June 2015

QuantiTect® Virus Handbook

QuantiTect Virus Kit — with master mix containing ROX passive reference dye

QuantiTect Virus +ROX™ Vial Kit — with separate tube of ROX passive reference dye, and master mix that does not contain ROX dye

For highly sensitive real-time singleplex or multiplex PCR or one-step RT-PCR using sequence-specific probes for detection of viral DNA and RNA and internal controls

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QIAGEN Sample and Assay Technologies

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Contents

Kit Contents

QuantiTect Virus Kit	**(50)**	**(200)**	**(2 x 500)**
Catalog no.	**211011**	**211013**	**211015**
Number of 50 µl reactions	**50**	**200**	**1000**
5x QuantiTect Virus Master Mix*	0.5 ml	2 x 1 ml	10 x 1 ml
100x QuantiTect Virus RT Mix†	25 µl	2 x 50 µl	10 x 50 µl
RNase-Free Water	2 ml	4 x 2 ml	20 x 2 ml
QuantiTect Nucleic Acid Dilution Buffer	1 vial	2 vials	6 vials
Handbook	1	1	1

* Contains HotStarTaq® *Plus* DNA Polymerase, QuantiTect Virus Buffer, dNTP mix (dATP, dCTP, dGTP, dTTP), and ROX passive reference dye.

† Contains a unique formulation of Sensiscript® Reverse Transcriptase.

QuantiTect Virus +ROX Vial Kit	**(50)**	**(200)**	**(2 x 500)**
Catalog no.	**211031**	**211033**	**211035**
Number of 50 µl reactions	**50**	**200**	**1000**
5x QuantiTect Virus NR Master Mix (without ROX dye)‡	0.5 ml	2 x 1 ml	10 x 1 ml
50x ROX Dye Solution	210 µl	210 µl	1050 µl
100x QuantiTect Virus RT Mix§	25 µl	2 x 50 µl	10 x 50 µl
RNase-Free Water	2 ml	4 x 2 ml	20 x 2 ml
QuantiTect Nucleic Acid Dilution Buffer	1 vial	2 vials	6 vials
Handbook	1	1	1

‡ Contains HotStarTaq *Plus* DNA Polymerase, QuantiTect Virus Buffer, and dNTP mix (dATP, dCTP, dGTP, dTTP).

§ Contains a unique formulation of Sensiscript Reverse Transcriptase.

Shipping and Storage

QuantiTect Virus Kits are shipped on dry ice. They should be stored immediately upon receipt at –15 to –30°C in a constant-temperature freezer and protected from light. The kits can be stored under these conditions until the expiration date on the kit box without showing any reduction in performance.

5x QuantiTect Virus Master Mix and 5x QuantiTect Virus NR Master Mix can be stored at 2–8°C for up to 2 months without showing any reduction in performance. 100x QuantiTect Virus RT Mix, however, must be stored at –15 to –30°C in order to guarantee performance.

Product Use Limitations

The QuantiTect Virus Kits are intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding QuantiTect Virus Kits or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Product Description

Component	Description
HotStarTaq *Plus* DNA Polymerase*†	HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus*, cloned into *E. coli* (Deoxynucleoside-triphosphate: DNA deoxynucleotidyl-transferase, EC 2.7.7.7). The enzyme is activated by a 5-minute, 95°C incubation step.
QuantiTect Virus Buffer*†	Novel PCR buffer for highly sensitive detection of viral nucleic acids, including multiplex-PCR–enabling Factor MP
dNTP mix*†	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
Passive reference fluorescent dye*	ROX
50x ROX Dye Solution‡	Separate tube of passive reference dye for normalization of fluorescent signals on Applied Biosystems® 7500 Real-Time PCR Systems and, optionally, on instruments from Agilent (formerly Stratagene); not required for Rotor-Gene® cyclers and instruments from Bio-Rad, Cepheid, Eppendorf, and Roche
100x QuantiTect Virus RT Mix	Contains a unique formulation of Sensiscript Reverse Transcriptase optimized for highly sensitive detection of viral RNA
QuantiTect Nucleic Acid Dilution Buffer	Proprietary buffer formulation for dilution and storage of nucleic acid standards
RNase-free water	Ultrapure quality, PCR-grade

* Included in 5x QuantiTect Virus Master Mix.

† Included in 5x QuantiTect Virus NR Master Mix (without ROX dye).

‡ Supplied with the QuantiTect Virus +ROX Vial Kit.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each component of QuantiTect Virus Kits is tested against predetermined specifications to ensure consistent product quality. See the quality-control label inside the kit box for lot-specific values.

Introduction

QuantiTect Virus Kits provide highly sensitive real-time PCR analysis of viral nucleic acids (RNA and/or DNA) and internal controls using sequence-specific probes. Reactions can be carried out with or without a reverse-transcription step, enabling flexible design of multiplex assays to detect RNA targets, DNA targets, or both RNA and DNA targets.

Each kit contains a highly concentrated 5x master mix, which allows use of larger volumes of template (up to 50% of the reaction volume) to increase the sensitivity of assays. Real-time PCR or RT-PCR using sequence-specific probes can be performed in either singleplex or multiplex format. Depending on the real-time cycler used, up to 6 probes labeled with different dyes can be analyzed simultaneously in the same well or tube, or one or more probes labeled with the same dye can be used.

Time-consuming optimization of multiplex assays, such as for inclusion of internal positive controls or analysis of multiple target nucleic acids, is not required, as the 5x master mix is already optimized. The kits are compatible with all types of probe chemistries, providing flexibility in the choice of probes for multiplex assays.

The kits have been optimized for use with TaqMan® probes and are available in 2 formats:

- **QuantiTect Virus Kit**: This kit is supplied with a master mix containing ROX passive reference dye, and is optimized for use with real-time cyclers that require a high concentration of ROX dye for fluorescence normalization (e.g., instruments from Applied Biosystems, but not Applied Biosystems 7500 Real-Time PCR Systems).
- **QuantiTect Virus +ROX Vial Kit**: This kit is supplied with a master mix that is free of ROX dye, and also includes a separate solution of ROX dye which the user can add to reactions, depending on the real-time cycler used. The kit is intended for use with cyclers that require a lower concentration of ROX dye for fluorescence normalization (e.g., Applied Biosystems 7500 Real-Time PCR Systems), for use with cyclers that allow optional use of ROX dye (e.g., instruments from Agilent), and for use with cyclers that do not require ROX dye (e.g., Rotor-Gene cyclers). Running reactions without ROX dye increases multiplexing capacity and allows greater flexibility when choosing reporter dyes for probes.

Flexibility to detect viral RNA and/or viral DNA

Use of 5x QuantiTect Virus Master Mix together with 100x QuantiTect Virus RT Mix allows both reverse transcription and PCR to take place in a single tube, enabling detection of viral RNA and viral DNA in the same vessel. All reagents required for both reactions are added at the beginning of the procedure, and there is no need to open the tube once the reverse-transcription reaction has been started. If only viral DNA is to be detected, 100x QuantiTect Virus RT Mix and the reverse-transcription step can be omitted, shortening the protocol.

100x QuantiTect Virus RT Mix

100x QuantiTect Virus RT Mix contains a unique formulation of Sensiscript Reverse Transcriptase optimized for highly sensitive detection of viral RNA. Sensiscript Reverse Transcriptase exhibits a high affinity for RNA, facilitating transcription through secondary structures that may inhibit other reverse transcriptases.

5x QuantiTect Virus Master Mixes

In contrast to current methods, QuantiTect Virus Kits eliminate the need for optimization of the concentrations of primers, Mg^{2+}, and DNA polymerase. 5x QuantiTect Virus Master Mixes are specifically optimized for highly sensitive detection of one or more viral nucleic acid targets and internal controls. The optimized master mixes ensure that the target sequences in a multiplex reaction are amplified with the same efficiency and sensitivity as the target sequences in corresponding singleplex reactions.

5x QuantiTect Virus NR Master Mix contains HotStarTaq *Plus* DNA Polymerase and QuantiTect Virus Buffer. 5x QuantiTect Virus Master Mix contains in addition ROX passive reference dye. Reverse transcriptases are supplied in a separate tube.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase. HotStarTaq *Plus* DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperatures. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity and accurate quantification. The enzyme is activated by a 5-minute, 95°C incubation step, which is easily incorporated into existing thermal cycling programs.

QuantiTect Virus Buffer

QuantiTect Virus Buffer has been specifically developed for highly sensitive detection of viral nucleic acids using sequence-specific probes. In addition to various salts and additives, the buffer also contains a specially optimized combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^{2+} is usually not required. The buffer also contains Factor MP, which facilitates multiplex PCR. This synthetic factor increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The combination of these different components of QuantiTect Virus Buffer prevents different amplification reactions from affecting each other.

The buffer composition also eliminates the need to determine a specific annealing temperature for each primer–probe set. This enables fast 2-step cycling with a combined annealing/extension step for all targets, with the advantage that several assays can be run in parallel on a single real-time cycler using the same cycling protocol.

ROX passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection. However, when performing multiplex, real-time PCR with these instruments, the presence of ROX passive reference dye will limit their multiplexing capability.

The use of ROX dye is necessary for instruments from Applied Biosystems and is optional for instruments from Agilent. When performing multiplex, real-time PCR with ROX passive reference dye on these instruments, we do not recommend using probes that have ROX or Texas Red® fluorophore as the reporter dye, since their performance in the presence of ROX passive reference dye is unpredictable. When performing reactions using probes labeled with ROX, Texas Red, or other equivalent fluorophore, use a real-time cycler that does not require ROX dye for fluorescence normalization.

The master mix supplied with the QuantiTect Virus Kit contains ROX dye at a concentration that is optimal for instruments from Applied Biosystems (models 7000, 7300, 7700, 7900HT, StepOne™, and StepOnePlus™, but not Applied Biosystems 7500 Real-Time PCR Systems).

For Applied Biosystems 7500 Real-Time PCR Systems and instruments from Agilent, ROX dye is required at a lower concentration. This is provided by the QuantiTect Virus +ROX Vial Kit, which requires the user to add the supplied ROX dye solution to the master mix during reaction setup. If desired, ROX dye can be premixed with an entire tube of master mix in a 1:10 ratio (e.g., 100 µl 50x ROX Dye Solution and 1 ml 5x QuantiTect Virus NR Master Mix). Remember to indicate on the tube that ROX dye has been added. Store the premixed solution at –15 to –30°C, protected from light.

Instruments from all other suppliers, which do not require ROX dye for fluorescence normalization, should be used with the QuantiTect Virus +ROX Vial Kit, which provides a master mix that does not contain ROX dye.

QuantiTect Nucleic Acid Dilution Buffer

QuantiTect Nucleic Acid Dilution Buffer is intended for dilution of nucleic acids (RNA or DNA) used to generate standard curves or as positive controls in real-time PCR or RT-PCR. The buffer stabilizes RNA and DNA standards during dilution and reaction setup and prevents loss of nucleic acids on plastic surfaces, such as tubes or pipet tips. The buffer is ready to use and is free of RNases and DNases. Proper use of the buffer enables safe and accurate dilution of the small amounts of nucleic acids typically used as standards for analysis of viral nucleic acids.

Aliquots of diluted standards can be stored in QuantiTect Nucleic Acid Dilution Buffer at –15 to –30°C for up to 6 months. Repeated freezing and thawing should be avoided.

Sequence-specific probes

QuantiTect Virus Kits can be used with all types of probe. This handbook contains optimized protocols for use with TaqMan probes, a major type of sequence-specific probe used in quantitative, real-time PCR (see below). For more details on sequence-specific probes, and their design and handling, see Appendix A, page 54.

TaqMan probes

TaqMan probes are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached (Figure 1). The fluorophore is at the 5' end of the probe, and the quencher moiety is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5'→3' exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher moiety. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

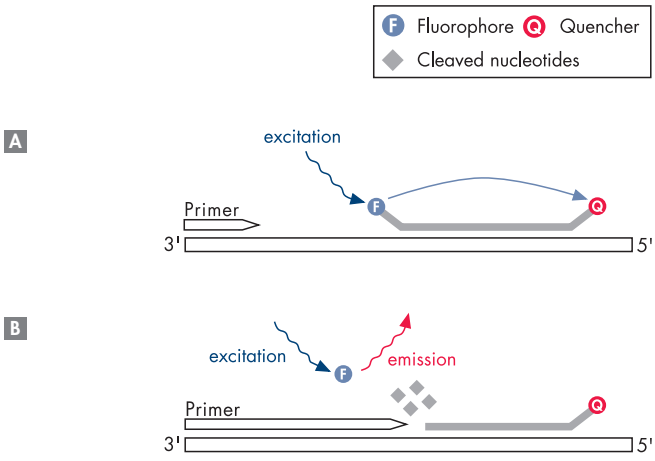

Figure 1. Principle of TaqMan probes in quantitative, real-time PCR. A Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorophore with the quencher results in efficient quenching of fluorescence from the fluorophore. B During the PCR extension step, *Taq* DNA polymerase extends the primer. When the enzyme reaches the TaqMan probe, its 5'→3' exonuclease activity cleaves the fluorophore from the probe. The fluorescent signal from the free fluorophore is measured. This signal is proportional to the amount of accumulated PCR product.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Primers and probes from an established oligonucleotide manufacturer. Primers should be of standard quality, and probes should be HPLC purified. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry (for details, see Appendix A, page 54). Primer and probe stock solutions should be stored in aliquots at –20°C. Probe stock solutions should be protected from exposure to light.
- Nuclease-free (RNase/DNase-free) consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of viral nucleic acids. See Appendix C, page 59, for details about avoiding nucleases during PCR setup.
- Cooling device or ice
- Real-time PCR thermal cycler (we recommend the Rotor-Gene Q for high-precision and flexibility in multiplex analysis; for details, visit www.qiagen.com/goto/Rotor-GeneQ)
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your real-time cycler)
- Optional: Trizma® base and EDTA for preparing TE buffer for storing primers and probes (see Appendix A, page 54). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.
- Optional: QIAgility® for rapid, high-precision automated PCR setup; for details, visit www.qiagen.com/goto/QIAgility. Please note that the number of reactions will be 10–20% lower than indicated on the kit packaging to guarantee process safety and optimal performance.

Important Notes

Selecting kits and protocols

To select the correct QuantiTect Virus Kit and protocol to use with your real-time cycler, refer to Table 1. Additional supplementary protocols (e.g., for real-time cyclers with 384-well PCR blocks) are continually being developed and are available from QIAGEN Technical Services (see back cover or visit www.qiagen.com) or at www.qiagen.com/goto/QTVirus.

In general, the following cyclers are not compatible with multiplex, real-time PCR: GeneAmp® 5700, MyiQ™, and DNA Engine Opticon® (i.e., the single-color machine). The capabilities of the LightCycler® 1.x for multiplex, real-time PCR are very limited due to its detection optics.

Table 1. Choosing the correct QuantiTect Virus Kit and protocol for your real-time cycler

Cycler	Kit	Singleplex/ duplex protocol	Triplex/ 4-plex protocol
ABI PRISM® 7000	QuantiTect Virus Kit	Protocol 1, page 34	Protocol 2, page 38*
ABI PRISM 7700†	QuantiTect Virus Kit	Protocol 1, page 34	–
ABI PRISM 7900HT (96 well)	QuantiTect Virus Kit	Protocol 1, page 34	Protocol 2, page 38*
Applied Biosystems 7300	QuantiTect Virus Kit	Protocol 1, page 34	Protocol 2, page 38*
Applied Biosystems 7500‡	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
Applied Biosystems 7900HT Fast	QuantiTect Virus Kit	Protocol 1, page 34	Protocol 2, page 38*

* Only triplex assays are possible.

† Only singleplex or duplex assays are possible due to hardware limitations.

‡ Includes the Applied Biosystems 7500 Fast Real-Time PCR System.

Table continues on next page.

Table 1. Continued

Cycler	Kit	Singleplex/ duplex protocol	Triplex/ 4-plex protocol
Applied Biosystems StepOne*	QuantiTect Virus Kit	Protocol 1, page 34	–
Applied Biosystems StepOnePlus	QuantiTect Virus Kit	Protocol 1, page 34	Protocol 2, page 38†
Chromo4	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
DNA Engine Opticon 2*	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	–
iCycler iQ® and iQ5	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
LightCycler 480 (96 well)	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
Mx3000P®, Mx3005P®, and Mx4000®	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
Rotor-Gene Q, Rotor-Gene 6000, and Rotor-Gene 3000	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
SmartCycler® II	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46
Other‡	QuantiTect Virus +ROX Vial Kit	Protocol 3, page 42	Protocol 4, page 46

* Only singleplex or duplex assays are possible due to hardware limitations.

† Only triplex assays are possible.

‡ Refer to manufacturer's instructions for multiplex capacity.

Guidelines for effective multiplex assays

QuantiTect Virus Kits work with most existing probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in quantitative, multiplex, real-time PCR, some considerations need to be made, including the choice of a compatible combination of reporter dyes (i.e., the fluorophores on the probes) and the quality of the primers and probes. Please read the following guidelines before starting.

- Check the functionality of each set of primers and probe in individual assays before combining the different sets in a multiplex assay.
- Choose compatible reporter dyes and quenchers. For details, see "Suitable combinations of reporter dyes", page 17.
- PCR products should be as short as possible, ideally 60–150 bp. In rare cases (e.g., amplification of difficult target sequences or PCR products >150 bp), different cycling conditions may improve results. For details, see Appendix F, page 64.
- Always use the same algorithm or software to design the primers and probes. For optimal results, only combine assays that have been designed using the same parameters (e.g., similar melting points [T_m]). For details, see Appendix A, page 54.
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix A, page 54.
- Check the real-time cycler user manual for **correct setup of the cycler for multiplex analysis** (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for each reporter dye used.
- Some real-time cyclers require you to perform **a calibration procedure for each reporter dye**. Check whether the reporter dyes you selected for your multiplex assay are part of the standard set of dyes already calibrated on your instrument. If they are not, perform a calibration procedure for each dye before using them for the first time (for details, refer to the manufacturer's instructions for your real-time cycler).
- Always start with the **cycling conditions specified in the protocol** you are following. The unique composition of QuantiTect Virus Buffer ensures specific annealing for each primer set. This enables fast 2-step cycling with a combined annealing/extension step for all targets.
- It is important to **use the hot-start step** for activation of HotStarTaq *Plus* DNA Polymerase.
- It is **not recommended** to shorten the denaturation step to less than 15 s.

- Optimal analysis settings (i.e., baseline settings and threshold values) for each reporter dye are a prerequisite for accurate quantification data. For details, check the literature from the manufacturer of your real-time cycler.
- Perform appropriate controls for evaluating the performance of your multiplex assays (e.g., amplifying each target individually and comparing the results with those for the multiplex assay).

Suitable combinations of reporter dyes

Multiplex, real-time PCR requires the simultaneous detection of different fluorescent reporter dyes (Table 2). For accurate detection, the fluorescence spectra of the dyes should be well separated or exhibit only minimal overlap. **Please read the general recommendations and instrument-specific recommendations on the next few pages before starting.**

Note: If there are no specific recommendations below for your real-time cycler, please refer to the user manual or other technical documentation for your instrument to find out which reporter dyes can be used in multiplex analysis.

Table 2. Dyes commonly used in quantitative, multiplex, real-time PCR

Dye	Excitation maximum (nm)	Emission maximum (nm)*
ATTO 390	390	479
AMCA-X	353	442
Marina Blue®	362	459
FAM™	494	518
TET™	521	538
JOE™	520	548
VIC®	538	552
Yakima Yellow®	526	552
HEX™	535	553
CAL Fluor® Orange 560	538	559
Bodipy® TMR	542	574
NED™	546	575
Cy®3	552	570
TAMRA™	560	582
Cy3.5	588	604
ROX	587	607

* Emission spectra may vary depending on the buffer conditions.

Table continues on next page.

Table 2. Continued

Dye	Excitation maximum (nm)	Emission maximum (nm)*
Texas Red	596	615
TYE™ 665	645	665
Cy5	643	667
Quasar® 705	690	705

* Emission spectra may vary depending on the buffer conditions.

General recommendations

- Before starting, choose suitable combinations of reporter dyes and quenchers that are compatible with multiplex analysis using the detection optics of your real-time cycler. Order the probes from an established oligonucleotide manufacturer.
- For optimal results, follow the recommended combinations of dyes shown in Tables 3–13 (pages 19–32).
- For duplex analysis, the use of nonfluorescent quenchers (e.g., Dark Quencher or Black Hole Quencher® [BHQ®] on TaqMan probes) is preferred over fluorescent quenchers (e.g., TAMRA fluorescent dye). TAMRA quencher can be used in duplex analysis if the 2 reporter dyes are 6-FAM dye and HEX, JOE, or VIC dye.
- **For triplex and 4-plex analyses, we strongly recommend using nonfluorescent quenchers.** Due to the detection optics of more recent real-time cyclers and the possible combinations of reporter dyes, triplex and 4-plex analyses may only be possible with nonfluorescent quenchers, especially with instruments from Applied Biosystems.

Recommendations for instruments from Applied Biosystems

Tables 3–8 on the next pages give specific recommendations for the ABI PRISM 7000, ABI PRISM 7700, Applied Biosystems 7300, Applied Biosystems 7500, Applied Biosystems 7900HT, StepOne, and StepOnePlus.

Table 3. Suitable reporter dyes — ABI PRISM 7000 or Applied Biosystems 7300

Type of assay	Filter A*	Filter B*	Filter C*	Filter D†
Duplex	6-FAM	HEX‡ JOE VIC		ROX (passive reference)
Duplex	6-FAM		Bodipy TMR‡ NED	ROX (passive reference)
Triplex§	6-FAM	HEX‡ JOE VIC	Bodipy TMR‡ NED	ROX (passive reference)

* Use filter A, filter B, and filter C to detect the least abundant target, the second least abundant target, and the most abundant target, respectively.

† Filter D is for detecting ROX passive reference dye, a component of 5x QuantiTect Virus Master Mix.

‡ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

§ Each probe in the triplex assay must contain a nonfluorescent quencher.

Table 4. Suitable reporter dyes — Applied Biosystems 7500

Type of assay	Filter A*	Filter B*	Filter C*†	Filter D‡	Filter E*
Duplex	6-FAM	HEX§ JOE VIC		ROX (passive reference)	
Duplex	6-FAM		Bodipy TMR§ NED	ROX (passive reference)	
Triplex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX (passive reference)	
4-plex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX (passive reference)	Cy5

* Use filter A, filter B, and filters C and E to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

† When using filter C in duplex, triplex, or 4-plex PCR, all probes in the reaction must be labeled with a nonfluorescent quencher instead of a fluorescent quencher such as TAMRA dye.

‡ Filter D is for detecting ROX passive reference dye, which is supplied as a separate solution with the QuantiTect Virus +ROX Vial Kit. The dye can be added to reactions, or premixed with 5x QuantiTect Virus NR Master Mix (without ROX dye).

§ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 5. Suitable reporter dyes — Applied Biosystems 7900HT

Type of assay	Target 1*	Target 2*	Target 3*†	Passive reference‡
Duplex	6-FAM	HEX§ JOE VIC		ROX
Duplex	6-FAM		Bodipy TMR§ NED	ROX
Triplex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX

* Target 1, target 2, and target 3 correspond to the least abundant target, the second least abundant target, and the most abundant target, respectively.

† When using a Bodipy TMR or NED labeled probe in duplex or triplex PCR, all probes in the reaction must be labeled with a nonfluorescent quencher instead of a fluorescent quencher such as TAMRA dye.

‡ ROX fluorescent dye is used as passive reference and is a component of 5x QuantiTect Virus Master Mix.

§ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 6. Suitable reporter dyes — Applied Biosystems StepOne*

Type of assay	Filter 1†	Filter 2†	Passive reference‡
Duplex	6-FAM	HEX§ JOE VIC	ROX

* This real-time cycler is only designed for duplex analysis with the standard calibration.

† Use filter 1 to detect the least abundant target.

‡ ROX fluorescent dye is used as passive reference and is a component of 5x QuantiTect Virus Master Mix.

§ Before using HEX dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 7. Suitable reporter dyes — Applied Biosystems StepOnePlus

Type of assay	Filter 1*	Filter 2*	Filter 3*†	Filter 4‡
Duplex	6-FAM	HEX§ JOE VIC		ROX (passive reference)
Duplex	6-FAM		Bodipy TMR§ NED	ROX (passive reference)
Triplex	6-FAM	HEX§ JOE VIC	Bodipy TMR§ NED	ROX (passive reference)

* Use filter 1, filter 2, and filter 3 to detect the least abundant target, the second least abundant target, and the most abundant target, respectively.

† When using filter 3 in duplex or triplex PCR, all probes in the reaction must be labeled with a nonfluorescent quencher instead of a fluorescent quencher such as TAMRA dye.

‡ Filter 4 is for detecting ROX passive reference dye, a component of 5x QuantiTect Virus Master Mix.

§ Before using HEX or Bodipy TMR dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Table 8. Suitable reporter dyes — ABI PRISM 7700*

Type of assay	Target 1†	Target 2†	Passive reference‡
Duplex	6-FAM	HEX§ JOE VIC	ROX

* This real-time cycler is only designed for duplex analysis with the standard calibration.

† Target 1 and target 2 correspond to the least abundant target and the second least abundant target, respectively.

‡ ROX fluorescent dye is used as passive reference and is a component of 5x QuantiTect Virus Master Mix.

§ Before using HEX dye, a pure dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services.

Recommendations for Mx3000P, Mx3005P, and Mx4000 systems

Mx3000P, Mx3005P, and Mx4000 systems allow the use of different combinations of excitation and emission filters. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using Mx3000P, Mx3005P, and Mx4000 systems are given in Table 9 (page 24).

- Before performing a multiplex assay on the Mx3000P, Mx3005P, or Mx4000 system:
  - Check which reporter dyes can be detected with the sets of excitation and emission filters installed on your instrument.

    View the installed filter sets on your instrument as follows. Make sure the computer is connected to the instrument, and start the instrument software. Click the "Options" menu and select "Optics Configuration" to open the "Optics Configuration" dialog box. Click the "Dye Assignment" tab: the 4 filter sets displayed correspond to the filter sets installed on your instrument.

    View the dyes assigned to the installed filter sets as follows. In the "Dye Assignment" tab, click "Additional Dye Information" to open the "Dye Information" dialog box. Select "Detected dyes" to display the filter sets installed on your instrument and the defined dyes that are compatible with them.
  - Ensure that each reporter dye is detected by a different filter set in a distinct optical path.
- Refer to the *Mx4000 Multiplex Quantitative PCR System Instruction Manual,* the *Mx3005P Real-Time PCR System Instruction Manual,* or the *Mx3000P Real-Time PCR System On-line Help Manual* for additional information on the detection optics and correctly setting up the instrument for multiplex analysis.
- Different detection filter sets are available for Mx3000P, Mx3005P, and Mx4000 systems. Note that only some combinations of filter sets are compatible with multiplex analysis using commonly used reporter dyes. For details, see Table 9 (page 24).

Table 9. Suitable reporter dyes — Mx3000P, Mx3005P, and Mx4000*

Type of assay	Optical path 1 (FAM filter set)†	Optical path 2 (HEX/ JOE filter set)†	Optical path 3 (ROX filter set)†	Optical path 4 (Cy5 filter set)†
Duplex	6-FAM	HEX JOE VIC		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			Cy5
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX	
Triplex	6-FAM	HEX JOE VIC		Cy5
Triplex	6-FAM		Texas Red ROX	Cy5
4-plex	6-FAM	HEX JOE VIC	ROX	Cy5

* The Mx3005P has 5 detection channels. This table shows the recommended dyes for use with four of the channels.

† Use optical path 1, optical path 2, and optical paths 3 and 4 to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

Recommendations for the iCycler iQ system

The iCycler iQ system is capable of using different combinations of excitation and emission filters. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using the iCycler iQ system are given in Table 10 (page 26).

- Before performing a multiplex assay on the iCycler iQ system:
  - Check that a filter set for each selected reporter dye is installed on the instrument. Ensure that each reporter dye is detected by a different filter set.
  - Calibrate each selected reporter dye on the instrument using a pure dye. The iCycler iQ Dye Calibrator Solution Kit (Bio-Rad, cat. no. 170-8792) may be used. Pure dye calibration data are used to separate the total fluorescence signal into the individual dyes. Pure dye calibration data are stored in the **RME.ini** file, which is stored at **C:\Program Files\Bio-Rad\iCycler\Ini**.
- Recalibrating the instrument (i.e., overwriting **RME.ini**) is required when changing the reaction volume, when switching from using caps to optical tape (or vice versa), or when adding new dyes for use in multiplex assays. If desired, the current **RME.ini** file can be archived before it is overwritten.
- The iCycler iQ system requires the collection of well factors before each run. If your sample plate does not contain the same dyes at the same concentrations in all wells, external well factors must be used. Collecting external well factors can be done using iCycler iQ External Well Factor Solution (Bio-Rad, cat. no. 170-8794).
- Refer to the *iCycler iQ Real-Time PCR Detection System Instruction Manual* for additional information on filter wheel setup, external well factors, selection and calibration of dyes, and correctly setting up the instrument for multiplex analysis.

Table 10. Suitable reporter dyes — iCycler iQ

Type of assay	Channel 1 (filter 490/530)*†	Channel 2 (filter 530/575)*†	Channel 3 (filter 575/620)*†	Channel 4 (filter 635/680)*†
Duplex	6-FAM	HEX JOE VIC		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			Cy5
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX	
Triplex	6-FAM	HEX JOE VIC		Cy5
Triplex	6-FAM		Texas Red ROX	Cy5
4-plex	6-FAM	HEX JOE VIC	Texas Red ROX	Cy5

* The numbers indicate the excitation/emission wavelengths of the detection filter set.

† Use channel 1, channel 2, and channels 3 and 4 to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

Recommendations for the Rotor-Gene 3000

The Rotor-Gene 3000 system uses a 4-channel light source and a detection filter wheel with 6 detection filters. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using the Rotor-Gene 3000 system are given in Table 11 (page 28).

- The Rotor-Gene 3000 system has 4 preset channels that provide the best choice for multiplex assays. Each channel detects a particular reporter dye (FAM, JOE, ROX, or Cy5) and is named after the dye it detects. Other reporter dyes with similar spectra can also be detected by these channels, and do not require calibration.
- The detection filter wheel contains three 10 nm band-pass filters (only signals within a certain wavelength band can pass through) and 3 high-pass filters (only signals with a wavelength above a certain limit can pass through). With regard to the 4 preset channels, the FAM, JOE, and ROX channels each use a 10 nm band-pass filter, while the Cy5 channel uses a high-pass filter. The band-pass filters are named after the wavelength they let through followed by the unit "nm": 510 nm, 555 nm, and 610 nm. The high-pass filter is named after its wavelength limit followed by "hp": 665 hp. There are 2 additional high-pass filters (610 hp and 585 hp), which can be used to detect dyes that cannot be detected using the preset channels. However, their use may be limited in multiplex assays.
- Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
- Refer to the Rotor-Gene Software Manual for additional information on setting up detection channels and correctly setting up the instrument for multiplex analysis.

Table 11. Suitable reporter dyes — Rotor-Gene 3000

Type of assay	Channel 1 (470/510)*†	Channel 2 (530/555)*†	Channel 3 (585/610)*†	Channel 4 (625/665)*†
Duplex	6-FAM	HEX JOE VIC		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			Cy5
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX	
Triplex	6-FAM	HEX JOE VIC		Cy5
Triplex	6-FAM		ROX‡ Cy3.5‡	Cy5
4-plex	6-FAM	HEX JOE VIC	ROX‡	Cy5

* The numbers in parentheses indicate the wavelengths of the excitation light source and the detection filter.

† Use channel 1, channel 2, and channels 3 and 4 to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

‡ We do not recommend using channel 3 to detect Texas Red dye if channel 4 is also being used. This is because Texas Red dye is partly detected by channel 4.

Recommendations for the Rotor-Gene Q and Rotor-Gene 6000

Rotor-Gene Q and Rotor-Gene 6000 systems have 6 detection channels and use a separate high-power LED as an excitation source for each channel. This provides flexibility when selecting reporter dyes for multiplex assays. However, care must be taken to select suitable combinations of reporter dyes and channels that exhibit minimal crosstalk. Suitable combinations of reporter dyes for multiplex assays using Rotor-Gene Q and Rotor-Gene 6000 systems are given in Table 12 (page 29).

- Rotor-Gene Q and Rotor-Gene 6000 systems have 4 preset channels that provide the best choice for multiplex assays: Green, Yellow, Orange, and Crimson. Each channel detects reporter dyes that emit light at a particular wavelength. For 5-plex and 6-plex assays, the Blue and Red channels can be additionally used.
- Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
- Refer to the user manual supplied with the Rotor-Gene cycler for additional information on setting up detection channels and correctly setting up the instrument for multiplex analysis.

Table 12. Suitable reporter dyes — Rotor-Gene Q and Rotor-Gene 6000

Type of assay	Blue channel	Green channel*	Yellow channel*	Orange channel*	Red channel	Crimson channel*
Duplex		6-FAM	HEX VIC Yakima Yellow CAL Fluor Orange 560			
Duplex		6-FAM		ROX		
Duplex		6-FAM				Quasar 705
Triplex		6-FAM	HEX VIC Yakima Yellow CAL Fluor Orange 560	ROX		

* Use the green channel, the yellow channel, and the orange and crimson channels to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

Table continues on next page.

Table 12. Continued

Type of assay	Blue channel	Green channel*	Yellow channel*	Orange channel*	Red channel	Crimson channel*
Triplex		6-FAM	HEX VIC Yakima Yellow CAL Fluor Orange 560			Quasar 705
Triplex		6-FAM		ROX		Quasar 705
4-plex		6-FAM	HEX VIC Yakima Yellow CAL Fluor Orange 560	ROX		Quasar 705
5-plex		6-FAM	HEX VIC Yakima Yellow CAL Fluor Orange 560	ROX	TYE 665	Quasar 705
6-plex	ATTO 390† AMCA-X† Marina Blue†	6-FAM	HEX VIC Yakima Yellow CAL Fluor Orange 560	ROX	TYE 665	Quasar 705

* Use the green channel, the yellow channel, and the orange and crimson channels to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively.

† One of the following quenchers tested by QIAGEN should be used: BHQ-0 or dabcyl.

Recommendations for the LightCycler 480 system

The LightCycler 480 system uses a xenon lamp as its light source and has 5 excitation filters and 6 emission filters, allowing the setup of 5 detection channels suitable for multiplex analysis. Suitable combinations of reporter dyes for multiplex assays using the LightCycler 480 system are given in Table 13 (page 32).

- Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
- Refer to the *LightCycler 480 Operator's Manual* for additional information on activating and deactivating detection channels and correctly setting up the instrument for multiplex analysis.
- Make sure to select suitable combinations of reporter dyes and filters that exhibit minimal crosstalk. There are 2 options for avoiding crosstalk on the LightCycler 480 system:
  - Dyes can be used that have widely separated emission spectra (e.g., FAM and Cy5). However, it is still recommended to determine the degree of crosstalk for these assays.
  - Alternatively, the LightCycler 480 system can use a color compensation file that contains information to correct crosstalk between different detection channels. Check that each selected reporter dye is compatible with one of the detection channels installed on the instrument. Ensure that each reporter dye is detected by a different channel.
  - A supplementary protocol which describes how to generate and use color compensation files for multiplex assays using TaqMan probes is available. Visit www.qiagen.com/literature, enter *PCR82* in the "Search" field, and then click "Search" to retrieve the protocol.

Table 13. Suitable reporter dyes — LightCycler 480

Type of assay	Channel 1 (450/500)*†	Channel 2 (483/533)*†	Channel 3 (523/568)*†	Channel 4 (558/610)*†	Channel 5 (615/670)*†
Duplex	Cyan 500	6-FAM			
Duplex		6-FAM	HEX‡		
Duplex		6-FAM		Texas Red ROX	
Duplex		6-FAM			Cy5
Triplex	Cyan 500	6-FAM	HEX‡		
Triplex	Cyan 500	6-FAM		Texas Red ROX	
Triplex	Cyan 500	6-FAM			Cy5
Triplex		6-FAM	HEX‡	Texas Red ROX	
Triplex		6-FAM	HEX‡		Cy5
Triplex		6-FAM		Texas Red ROX	Cy5
4-plex	Cyan 500	6-FAM	HEX‡	Texas Red ROX	
4-plex	Cyan 500	6-FAM	HEX‡		Cy5
4-plex	Cyan 500	6-FAM		Texas Red ROX	Cy5
4-plex		6-FAM	HEX‡	Texas Red ROX	Cy5

* The numbers in parentheses indicate the wavelengths of the excitation and emission filters on the first-generation LightCycler 480. Newer versions of the LightCycler 480 have slightly different filters installed: please check the user manual supplied with your instrument. All reporter dye combinations shown have been successfully tested by QIAGEN on the first-generation LightCycler 480 only.

† Use channel 2, channel 3, and channels 1, 4 and 5 to detect the least abundant target, the second least abundant target, and the most abundant targets, respectively.

‡ JOE or VIC dye (not tested by QIAGEN) can be used instead of HEX dye.

Controls

No template control (NTC)

At least one NTC reaction should be included in each PCR run, containing all the components of the reaction except for the template. This enables detection of contamination in the reagents.

No RT control

Depending on your sample type and the life cycle of the virus species detected, you may want to include a control in RT-PCR without reverse transcriptases. This enables testing for contaminating DNA, such as DNA from viral sequences integrated into the host genome. The control reaction contains all components including template RNA, except for 100x QuantiTect Virus RT Mix (which contains reverse transcriptases). Reverse transcription therefore cannot take place, and the only template available is contaminating DNA.

Alternatively, DNA in the sample can be removed by digestion with DNase* before RT-PCR amplification.

Positive control

In some cases, it may be necessary to perform a positive control reaction in the PCR run, containing a known concentration or copy number of template. Positive controls can be absolute standards or known positive samples.

Absolute standards include commercially available standards and in-lab standards, such as a plasmid containing cloned viral sequences or in vitro transcribed viral RNA. Absolute standards are used at a known copy number and provide quantitative information.

A positive sample is usually a substitute for an absolute standard and is used only to test for presence or absence of the target.

Internal positive control

An internal, positive control (detected with a second, differently labeled probe) can be used to test for the presence of PCR inhibitors. This control should be either DNA or RNA, depending on the viral nucleic acid being detected. Ensure that the internal positive control is used at a high enough copy number for accurate detection.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Protocol 1: Singleplex or Duplex Analysis on Most Applied Biosystems Cyclers

This protocol is optimized for use of the QuantiTect Virus Kit with TaqMan probes and real-time cyclers from Applied Biosystems **except Applied Biosystems 7500 Real-Time PCR Systems**. For further information, see "ROX passive reference dye", page 11.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.** The cycling is optimized for PCR products between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 64.
- **Use the primer concentrations specified in this protocol.**
- We strongly recommend testing the performance of new primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 16. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- See the user manual for the real-time cycler for correct instrument setup for multiplex analysis (e.g., setting up detection of multiple dyes in the same well). Make sure to activate the detector for each reporter dye used. Depending on the instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.
- If using an already established duplex assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer-limiting concentrations again.
- **After reverse transcription, the PCR must start with an initial incubation step of 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase.**
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61). Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E (page 62).

Procedure

1. **Thaw 5x QuantiTect Virus Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral nucleic acids), optional standards, and references. Mix the individual solutions.**

   Standards should be diluted in QuantiTect Nucleic Acid Dilution Buffer at an appropriate concentration to enable use of 5–10 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 14 (page 36). It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

   Note: For RT-PCR, 100x QuantiTect Virus RT Mix should be taken from –15 to –30°C immediately before use, always kept on ice, and returned to –15 to –30°C immediately after use.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

   Note: Keep the tubes or plate on ice.

4. **Add template nucleic acids to the individual PCR tubes or wells, and mix thoroughly.**

   Note: Ensure that the reaction mix and template are thoroughly mixed.

5. **Program the real-time cycler according to Table 15 (page 37).**

Table 14. Reaction setup

Component	Volume*	Final concentration
5x QuantiTect Virus Master Mix	10 µl	1x
20x primer–probe mix 1†	2.5 µl	0.4 µM forward primer 1‡ 0.4 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	2.5 µl	0.4 µM forward primer 2‡ 0.4 µM reverse primer 2‡ 0.2 µM probe 2§
For RT-PCR only: 100x QuantiTect Virus RT Mix	0.5 µl	1x
RNase-free water	Variable	–
Template DNA or RNA (added at step 4)	Variable	Maximum up to 50% of final reaction volume
Total reaction volume	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of master mix and all other reaction components accordingly.

† For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61).

‡ A final primer concentration of 0.4 µM is optimal. Before adapting primer concentration, verify the concentration of your primer solutions.

§ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 15. Cycling conditions

Step	Time	Temperature	Additional comments
For RT-PCR only: Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA. Omit this step if you are analyzing DNA targets.
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions.**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**45 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection, optimized for PCR products up to 150 bp. For PCR products >150 bp, different cycling conditions may improve results in some cases. For details, see Appendix F, page 64.
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or RNA.

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. For each probe, select the analysis settings (i.e., baseline settings and threshold values). Note that optimal analysis settings are a prerequisite for accurate quantification data.

Protocol 2: Triplex Analysis on Most Applied Biosystems Cyclers

This protocol is optimized for use of the QuantiTect Virus Kit with TaqMan probes and real-time cyclers from Applied Biosystems **except Applied Biosystems 7500 Real-Time PCR Systems**. For further information, see "ROX passive reference dye", page 11.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.** Please note that these cycling conditions differ from those described in the protocol for duplex assays (page 34). The cycling is optimized for PCR products between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 64.
- **Use the primer concentrations specified in this protocol.** Please note that these primer concentrations differ from those described in the duplex assays protocol (page 34).
- We strongly recommend testing the performance of new primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 16. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established multiplex assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer-limiting concentrations again.
- **After reverse transcription, the PCR must start with an initial incubation step of 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase.**
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61). Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E (page 62).

Procedure

1. **Thaw 5x QuantiTect Virus Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral nucleic acids), optional standards, and references. Mix the individual solutions.**

   Standards should be diluted in QuantiTect Nucleic Acid Dilution Buffer at an appropriate concentration to enable use of 5–10 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 16 (page 40). It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

   Note: For RT-PCR, 100x QuantiTect Virus RT Mix should be taken from –15 to –30°C immediately before use, always kept on ice, and returned to –15 to –30°C immediately after use.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

   Note: Keep the tubes or plate on ice.

4. **Add template nucleic acids to the individual PCR tubes or wells, and mix thoroughly.**

   Note: Ensure that the reaction mix and template are thoroughly mixed.

5. **Program the real-time cycler according to Table 17 (page 41).**

Table 16. Reaction setup

Component	Volume*	Final concentration
5x QuantiTect Virus Master Mix	10 µl	1x
20x primer–probe mix 1†	2.5 µl	0.2 µM forward primer 1‡ 0.2 µM reverse primer 1‡ 0.2 µM probe 1§
20x primer–probe mix 2†	2.5 µl	0.2 µM forward primer 2‡ 0.2 µM reverse primer 2‡ 0.2 µM probe 2§
20x primer–probe mix 3†	2.5 µl	0.2 µM forward primer 3‡ 0.2 µM reverse primer 3‡ 0.2 µM probe 3§
For RT-PCR only: 100x QuantiTect Virus RT Mix	0.5 µl	1x
RNase-free water	Variable	–
Template DNA or RNA (added at step 4)	Variable	Maximum up to 50% of final reaction volume
Total reaction volume	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl, adjust the amount of master mix and all other reaction components accordingly.

† For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61).

‡ A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions. In some cases, other primer concentrations between 0.1 µM and 0.3 µM may improve performance.

§ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 17. Cycling conditions

Step	Time	Temperature	Additional comments
For RT-PCR only: Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA. Omit this step if you are analyzing DNA targets.
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions.**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**75 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection, optimized for PCR products up to 150 bp. For PCR products >150 bp, different cycling conditions may improve results in some cases. For details, see Appendix F, page 64.
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or RNA.

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. For each probe, select the analysis settings (i.e., baseline settings and threshold values). Note that optimal analysis settings are a prerequisite for accurate quantification data.

Protocol 3: Singleplex or Duplex Analysis on the Applied Biosystems 7500 and Other Cyclers

This protocol is optimized for use of the QuantiTect Virus +ROX Vial Kit with TaqMan probes on Applied Biosystems 7500 Real-Time PCR Systems, Rotor-Gene cyclers, and instruments from Bio-Rad, Cepheid, Roche, and Agilent. For further information, see "ROX passive reference dye", page 11.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.** The cycling is optimized for PCR products between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 64.
- **Use the primer concentrations specified in this protocol.**
- We strongly recommend testing the performance of new primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 16. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established duplex assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer-limiting concentrations again.
- **After reverse transcription, the PCR must start with an initial incubation step of 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase.**
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61). Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E (page 62).

Procedure

1. **Thaw 5x QuantiTect Virus NR Master Mix, 50x ROX Dye Solution, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral nucleic acids), optional standards, and references. Mix the individual solutions.**

   Standards should be diluted in QuantiTect Nucleic Acid Dilution Buffer at an appropriate concentration to enable use of 5–10 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 18 (page 44). It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

   Note: For RT-PCR, 100x QuantiTect Virus RT Mix should be taken from –15 to –30°C immediately before use, always kept on ice, and returned to –15 to –30°C immediately after use.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

   Note: Keep the tubes or plate on ice.

4. **Add template nucleic acids to the individual PCR tubes or wells, and mix thoroughly.**

   Note: Ensure that the reaction mix and template are thoroughly mixed.

5. **Program the real-time cycler according to Table 19 (page 45).**

Table 18. Reaction setup

Component	Volume/reaction 50 µl*	20 µl*†	Final concentration
5x QuantiTect Virus NR Master Mix	10 µl	4 µl	1x
50x ROX Dye Solution‡	1 µl	0.4 µl	1x
20x primer–probe mix 1§	2.5 µl	1 µl	0.4 µM forward primer 1¶ 0.4 µM reverse primer 1¶ 0.2 µM probe 1**
20x primer–probe mix 2§	2.5 µl	1 µl	0.4 µM forward primer 2¶ 0.4 µM reverse primer 2¶ 0.2 µM probe 2**
For RT-PCR only: 100x QuantiTect Virus RT Mix	0.5 µl	0.2 µl	1x
RNase-free water	Variable	Variable	–
Template DNA or RNA (added at step 4)	Variable	Variable	Maximum up to 50% of final reaction volume
Total reaction volume	**50 µl***	**20 µl*†**	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly.

† Refers to the Applied Biosystems 7500 Fast Real-Time PCR System.

‡ For real-time cyclers that do not require ROX dye, add RNase-free water instead.

§ For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61).

¶ A final primer concentration of 0.4 µM is optimal. Before adapting primer concentration, verify the concentration of your primer solutions.

** A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 19. Cycling conditions

Step	Time	Temperature	Additional comments
For RT-PCR only: Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA. Omit this step if you are analyzing DNA targets.
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions.**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**45 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection, optimized for PCR products up to 150 bp. For PCR products >150 bp, different cycling conditions may improve results in some cases. For details, see Appendix F, page 64.
Number of cycles	40–50		The number of cycles depends on the amount of template RNA.

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. For each probe, select the analysis settings (i.e., baseline settings and threshold values). Note that optimal analysis settings are a prerequisite for accurate quantification data.

Protocol 4: Triplex or 4-plex Analysis on the Applied Biosystems 7500 and Other Cyclers

This protocol is optimized for use of the QuantiTect Virus +ROX Vial Kit with TaqMan probes on Applied Biosystems 7500 Real-Time PCR Systems, Rotor-Gene cyclers, and instruments from Bio-Rad, Cepheid, Roche, and Agilent. For further information, see "ROX passive reference dye", page 11.

Important points before starting

- **Always start with the cycling conditions specified in this protocol.** Please note that these cycling conditions differ from those described in the protocol for duplex assays (page 42). The cycling is optimized for PCR products between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 64.
- **Use the primer concentrations specified in this protocol.** Please note that these primer concentrations differ from those described in the protocol for duplex assays (page 42).
- We strongly recommend testing the performance of new primer–probe sets in individual assays before combining them in a multiplex assay.
- Read "Guidelines for effective multiplex assays", page 16. Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established multiplex assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol. It is not necessary to determine primer-limiting concentrations again.
- **After reverse transcription, the PCR must start with an initial incubation step of 5 min at 95°C to activate HotStarTaq *Plus* DNA Polymerase.**
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61). Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E (page 62).

Procedure

1. **Thaw 5x QuantiTect Virus NR Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral nucleic acids), optional standards, and references. Mix the individual solutions.**

   Standards should be diluted in QuantiTect Nucleic Acid Dilution Buffer at an appropriate concentration to enable use of 5–10 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 20 (page 48). It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep the reagents, samples, and controls on ice or in a cooling device.

   Note: For RT-PCR, 100x QuantiTect Virus RT Mix should be taken from –15 to –30°C immediately before use, always kept on ice, and returned to –15 to –30°C immediately after use.

3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

   Note: Keep the tubes or plate on ice.

4. **Add template nucleic acids to the individual PCR tubes or wells, and mix thoroughly.**

   Note: Ensure that the reaction mix and template are thoroughly mixed.

5. **Program the real-time cycler according to Table 21 (page 49).**

Table 20. Reaction setup

Component	Volume/reaction 50 µl*	20 µl*†	Final concentration
5x QuantiTect Virus NR Master Mix	10 µl	4 µl	1x
50x ROX Dye Solution‡	1 µl	0.4 µl	1x
20x primer–probe mix 1§	2.5 µl	1 µl	0.2 µM forward primer 1¶ 0.2 µM reverse primer 1¶ 0.2 µM probe 1**
20x primer–probe mix 2§	2.5 µl	1 µl	0.2 µM forward primer 2¶ 0.2 µM reverse primer 2¶ 0.2 µM probe 2**
20x primer–probe mix 3§	2.5 µl	1 µl	0.2 µM forward primer 3¶ 0.2 µM reverse primer 3¶ 0.2 µM probe 3**
For 4-plex assays only: 20x primer–probe mix 4§	2.5 µl	1 µl	0.2 µM forward primer 4¶ 0.2 µM reverse primer 4¶ 0.2 µM probe 4**
For RT-PCR only: 100x QuantiTect Virus RT Mix	0.5 µl	0.2 µl	1x
RNase-free water	Variable	Variable	–
Template DNA or RNA (added at step 4)	Variable	Variable	Maximum up to 50% of final reaction volume
Total reaction volume	**50 µl***	**20 µl*†**	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly.

† Refers to the Applied Biosystems 7500 Fast Real-Time PCR System.

‡ For cyclers which do not require ROX dye, add RNase-free water instead.

§ For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. See Appendix D (page 61).

¶ A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, check the concentration of your primer solutions. In some cases, other primer concentrations between 0.1 µM and 0.3 µM may improve performance.

** A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 21. Cycling conditions

Step	Time	Temperature	Additional comments
For RT-PCR only: Reverse transcription	**20 min**	**50°C**	RNA is reverse transcribed into cDNA. Omit this step if you are analyzing DNA targets.
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions.**
Denaturation	**15 s**	**95°C**	
Annealing/extension	**75 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection, optimized for PCR products up to 150 bp. For PCR products >150 bp, different cycling conditions may improve results in some cases. For details, see Appendix F, page 64.
Number of cycles	40–50		The number of cycles depends on the amount of template DNA or RNA.

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. For each probe, select the analysis settings (i.e., baseline settings and threshold values). Note that optimal analysis settings are a prerequisite for accurate quantification data.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and suggestions
No signal, or one or more signals detected late in PCR		
a)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols.
b)	HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq *Plus* DNA Polymerase activation step (5 min at 95°C) as described in the protocols.
c)	Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See Appendix A, page 54, for details on evaluating the concentration of primers and probes. Repeat the assay.
d)	Wrong or no detection step	Ensure that fluorescence detection takes place during the combined annealing/extension step when using TaqMan probes.
e)	Primer or probe concentration not optimal	Use optimal primer concentrations. For duplex assays on all real-time cyclers, use each primer at 0.4 µM. For triplex or 4-plex assays on all real-time cyclers, use each primer at 0.2 µM. In most cases, a probe concentration of 0.2 µM gives satisfactory results. Depending on the quality of your probe, results may be improved by adjusting probe concentration within the range of 0.1–0.4 µM. Check the concentrations of primers and probes by spectrophotometry (see Appendix A, page 54).

		Comments and suggestions
f)	Problems with starting template	Check the concentration, storage conditions, and quality of the starting nucleic acids or standard control template nucleic acids. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the assay using the new dilutions. Efficient removal of PCR inhibitors is essential for optimal results. Purify nucleic acids from your sample using an appropriate purification method (see "Ordering Information", page 66). Ensure that all reagents, buffers, and solutions used for isolating and dilution of template nucleic acids are free of nucleases (RNases/DNases).
g)	Insufficient amount of starting template	Increase the amount of template if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
h)	Insufficient number of cycles	Increase the number of cycles.
i)	Probe design not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review the probe design guidelines (see Appendix A, page 54).
j)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Check whether the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.
k)	Fluorescence crosstalk	Check that the reporter dyes used in your assay are suitable for multiplex analysis on your instrument. Run appropriate controls to estimate potential crosstalk effects.

Differences in C_T values or in PCR efficiencies between a multiplex assay and the corresponding singleplex assays

a)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b)	Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.
c)	Imprecise spectral separation of reporter dyes	Since multiplex assays use multiple probes, each with a fluorescent dye, the increased fluorescent background may affect the shape of the amplification plots obtained with some real-time cyclers. This may lead to differences in C_T values of up to 5% between the multiplex assay and the corresponding singleplex assays; this can usually be avoided by using optimal threshold settings. If using the ABI PRISM 7700, perform analysis with and without spectral compensation.

No linearity in ratio of C_T value/crossing point to log of the template amount

a)	Template amount too high	When signals are coming up at very early C_T values, adjust the analysis settings accordingly.
b)	Template amount too low	Increase template amount if possible. Note that detection of very low starting copy numbers may not be in the linear range of a standard curve.

Increased fluorescence or C_T value for "No Template" control

a)	Contamination of reagents	Discard all the components of the multiplex assay (e.g., master mix, primers, and probes). Repeat the multiplex assay using new components.
b)	Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.

Varying fluorescence intensity

a)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
c)	Wavy curve at high template amounts for highly expressed targets	In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template.
d)	**ABI PRISM 7000**: Uneven curves or high standard deviations	Do not use reaction volumes smaller than 25 µl and always use optical adhesive covers to seal plates. In some cases, increasing the reaction volume to 50 µl may improve results.

Appendix A: Assay Design and Handling Primers and Probes

Important factors for success in quantitative, multiplex, real-time PCR include the design of optimal primer pairs and probes, the use of appropriate primer and probe concentrations, and the correct storage of primers and probes.

Assay design

Guidelines for the optimal design of primers and probes are given below. It is particularly important to minimize nonspecific annealing of primers and probes. This can be achieved through careful assay design.

T_m of primers for TaqMan assays

- Use specialized design software (e.g., Primer Express® Software) to design primers and probes.
- T_m of all primers should be 58–62°C and within 2°C of each other.
- T_m of probes should be 8–10°C higher than the T_m of the primers.
- Avoid a guanidine at the 5' end of probes, next to the reporter, since this causes quenching.
- Avoid runs of 4 or more of the same nucleotide, especially of guanidine.
- Choose the binding strand so that the probe has more C than G bases.
- All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension).

Primer sequence

- Length: 18–30 nucleotides.
- GC content: 30–70%.
- Always check the specificity of primers by performing a BLAST® search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence.
- Check that primers and probes are not complementary to each other.
- Try to avoid highly repetitive sequences.
- Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to minimize primer–dimer formation.
- Avoid mismatches between the 3' end of primers and the template sequence.
- Avoid runs of 3 or more Gs and/or Cs at the 3' end.

- Avoid complementary sequences within a primer sequence and between the primer pair.

Product size

Ensure that the length of PCR products is 60–150 bp. Some longer amplicons may amplify efficiently in multiplex PCR, with minimal optimization. See Appendix F (page 64).

Special considerations for design of assays for viral nucleic acids

- Design primers or probes in a conserved region of the virus.
- Perform appropriate database analysis to identify such a region and verify the functionality of the assay using isolates of a different origin.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given below. For optimal results, we recommend only combining primers of comparable quality.

Storage buffer

Lyophilized primers and probes should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). We recommend using TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes.

However, probes labeled with fluorescent dyes such as Cy3, Cy3.5, Cy5, and Cy5.5 should be stored in TE buffer, pH 7.0, since they tend to degrade at higher pH.

Storage

Primers should be stored in sterile, nuclease-free TE buffer in small aliquots at –20°C. Standard primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6–9 months. Repeated freeze–thaw cycles should be avoided, since they may lead to degradation.

For easy and reproducible handling of primer–probe sets used in multiplex assays, we recommend preparing 20x primer–probe mixes, each containing 2 primers and 1 probe for a particular target at the suggested concentrations (see protocols).

Dissolving primers and probes

Before opening a tube containing lyophilized primer or probe, spin the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of sterile, nuclease-free TE buffer, mix, and leave for 20 minutes to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.

We do not recommend dissolving primers and probes in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.

Concentration

Spectrophotometric conversion for primers and probes:

1 A_{260} unit = 20–30 µg/ml

To check primer concentration, the molar extinction coefficient (ε_{260}) can be used:

$A_{260} = \varepsilon_{260}$ x molar concentration of primer or probe

If the ε_{260} value is not given on the data sheet supplied with the primers or probes, it can be calculated from the primer sequence using the following formula:

ε_{260} = 0.89 x [(A x 15,480) + (C x 7340) + (G x 11,760) + (T x 8850)]

Example

Concentration of diluted primer: 1 µM = 1 x 10^{-6} M

Primer length: 24 nucleotides with 6 each of A, C, G, and T bases

Calculation of expected A_{260}: 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] x (1 x 10^{-6}) = 0.232

The measured A_{260} should be within ±30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers or probes, or having the primers or probes resynthesized.

For probes, the fluorescent dye does not significantly affect the A_{260} value.

Primer and probe quality

The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel;* a single band should be seen. Please contact QIAGEN Technical Services or your local distributor for a protocol.

Probe quality

The quality of the fluorescent label and the purity of TaqMan probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 units DNase* at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix B: Data Analysis

When carrying out data analysis, follow the recommendations provided by the manufacturer of your real-time cycler. Fundamental guidelines for data analysis and some important considerations are given below. Further information can be found in *Critical Factors for Successful Real-Time PCR*. To obtain a copy, contact QIAGEN Technical Services, or visit www.qiagen.com/literature/brochures to download a PDF.

General considerations for multiplex data analysis

Real-time PCR data are produced as sigmoidal-shaped amplification plots (when using a linear scale), in which fluorescence is plotted against the number of cycles (Figure 2, page 58).

- The threshold cycle (C_T value) serves as a tool for calculation of the starting template amount in each sample. This is the cycle in which there is the first detectable significant increase in fluorescence.
- The optimal threshold setting depends on the reaction chemistries used for PCR. Therefore, an optimal threshold setting established for another kit may not be suitable for the QuantiTect Virus Kit you are using, and may need to be adjusted.
- The method for determination of C_T values differs depending on the real-time cycler used. Check the handbook or the software help file for your real-time cycler for details on threshold settings.
- Most real-time cyclers contain a function that determines the noise level in early cycles, where there is no detectable increase in fluorescence due to PCR products (usually referred to as the baseline settings). Adjust the settings for this function.
- For multiplex assays, the analysis settings need to be adjusted for each of the reporter dyes used.
- Depending on your real-time cycler, low levels of signal crosstalk, even between apparently well separated reporter dyes, may influence multiplex results in rare cases. In most cases, low levels of crosstalk can be overcome by optimal analysis settings. If this is not successful, repeat the multiplex assay using the optimal combination of reporter dyes recommended for your real-time cycler (see "Suitable combinations of reporter dyes", page 17).

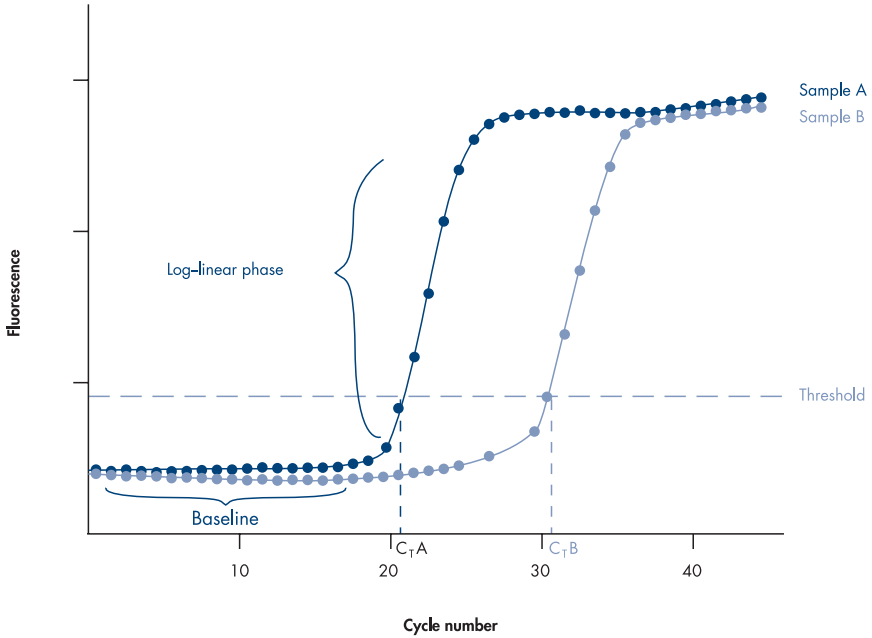

Figure 2. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (Sample A and Sample B).

Appendix C: General Remarks for Working with Viral RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the viral RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with viral RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with viral RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and viral RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible.

To remove RNase contamination from bench surfaces, nondisposable plasticware, and laboratory equipment (e.g., pipets and electrophoresis tanks), use of RNaseKiller (cat. no 2500080) from 5 PRIME (www.5prime.com) is recommended. RNase contamination can alternatively be removed using general laboratory reagents. To decontaminate plasticware, rinse with 0.1 M NaOH, 1 mM EDTA* followed by RNase-free water (see "Solutions", page 60), or rinse with chloroform* if the plasticware is chloroform-resistant. To decontaminate electrophoresis tanks, clean with detergent (e.g., 0.5% SDS),* rinse with RNase-free water, rinse with ethanol (if the tanks are ethanol-resistant), and allow to dry.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for viral RNA work should be cleaned with a detergent,* thoroughly rinsed, and oven baked at 240°C for at least 4 hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate), as described in "Solutions" below.

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris* buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carbethoxylation. Carbethoxylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: QuantiTect Virus buffers and RNase-free water are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Appendix D: Preparing a 20x Primer–Probe Mix for Each Target

For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. For singleplex and duplex assays, see Table 22. For triplex and 4-plex assays, see Table 23. Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, see Appendix E (page 62).

Table 22. Preparing 20x primer–probe mix for singleplex/duplex assays

Component	Concentration (20x)	Final concentration
Forward primer*	8 µM	0.4 µM†
Reverse primer*	8 µM	0.4 µM†
Probe	4 µM	0.2 µM‡
TE buffer	–	–

* If using more than one forward or reverse primer per target, use an 8 µM concentration for each primer.

† A final primer concentration of 0.4 µM is optimal. Before adapting primer concentration, verify the concentration of your primer solutions.

‡ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 23. Preparing 20x primer–probe mix for triplex/4-plex assays

Component	Concentration (20x)	Final concentration
Forward primer*	4 µM	0.2 µM†
Reverse primer*	4 µM	0.2 µM†
Probe	4 µM	0.2 µM‡
TE buffer	–	–

* If using more than one forward or reverse primer per target, use a 4 µM concentration for each primer.

† A final primer concentration of 0.2 µM is optimal. Before adapting primer concentration, verify the concentration of your primer solutions. In some cases, other primer concentrations between 0.1 µM and 0.3 µM may improve performance.

‡ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Appendix E: Customized Pipetting Scheme for Separate Primer and Probe Solutions

For ease of use, we recommend preparing for each of your targets a 20x primer–probe mix containing target-specific primers and probe. However, in some cases, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions this way, it may be helpful to copy and fill in Table 24 (page 63) with the calculated volumes of each primer to use.

Table 24. Preparing reaction mix for multiplex assays using separate primer and probe solutions

	Volume per reaction		
Component*	**50 µl**	**20 µl**	**Other: ____ µl**
5x QuantiTect Virus Master Mix ☐ 1 ☐ 2	10 µl	4 µl	_____ µl
50x ROX Dye Solution	☐ 0 µl ☐ 1 µl ☐ ___ µl	☐ 0 µl ☐ 0.4 µl ☐ ___ µl	_____ µl
Forward primer 1 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Reverse primer 1 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Probe 1 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 2 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Reverse primer 2 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Probe 2 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 3 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Reverse primer 3 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Probe 3 (0.2 µM)	_____ µl	_____ µl	_____ µl
Forward primer 4 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Reverse primer 4 ☐ 0.4 µM ☐ 0.2 µM	_____ µl	_____ µl	_____ µl
Probe 4 (0.2 µM)	_____ µl	_____ µl	_____ µl
For RT-PCR only: 100x QuantiTect Virus RT Mix	0.5 µl	0.2 µl	_____ µl
RNase-free water	_____ µl	_____ µl	_____ µl
Template DNA or RNA (added at step 4)	_____ µl	_____ µl	_____ µl
Total reaction volume	**50 µl**	**20 µl**	**_____ µl**

* The concentrations of primers and probe shown in this column represent their final concentrations in the reaction, not the concentrations of the stock solutions.

Appendix F: Customized Cycling Conditions

We recommend to always start with the cycling conditions specified in the protocols. It is especially important to include the initial PCR activation step (5 minutes at 95°C) to activate HotStarTaq *Plus* DNA Polymerase. Longer or shorter activation times are not recommended.

In rare cases (e.g., amplification of difficult PCR products or PCR products >150 bp), different cycling conditions may improve results. In these cases:

- Increase the annealing/extension time to 75 s (at 60°C), or
- Include a 3-step cycling protocol with a separate 15 s extension step at 72°C, or
- Do both of the above

If you have found different cycling conditions that are optimal for the probes and primers used, it may be helpful to copy and fill in Table 25 (page 65) with the optimized cycling conditions.

Table 25. Customized cycling conditions for multiplex assays

Step	Time	Temperature	Additional comments
For RT-PCR only: Reverse transcription	☐ **20 min** ☐ **__ min**	☐ **50°C** ☐ **__°C**	RNA is reverse transcribed into cDNA. Omit this step if you are analyzing DNA targets.
Initial PCR activation step	☐ **5 min**	☐ **95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step.
2-step cycling:			
Denaturation	☐ **15 s** ☐ **__ s**	☐ **95°C** ☐ **__°C**	
Annealing/extension	☐ **45 s** ☐ **75 s** ☐ **__ s**	☐ **60°C** ☐ **__°C**	Combined annealing/ extension step with fluorescence data collection
For 3-step cycling only: Extension	☐ **15 s** ☐ **__ s**	☐ **72°C** ☐ **__°C**	Separate extension step only if performing 3-step cycling (see Appendix F, page 64).
Number of cycles	☐ 40 ☐ 45 ☐ 50 ☐ __		The number of cycles depends on the amount of template DNA or RNA.

Ordering Information

Product	Contents	Cat. no.
QuantiTect Virus Kit (50)	For 50 x 50 µl reactions: 0.5 ml Master Mix (with ROX dye), 25 µl RT Mix, 2 ml RNase-Free Water, 1 Vial of Nucleic Acid Dilution Buffer	211011
QuantiTect Virus Kit (200)	For 200 x 50 µl reactions: 2 x 1 ml Master Mix (with ROX dye), 2 x 50 µl RT Mix, 4 x 2 ml RNase-Free Water, 2 Vials of Nucleic Acid Dilution Buffer	211013
QuantiTect Virus Kit (1000)	For 1000 x 50 µl reactions: 10 x 1 ml Master Mix (with ROX dye), 10 x 50 µl RT Mix, 20 x 2 ml RNase-Free Water, 6 Vials of Nucleic Acid Dilution Buffer	211015
QuantiTect Virus +ROX Vial Kit (50)	For 50 x 50 µl reactions: 0.5 ml Master Mix (without ROX dye), 210 µl ROX Dye Solution, 25 µl RT Mix, 2 ml RNase-Free Water, 1 Vial of Nucleic Acid Dilution Buffer	211031
QuantiTect Virus +ROX Vial Kit (200)	For 200 x 50 µl reactions: 2 x 1 ml Master Mix (without ROX dye), 210 µl ROX Dye Solution, 2 x 50 µl RT Mix, 4 x 2 ml RNase-Free Water, 2 Vials of Nucleic Acid Dilution Buffer	211033
QuantiTect Virus +ROX Vial Kit (1000)	For 1000 x 50 µl reactions: 10 x 1 ml Master Mix (without ROX dye), 1050 µl ROX Dye Solution, 10 x 50 µl RT Mix, 20 x 2 ml RNase-Free Water, 6 Vials of Nucleic Acid Dilution Buffer	211035
Related products		
QIAamp® MinElute® Virus Kits — for simultaneous purification of viral DNA and RNA from plasma, serum, and cell-free body fluids		
QIAamp MinElute Virus Vacuum Kit (50)	For 50 preps: 50 QIAamp MinElute Spin Columns, QIAGEN Protease, Carrier RNA, Buffers, Extension Tubes, Collection Tubes	*57714*

Ordering Information

Product	Contents	Cat. no.
QIAamp MinElute Virus Spin Kit (50)	For 50 preps: 50 QIAamp MinElute Spin Columns, QIAGEN Protease, Carrier RNA, Buffers, Collection Tubes; fully automatable on the QIAcube®	57704
QIAamp UltraSens® Virus Kit — for concentration and purification of viral DNA and RNA from serum and plasma		
QIAamp UltraSens Virus Kit (50)*	For 50 preps: 50 QIAamp Mini Spin Columns, Proteinase K, Carrier RNA, Collection Tubes, Buffers	53704
QIAamp Viral RNA Mini Kit — for purification of viral RNA from cell-free body fluids		
QIAamp Viral RNA Mini Kit (50)*	For 50 preps: 50 QIAamp Mini Spin Columns, Carrier RNA, Collection Tubes, RNase-Free Buffers	52904
QIAamp DSP Virus Kit — for purification of viral nucleic acids from human plasma and serum for in vitro diagnostic purposes		
QIAamp DSP Virus Kit	For 50 preps: QIAamp MinElute Spin Columns, Buffers, Reagents, Tubes, Column Extenders, VacConnectors; not available in all countries	60704

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* Larger kit sizes available; please inquire.

Notes

Notes

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www.qiagen.com

Australia ▪ techservice-au@qiagen.com

Austria ▪ techservice-at@qiagen.com

Belgium ▪ techservice-bnl@qiagen.com

Brazil ▪ suportetecnico.brasil@qiagen.com

Canada ▪ techservice-ca@qiagen.com

China ▪ techservice-cn@qiagen.com

Denmark ▪ techservice-nordic@qiagen.com

Finland ▪ techservice-nordic@qiagen.com

France ▪ techservice-fr@qiagen.com

Germany ▪ techservice-de@qiagen.com

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India ▪ techservice-india@qiagen.com

Ireland ▪ techservice-uk@qiagen.com

Italy ▪ techservice-it@qiagen.com

Japan ▪ techservice-jp@qiagen.com

Korea (South) ▪ techservice-kr@qiagen.com

Luxembourg ▪ techservice-bnl@qiagen.com

Mexico ▪ techservice-mx@qiagen.com

The Netherlands ▪ techservice-bnl@qiagen.com

Norway ▪ techservice-nordic@qiagen.com

Singapore ▪ techservice-sg@qiagen.com

Sweden ▪ techservice-nordic@qiagen.com

Switzerland ▪ techservice-ch@qiagen.com

UK ▪ techservice-uk@qiagen.com

USA ▪ techservice-us@qiagen.com

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