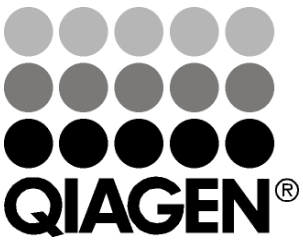
July 2011

QuantiFast® Pathogen PCR +IC Handbook

For sensitive real-time PCR for detection of viral or bacterial DNA and an internal control, using sequence-specific probes



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Contents

Kit Contents

QuantiFast Pathogen PCR +IC Kit	(100)	(400)
Catalog no.	211352	211354
Number of 25 µl reactions	100	400
5x QuantiFast Pathogen Master Mix*	0.5 ml	2 x 1 ml
50x ROX™ Dye Solution	210 µl	210 µl
50x High-ROX Dye Solution	210 µl	210 µl
RNase-Free Water	1.9 ml	3 x 1.9 ml
QuantiTect® Nucleic Acid Dilution Buffer	2 x 1.5 ml	5 x 1.5 ml
Buffer TE	2 ml	2 ml
Internal Control Assay	1 vial	1 vial
Internal Control DNA	1 vial	1 vial
Handbook	1	1

* Contains HotStarTaq® *Plus* DNA Polymerase, QuantiFast Pathogen Buffer, and dNTP mix (dATP, dCTP, dGTP, dTTP).

Shipping and Storage

The QuantiFast Pathogen PCR +IC Kit is shipped on dry ice. It should be stored immediately upon receipt at –15 to –30°C in a constant-temperature freezer and protected from light. The kit can be stored under these conditions until the expiration date on the kit box without showing any reduction in performance.

5x QuantiFast Pathogen Master Mix can be stored at 2–8°C for up to 2 months without showing any reduction in performance.

Internal Control Assay should be stored at –20°C, either lyophilized or reconstituted. Avoid repeated (>6 times) freeze–thaw cycles.

Internal Control DNA should be stored at –20°C, either lyophilized or reconstituted (see next two paragraphs). Reconstitute the Internal Control DNA soon after receipt. Avoid repeated (>6 times) freeze–thaw cycles.

For information on the correct reconstitution of Internal Control Assay and Internal Control DNA, see "Reconstitution and use of 10x Internal Control Assay and Internal Control DNA", page 24.

Product Use Limitations

The QuantiFast Pathogen PCR +IC Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a 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 the QuantiFast Pathogen PCR +IC Kit 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 material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

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.
QuantiFast Pathogen Buffer*	Novel PCR buffer for highly sensitive detection of viral nucleic acids, including multiplex PCR enabled by Factor MP and fast cycling enabled by Q-Bond® technology.
dNTP mix*	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality.
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).
50x High-ROX Dye Solution	Separate tube of passive reference dye for normalization of fluorescent signals on Applied Biosystems 7900 and StepOne™ Real-Time PCR Systems.
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 QuantiFast Pathogen Master Mix.

Quality Control

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

Introduction

The QuantiFast Pathogen PCR +IC Kit provides sensitive and rapid real-time PCR analysis of viral, bacterial, or fungal DNA using sequence-specific probes. To enable high process safety of pathogen detection through correct interpretation of negative detection results, each kit contains reagents for duplex real-time detection of a user-defined target with the QIAGEN Internal Control.

The exogenous Internal Control is detected in the same tube with the viral or bacterial DNA target in a duplex PCR to test for successful amplification (e.g., exclusion of PCR inhibitors). Alternatively, the Internal Control DNA can be added to the purification procedure to control both the purification process and PCR amplification.

The QuantiFast Pathogen PCR +IC Kit provides a ready-to-use Internal Control for universal use with different pathogen assays without the need for optimization. Each QuantiFast Pathogen PCR +IC Kit includes the Internal Control Assay (primer/probe set) targeting the Internal Control DNA for use as an amplification control via direct addition to the reaction mix. For addition of the Internal Control to the purification procedure, a highly concentrated Internal Control DNA (High conc.) can be ordered separately (see Ordering Information, page 59).

For simplified use, the QIAGEN Internal Control assay design eliminates the need for optimization of duplex detection with the target. The use of one universal exogenous Internal Control for all pathogen assays allows parallel read-out of different pathogen targets and easy implementation of new pathogen assays.

The QuantiFast Pathogen PCR +IC Kit has been optimized for use with TaqMan® probes in duplex, real-time detection of a viral or bacterial DNA target and the Internal Control DNA. The kit also provides the basis for multiplexing more than one target.

Each kit is supplied with a master mix that is free of ROX dye. The kit includes two separate vials of ROX dye solutions of different concentrations, which can be added to reactions, depending on the real-time cycler used.

The kits are compatible with the following cyclers and conditions for fluorescence normalization:

- Lower concentration of ROX dye (e.g., Applied Biosystems 7500 Real-Time PCR Systems)
- Higher ROX concentration (e.g., Applied Biosystems 7900 and StepOne Real-Time PCR Systems)
- Optional use of ROX dye (e.g., Agilent instruments)
- No requirement for 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.

The QuantiFast Pathogen PCR +IC Kit contains a highly concentrated 5x master mix, which allows use of larger volumes of template (up to 50% of the reaction volume) in order to increase the sensitivity of assays.

Time-consuming optimization of the duplex assay for inclusion of the Internal Control is not required, as the 5x master mix and the Internal Control assay are already optimized.

QuantiFast Pathogen Master Mix

In contrast to current methods, the QuantiFast Pathogen PCR +IC Kit eliminates the need for optimization of the concentrations of primers, Mg^{2+}, or DNA polymerase. 5x QuantiFast Pathogen Master Mix is specifically optimized for sensitive detection of pathogen nucleic acid targets in combination with the QIAGEN Internal Control. The optimized master mix ensures that the target sequence in the duplex reaction is amplified with the same efficiency and sensitivity as the target sequence in a corresponding singleplex reaction.

5x QuantiFast Pathogen Master Mix contains HotStarTaq *Plus* DNA Polymerase and QuantiFast Pathogen Buffer. 5x QuantiFast Pathogen Master Mix does not contain ROX passive reference dye; this is supplied in separate tubes.

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.

QuantiFast Pathogen Buffer

QuantiFast Pathogen Buffer has been specifically developed for sensitive and rapid detection of pathogen nucleic acids using sequence-specific probes.

To allow for fast-cycling, duplex PCR, a novel additive in the buffer, Q-Bond, allows short cycling times on both standard cyclers and on fast cyclers with rapid ramping rates. Q-Bond increases the affinity of *Taq* DNA polymerases for short single-stranded DNA, reducing the time required for primer/probe annealing to a few seconds. This allows a combined annealing/extension step of only 30 seconds in duplex PCR. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times.

In addition to various salts and additives, the buffer also contains a specially optimized combination of K^+ and NH_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 QuantiFast Pathogen 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 two-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 Agilent instruments. 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 QuantiFast Pathogen PCR +IC Kit does not contain ROX dye and can be used directly with all instruments that do not require ROX dye for fluorescence normalization. No ROX is required for Rotor-Gene cyclers and instruments from Bio-Rad, Agilent, and Roche.

Each QuantiFast Pathogen PCR +IC Kit also includes two separate vials of ROX dye solutions of different concentrations, which can be added to reactions, depending on the real-time cycler used.

The 50x ROX Dye solution 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) and for use with cyclers that allow optional use of ROX dye (e.g., Agilent instruments).

The 50x High-ROX Dye Solution is provided at a higher concentration that is optimal for other instruments from Applied Biosystems (models 7000, 7300, 7700, 7900HT, StepOne, and StepOnePlus™).

For use with instruments requiring ROX, the user must add the 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 (Table 1, page 12). Store the premixed solution at –15 to –30°C, protected from light. Remember to label the tube to show that ROX dye has been added.

Table 1. Addition of ROX dye to master mix for long-term storage*

Kit	50x ROX Dye Solution or 50x High-ROX Dye Solution	5x QuantiFast Pathogen Master Mix
QuantiFast Pathogen PCR +IC Kit (100)	50 µl	0.5 ml
QuantiFast Pathogen PCR +IC Kit (400)	100 µl	1 ml

* **Note**: If ROX dye is premixed with the 5x QuantiFast Pathogen Master Mix as indicated above, the premixed master mix has a 4.55x concentration. Adjust the volume of master mix to be added to the reaction accordingly (add 5.5 µl premixed Mastermix to each 25 µl reaction).

Internal Control DNA

With each QuantiFast Pathogen PCR +IC Kit, a DNA template of the Internal Control is supplied in a lyophilized format (Internal Control DNA [400]). This allows the user to control the amplification reaction and identify possible PCR inhibition enabling the correct interpretation of negative detection results.

The QIAGEN Internal Control DNA is a synthetic DNA construct with a unique and artificial sequence. The sequence targeted by the Internal Control Assay lacks homology with any sequences in the GenBank database.

To obtain a 10x solution for control of the amplification reaction, dissolve the Internal Control DNA (400) in QuantiTect Nucleic Acid Dilution Buffer as described in section "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24.

To control both the pathogen nucleic acid isolation procedure and the PCR amplification, a highly concentrated Internal Control can be used (Internal Control DNA [High conc.]). This is not provided in the kit, but can be ordered separately (see Ordering Information, page 59). To obtain a highly concentrated solution for control of purification and amplification, dissolve the Internal Control DNA (High conc.) in QuantiTect Dilution Buffer as described in section "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24.

Internal Control Assay

The Internal Control Assay allows simultaneous amplification of the Internal Control DNA and a user-defined pathogen target in duplex, real-time PCR using sequence-specific probes. The premixed Internal Control Assay contains a forward and reverse primer and a TaqMan probe for detection of the Internal Control DNA and has been specifically optimized to prevent interference with target primers. The Internal Control Assay employs MAX™ NHS Ester as a reporter dye. With excitation/emission maxima of 524/557 nm, the MAX dye has a spectral profile allowing detection in the same channel as HEX™, JOE®, or VIC®, and therefore can be used with most real-time cyclers.

To guarantee optimal performance of duplex amplification of the pathogen target and the Internal Control, we recommend the pathogen target assay design to follows certain design specifications. These specifications are in accordance with general recommendations for the design of optimal real-time PCR primers and probes, and are therefore likely to already apply for the majority of proven and established (e.g., literature-derived) real-time PCR assays. For more details on pathogen target assay design, see Appendix A, page 43.

QuantiTect Nucleic Acid Dilution Buffer

QuantiTect Nucleic Acid Dilution Buffer is intended for dilution of nucleic acids used to generate standard curves or as positive controls in real-time 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 pathogen 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. Avoid repeated freezing and thawing.

QuantiTect Nucleic Acid Dilution Buffer is also intended for reconstitution of the Internal Control DNA (see “Reconstitution and use of Internal Control Assay and Internal Control DNA”, page 24).

Sequence-specific probes

The QuantiFast Pathogen PCR +IC Kit is optimized for use with hydrolysis (TaqMan) probes, a major type of sequence-specific probes used in real-time PCR (see below). For more details about sequence-specific probes and their design and handling, see Appendix A, page 43.

Hydrolysis probes

TaqMan probes are sequence-specific oligonucleotides with a fluorophore and a quencher moiety attached (see Figure 1, page 14). 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 proportional to the amount of accumulated PCR product.

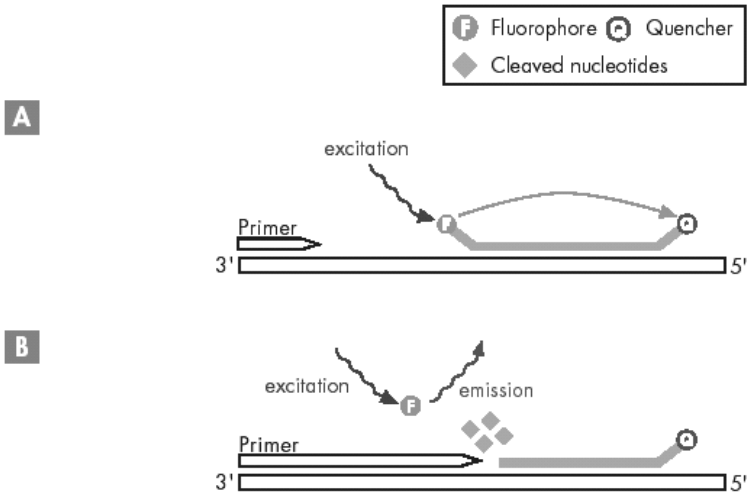


Figure 1. Principle of TaqMan probes in quantitative, real-time PCR. **A** Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorophore with the quencher results in efficient quenching of fluorescence from the fluorophore. **B** 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 material safety data sheets (MSDSs), available from the product supplier.

- Primers and TaqMan probes for detection of the pathogen target from an established oligonucleotide manufacturer. Primers should be of standard quality or high performance liquid chromatography (HPLC) pure, and probes should be highly purified, e.g. 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 43). Primer and probe stock solutions should be stored in aliquots at –20°C. Probe stock solutions should be protected from exposure to light. Typically, the target specific probe should be labeled with FAM™ and a non-fluorescent quencher.
- Optional: Internal Control DNA (High conc.). This highly concentrated Internal Control template is available separately and can be used for optional control of the purification procedure by addition to the sample lysate or lysis buffer (see Ordering Information, page 59).
- 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.
- Cooling device or ice
- Real-time PCR thermal cycler (we recommend the Rotor-Gene Q for high-precision results; 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 preparation of TE buffer for storing primers and probes (see Appendix A, page 43). Use RNase/DNase-free water and plastic consumables to prepare TE buffer.
- Optional: QIAsymphony® SP/AS instruments for fully integrated automation of complete workflows, from sample preparation to assay setup; for details, visit www.qiagen.com/goto/QIAsymphony.

Important Notes

Selecting kits and protocols

To select the correct QuantiFast Pathogen PCR +IC Kit and protocol to use with your real-time cycler, see Table 2.

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 2. Choosing the correct QuantiFast Pathogen PCR +IC protocol for real-time PCR

Cycler	Protocol
Rotor-Gene Q, Rotor-Gene 6000, and Rotor-Gene 3000	Protocol 1, page 26
Applied Biosystems 7500*	Protocol 2, page 30
Applied Biosystems 7300 and 7900, ABI PRISM® 7900HT (96 well), 7000 and 7700, Applied Biosystems StepOne and StepOnePlus	Protocol 3, page 34
Mx3000P®, Mx3005P®, and Mx4000®	Protocol 1, page 26
iCycler iQ® and iQ5, Chromo4, DNA Engine Opticon 2, CFX96™ and CFX384™ Real-Time PCR Detection Systems	Protocol 1, page 26
LightCycler 480 (96 well)	Protocol 1, page 26
SmartCycler® II	Protocol 1, page 26
Others	Protocol 1, page 26

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

Guidelines for effective duplex detection of pathogen and Internal Control

The QuantiFast Pathogen PCR +IC Kit works with most existing TaqMan probe systems that have been designed using standard design methods. However, for optimal performance of a probe system in duplex, real-time PCR with the Internal Control Assay, some considerations need to be made, including considerations on primer and probe design and quality. Please read the following guidelines before starting.

- Check the functionality of the pathogen-specific set of primers and probe in singleplex setup before combining the pathogen assay with the Internal Control Assay in duplex PCR.
- Choose a reporter dye and quencher compatible with the Internal Control Assay. Typically, the target-specific probe should be labeled with FAM and a non-fluorescent quencher. For details, see "Selecting dyes and instrument setup", page 19.
- In general, PCR products for real-time PCR using sequence-specific probes should be as short as possible; ideally 60–150 bp. The Internal Control Assay has been specifically optimized for duplexing with typical pathogen target systems with amplicon lengths of up to 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 A, page 43.
- Always use the same algorithm or software to design the primers and probes. For optimal results, use pathogen assays that have been designed using standard software with standard algorithm parameters and reaction conditions. This will typically result in melting points (T_m) suitable for duplexing with the Internal Control. For details, see Appendix A, page 43.
- Check the concentration and integrity of primers and probes before starting. For details, see Appendix A, page 43.
- Check the real-time cycler user manual for **correct setup of the cycler for duplex analysis** (e.g., setting up detection of two different dyes from the same well). Be sure to activate the detector for both reporter dyes used.
- Some real-time cyclers require **a calibration procedure for each reporter dye**. Check whether the reporter dye of the pathogen assay is part of the standard set of dyes already calibrated on the instrument. If it is not, perform a calibration procedure for the dye before using it for the first time (for details, refer to the manufacturer's instructions for the real-time cycler).

- The Internal Control Assay employs MAX as a reporter dye. With excitation/emission maxima of 524/557 nm, MAX dye has a spectral profile allowing detection in the same channel as HEX, JOE, or VIC.

  If using MAX dye for the first time on an instrument, ensure that detection is carried out using the correct channel or filter. For details, see Table 3, page 19.

- Always start with the **cycling conditions specified in the protocol** you are following. The unique composition of QuantiFast Pathogen Buffer ensures specific annealing for each primer set. This enables fast two-step cycling with a combined annealing/extension step for pathogen target and Internal Control.

- 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 both reporter dyes are a prerequisite for accurate detection data. For details, check the literature from the manufacturer of your real-time cycler.

- Perform appropriate tests for evaluating the performance of your duplex assay of pathogen target and Internal Control (e.g., amplifying the pathogen target individually and comparing the results with those for the duplex assay).

- Include appropriate controls in each real-time PCR run to give additional information for interpretation of results. For details, see "Controls", page 22 and Appendix C, page 50.

- On some real-time cyclers, ROX must be additionally added to the reaction as a reference dye. The required ROX concentration may vary between different cyclers. See Table 4, page 21, for recommendations for different instruments. **Note**: If there are no specific recommendations listed for your real-time cycler; refer to the user manual or other technical documentation for the instrument to determine the ROX concentration needed for duplex analysis.

Selecting dyes and instrument setup

Duplex, real-time PCR requires the simultaneous detection of two different fluorescent reporter dyes (Table 3). The Internal Control Assay provided with the QuantiFast Pathogen PCR +IC Kit uses the reporter dye MAX which has excitation/emission maxima of 524/557 nm, and a non-fluorescent quencher (Iowa Black®). For accurate detection, the fluorescence spectrum of the pathogen assay dye must be well separated from the MAX spectrum or exhibit only minimal overlap. Please read the general recommendations and instrument-specific recommendations on the next 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 filters can be used for the recommended reporter dyes in duplex analysis.

Table 3. Dyes commonly used in quantitative, multiplex, real-time PCR

Dye	Excitation maximum (nm)	Emission maximum (nm)*
FAM	494	518
TET™	521	538
JOE	520	548
VIC	538	552
MAX	524	557
Yakima Yellow®	526	552
HEX	535	553
Bodipy® TMR	542	574
NED™	546	575
Cy®3	552	570
TAMRA™	560	582
Cy3.5	588	604
ROX	587	607
Texas Red	596	615
Cy5	643	667

* Emission spectra may vary depending on the buffer conditions.

- **For duplex analysis of pathogen assay and Internal Control Assay, we recommend using FAM as a reporter dye for the pathogen assay.** This will guarantee optimal duplex performance with the Internal Control Assay which employs MAX as a reporter dye. Other dyes detected in a different detection channel than MAX may also be suitable. Order the probes for your pathogen assay from an established oligonucleotide manufacturer.
- **For optimal results, we recommend using a nonfluorescent quencher for the pathogen assay** (e.g., Dark Quencher, Black Hole Quencher® [BHQ®] or Iowa Black Quencher). It is **not recommended** to use fluorescent quenchers (e.g., TAMRA fluorescent dye). Due to their own native fluorescence, fluorescent quenchers contribute to an overall increase in background and reduce the signal-to-noise ratio.
- Before starting, choose the suitable filter or channel for detection of pathogen and Internal Control reporter dyes using the detection optics of your real-time cycler. If you have not used the reporter dyes FAM or MAX on the real-time cycler before, note that some instruments require a calibration procedure to be performed for a reporter dye when using it for the first time. Additionally, check whether the instrument requires the addition of ROX to the duplex reactions as a reference dye. For recommendations on filters, calibration, and ROX dye, see Table 4, page 21.

Table 4. Instrument-specific requirements for filter or channel, calibration, and ROX concentration

Instrument	Filter or channel for detection of pathogen target assay (FAM)	Filter or channel for detection of Internal Control assay (MAX)	Calibration	ROX dye solution*
Rotor-Gene Q†, Rotor-Gene 6000†	Green channel	Yellow channel	Not required	Not required
Rotor-Gene 3000†	Channel 1 (470/510)	Channel 2 (530/555)	Not required	Not required
Applied Biosystems 7500	FAM/SYBR® Green	VIC/JOE	Required for new dyes‡	ROX Dye Solution
Applied Biosystems 7900HT, StepOne and StepOnePlus, ABI PRISM 7700, 7000 and 7300	FAM/SYBR Green	VIC/JOE	Required for new dyes‡	High-ROX Dye Solution
Mx3000P, Mx3005P, Mx4000	FAM/SYBR Green (492-516)	HEX/JOE/VIC (535-555)	Not required	Not required/ optional§
iCycler iQ System¶	490/530	530/575	Required for new dyes	Not required
CFX96, CFX384	FAM	VIC	Not required	Not required
LightCycler 480**	Channel 2 FAM (483/533)	Channel 3 HEX (523/568)	Use color compensation file	Not required

* The master mix supplied with the QuantiFast Pathogen PCR +IC Kit does not contain ROX dye and can be used directly with all instruments that do not require ROX dye for fluorescence normalization. Each QuantiFast Pathogen PCR +IC Kit also includes two separate vials of ROX dye solutions of different concentrations, which can be added to reactions, depending on the real-time cycler used.

† 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. See Appendix B, page 48, for information on Rotor-Gene setup for adjustment of fluorescence channel sensitivity.

‡ Before using a reporter dye for the first time on the instrument, a pure dye calibration of the real-time cycler must be performed. See the manufacturer's manual for details on calibration. If the instrument has been calibrated for VIC, this calibration can be used for the detection of MAX. In this case, create a new detector for the detection of the Internal Control

Assay ("MAX/IowaBlack") and assign VIC as the reporter dye. For the quencher dye, select "None".

§ Instruments from Agilent can be used without ROX or, optionally, with a lower concentration of ROX dye contained in the 50x ROX Dye Solution.

¶ 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.

**It is recommended to use a color compensation file on the LightCycler 480 system that contains information to correct crosstalk between the different detection channels. Refer to the *LightCycler 480 Instrument Operator's Manual* for additional information on activating and deactivating detection channels and correctly setting up the instrument for multiplex analysis.

Controls

- Make sure that at least one positive control, as well as one negative control (Water; PCR grade), are included per PCR run.
- For interpretation of PCR results for samples and controls, see Appendix C, "Data Analysis", page 50.

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 pathogen template. This enables detection of contamination in the reagents.

Please note that the Internal Control DNA will be detected in the NTCs if added to the reaction mix as an amplification control (see "Internal Control" page 23).

Positive control

When performing PCR on unknown samples, it is recommended to perform a positive control reaction in the PCR run, containing a sample that is known to include the targeted viral or bacterial DNA. A positive control serves to prove the functionality of the pathogen assay, e.g., the correct setup of the reaction mix. 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 or bacterial sequences. 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 control

For increased process safety in pathogen-detection assays, an Internal Control (internal positive control) is detected in the same tube as the viral or bacterial DNA target in a duplex PCR. The Internal Control tests for successful amplification and excludes, e.g., the presence of PCR inhibitors. Alternatively, the Internal Control can be added to the purification procedure to control both purification and amplification. The QuantiFast Pathogen PCR +IC Kit provides a ready-to-use Internal Control for universal use with different pathogen assays, without the need for optimization. Each QuantiFast Pathogen PCR +IC Kit includes the Internal Control Assay and Internal Control DNA for use as an amplification control by direct addition to the reaction mix. For addition of the Internal Control to the purification procedure, a separate Internal Control DNA (High conc.) can be ordered separately (see Ordering Information, page 59).

Please note that adding the Internal Control DNA to the reaction mix as an amplification control will result in positive Internal Control signals in NTCs. These signals serve as a reference to assure that the Internal Control DNA has been successfully amplified. If the Internal Control signal is detected in the NTCs, but not in sample reactions, this may indicate the presence of inhibitors or other disturbances of the sample reaction.

If the Internal Control DNA (High conc.) has been added to the sample lysate or lysis buffer as a purification control, no Internal Control signals will be detected in the NTCs. As a reference for successful amplification of the Internal Control DNA, a separate, individual Internal Control reaction can be prepared in which 2.5 µl of the reconstituted Internal Control DNA (400) is added to the reaction mix instead of sample DNA. If necessary, adjust the final reaction volume by adding RNase-free water.

For guidelines on data interpretation of pathogen and Internal Control detection, see Appendix C, page 50.

Reconstitution and use of Internal Control Assay and Internal Control DNA

Reconstitution of the Internal Control Assay

To reconstitute a tube of 10x Internal Control Assay, briefly centrifuge the tube, add Buffer TE (provided with the kit), and mix by vortexing the tube 4–6 times (Table 5). If necessary, gently warm the tube to help the primers and probe dissolve. We recommend freezing the reconstituted primers in aliquots in order to avoid repeated freezing and thawing.

Table 5. Reconstituting 10x Internal Control Assay

Product	Volume of Buffer TE per tube
Internal Control Assay (100)	275 µl
Internal Control Assay (400)	1.1 ml

Reconstitution of the Internal Control DNA

To reconstitute a tube of Internal Control DNA, briefly centrifuge the tube, add 1.1 ml QuantiTect Nucleic Acid Dilution Buffer (supplied) and mix by vortexing the tube 4–6 times.
We recommend freezing the reconstituted Internal Control DNA in aliquots in order to avoid repeated freezing and thawing. Reconstituted Internal Control should be stored at –20°C. See "Shipping and Storage", page 4, for details.

Table 6. Reconstituting Internal Control DNA

Product	Volume of QT Dilution Buffer per tube	Concentration/ application
Internal Control DNA (400)*	1.1 ml	10x Solution for amplification control (add directly to reaction mix).
Internal Control DNA (High conc.) †	1.1 ml	Highly concentrated solution for purification and amplification control (add to sample lysate or lysis buffer).

* Supplied in the kit.

† Not supplied in the kit; to be ordered separately, see Ordering Information, page 59.

Use of Internal Control DNA (400)

After reconstitution of the lyophilized Internal Control DNA (400) in QuantiTect Nucleic Acid Dilution Buffer, add the resulting 10x Internal Control DNA solution directly to the reaction mixture (see Protocols 1–3, pages 26–34).

Successful amplification results in a C_T value of 30±3 for the Internal Control, the deviation being based on variance of instrument and data analysis.

Use of Internal Control DNA (High conc.)*

After reconstitution of the lyophilized Internal Control DNA in QuantiTect Nucleic Acid Dilution buffer, add the resulting highly concentrated Internal Control DNA solution to the sample lysate (or lysis buffer) at a ratio of 0.1 µl per 1 µl elution volume. As an example, if the QIAamp® MinElute® Virus Kit is used with DNA elution in 50 µl Buffer AVE, then 5 µl of the Internal Control should be added to the sample lysate.

Note: With this application, no Internal Control DNA is added to the reaction mixture.

The Internal Control DNA should be added only to the mixture of lysis buffer and sample material or directly to the lysis buffer. The Internal Control DNA must not be added to the sample material directly. If added to the lysis buffer, note that the mixture of Internal Control DNA and lysis buffer must be freshly prepared and used immediately (storage of the mixture at room temperature or at 2–8°C for even just a few hours may lead to internal control failure and a reduced extraction efficiency). Do not add the Internal Control DNA directly to the sample material.

Generally, the quantity of Internal Control DNA to be added to the purification depends only on the elution volume. Successful purification and amplification should result in a C_T value of the Internal Control of 30±3. Depending on the purification method used, the purification efficiency for the Internal Control DNA may vary. In case of lower purification efficiency, increase the volume of Internal Control DNA per sample to more than 0.1 µl per 1 µl elution volume. If necessary, evaluate the appropriate amount of Internal Control DNA to be added to the purification to obtain a C_T value in the range of 30±3 by testing the extraction of the Internal Control DNA from a negative purification control. Note that the use of **carrier RNA** is critical for the extraction efficiency if cell-free fluids are processed.

* Not supplied in the kit, but can be ordered separately. See Ordering Information, page 59.

Protocol 1: Duplex PCR Using TaqMan Probes with Rotor-Gene Cyclers and Other Cyclers Not Requiring ROX Reference Dye

This protocol is optimized for use of the QuantiFast Pathogen PCR +IC Kit with TaqMan probes on Rotor-Gene real-time cyclers and other cyclers not requiring ROX reference dye, such as cyclers from Agilent, Bio-Rad, and Roche. 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 and the Internal Control Assay have been optimized for use with pathogen assays generating amplicons between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 57.
- Use the primer and probe concentrations specified in this protocol.
- We strongly recommend testing the performance of new pathogen-specific primer–probe sets in individual assays before combining them with the Internal Control in a duplex assay.
- Read "Guidelines for effective duplex detection of pathogen and Internal Control", page 17.
- Make sure that at least one positive control, as well as one negative control (water, PCR grade), are included per PCR run.
- See the user manual for the real-time cycler to ensure correct instrument setup for duplex analysis (e.g., setting up detection of two different dyes in the same well or tube). Be 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. For details on channels and calibration see "Selecting dyes and instrument setup", page 19.
- 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, 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 a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe (see Appendix D, page 54). 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 55.

Procedure

1. **Thaw 5x QuantiFast Pathogen Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral or bacterial DNA), 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 5–12.5 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 7 (page 28). It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Note: If Internal Control DNA (High conc.) has been added to the sample lysate or lysis buffer to control for both purification and amplification, do not add Internal Control DNA to the reaction mix, but add water instead.

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

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

   For optimal duplex performance of pathogen and Internal Control assays, we recommend using a total reaction volume of 25 µl. If your real-time cycler requires a different reaction volume, adjust the amount of reaction mix including all reaction components accordingly. Make sure to evaluate the performance of pathogen detection in duplex amplification with the Internal Control in relation to performance of the pathogen-specific primer–probe set as a singleplex assay.

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 8, page 29.**

   Data acquisition should be performed during the combined annealing/ extension step.

   For the Rotor-Gene Q, we recommend determining the fluorescence range for your pathogen target. For the Internal Control Assay, a fixed gain of +9 should be employed. For details on adjusting fluorescence sensitivity on Rotor-Gene Q cyclers, see Appendix B, page 48.

Table 7. Reaction setup

Component	Volume*	Final concentration
5x QuantiFast Pathogen Master Mix	5 µl	1x
10x pathogen specific primer–probe mix†	2.5 µl	0.4 µM forward primer 1‡ 0.4 µM reverse primer 1‡ 0.2 µM probe 1§
10x Internal Control Assay¶	2.5 µl	1x
10x Internal Control DNA¶	2.5 µl	1x
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable	Variable
Total reaction volume	**25 µl***	–

* If the real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† For ease of use, we recommend preparing a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe. See Appendix D, page 54.

‡ A final primer concentration of 0.4 µM is optimal in most cases. Depending on assay design and pathogen target sequence, performance may be improved by increasing the primer concentration to 0.5 to 1.0 µM. Before adapting primer concentration, verify the concentration of the 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.

¶ See, "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24.

Note: If the Internal Control DNA has been added to the sample lysate or lysis buffer to control purification and amplification, do not add Internal Control DNA to the reaction mix, but add RNase-free water instead.

Table 8. Cycling conditions

Step	Time	Temperature	Additional comments
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*	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection.
Number of cycles	40–45		The number of cycles depends on the amount of template DNA.

* On the Rotor-Gene Q, perform "gain optimization before first acquisition" in the green channel to adjust fluorescence channel sensitivity for your pathogen assay. Set a fixed gain of +9 in the yellow channel to ensure optimal fluorescence gain for the Internal Control Assay. See Appendix B, page 48, for detailed information.

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. Select the analysis settings (i.e., baseline settings and threshold values) separately for pathogen and IC assay. Note that optimal analysis settings are a prerequisite for accurate detection data.

Protocol 2: Duplex PCR Using TaqMan Probes with ABI 7500 Cyclers

This protocol is optimized for use of the QuantiFast Pathogen PCR +IC Kit with TaqMan probes on Applied Biosystems 7500 real-time cyclers. 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 and the Internal Control Assay have been optimized for use with pathogen assays generating amplicons between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 57.
- Use the primer and probe concentrations specified in this protocol.
- We strongly recommend testing the performance of new pathogen-specific primer–probe sets in individual assays before combining them with the Internal Control in a duplex assay.
- Read "Guidelines for effective duplex detection of pathogen and Internal Control", page 17.
- Make sure that at least one positive control, as well as one negative control (water, PCR grade), are included per PCR run.
- See the user manual for the real-time cycler to ensure correct instrument setup for duplex analysis (e.g., setting up detection of two different dyes in the same well or tube). Be 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. For details on channels and calibration see "Selecting dyes and instrument setup", page 19.
- 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, 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 a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe (see Appendix D, page 54). 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 55.

Procedure

1. **Thaw 5x QuantiFast Pathogen Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral or bacterial DNA), 50x ROX Dye Solution, 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 5–12.5 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 9 (page 32). It is recommended to prepare a volume of reaction mix 10% greater than that required for the total number of reactions to be performed.**

   Note: If Internal Control DNA (High conc.) has been added to the sample lysate or lysis buffer to control both purification and amplification, do not add Internal Control DNA to the reaction mix, but add water instead.

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

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

   For optimal duplex performance of pathogen and Internal Control assays, we recommend using a total reaction volume of 25 µl. If your real-time cycler requires a different reaction volume, adjust the amount of reaction mix including all reaction components accordingly. Make sure to evaluate the performance of pathogen detection in duplex amplification with the Internal Control in relation to performance of the pathogen-specific primer–probe set as a singleplex assay.

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 10 (page 33).**

Table 9. Reaction setup

Component	Volume*	Final concentration
5x QuantiFast Pathogen Master Mix	5 µl	1x
50x ROX Dye Solution	0.5 µl	1x
10x pathogen specific primer–probe mix[†]	2.5 µl	0.4 µM forward primer 1[‡] 0.4 µM reverse primer 1[‡] 0.2 µM probe 1[§]
10x Internal Control Assay[¶]	2.5 µl	1x
10x Internal Control DNA[¶]	2.5 µl	1x
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable	Variable
Total reaction volume	**25 µl***	–

* If the real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† For ease of use, we recommend preparing a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe. See Appendix D, page 54.

‡ A final primer concentration of 0.4 µM is optimal in most cases. Depending on assay design and pathogen target sequence, performance may be improved by increasing the primer concentration to 0.5 to 1.0 µM. Before adapting primer concentration, verify the concentration of the 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.

¶ See, "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24.

Note: If the Internal Control DNA has been added to the sample lysate or lysis buffer to control purification and amplification, do not add Internal Control DNA to the reaction mix, but add RNase-free water instead.

Table 10. Cycling conditions

Step	Time	Temperature	Additional comments
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	**30 s***	**60°C**	Combined annealing/ extension step with fluorescence data collection.
Number of cycles	40–45		The number of cycles depends on the amount of template DNA.

* On some Applied Biosystems 7500 models, the minimum time for annealing/extension may be 32 seconds.

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. Select the analysis settings (i.e., baseline settings and threshold values) separately for pathogen and IC assay. Note that optimal analysis settings are a prerequisite for accurate detection data.

Protocol 3: Duplex PCR Using TaqMan Probes with Most Applied Biosystems Cyclers

This protocol is optimized for use of the QuantiFast Pathogen PCR +IC Kit with TaqMan probes on most Applied Biosystems real-time, **with the exception of Applied Biosystems 7500 Real-Time PCR Systems**. For further information, see "ROX passive reference dye", page 11 and "Selecting Kits and Protocols", page 16.

Important points before starting

- Always start with the cycling conditions specified in this protocol. The cycling and the Internal Control Assay have been optimized for use with pathogen assays generating amplicons between 60 and 150 bp. For PCR products >150 bp, different cycling conditions may improve results. For details, see Appendix F, page 57.
- Use the primer and probe concentrations specified in this protocol.
- We strongly recommend testing the performance of new pathogen-specific primer–probe sets in individual assays before combining them with the Internal Control in a duplex assay.
- Read "Guidelines for effective duplex detection of pathogen and Internal Control", page 17.
- Make sure that at least one positive control, as well as one negative control (water, PCR grade), are included per PCR run.
- See the user manual for the real-time cycler to ensure correct instrument setup for duplex analysis (e.g., setting up detection of two different dyes in the same well or tube). Be 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. For details on channels and calibration see "Selecting dyes and instrument setup", page 19.
- 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, 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 a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe (see Appendix D, page 54). 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 55.

Procedure

1. **Thaw 5x QuantiFast Pathogen Master Mix, primer and probe solutions, RNase-free water, template nucleic acids (isolated viral or bacterial DNA), 50x High-ROX Dye Solution, 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 5–12.5 µl per reaction.

2. **Prepare a reaction mix for the required number of reactions according to Table 11 (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.**

   Note: If Internal Control DNA (High conc.) has been added to the sample lysate or lysis buffer to control both purification and amplification, do not add Internal Control DNA to the reaction mix, but add water instead.

   Typically, reaction setup can be done at room temperature (15–25°C). However, it is recommended to keep samples, controls, and Internal Control DNA on ice.

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

   For optimal duplex performance of pathogen and Internal Control assays, we recommend using a total reaction volume of 25 µl. If your real-time cycler requires a different reaction volume, adjust the amount of reaction mix including all reaction components accordingly. Make sure to evaluate the performance of pathogen detection in duplex amplification with the Internal Control in relation to performance of the pathogen-specific primer–probe set as a singleplex assay.

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 12 (page 37).**

Table 11. Reaction setup

Component	Volume*	Final concentration
5x QuantiFast Pathogen Master Mix	5 µl	1x
50x High-ROX Dye Solution	0.5 µl	1x
10x pathogen specific primer–probe mix†	2.5 µl	0.4 µM forward primer 1‡ 0.4 µM reverse primer 1‡ 0.2 µM probe 1§
10x Internal Control Assay¶	2.5 µl	1x
10x Internal Control DNA¶	2.5 µl	1x
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable	Variable
Total reaction volume	**25 µl***	–

* If the real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly.

† For ease of use, we recommend preparing a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe. See Appendix D, page 54.

‡ A final primer concentration of 0.4 µM is optimal in most cases. Depending on assay design and pathogen target sequence, performance may be improved by increasing the primer concentration to 0.5 to 1.0 µM. Before adapting primer concentration, verify the concentration of the 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.

¶ See, "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24.

Note: If the Internal Control DNA has been added to the sample lysate or lysis buffer to control purification and amplification, do not add Internal Control DNA to the reaction mix, but add RNase-free water instead.

Table 12. Cycling conditions

Step	Time	Temperature	Additional comments
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	**30 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection.
Number of cycles	40–45		The number of cycles depends on the amount of template DNA.

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. Select the analysis settings (i.e., baseline settings and threshold values) separately for pathogen and IC assay. Note that optimal analysis settings are a prerequisite for accurate detection 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).

For information on the correct interpretation of pathogen target and Internal Control signals, refer to "Controls" (page 22) and to Appendix C, "Data Analysis" (page 50).

	Comments and suggestions
Target signal not detected (or detected late) including target signal in positive controls and/or Internal Control signal not detected (or detected late) including Internal Control signals in NTCs.*	
a) Incorrect 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 43, for details on evaluating the concentration of primers and probes. Repeat the assay.
d) Incorrect or no detection step	Ensure that fluorescence detection takes place during the during the combined annealing/ extension step when using TaqMan probes.

* Note that no Internal Control Signal will be detected in NTCs if the Internal Control DNA was added during to the purification procedure. For details on the interpretation of pathogen target and Internal Control signals, refer to "Controls" page 22) and to Appendix C, "Data Analysis" (page 50).

	Comments and suggestions
e) Primer or probe concentration not optimal*	Use optimal primer concentrations. For the pathogen target assays on all real-time cyclers, use each primer at 0.4 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 43). Ensure the correct handling and storage of the Internal Control Assay (see Shipping and Storage, page 4 and "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24).
f) Problems with control templates	Check the concentration, storage conditions, and quality of the pathogen positive control template nucleic acids. If necessary, make new serial dilutions of control nucleic acid from the stock solutions. Repeat the assay using the new dilutions. Ensure that sufficient copies of the target nucleic acids are present in your positive control samples. Ensure the correct handling and storage of the Internal Control DNA (see Shipping and Storage, page 4 and "Reconstitution and use of Internal Control Assay and Internal Control DNA", page 24).
g) Insufficient number of cycles	Increase the number of cycles.
h) 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 43).

* Note that no Internal Control Signal will be detected in NTCs if the Internal Control DNA was added during to the purification procedure. For details on the interpretation of pathogen target and Internal Control signals, refer to "Controls" page 22) and to Appendix C, "Data Analysis" (page 50).

	Comments and suggestions
i) Incorrect 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.

Target signal and IC signal not detected (or late detected) in samples while target signal is detected in positive controls and Internal Control signal is detected in NTCs.*

Presence of inhibitory substances or nucleases in the samples	Check the concentration, storage conditions, and quality of the pathogen starting template nucleic acids. 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 59). If containing low amounts of inhibitors, samples may still be used successfully in PCR by adding less sample (and therefore less inhibitor), to the reaction. Add a smaller sample volume to the reactions or dilute samples (e.g., 5- and 10-fold) before use in PCR. Ensure that all reagents, buffers, and solutions used for isolating and dilution of template nucleic acids are free of nucleases (RNases/DNases).

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

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.

* Note that no Internal Control Signal will be detected in NTCs if the Internal Control DNA was added during to the purification procedure. For details on the interpretation of pathogen target and Internal Control signals, refer to "Controls" page 22) and to Appendix C, "Data Analysis" (page 50).

	Comments and suggestions
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 pathogen target in "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.

	Comments and suggestions
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) For ABI PRISM 7000 only: 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.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Appendix A: Assay Design and Handling Primers and Probes

Important factors for success in 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 pathogen specific primers for duplex real-time PCR detection of pathogen target and Internal Control

- Use specialized design software (e.g., Primer Express® Software or Primer3 Software) to design primers and probes. Already existing assays designed with this software under standard salt settings typically work fine as they are.
- For new assay designs, the following guidelines work efficiently:
  - Using standard algorithm parameters and reaction conditions of Primer Express or Primer3 Software, the T_m of both primers should be 58–63°C and within 2°C of each other.
  - T_m of probes should be 5–10°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 guanidine.
  - Choose the binding strand so that the probe has more C than G bases.

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 probe 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

Cycling and Internal Control Assay have been optimized for use with pathogen assays generating amplicons of up to approximately 150 bp. If amplifying longer PCR products, see Appendix F for recommendations (page 57).

Special considerations for design of assays for pathogen nucleic acids

- Design primers or probes in a conserved region of the pathogen.
- 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.

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.

The lyophilized Internal Control Assay, containing primers and probe for detection of the Internal Control, should be dissolved in Buffer TE provided with the QuantiFast Pathogen PCR +IC Kit as described in section "Reconstitution and Use of Internal Control Assay and Internal Control DNA", page 24.

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 pathogen-specific primer–probe sets, we recommend preparing a 10x primer–probe mix, containing the primers and the probe for your pathogen target at the suggested concentrations (see protocols). See Appendix D (page 54).

Dissolving pathogen primers and probes

For information on reconstitution of the Internal Control Assay, see "Reconstitution and Use of Internal Control Assay and Internal Control DNA", page 24.

For reconstitution of your pathogen specific primers and probe, we recommend to spin the tubes containing lyophilized primer or probe briefly before opening, 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 (not supplied in the kit), 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:

$\varepsilon_{260} = 0.89 \times [(A \times 15{,}480) + (C \times 7340) + (G \times 11{,}760) + (T \times 8850)]$

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}: $0.89 \times [(6 \times 15{,}480) + (6 \times 7340) + (6 \times 11{,}760) + (6 \times 8850)] \times (1 \times 10^{-6}) = 0.232$

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 hydrolysis 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 material safety data sheets (MSDSs), available from the product supplier.

Appendix B: Rotor-Gene Q Setup for Adjustment of Fluorescence Channel Sensitivity

Adjustment of fluorescence channel sensitivity for the Internal Control Assay

We recommend setting the detection range of the yellow channel for the Internal Control Assay on a fixed gain to ensure optimal fluorescence gain. Click "Edit Gain" in the "New Run Wizard" dialog box (Figure 2) to open the "Gain for Yellow" dialog box. Set the gain for the yellow channel to a value of 9 (Figure 2).

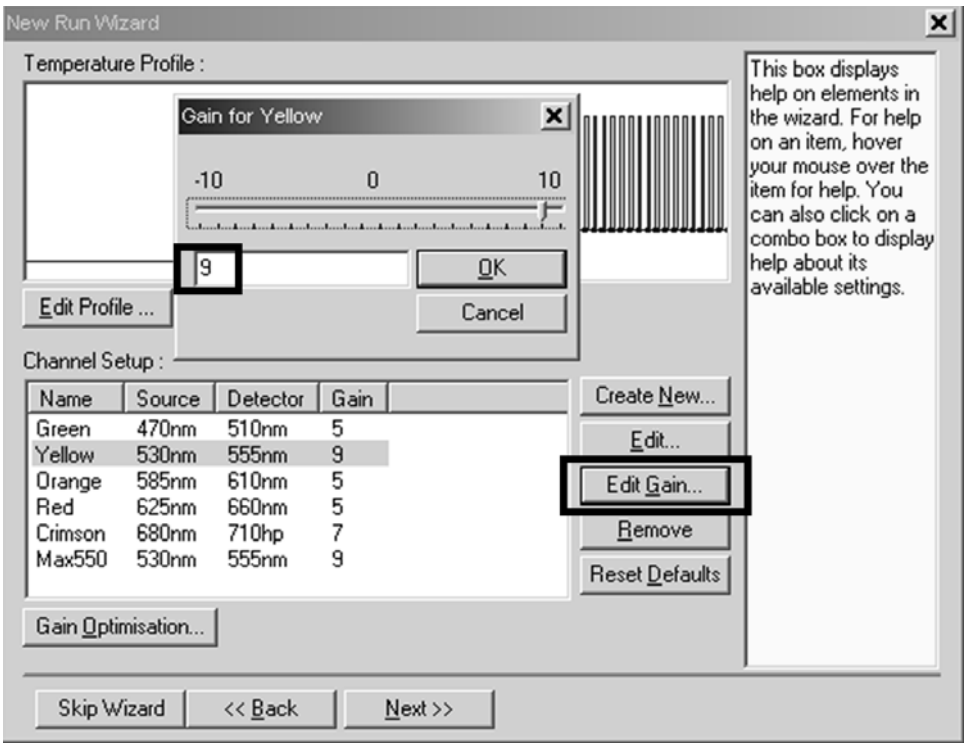
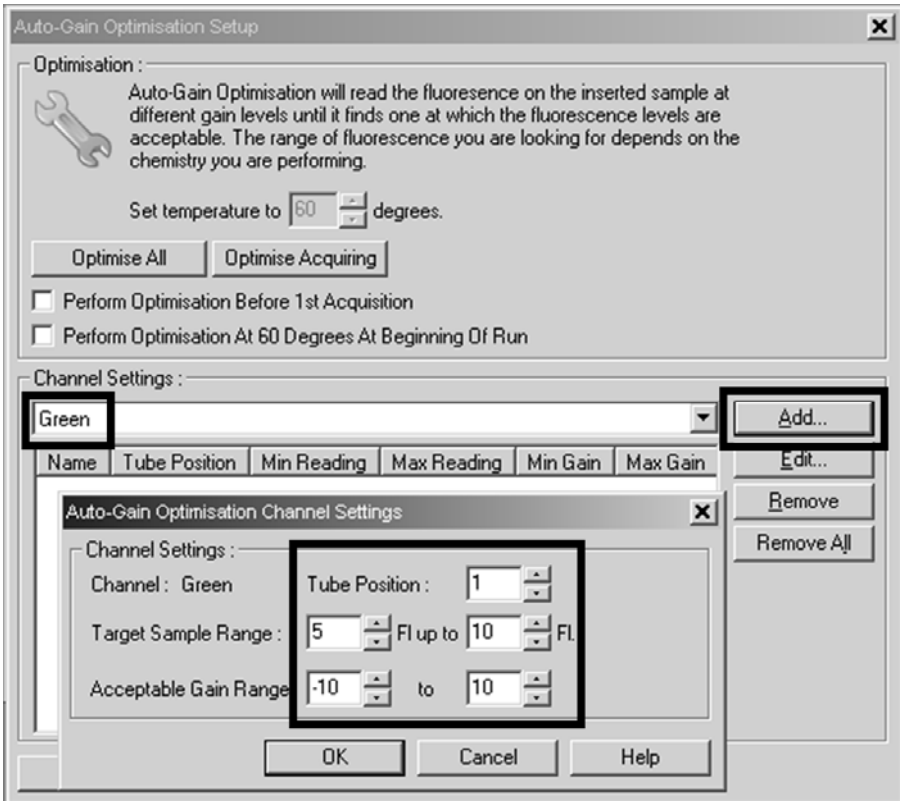


Figure 2. Setting a fixed gain for the Internal Control Assay (yellow channel).

Adjustment of fluorescence channel sensitivity for the pathogen assay

We recommend determining the detection range of the green channel for the pathogen assay according to the fluorescence intensities in the PCR tubes. Click "Gain Optimisation" in the "New Run Wizard" dialog box (Figure 2) to open the "Auto-Gain Optimisation Setup" dialog box. Add channel "Green" from the drop-down menu and adapt the "Auto-Gain Optimisation Settings" as shown in Figure 3A). Adjust the calibration temperature to 60 degrees to match the annealing temperature of the amplification program, and check the box "Perform Optimisation Before 1st Acquisition" (Figure 3B).

A



B

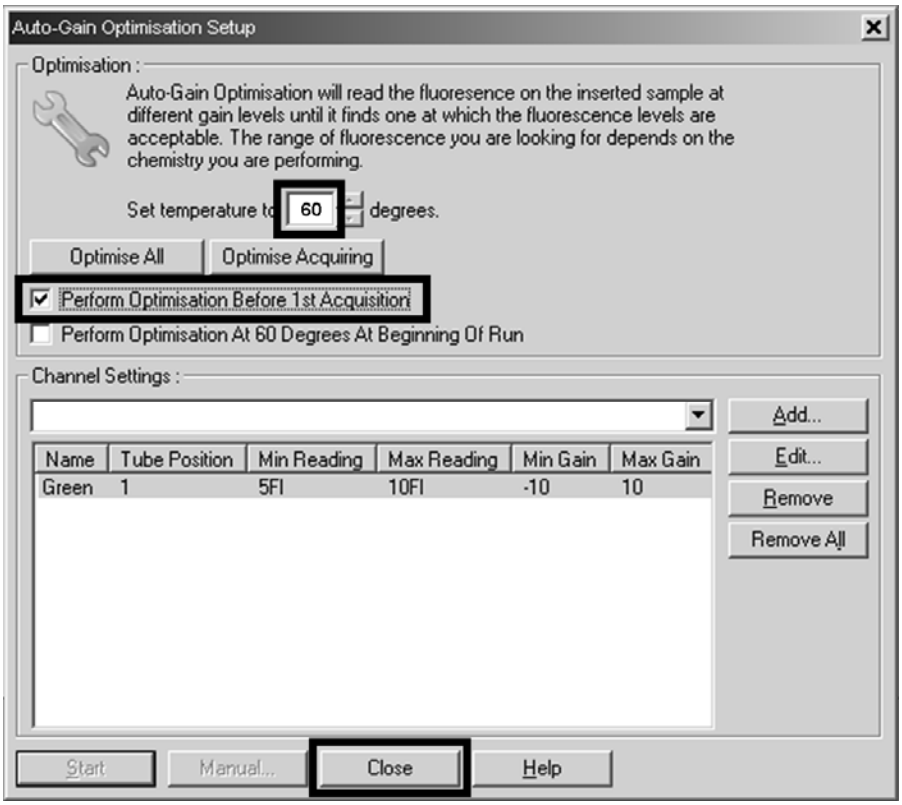


Figure 3. Adjusting the fluorescence channel sensitivity for the pathogen assay (green channel) in the "Auto-Gain Optimisation Setup" dialog box.

Appendix C: Data Analysis

When carrying out data analysis, follow the recommendations provided by the manufacturer of the 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.

Considerations for duplex 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 4, page 51).

- The quantification 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 QuantiFast Pathogen PCR +IC Kit, and may need to be adjusted. On the Rotor-Gene Q, a threshold value for the Internal Control Assay (Yellow channel) of 0.05 will give satisfactory results in most cases.
- 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 duplex or 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 duplex results in rare cases. In most cases, low levels of crosstalk can be overcome by optimal analysis settings.

Data interpretation of pathogen and Internal Control detection

- When performing PCR on unknown samples, we recommend including appropriate controls in each run in addition to the Internal Control provided with the QuantiFast Pathogen PCR +IC Kit. For more information, see “Controls”, page 22.
- After the run is finished, analyze the data. The following results of qualitative pathogen detection are possible:
  - **A signal for the pathogen target is detected in the sample**

    The result of the analysis is positive: the sample contains pathogen target DNA. In this case, the detection of a signal for MAX is dispensable. Note that high initial concentrations of the target pathogen DNA resulting in a strong positive signal for the pathogen target can lead to a reduced or absent fluorescence signal of the Internal Control (MAX signal) due to competition. On the other hand, depending on real-time cycler and on the spectral profile of the probe employed in the pathogen assay, a very high fluorescence intensity for the pathogen target may slightly increase the Internal Control signal due to cross-talk.

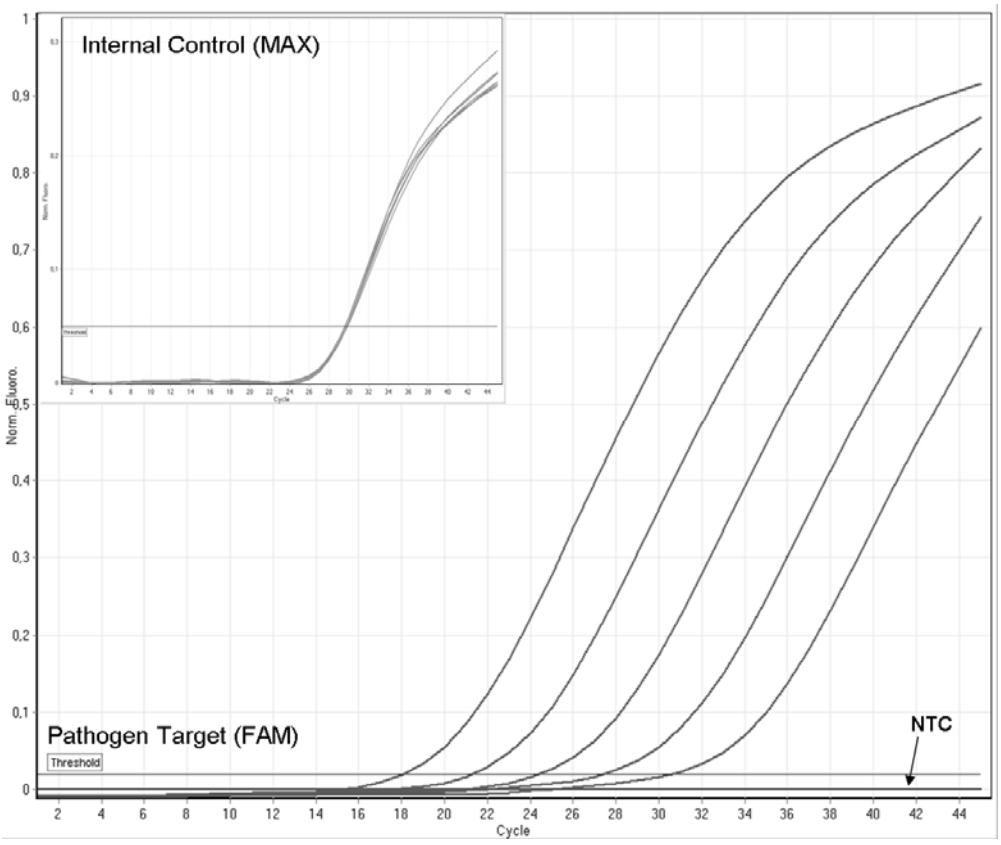


Figure 4. Typical amplification plots on the Rotor-Gene Q for different concentrations of a pathogen target and for the Internal Control (inset).

In the sample, no signal is detected for the pathogen target. At the same time, a signal for the Internal Control appears

This indicates that no pathogen DNA is detectable in the sample. It can be considered negative, provided that a positive control reaction has been performed and proven the functionality of the pathogen assay. In the case of a negative pathogen PCR, the detected signal of the Internal Control rules out the possibility of PCR inhibition.

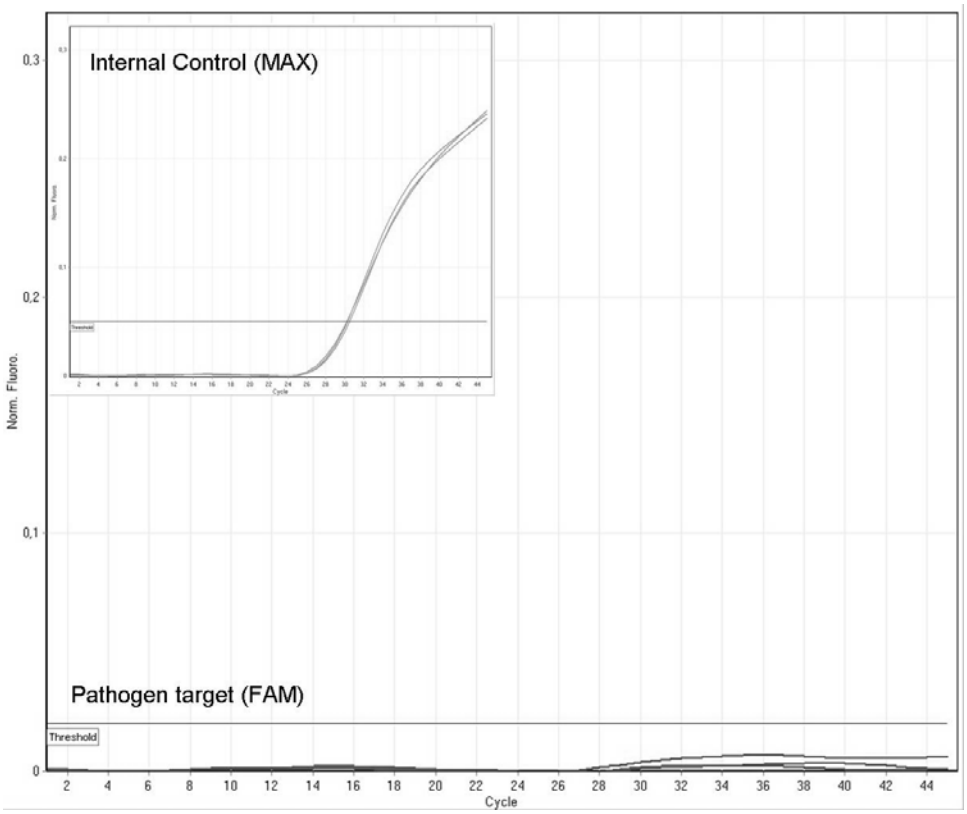


Figure 5. Typical amplification plots on the Rotor-Gene Q showing no signal for three replicates of a pathogen target with successful amplification of the Internal Control (inset) at the same time, indicating absence of the pathogen target in the sample.

No signal is detected for pathogen target and Internal Control

No result can be concluded. If additional controls have been included in the PCR run, this may provide additional information on the reason for a negative signal of both pathogen target and Internal Control in the sample.

If a signal for the pathogen target is detected in the positive control, and a signal for the Internal Control is detected in the NTC, but no signals for pathogen target and Internal Control are detected in the sample, this indicates that inhibition may have occurred in the sample reactions.

If no signals for the pathogen target and the Internal Control are detected in the sample, positive control and NTC, the PCR run may have failed. Information regarding error sources and their solution can be found in “Troubleshooting Guide”, page 38.

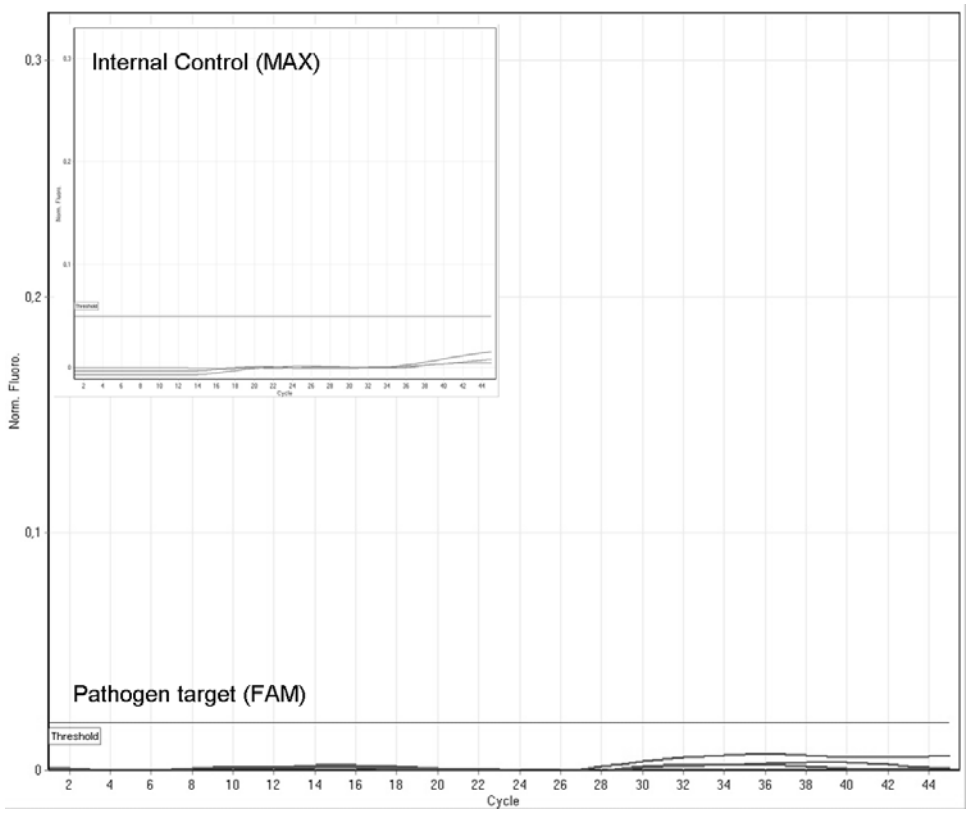


Figure 6. Amplification curves on the Rotor-Gene Q do not exceed the threshold for either of the pathogen target and the Internal Control (inset), indicating that amplification has failed.

Appendix D: Preparing a 10x Primer–Probe Mix for the Pathogen Target Assay

For ease of use, we recommend preparing a 10x primer–probe mix for the pathogen assay containing target-specific primers and probe (Table 13). Alternatively, it may be preferable to prepare the reaction mix with separate primer and probe solutions. If you commonly set up reactions in this way, see Appendix E (page 55).

Table 13. Preparing 10x primer–probe mix for pathogen assay

Component	Concentration (10x)	Final concentration
Forward primer*	4 µM	0.4 µM†
Reverse primer*	4 µM	0.4 µM†
Probe	2 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 0.4 µM is optimal in most cases. Depending on assay design and pathogen target sequence, performance might be improved by increasing the primer concentration to 0.5 to 1.0 µM. Before adapting primer concentration, verify the concentration of the 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.

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

For ease of use, we recommend preparing a 10x primer–probe mix containing target-specific primers and probe for each of the targets. 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 in this way, it may be helpful to copy and complete Table 14 (page 56) with the calculated volumes of each primer to use.

Table 14. Preparing reaction mix using separate primer and probe solutions

	Volume per reaction	
Component*	**25 µl**	**Other: ____ µl**
5x QuantiFast Pathogen Master Mix	5 µl	_____ µl
50x ROX Dye Solution	☐ 0 µl ☐ 0.5 µl ☐ ___ µl	_____ µl
50x High-ROX Dye Solution	☐ 0 µl ☐ 0.5 µl ☐ ___ µl	_____ µl
Forward primer Pathogen Assay ☐ 0.4 µM ☐ ___ µM	_____ µl	_____ µl
Reverse primer Pathogen Assay ☐ 0.4 µM ☐ ___ µM	_____ µl	_____ µl
Probe Pathogen Assay ☐ 0.2 µM ☐ ___ µM	_____ µl	_____ µl
10x Internal Control Assay	2.5 µl	_____ µl
10x Internal Control DNA	2.5 µl	_____ µl
RNase-free water	_____ µl	_____ µl
Template DNA (added at step 4)	_____ µl	_____ µl
Total reaction volume	**25 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 to 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 45–75 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
- For multiplexing of more than one pathogen target and the Internal Control increase the annealing/extension time to 75 s (at 60°C) using a 0.2 µM concentration for each primer employed in the pathogen assays.

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 15 (page 58) with the optimized cycling conditions.

Table 15. Customized cycling conditions

Step	Time	Temperature	Additional comments
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	☐ 30 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 57).
Number of cycles	☐ 40 ☐ 45 ☐		The number of cycles depends on the amount of template DNA.

Ordering Information

Product	Contents	Cat. no.
QuantiFast Pathogen PCR +IC Kit (100)	For 100 x 25 µl reactions: Master Mix, lyophilized Internal Control Assay, lyophilized Internal Control DNA, ROX Dye Solution, High-ROX Dye Solution, RNase-Free Water, Nucleic Acid Dilution Buffer, Buffer TE	211352
QuantiFast Pathogen PCR +IC Kit (400)	For 400 x 25 µl reactions: Master Mix, lyophilized Internal Control Assay, lyophilized Internal Control DNA, ROX Dye Solution, High-ROX Dye Solution, RNase-Free Water, Nucleic Acid Dilution Buffer, Buffer TE	211354
Internal Control DNA (High conc.)	For approximately 200 sample preps (depending on elution volume): Lyophilized Internal Control DNA, Nucleic Acid Dilution Buffer	211392
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
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

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
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
QIAsymphony Virus/Bacteria Mini Kit — for automated purification of viral nucleic acids and bacterial DNA from a broad range of sample types		
QIAsymphony Virus/Bacteria Mini Kit (192)	For 192 preps: 2 reagent cartridges and enzyme racks and accessories	931036
BioSprint® 96 One-For-All Vet Kit — for automated purification of viral nucleic acids and bacterial DNA from a broad range of veterinary sample types		
BioSprint 96 One-For-All Vet Kit (384)	For 384 preps: Large 96-Rod Covers, 96-Well Microplates MP, S-Blocks, MagAttract® Suspension G, Buffers and Reagents	947057

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Notes

Notes

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Canada ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

China ■ Orders 86-21-3865-3865 ■ Fax 86-21-3865-3965 ■ Technical 800-988-0325

Denmark ■ Orders 80-885945 ■ Fax 80-885944 ■ Technical 80-885942

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Italy ■ Orders 800-789-544 ■ Fax 02-334304-826 ■ Technical 800-787980

Japan ■ Telephone 03-6890-7300 ■ Fax 03-5547-0818 ■ Technical 03-6890-7300

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Switzerland ■ Orders 055-254-22-11 ■ Fax 055-254-22-13 ■ Technical 055-254-22-12

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