

Select a gene expression assay

You can design your experiment using the following types of gene expression assays:

- TaqMan® Gene Expression Assays (this page)
- Custom TaqMan® Gene Expression Assays (page 14)
- User-designed assays (page 14)

Note: The term *assay* refers to the primer and probe set.

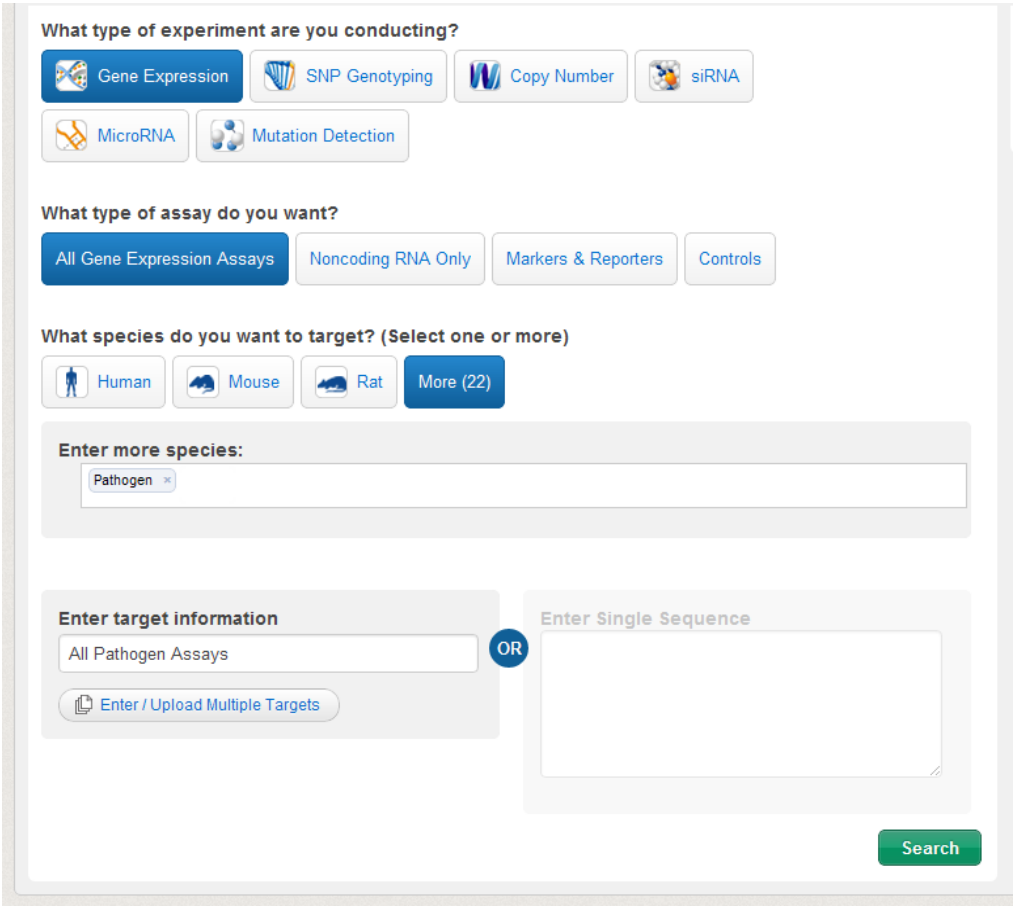
The Reverse Transcriptase enzyme contained in this kit is produced using an *E. coli* expression vector containing a proprietary version of the MMLV *pol* gene (GenBank accession no. J02255) expressed from pET-24(+). It is possible that a minimal amount of the expression vector could be carried over into the final mastermix formulation. If you are targeting MMLV, a related virus, or any of the plasmid sequence, we recommend designing primer sequences not contained in the expression vector.

TaqMan® Gene Expression Assays

1. Go to:
   www.lifetechnologies.com/taqmangeneexpression.com

2. In the TaqMan® Gene Expression Assays product page, select the **order predesigned assays** tab, select your search criteria, then click **Search**.

   Note: To search for TaqMan® Gene Expression Assays for Pathogens: Make sure that the **Gene Expression** and **All Gene Expression Assays** tabs are selected. In the Choose Species section, select the **More** tab, click in the **Enter more species** box and start typing "Pathogen" directly in the box**.** Select **Pathogen**, then click **Search**.



3. If required, log in to the online store.

Custom TaqMan® Gene Expression Assays

1. Go to:
   www.lifetechnologies.com/taqmangeneexpression
2. In the TaqMan® Gene Expression Assays product page, select the **order custom assays** tab.
3. Use the **Custom TaqMan® Assay Design Tool** to input sequences, submit for design, and order Custom Assays.

User-designed assays

You can use Primer Express® Software v3.0 to design your own assays (primer and probe sets) for use with the TaqMan® Fast Virus 1-Step Master Mix. For more information, see Appendix A, "Guidelines for Custom-Designed Assays" on page 23.

Select your experiment type

Select one of the following experiment types:

- Presence/absence
- Standard curve

Determine the number of reactions

Determine the total number of reactions in your experiment. For each experiment type, you need the following types of reactions:

Experiment type	Reaction type	Description
Presence/ absence	Unknown	A well that can contains: • Sample (DNA or RNA in which the presence of a target is unknown) • TaqMan® Fast Virus 1-Step Master Mix • Gene expression assay of choice
	Exogenous Internal positive control (IPC)	A short synthetic DNA template that you can add to the PCRs to distinguish between true negative results and reactions affected by PCR inhibitors, incorrect assay setup, or a reagent or instrument failure. **Note:** Life Technologies recommends the TaqMan® Exogenous Internal Positive Control Reagents (Cat. no. 4308323) for this purpose.
	No amplification control (NAC)	A well that contains all reaction components, except the unknown sample and IPC. Alternatively, the well may contain the IPC plus a blocking agent for the IPC. No amplification should occur in NAC wells.
	No template control (NTC)	A well that contains all PCR components, except the unknown sample. Only the IPC should amplify in NTC wells.
	Replicate	A reaction that is identical to another; it contains identical components and volumes. Life Technologies recommends performing at least three replicates of each reaction.
Standard curve	Unknown	A well that can contains: • Sample (DNA or RNA in which the quantity of the target is unknown) • TaqMan® Fast Virus 1-Step Master Mix • Gene expression assay of choice
	Standard	A reaction that contains known standard quantities; used in quantitation experiments to generate standard curves. **Note:** You can perform a standard curve experiment without running standards, if you only want to collect the C_T values.
	Standard dilution series	A set of standards containing a range of known quantities. The standard dilution series is prepared by serially diluting standards.
	No template control (NTC)	A negative control well that contains water or buffer instead of sample. No amplification of the target should occur in negative control wells.
	Replicate	A reaction that is identical to another; it contains identical components and volumes. Life Technologies recommends performing at least three replicates of each reaction.

Prepare the samples

Starting template

You can use this protocol for both RNA and DNA targets. During thermal cycling, the reverse transcription step will not affect performance with DNA targets.

Prepare the samples

Isolate and purify the target nucleic acid samples according to your laboratory practices. Life Technologies recommends the following kits:

- A MagMAX™ RNA or DNA Isolation Kit that is appropriate for your sample type.
- A MagMAX™ Viral RNA Isolation Kit, when isolating viral RNA from cell-free research samples such as serum. The viral RNA isolation kits include carrier RNA to maximize RNA recovery.

Examples of available MagMAX™ kits are listed under "Reagents" on page 9. To find all currently available kits, go to:

www.lifetechnologies.com/magmax

For manual column-based extraction, Life Technologies recommends the PureLink™ Viral RNA/DNA Mini Kit (Cat. no. 12280-050).

Storage conditions

Store the prepared samples at –10°C to –86°C in RT-PCR Grade Water.

If you dilute your samples, use TE buffer or RT-PCR Grade Water as the diluent.

Prepare and run the RT-PCRs

Guidelines

Item	Guideline
Assays (primer and probe set)	Keep all TaqMan® or Custom TaqMan® Gene Expression Assays in the freezer, protected from light, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
	Just before use, allow the assays to thaw on ice.
	At initial use, aliquot the assays to avoid multiple freeze/thaw cycles.
TaqMan® Fast Virus 1-Step Master Mix	Keep the master mix in the freezer, protected from light, until you are ready to use it.
	Just before use, allow the master mix to thaw on ice. **Note:** The master mix does not freeze at –10°C to –30°C but gelling may occur. Thawing the master mix on ice allows the master mix to return to its liquid state.
Storing combined master mix and assay	You can combine the TaqMan® Fast Virus 1-Step Master Mix and the gene expression assay ahead of time and store the solution for short periods. Follow these guidelines: • Store the solution at –10°C to –30°C. • To achieve the correct ratio of master mix to assay, you may be able to add the assay directly to the shipped master mix tubes. (The shipped tubes are filled with automated fillers that are as precise as a pipette. The target fill volume for the 1-mL tubes is 1.05 mL; the target fill volume for the 10-mL tubes is 10.3 mL.)
(For standard curve experiments) Standards	Standards are critical for accurate analysis of run data. Mistakes or inaccuracies in making the dilutions directly affect the quality of the results. The quality of pipettes and tips and the care used in measuring and mixing dilutions affect accuracy. Use TE buffer or RT-PCR Grade Water to prepare the standard dilution series.
No-RT control	If you are concerned that your one-step real-time RT-PCR is detecting genomic DNA rather than a particular RNA species, you can run a no-RT control reaction with the TaqMan® Fast Virus 1-Step Master Mix. To run a no-RT control: Heat-kill the RT enzyme by heating one aliquot of master mix at 95°C for 5 minutes before mixing it with the assay and sample. The PCR hot-start mechanism will reactivate after the master mix has cooled to room temperature.
Thermal-cycling temperature ranges	The optimal temperatures for reverse transcription (RT) and annealing are recommended in this protocol (see "Set up a plate document or experiment" on page 20). However, in some instances you may wish to alter the temperatures; testing has shown that the: • RT enzyme will function best in the range of 48 to 55°C • Annealing temperature should be in the range of 56 to 62°C **Note:** Be sure the annealing temperature is consistent with the melting temperature (T_m) of your primer designs. For guidelines on designing primers, see "Probe and primer design" on page 24.
Multiplexing	If you are multiplexing, the TaqMan® Fast Virus 1-Step Master Mix is designed to accommodate multiple assays. For guidelines on designing multiplex reactions, go to **www.lifetechnologies.com**, then search for the application note *Factors Influencing Multiplex Real-Time PCR* (Part no. 136AP04-01).

Item	Guideline
Adding UDG (Uracil-DNA Glycosylase) to the RT-PCRs	You can add UDG to the RT-PCRs, or you can use TaqMan® Fast Virus 1-Step Master Mix that contains UDG. Life Technologies recommends 0.01 units per 1 µL of reaction (for example, 0.2 units for a 20-µL reaction). The UDG step is performed at the same time and temperature (50°C) as the RT step, using the same thermal-cycling conditions as reactions without UDG. **Note:** The TaqMan® Fast Virus 1-Step Master Mix does not contain dUTP, so amplicons generated by this master mix will not digest with UDG. UDG can prevent false signals caused by dUTP-containing amplicon cross-contamination from occurring in your real-time PCRs.

Prepare the RT-PCR mix in a reaction plate

For Fast real-time PCR systems

For Fast real-time PCR systems, you must use a volume of ≤30 µL per reaction.

1. Thaw all reagents on ice.
2. Gently invert the TaqMan® Fast Virus 1-Step Master Mix 2 to 3 times to ensure a homogenous solution.
3. Per the table below, calculate the total volume required for each reaction component:
   volume for 1 reaction × total no. of reactions (from page 15) + 10%

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

Component	Volume for one 20-µL reaction	Notes
4X TaqMan® Fast Virus 1-Step Master Mix	5 µL	—
TaqMan® or Custom TaqMan® Gene Expression Assay (20X)	1 µL	If you are not using pre-formulated TaqMan® Gene Expression Assays, Life Technologies recommends primer concentrations of 400 to 900 nM and a probe concentration of 100 to 250 nM.
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**20 µL**	**—**

4. Working on ice, add the components directly to each well of an optical reaction plate.
5. Cover the reaction plate with an optical adhesive cover, vortex the plate briefly (5 to 10 seconds), then centrifuge at 1200 rpm for 1 minute to spin down the contents and eliminate air bubbles.

   IMPORTANT! The TaqMan® Fast Virus 1-Step Master Mix is a 4X formulation and is more viscous than most master mixes. Be sure that all of the components are thoroughly mixed before proceeding.

For Standard real-time PCR systems

1. Thaw all reagents on ice.
2. Gently invert the TaqMan® Fast Virus 1-Step Master Mix 2 to 3 times to ensure a homogenous solution.
3. Per the table below, calculate the total volume required for each reaction component:

   volume for 1 reaction × total no. of reactions (from page 15) + 10%

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

Component	Volume for one 50-µL reaction	Notes
4X TaqMan® Fast Virus 1-Step Master Mix	12.5 µL	—
TaqMan® or Custom TaqMan® Gene Expression Assay (20X)	2.5 µL	If you are not using pre-formulated TaqMan® Gene Expression Assays, Life Technologies recommends primer concentrations of 400 to 900 nM and a probe concentration of 100 to 250 nM.
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**50 µL**	**—**

4. Working on ice, add the components directly to each well of an optical reaction plate.
5. Cover the reaction plate with an optical adhesive cover, vortex the plate briefly (5 to 10 seconds), then centrifuge at 1200 rpm for 1 minute to spin down the contents and eliminate air bubbles.

   IMPORTANT! The TaqMan® Fast Virus 1-Step Master Mix is a 4X formulation and is more viscous than most master mixes. Be sure that all of the components are thoroughly mixed before proceeding.

Set up a plate document or experiment

For Fast real-time PCR systems

In the real-time PCR system software, set up a plate document or experiment using the following parameters:

- Sample volume: 20-µL
- Auto Increment Settings: Accept the default value
- Data Collection: Accept the default value
- Ramp Rate Settings: Accept the default value
- Run mode: Use the default run mode for your system and sample block module (that is, Fast mode on Fast instruments and standard mode on standard instruments).
- Thermal-cycling conditions for sample volumes ≤30 µL:

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	3 seconds
			60°C	30 seconds

† Reverse transcription works best between 48°C and 55°C.

For Standard real-time PCR systems

In the real-time PCR system software, set up a plate document or experiment using the following parameters:

- Sample volume: 50-µL
- Auto Increment Settings: Accept the default value
- Data Collection: Accept the default value
- Ramp Rate Settings: Accept the default value
- Run mode: Standard
- Thermal-cycling conditions for sample volumes >30 µL:

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	15 seconds
			60°C	60 seconds

† Reverse transcription works best between 48°C and 55°C.

Run the RT-PCR plate

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

Analyze the data

Data analysis varies depending on the real-time PCR system that you use. In general, to analyze the data in the real-time PCR system software, you:

1. View the amplification plot, and modify as needed:
   a. Set the baseline and threshold values. See "About baseline and threshold values" below.
   b. Remove outliers from the analysis.
2. In the well table or results table, view the C_T values for each well and for each replicate group.
3. (For standard curve experiments) View the standard curve for:
   - Slope
   - Amplification efficiency
   - R2 values
   - Y-intercept
   - C_T values
   - Outliers

About baseline and threshold values

You can use the real-time PCR system software to set the baseline and threshold values for the amplification plot, 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 signal and within the exponential growth phase of the amplification curve.

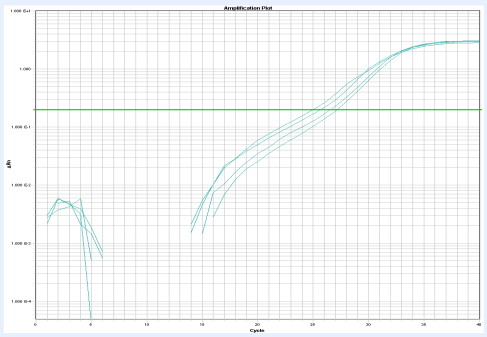
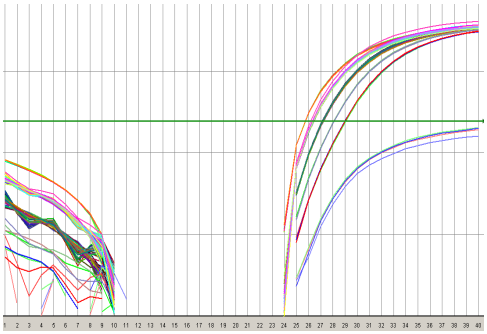
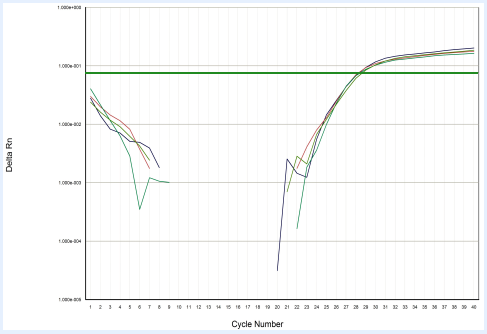
For more information

For more information on data analysis, refer to the appropriate Getting Started Guide for your instrument. For document titles and part numbers, see "Documentation and Support" on page 35.

Troubleshooting

The table below provides troubleshooting information for the TaqMan® Fast Virus 1-Step Master Mix. For general real-time PCR troubleshooting information refer to the appropriate guide for your instrument. For document titles and part numbers, see "Documentation and Support" on page 35.

Troubleshooting table

Observation	Possible cause	Recommended action
The amplification plot displays S-shaped curves. 	The RT-PCR mix was not thoroughly combined.	When preparing the RT-PCR mix (page 18), be sure to vortex the reaction plate briefly (5 to 10 seconds) before centrifuging.
The amplification plot is truncated. 	The baseline was set too high.	When analyzing the data (page 21), manually reset the baseline, or use the automatic baseline function.
The C_T is low, but merges with the background signal (noise). 	When automatic baseline is used, the software raises the threshold bar to avoid the elevated baseline.	When analyzing the data (page 21), change to manual C_T, then manually adjust the threshold bar.

Guidelines for Custom-Designed Assays

This appendix covers:

Amplicon site selection

Using Primer Express® Software

Using Primer Express® Software, select an *amplicon site* (segment of cDNA) within the target sequence. For detailed information, refer to the *Primer Express® Version 3.0 Getting Started Guide* and *Software Help*.

General amplicon site selection guidelines

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 primer pair must be specific to the target gene; the primer pair must not amplify pseudogenes or other related genes.
- Design primer pairs according to Primer Express® Software guidelines.
- 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 control 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

Using Primer Express® Software, design a probe to detect amplification of the target sequence, then design primers to amplify the target sequence. For detailed information, refer to the *Primer Express® Version 3.0 Getting Started Guide* and *Software Help*.

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°C to 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 oligonucleotide 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

With your custom-designed assay, determine the primer concentrations to use to obtain the earliest threshold cycle (C_T) and the maximum baseline-corrected normalized reporter (ΔR_n).

Primer concentrations to test

Use the TaqMan® Fast Virus 1-Step Master Mix to prepare four technical replicates of each of the four conditions shown below:

Forward primer final concentration (nM)	Reverse primer final concentration (nM)	
	400	**900**
400	400/400	400/900
900	900/400	900/900

Prepare and run the RT-PCRs

1. Prepare the 16 RT-PCRs:

Component	Volume for one 20-µL reaction	Notes
4× TaqMan® Fast Virus 1-Step Master Mix	5 µL	—
Forward primer (400 or 900 nM final)	1 µL	—
Reverse primer (400 or 900 nM final)		
TaqMan® probe (250 nM final)		
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total Volume	**20.0 µL**	**—**

2. Run the RT-PCRs:
   - Sample volume: 20 µL
   - Auto Increment Settings: Accept the default value
   - Data Collection: Accept the default value
   - Ramp Rate Settings: Accept the default value
   - Run mode: Use the default run mode for your system and sample block module (that is, Fast mode on Fast instruments and standard mode on standard instruments).
   - Thermal-cycling conditions for sample volumes ≤30 µL:

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	3 seconds
			60°C	30 seconds

† Reverse transcription works best between 48 °C and 55 °C.

Evaluate the results

1. Review the ΔR_n values to identify the optimal primer concentrations for PCR yield.

2. Review the C_T values to identify the optimal primer concentrations for C_T and detect any potential nonspecific amplification in the negative controls.

3. Select the forward primer and reverse primer combination that produces the earliest C_T and the highest ΔR_n.

Determine optimal probe concentration

With your custom-designed assay, determine the probe concentration to use to obtain the earliest threshold cycle (C_T) for the target sequence.

Probe concentrations to test

Use the TaqMan® Fast Virus 1-Step Master Mix to prepare four replicates of reactions with the following final probe concentrations: 100 nM and 250 nM. Use the optimal primer concentrations you determined in the experiment you performed on page 27.

Prepare and run the RT-PCRs

1. Prepare the 8 RT-PCRs:

Component	Volume for one 20-µL reaction	Notes
4X TaqMan® Fast Virus 1-Step Master Mix	5 µL	—
Forward primer (optimal)	1 µL	—
Reverse primer (optimal)		
TaqMan® probe (100 or 250 nM final)		
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total Volume	**20.0 µL**	**—**

2. Run the RT-PCRs:
   - Sample volume: 20 µL
   - Auto Increment Settings: Accept the default value
   - Data Collection: Accept the default value
   - Ramp Rate Settings: Accept the default value
   - Run mode: Use the default run mode for your system and sample block module (that is, Fast mode on Fast instruments and standard mode on standard instruments).
   - Thermal-cycling conditions for sample volumes ≤30 µL:

Step	Stage	No. of cycles	Temperature	Time
Reverse transcription	1	1	50°C†	5 minutes
RT inactivation/initial denaturation	2	1	95°C	20 seconds
Amplification	3	40	95°C	3 seconds
			60°C	30 seconds

† Reverse transcription works best between 48 °C and 55 °C.

Evaluate the results

1. Review the ΔR_n values to identify the optimal probe concentration for PCR yield.
2. Review the C_T values to identify the optimal probe concentration for C_T and detect any potential nonspecific amplification in the negative controls.
3. Select the probe concentration that produces the earliest C_T and the highest ΔR_n.

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).
- 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. 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

Product documentation

A portable document format (PDF) version of the document listed below is available at **www.lifetechnologies.com**

Note: To open the PDF version, use the Adobe Acrobat Reader software available from **www.adobe.com**

Document	Part number
TaqMan® Fast Virus 1-Step Master Mix *Protocol*	4453800

Related documentation

To obtain the documents listed in this section or additional documentation, see "Obtaining support" on page 37.

Chemistry/reagent documentation

Application note: *Factors Influencing Multiplex Real-Time PCR*	136AP04-01
Applied Biosystems® Real-Time PCR Systems Reagent Guide	4387787
Applied Biosystems® StepOne™ and StepOnePlus™ Real-Time PCR Systems Reagent Guide	4379704
Bioinformatic Evaluation of a Sequence for Custom TaqMan® Gene Expression Assays Tutorial	–
Custom TaqMan® Assays: Design and Ordering Guide	4367671
Custom TaqMan® Gene Expression Assays Protocol	4334429
Primer Express® Software Version 3.0 Getting Started Guide	4362460
Real-Time PCR Systems Chemistry Guide: Applied Biosystems® 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems	4348358
TaqMan® Gene Expression Assays Protocol	4333458

Instrument documentation

7500/7500 Fast system	*Applied Biosystems® 7500/7500 Fast Real-Time PCR System Getting Started Guide for Presence/Absence Experiments*	4387785
	Applied Biosystems® 7500/7500 Fast Real-Time PCR System Getting Started Guide for Standard Curve Experiments	4387779
7900HT/7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Module)	*Applied Biosystems® 7900HT Fast Real-Time PCR System Quick Reference Card: Performing Fast Gene Quantification*	4351892
	Applied Biosystems® 7900HT Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide	4364014
	Applied Biosystems® 7900HT Fast Real-Time PCR System User Bulletin: Performing Fast Gene Quantitation with 384-Well Plates	4369584
	Applied Biosystems® 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide	4351684
StepOne™ and StepOnePlus™ systems	*Applied Biosystems® StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Presence/Absence Experiments*	4376787
	Applied Biosystems® StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4376784
ViiA™ 7 Real-Time PCR System	*Applied Biosystems® ViiA™ 7 Real-Time PCR System Getting Started Guides*	4441434

Obtaining SDSs

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

Note: For the SDSs of chemicals not distributed by Life Technologies Corporation, 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
- 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 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 Corporations' website at www.lifetechnologies.com/termsandconditions. If you have any questions, please contact Life Technologies at www.lifetechnologies.com/support.

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