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RespiFast RG Panel Handbook



Version 1

IVD

Qualitative in vitro diagnostics

For use with Rotor-Gene® Q instruments

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REF

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Intended Use

The RespiFast RG Panel is a qualitative multiplex PCR test to detect and differentiate 16 RNA viruses,* 2 DNA viruses, and 4 bacteria, all of which can cause respiratory tract infections in humans: adenovirus, bocavirus, coronavirus OC43, coronavirus 229E, coronavirus NL63/HKU1, human metapneumovirus (hMPV), influenza A virus, influenza B virus, influenza A virus subtype H1N1pdm09, parainfluenza virus type 1, parainfluenza virus type 2, parainfluenza virus type 3, parainfluenza virus type 4, rhinovirus/enterovirus, respiratory syncytial virus type A (RSVA), respiratory syncytial virus type B (RSVB), *Bordetella pertussis*, *Chlamydomphila pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*.

The RespiFast RG Panel aids in the diagnosis of respiratory tract infection when used in conjunction with other clinical and laboratory findings. Negative results do not necessarily indicate absence of viral or bacterial respiratory tract infection; negative results should not be used as the sole basis for diagnosis, therapy, or other treatment decisions. Positive results do not exclude co-infection with other pathogens. The pathogen(s) detected may not be the definite cause of disease.

Other laboratory testing and assessment of clinical presentation must be included in the final diagnosis. The product is for use by laboratory professionals only.

Summary and Explanation

Acute respiratory tract infection is the most widespread type of acute infection in adults and children and is a significant cause of disease in immunocompromised patients. Respiratory tract infections (RTI) are commonly divided into upper respiratory tract infections (URTI) and

* The RespiFast RG Panel detects 16 RNA viruses but does not differentiate rhinovirus from enterovirus and coronavirus HKU1 from coronavirus NL63.

lower respiratory tract infections (LRTI). The URTI include rhinorrhea, conjunctivitis, pharyngitis, otitis media, and sinusitis. LRTI include pneumonia, bronchiolitis, and bronchitis. Both viruses and bacteria can cause acute RTI, and the number of causative agents is large as well as diverse, providing a great challenge for diagnostics.

Nucleic acid amplification tests have proven to be rapid, sensitive, and specific alternatives in either singleplex or multiplex format. Multiplex assays allow the co-amplification of more than one target, providing insight into the significance of mixed infections and the prognosis and recrudescence of the respiratory disease.

Principles of the Procedure

The RespiFast RG Panel is based on the SmartFinder® technology that allows a highly complex analysis of up to 14 targets in a single real-time PCR reaction. The RespiFast RG Panel contains 23 different RespiFast primer sets combined with 15 fluorescent labeled SMART (single-tube multiplex amplification in real time) probes that enable the detection of 22 different pathogens plus an internal control and 2 amplification controls. The reaction starts with a pre-amplification that combines a reverse transcription step with a PCR step to amplify the target cDNA or DNA. Subsequently, a part of the pre-amplification reaction is transferred to two PCR tubes. Two separate Step 2 reactions are performed. The detection is performed using a Melting Curve analysis. A schematic representation of the assay workflow is shown in **Figure 1**.

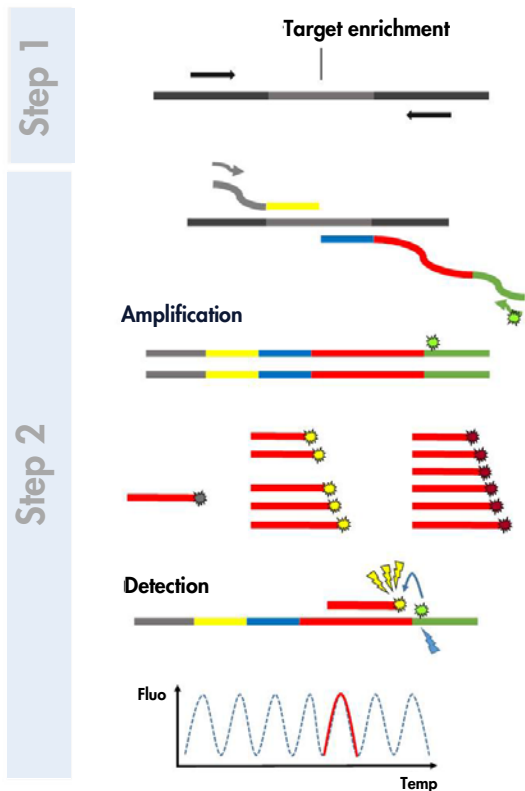


Figure 1. Overview of the workflow with the RespiFast RG Panel.

The RespiFast RG Panel uses the Rotor-Gene Q MDx or Rotor-Gene Q instrument for the detection of the pathogen(s) nucleic acids.

An internal control (IC) is included in the assay to discriminate between true negative samples and false negative samples due to nucleic acid degradation, PCR inhibition, or test failure. RespiFast RG Panel additionally contains 2 amplification controls (AC) which enable to contingently discriminate between extraction failure and amplification failure in the assay.

The input sample is total nucleic acids extracted and purified from nasopharyngeal swabs. Preparation of samples is a separate process from the scope of the panel. Use suitable methods or products to handle specimens and extract and purify nucleic acids (see “Specimen Handling and Storage”, page 14, and “Nucleic acid extraction and preparation”, page 14).

Target genes

Target genes used for probe and primer design are shown in Table 1.

Table 1. Target genes of the RespiFast RG Panel

Target	Gene	Pathogen code
Control		
Internal control	MS2 phage lysis protein gene	IC
Amplification control 1	unique artificial sequence	AC1
Amplification control 2	unique artificial sequence	AC2
Viruses		
Adenovirus	Hexon (H) gene	Adeno
Bocavirus	Noncapsid (NP1) gene	Boca
Coronavirus 229E	Nucleocapsid protein (NP) gene	229E
Coronavirus HKU1	Nucleocapsid phosphoprotein (N) gene	HKU1
Coronavirus NL63	Nucleocapsid protein (NP) gene	NL63
Coronavirus OC43	Nucleocapsid protein (NP) gene	OC43
Human metapneumovirus	Nucleocapsid protein (NP) gene	hMPV
Influenza A virus	Matrix protein (M1) gene	InfA
Influenza B virus	Matrix protein (M1) gene	InfB
Influenza A virus subtype H1N1pdm09	Neuraminidase gene	H1N1
Parainfluenza virus type 1	Hemagglutinin-neuraminidase (HN) gene	PIV1
Parainfluenza virus type 2	Hemagglutinin-neuraminidase (HN) gene	PIV2
Parainfluenza virus type 3	Hemagglutinin-neuraminidase (HN) gene	PIV3
Parainfluenza virus type 4	Major nucleocapsid protein (N) gene	PIV4
Rhinovirus/ Enterovirus	5' untranslated region polyprotein (PP) gene	Rhino/Enterovirus
Respiratory syncytial virus type A	Nonstructural protein gene	RSVA
Respiratory syncytial virus type B	Nucleoprotein gene	RSVB
Bacteria		
<i>Bordetella pertussis</i>	Insertion sequence 481 (IS481)	B. pert
<i>Chlamydomphila pneumoniae</i>	Major outer membrane (OmpA) gene	C. pneu
<i>Legionella pneumophila</i>	Macrophage inhibitor potentiator (Mip) gene	L. pneu
<i>Mycoplasma pneumoniae</i>	Cytadhesin protein (P1) gene	M. pneu

For each sample, one pre-amplification (Step 1) and two Step 2 reactions are performed. The pathogens, the labels, and the indicated T_m of the corresponding SMART hybridization probes in the 2 RespiFast reactions are shown in Table 2.

Table 2. Targets and corresponding T_m values of SMART probes

Label	SMART probe	T_m (°C) acceptance range	Pathogen code (mix 1)	Pathogen code (mix 2)
Cy5 [®] 5	Cy5 probe 1	50.5–53.5*	L. pneu	OC43
	Cy5 probe 2a	55–58	B. pert	–
	Cy5 probe 2b	52.5–55.5	–	HKU1/NL63
	Cy5 probe 3	60.5–63.5	Rhino/Entero	229E
		58.5–61.5		
	Cy5 probe 4	66.5–69.5	C. pneu	–
	Cy5 probe 5	70.5–73.5	M. pneu	–
Cy5 probe 6	76–79	–	H1N1	
ROX [™]	ROX probe 1	53.5–56.5	RSVA	PIV1
	ROX probe 2	58–61	Adeno	PIV2
	ROX probe 3	62.5–65.5	hMPV	PIV3
	ROX probe 4	66.5–69.5	RSVB	PIV4
	ROX probe 5	72.5–75.5	InfA	Boca
	ROX probe 6	76.5–79.5	InfB	–
BHQ [®] 1	BHQ1 probe 1	70.5–73.5	IC	IC
	BHQ1 probe AC1	61–64	AC1	–
	BHQ1 probe AC2	55.5–58.5	–	AC2

* The Cy5 probe 1 peaks sometimes appears as a double peak. The T_m value of the second peak is the correct one.

Materials Provided

Kit contents

RespiFast RG Panel		(25)
Catalog no.		4693163
Number of reactions		25
Contents	Cap color	Volume
Pre-amplification master mix	Purple	>160 µl
Pre-amplification primer mix	White	>220 µl
RespiFast buffer 1 (contains AC1)	Red	>500 µl
RespiFast buffer 2 (contains AC2)	Blue	>500 µl
RespiFast enzyme	Orange	>55 µl
Internal Control (IC)	Black	>450 µl
Positive Control (PC)	Green	>50 µl
Dilution buffer	Transparent	>1500 µl
<i>RespiFast RG Panel Handbook</i> (English)		1 pc

Materials Required but Not Provided

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

Reagents

- RNA/DNA extraction kit (see “Nucleic acid extraction and preparation”, page 14)
- RNase/DNase-free water

Consumables

- Disposable tips containing hydrophobic filters
- Disposable low retention tips containing hydrophobic filters
- RNase/DNase-free 1.5 ml tubes
- RNase/DNase-free 0.2 ml PCR tubes (single tubes with caps or strip tubes with individual caps)
- Strip Tubes and Caps, 0.1 ml (cat. no. 981103 or 981106) for use with Rotor-Gene Q instruments

Equipment

- Adjustable pipets: * 0.1–2 µl, 2–20 µl, 20–200 µl
- Vortex mixer*
- Benchtop centrifuge* with a rotor for 2 ml tubes
- Centrifuge* for 0.2 ml reaction tubes
- Cooling block or ice
- Thermal cycler* for 0.2 ml PCR tubes with a heated lid and ramp rates of 1–5°C/second (for pre amplification; e.g., GeneAmp® PCR System 9700 block-based thermal cycler)
- Rotor-Gene Q MDx 5plex HRM* or Rotor-Gene Q 5plex HRM* instrument and accessories

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Warnings and Precautions

For in vitro diagnostic use

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

For safety information for the purification kit used, see the relevant kit handbook. For safety information regarding instruments, see the relevant instrument user manual.

Discard sample and assay waste according to your local safety regulations.

General precautions

Always pay attention to the following:

- This molecular assay should only be performed by qualified laboratory personnel.
- Store and extract positive materials (specimens and positive controls) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Wear disposable gloves when performing the assay.
- Use disposable tips containing hydrophobic filters to prevent cross-contamination.
- Use disposable low retention tips containing hydrophobic filters when handling the pre-amplification master mix, and RespiFast enzyme.
- Use RNase/DNase-free PCR vials.
- Always thaw RNA/DNA samples on ice and keep them on ice or in a cooling block.

- Always keep enzymes on ice or in a cooling block when taken out of the freezer. Handle enzymes with care and mix very gently.
- When thawed, centrifuge the reagents for 5 seconds in a centrifuge and mix by gently pipetting up and down.
- All cycling programs should be entered in the Rotor-Gene Q software before performing the assay.
- Always centrifuge PCR vials and plates briefly and open with care to avoid aerosols.

Note: To prevent contamination, we strongly recommend performing the experimental activities, in PCR cabinets, in the following 3 separate areas:

Area 1: Prepare master mixes for pre-amplification and step 2/melting analysis.

Area 2: Add RNA/DNA sample to the mix.

Area 3: Perform the pre-amplification step 2 and melting curve analysis in 2 steps:

- Perform the pre-amplification.
- Add pre-amplification products to step 2 mix (preferably inside a PCR cabinet) and perform step 2 and melting curve analysis.

Reagent Storage and Handling

The components of the RespiFast RG Panel should be stored in the dark at -30 to -15°C and are stable until the expiration date stated on the label. Repeated thawing and freezing (>5 x) should be avoided, as this may reduce assay performance.

Specimen Handling and Storage

Respiratory pathogen detection depends on the collection of high-quality specimens, their rapid transport to the laboratory, and appropriate storage before laboratory testing. Nasopharyngeal swabs are suitable for the detection of viral and/or bacterial infections of the respiratory tract.

Clinical specimens should be transported to the laboratory as soon as possible, aliquoted, and processed. The specimens should be kept at 4°C. If specimens cannot be processed within 48 hours, they should be kept frozen at or below -20°C, preferably -70°C.

Procedure

Nucleic acid extraction and preparation

The QIAamp® MinElute® Virus Spin Kit and QIASymphony® DSP Virus/Pathogen Mini Kit from QIAGEN, shown in Table 3, are validated for DNA and RNA purification from the indicated human sample type for use with the RespiFast RG Panel. Carry out the nucleic acid purification according to the instructions in the kit handbook.

Table 3. Purification kit validated for use with the RespiFast RG Panel

Sample type	Nucleic acid isolation kit	Catalog number (QIAGEN)
Nasopharyngeal swabs	QIAamp MinElute Virus Spin Kit (50)	57704
	QIASymphony DSP Virus/Pathogen Mini Kit with Complex 200 protocol	937036

Use 200 µl of sample material and elute in 60 µl elution buffer. We strongly recommend not to attempt to concentrate the nucleic acids as this might increase the risk of inhibition of the

RT-PCR enzymes. An internal control is added to the lysed sample (see “Using an internal control and carrier RNA”, page 15).

When using the QIAamp MinElute Virus Spin Kit, we recommend adding lysis buffer to the sample in a laminar airflow cabinet. The extraction procedure can be continued on a bench once the sample is lysed. It is advisable to decontaminate the laminar airflow cabinet after use with a decontamination solution (e.g., 1000 ppm sodium hypochlorite)* and running the UV light for 30 minutes.

Prevent freeze-thaw cycles of the extracted DNA/RNA as much as possible and store nucleic acid extracts at 4°C when processed within one day. For longer periods, store the extracted RNA/DNA at -20°C or -70°C.

Using a negative control



Each run should include a negative control. A negative control consists of 200 µl PCR-grade water plus internal control (IC). This negative control is treated as a regular sample, including the nucleic acid extraction procedure. In the final analysis, the negative control signal serves as a reference for the background signal in FAM/ROX and FAM/Cy5 channels and for the positive IC and AC signals in the Green channel.

Using an internal control and carrier RNA

Using the QIAamp MinElute Virus Spin Kit or QIAasymphony DSP Virus/Pathogen Mini Kit in combination with the RespiFast RG Panel requires introduction of the internal control (IC) into the purification procedure to monitor the efficiency of sample preparation and downstream

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

assay. In addition, both the QIAamp MinElute Virus Spin Kit and QIAasymphony DSP Virus/Pathogen Mini Kit require the preparation of carrier RNA.

The IC is a MS2 phage and is supplied as a control for lysis, RNA/DNA extraction, the RespiFast RG Panel assay performance, and to check for possible PCR inhibition. The IC is added together with the carrier RNA to the lysis buffer prior to adding to the sample. The amount of spiked IC is independent of the initial volume of the sample.

The amount of spiked IC is dependent on the elution volume. Using the QIAamp MinElute Virus Spin Kit, the extracted RNA/DNA will be eluted in 60 µl. The volume of IC to be added to Buffer AL is 5.5 µl per sample (see Table 4).

In case of a strong infection and/or multiple infections, the IC melting peak might not be visible in the final analysis. This is explained by the fact that high amounts of pathogenic nucleic acids consume most of the reagents in the assay. Consequently, when the IC signal is absent in the presence of one or more specific melting peaks indicating an infection, the assay is still valid.

Table 4. Volumes of Buffer AL, carrier RNA–Buffer AVE mix, and IC required with QIAamp MinElute Virus Spin Kit extraction

Number of samples	Volume Buffer AL (ml)	Volume carrier RNA–AVE (µl)	Volume internal control (µl)
1	0.22	6.2	5.5
2	0.44	12.3	11
3	0.66	18.5	16.5
4	0.88	24.6	22
5	1.1	30.8	27.5
6	1.32	36.9	33
7	1.54	43.1	38.5
8	1.76	49.2	44
9	1.98	55.4	49.5
10	2.2	61.5	55
11	2.42	67.7	60.5
12	2.64	73.8	66

Using the QIAasymphony DSP QIAasymphony DSP Virus/Pathogen Mini Kit in combination with the RespiFast RG Panel requires introduction of the internal control into the purification procedure to monitor the efficiency of sample preparation and the downstream assay.

For internal control (IC) calculation, the “IC Calculator” within the QIAasymphony Management Console (QMC) should be used.

The guaranteed elution volume of 60 µl needs an actual volume of 90 µl elution buffer that is added by the instrument to the sample. The amount of added IC per sample is 7.5 µl. Therefore set the internal control ratio/sample to 7.5 within the “IC Calculator” of the QIAasymphony Management Console (QMC).

Table 5 represents the addition of internal control to the sample at a ratio of 7.5 μl per sample. It is recommended to prepare fresh mixtures for each run just before use.

Table 5. Preparation of carrier RNA and internal control

Component	n = number of samples and controls	
	n ≤ 13	n ≥ 13
	Volume (μl) (Sarstedt tubes)*	Volume (μl) (CO tubes)†
Stock carrier RNA (CARRIER)	$(n + 3) \times 3$	$(n + 5) \times 3$
Internal control	$(n + 3) \times 7.5$	$(n + 5) \times 7.5$
Buffer AVE	$(n + 3) \times 109.5$	$(n + 5) \times 109.5$
Final volume per sample (excluding dead volume)	120	120
Total volume for n samples	$(n + 3) \times 120$	$(n + 5) \times 120$

* Micro tubes 2.0 ml Type H and Micro tubes 2.0 ml Type I (Sarstedt, cat. nos. 72.693 and 72.694). If preparing IC as a stock solution in a larger tube, multiply the total volume of each component by the number of IC tubes used. Internal control mixture corresponding to 3 additional samples (i.e. 360 μl) is required. Do not fill more than 1.92 ml total volume (corresponding to a maximum of 13 samples).

If using more than 13 reactions in Micro tubes 2.0 ml, set up the reactions in a larger tube and load in multiple tubes. Make sure that for each tube the required excess volume of 3 additional reactions is added.

† Tubes 14 ml, 17 x 100 mm polystyrene round-bottom (Corning former Becton Dickinson, cat. no. 352051). Internal control mixture corresponding to 5 additional samples (i.e. 600 μl) is required. Do not fill more than 13.92 ml total volume.

Using the amplification control

Both RespiFast buffers 1 and 2 contain an amplification control (AC) specific for that RespiFast buffer. The AC controls the second part of the RespiFast protocol: correct addition of reagents and applying the correct PCR and Melting Analysis protocol. AC 1 and AC 2 are detected by a BHQ1 labelled detection probe and display different T_m values, which enables detection of mixing up mix 1 and mix 2 reactions.

The AC reaction can be out-competed in the reaction whenever a strong infection is detected. If no pathogen is detected in the reaction, the AC must reveal a melting peak, to validate the absence of pathogen reactions in that reaction.

When a strong infection is present in one of the RespiFast mixes of a sample, the IC may remain undetected in that RespiFast mix and in the corresponding RespiFast mix because it was outcompeted by the pathogen during the pre-amplification. In that case, the presence of the AC in the corresponding RespiFast mix ensures that proper amplification occurred in both RespiFast mixes.

Using the positive control

RespiFast RG Panel contains a positive control consisting in a DNA plasmid containing the target sequences of 4 pathogens that can be detected with the assay. Table 6 gives an overview of the content of the positive control. The positive control does not need to be extracted and is further handled as a regular sample in the RespiFast RG protocol. Using the positive control in a RespiFast RG Panel run is recommended but not mandatory.

Table 6. Positive control

Target	Detection mix	Detection channel	Average T_m
Influenza A	Mix 1	FAM/ROX	74°C
<i>Bordetella pertussis</i>	Mix 1	FAM/Cy5	56.5°C
Parainfluenza virus type 2	Mix 2	FAM/ROX	59.5°C
Coronavirus 229E	Mix 2	FAM/Cy5	60°C

Protocol: PCR Amplification and Melt Analysis

Things to do before starting

- Thaw template RNA/DNA (if frozen) and all reagents, and keep tubes on ice.
- Prepare slightly larger than required amounts of the various mixes so as to compensate for pipetting losses.

Procedure

Pre-amplification

1. Invert the pre-amplification master mix several times to ensure complete thawing and homogenization of the master mix. Centrifuge the tube briefly. Prepare the pre-amplification reaction mix according to Table 7 and keep it on ice.

The reaction mix typically contains all of the components needed for PCR except the sample.

Note: Step 2 mixes can be prepared at the same time as the pre-amplification mix and should be stored in the dark at 4°C until use. Simultaneous preparation of the mixes can only be done when the complete protocol is followed without delay; no pauses are allowed between the two steps. Stability of the mixes is not guaranteed after longer storage (such as overnight).

Table 7. Preparation of the pre-amplification reaction mix

Number of samples (cap color of reagent tube)	1	12
Pre-amplification master mix (purple)	6.25 µl	75 µl
Pre-amplification primer mix (white)	8.75 µl	105 µl
Total volume	15 µl	180 µl

2. Mix the pre-amplification reaction mix gently but thoroughly, and pipet 15 μ l of the mix into each 0.2 ml PCR tube. Then add 10 μ l of the eluted sample DNA/RNA, negative control or positive control (see Table 8).
3. Close the PCR tubes. Mix well. Centrifuge the tubes briefly, and place the tubes back on ice or in a cooling block.

Table 8. Preparation of the pre-amplification reactions

Number of samples	1	12
Pre-amplification reaction mix	15 μ l	15 μ l each
Sample, negative or positive control	10 μ l	10 μ l each
Total volume	25 μl	25 μl each

4. Program the block-based thermal cycler according to Table 9.

Table 9. Thermal cycler program for pre-amplification

Reverse transcription:	10 minutes	50°C
Initial activation step:	2 minutes	95°C
3-step cycling:		
Denaturation	20 seconds	94°C
Annealing	20 seconds	55°C
Extension	35 seconds	72°C
Number of cycles	40	
Hold:	–	20°C

5. Start the pre-amplification program while the PCR tubes are still on ice. Wait until the thermal cycler reaches 50°C. Then place the tubes in the block of the thermal cycler and close the heated lid.

RespiFast RG Panel Step 2

6. Create a temperature profile for the Rotor-Gene Q instrument according to the following steps. An overview of the thermal cycler program is shown in Table 10.

Setting the general assay parameters	Figure 2, Figure 3
Creating new channels	Figure 4 to Figure 7
Editing the gain	Figure 8, Figure 9
Programming the temperature profile	Figure 10 to Figure 15
Programming the melt program	Figure 16, Figure 17
Summary and saving the template	Figure 18, Figure 19
Starting the run	Figure 20

All specifications refer to the Rotor-Gene Q software version 2.3. Please find further information on programming Rotor-Gene instruments in the instrument user manual. In the illustrations these settings are framed in bold black. Illustrations are included for Rotor-Gene Q instruments.

Table 10. Overview of thermal cycler program for Step 2 cycling

			Comments
Initial denaturation step:	2 minutes	95°C	
PCR 1: 3-step cycling:			High-specificity PCR
Denaturation	15 seconds	94°C	
Annealing	15 seconds	55°C	
Extension	15 seconds	72°C	
Number of cycles	10		
PCR 2: 3-step cycling:			
Denaturation	15 seconds	94°C	
Annealing	15 seconds	50°C	Acquisition*
Denaturation	15 seconds	72°C	
Number of cycles	23		
Denaturation:	2 minutes	95°C	
Melt program:	–	40–90°C	Acquisition†

* Acquiring channels: Green, gain 4; Red, gain 1; FAM™/ROX, gain 8; and FAM/Cy5, gain 10.

† Acquiring channels: Green, Red, FAM/ROX, and FAM/Cy5. Use “Optimize gain before melt on all tubes”. “The gain giving the highest fluorescence less than 95 will be selected”.

7. First, open the “New Run Wizard” dialog box (**Figure 2**). Check the “Locking Ring Attached” box and click “Next”.

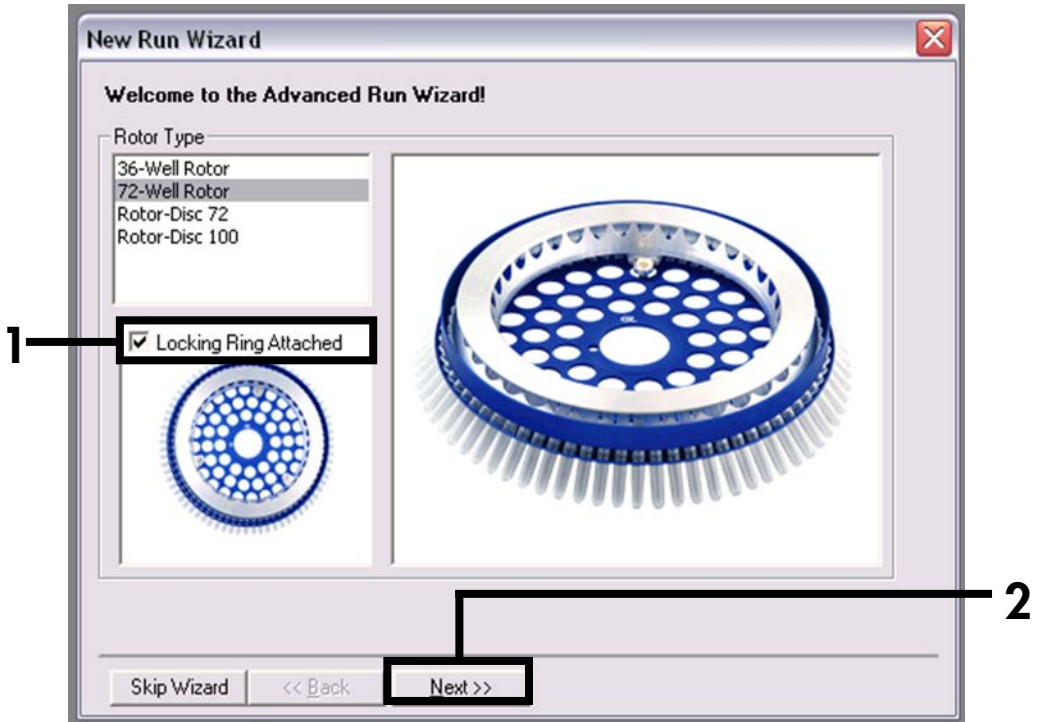


Figure 2. The “New Run Wizard” dialog box.

8. Select 25 for the reaction volume and click “Next” (Figure 3).

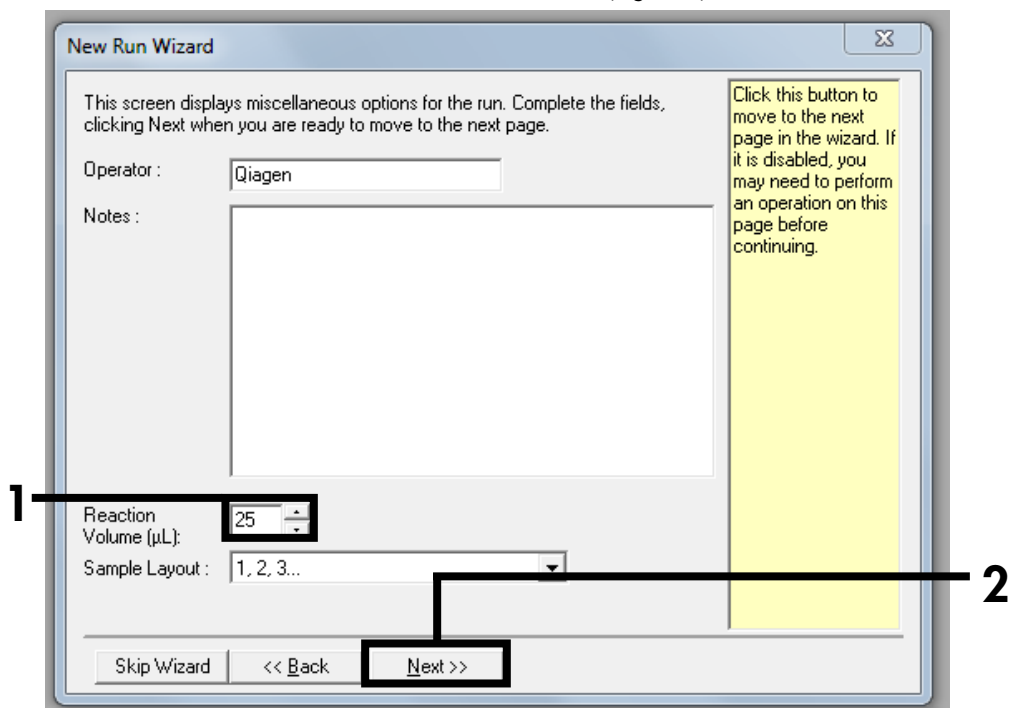


Figure 3. Setting the general assay parameters.

9. Click the “Create New” button in the next “New Run Wizard” dialog box (**Figure 4**), and program 2 additional channels, as shown in **Figure 5** to **Figure 7**.

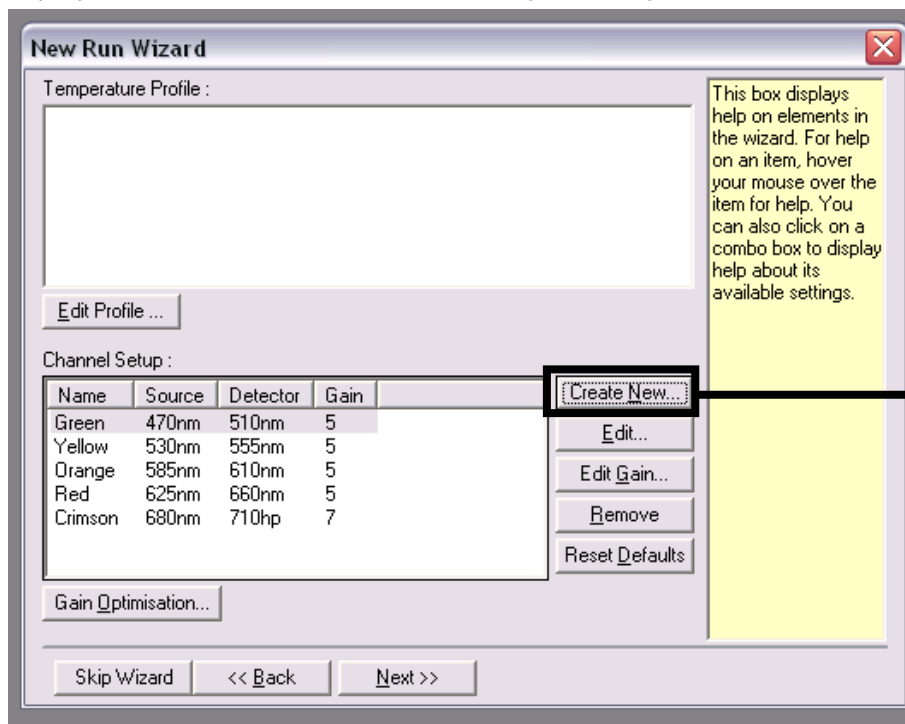


Figure 4. Create new channels.

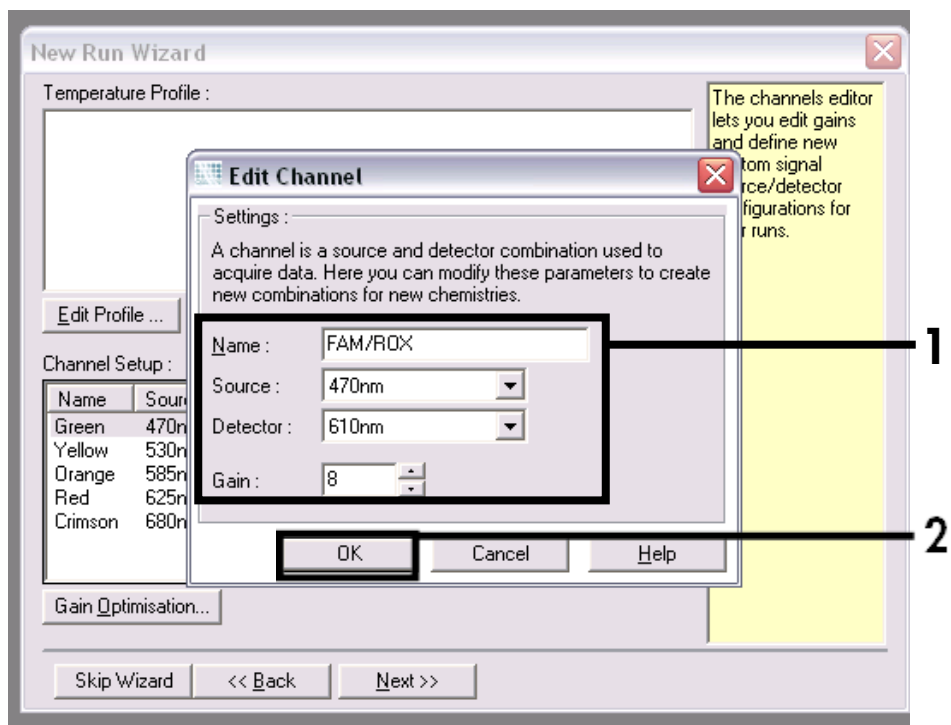


Figure 5. Create new channel "FAM/ROX".

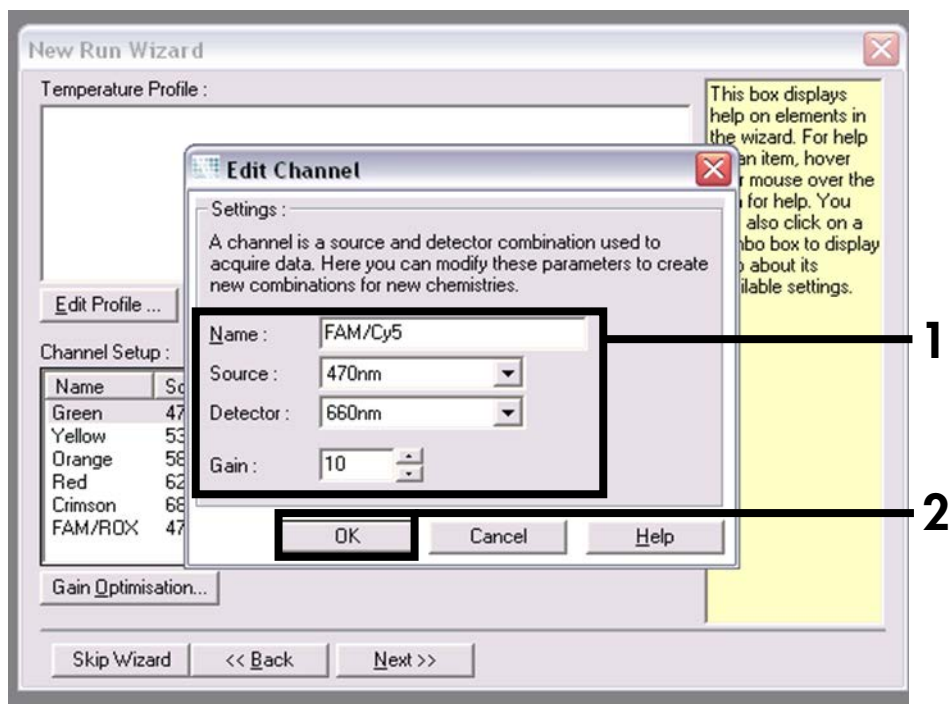


Figure 6. Create new channel "FAM/Cy5".

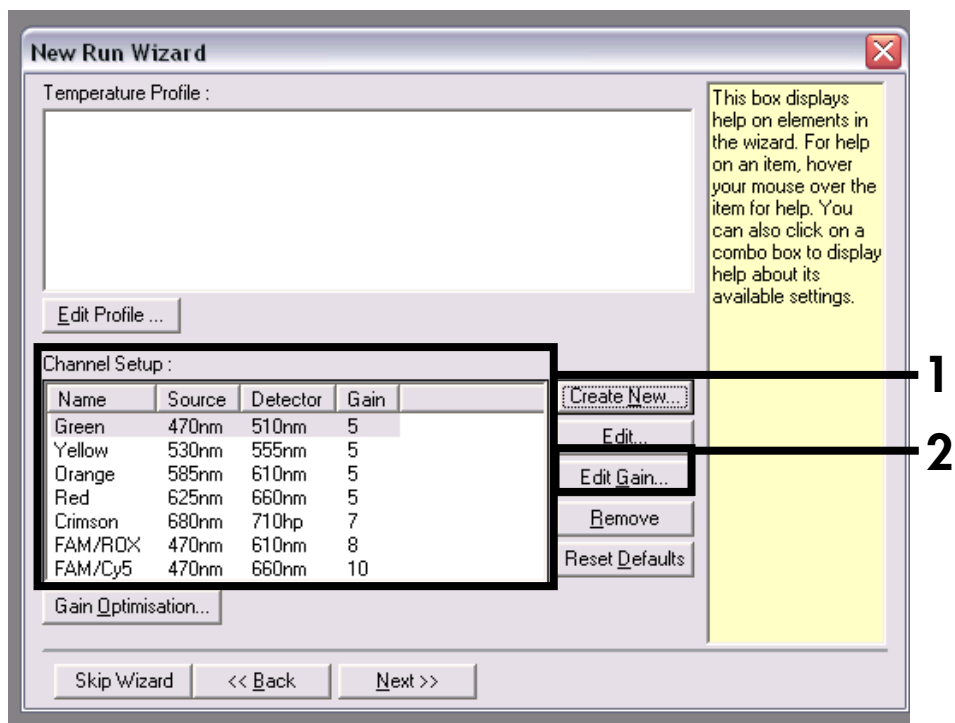


Figure 7. New channels included.

10. Edit the gain for each channel as shown in **Figure 8**. The final gain settings for each channel are indicated in **Figure 9**.

The gain settings for the Rotor-Gene Q instrument are selected to prevent saturation in the detection channels. However, if saturation is observed in any of the detection channels, the gain settings for this saturated channel should be adapted and a new melt curve analysis should be performed.

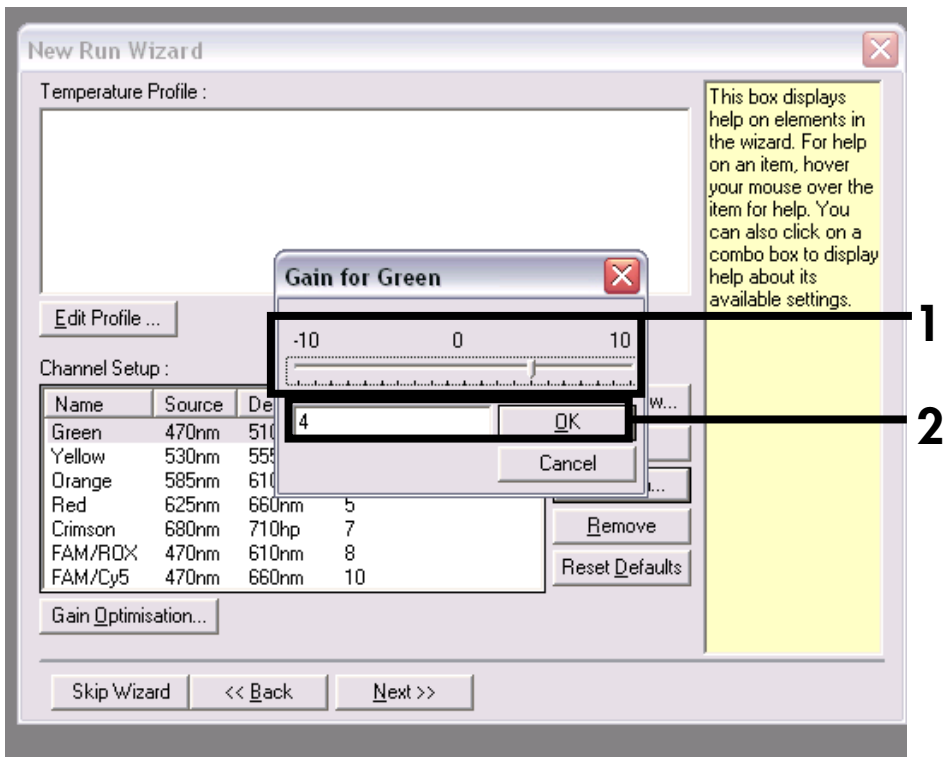


Figure 8. Editing the gain. As an example, the figure shows setting the gain to 4 for the Cycling Green channel.

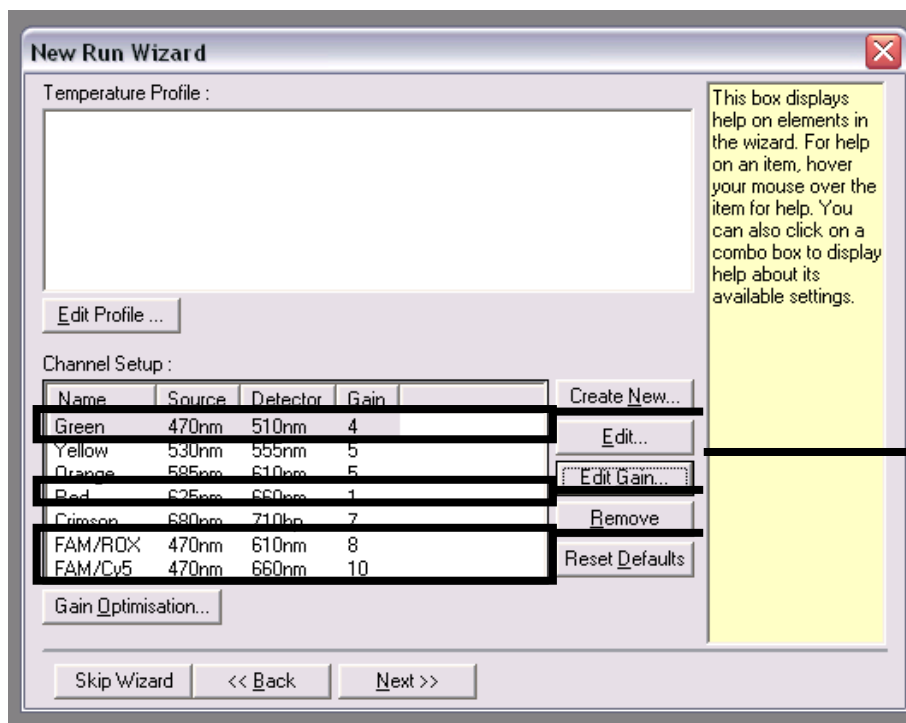


Figure 9. Final gain settings for all channels. Channels required are: Green, gain 4; Red, gain 1; FAM/ROX, gain 8; and FAM/Cy5, gain 10.

11. Click the “Edit Profile” button in the next “New Run Wizard” dialog box (Figure 10), and program the temperature profile as shown in Figure 11 to Figure 16.

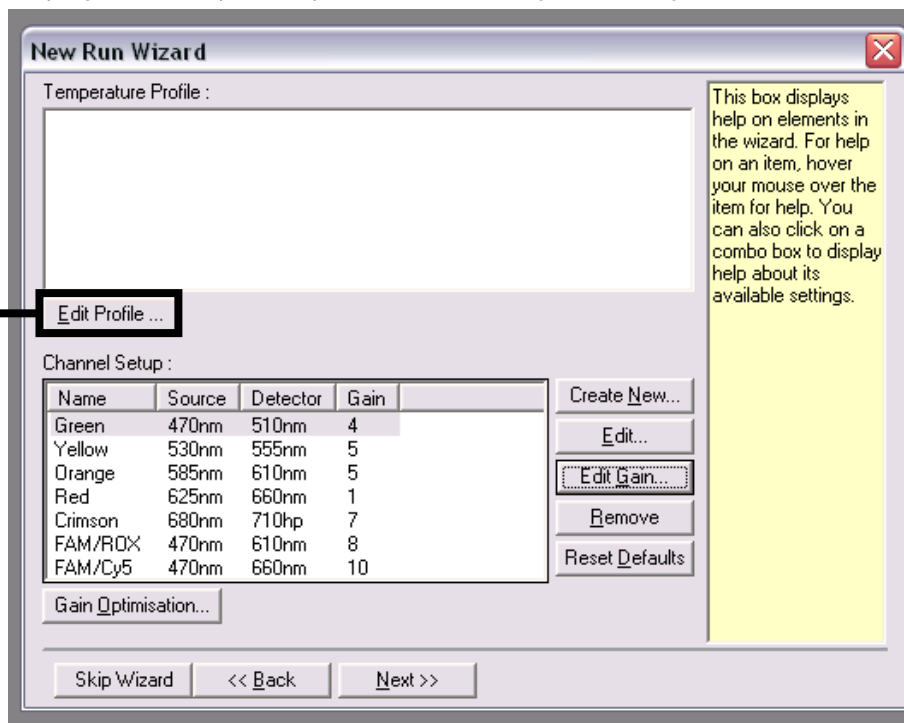


Figure 10. Editing the profile.

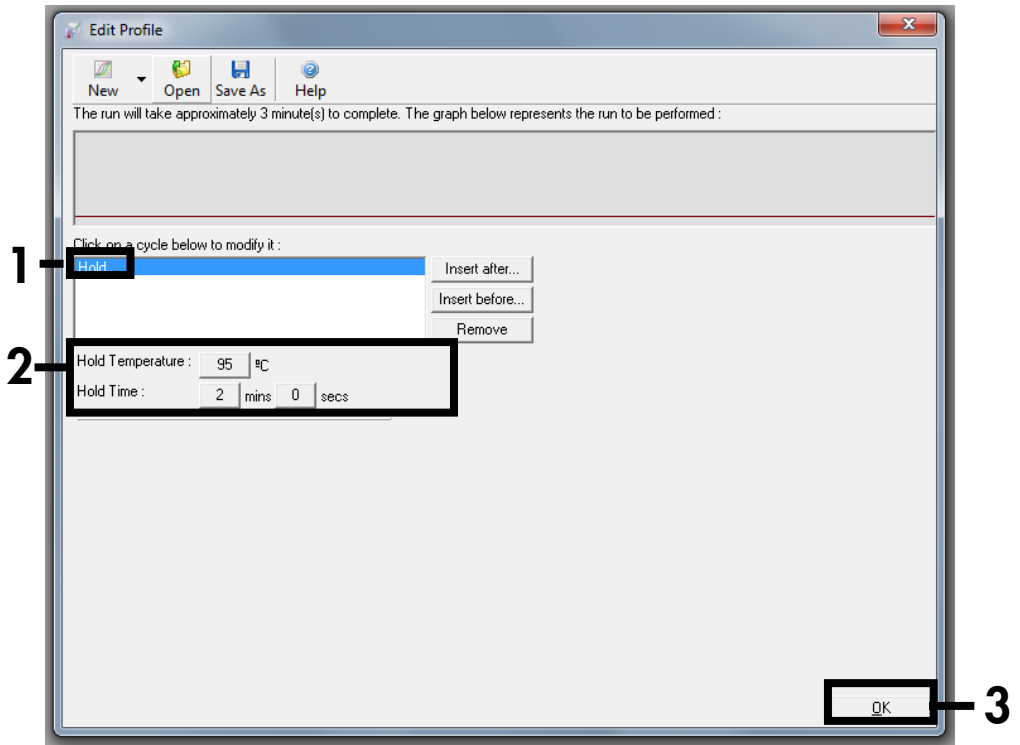


Figure 11. Initial denaturation step.

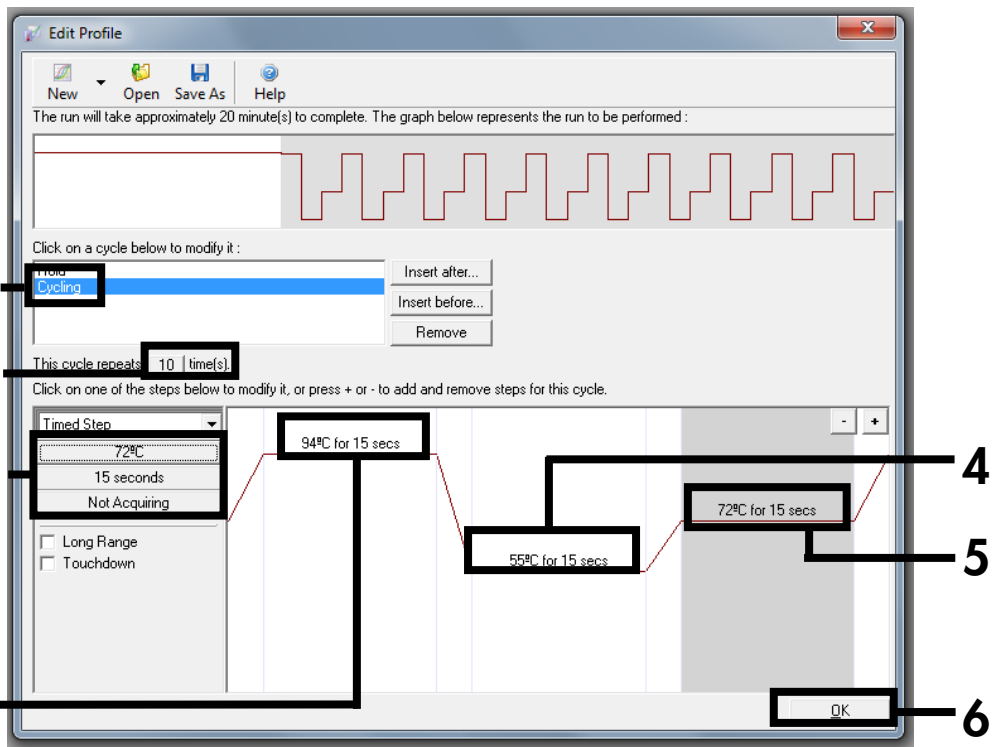


Figure 12. PCR 1.

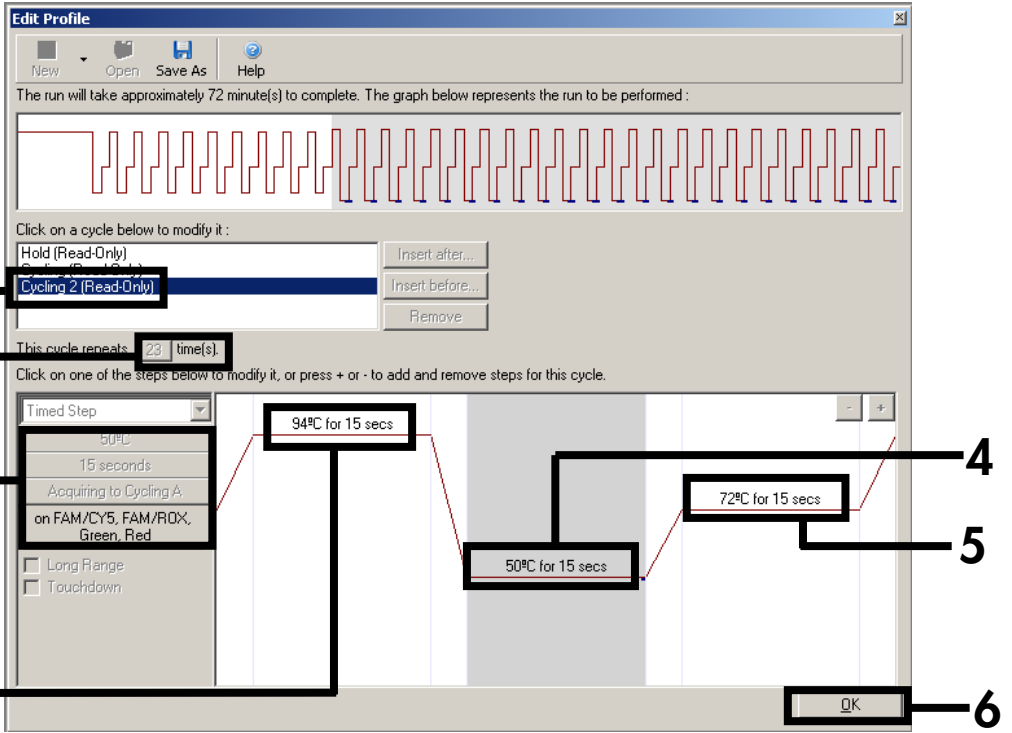


Figure 13. PCR 2 including signal acquisition at 50°C. Channels needed for acquisition are shown in Figure 14.

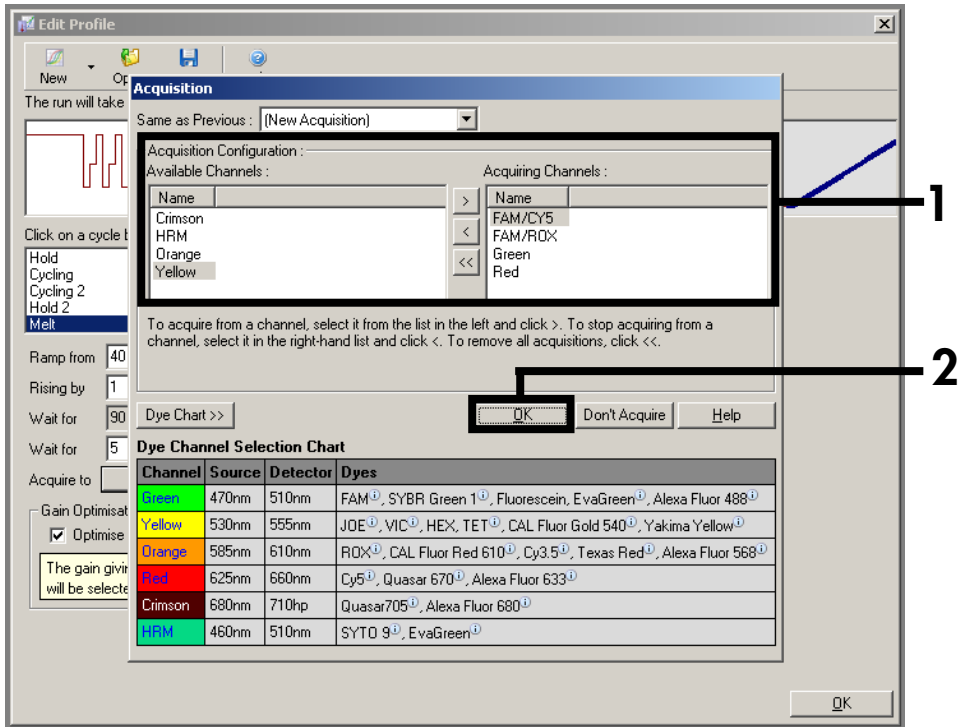


Figure 14. Selection of all channels needed for acquisition.

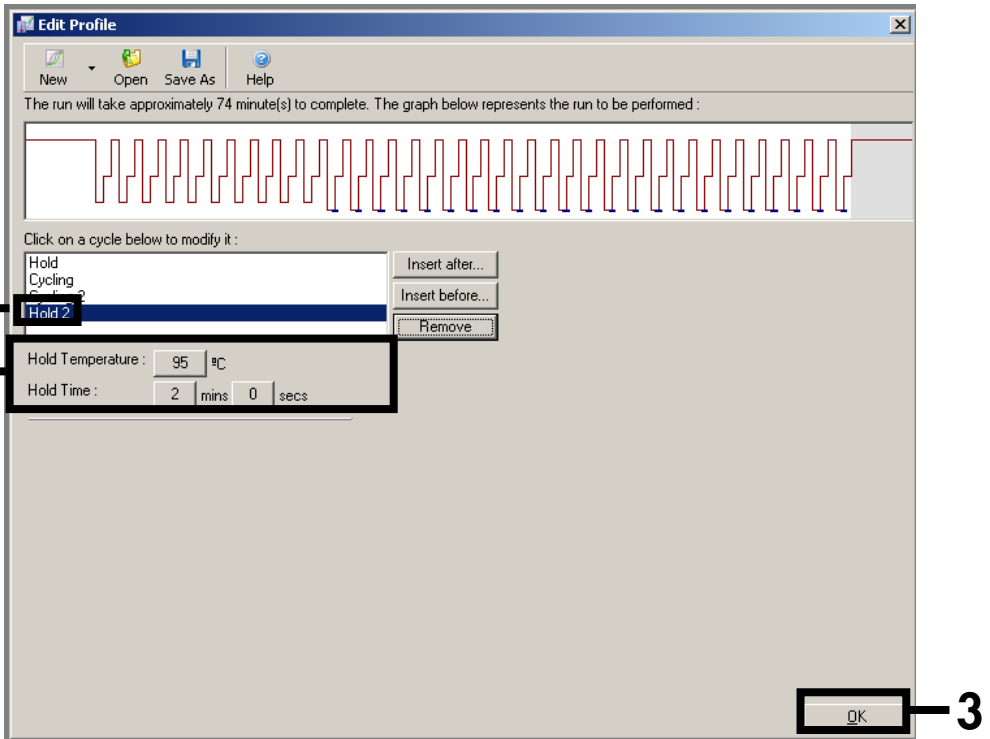


Figure 15. Denaturation of PCR products.

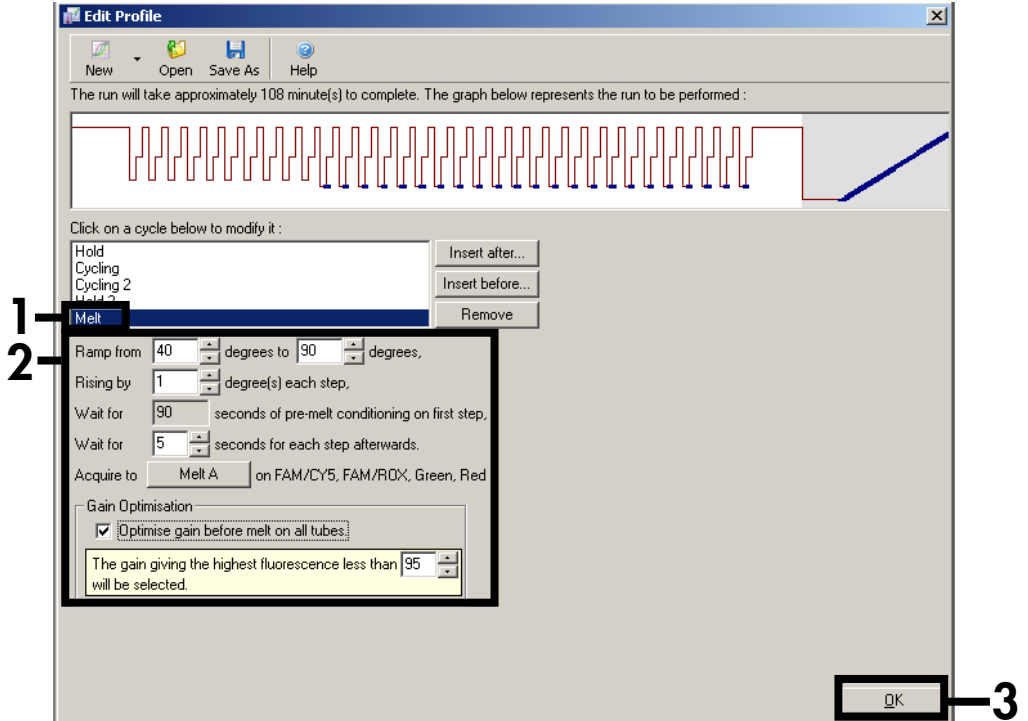


Figure 16. Melt program.

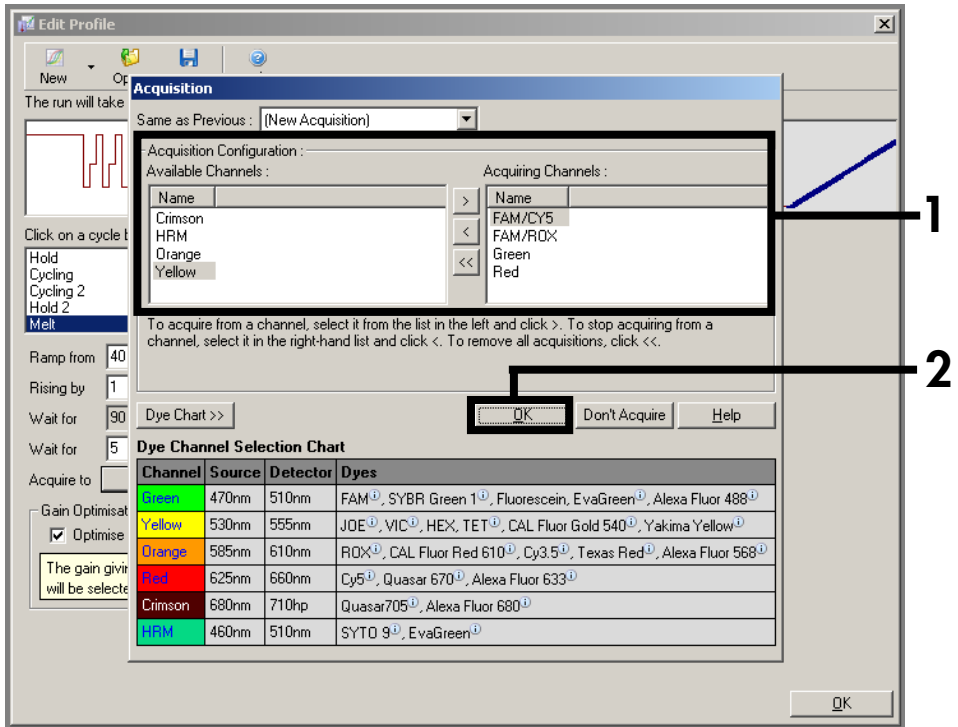


Figure 17. Acquisition during melt program.

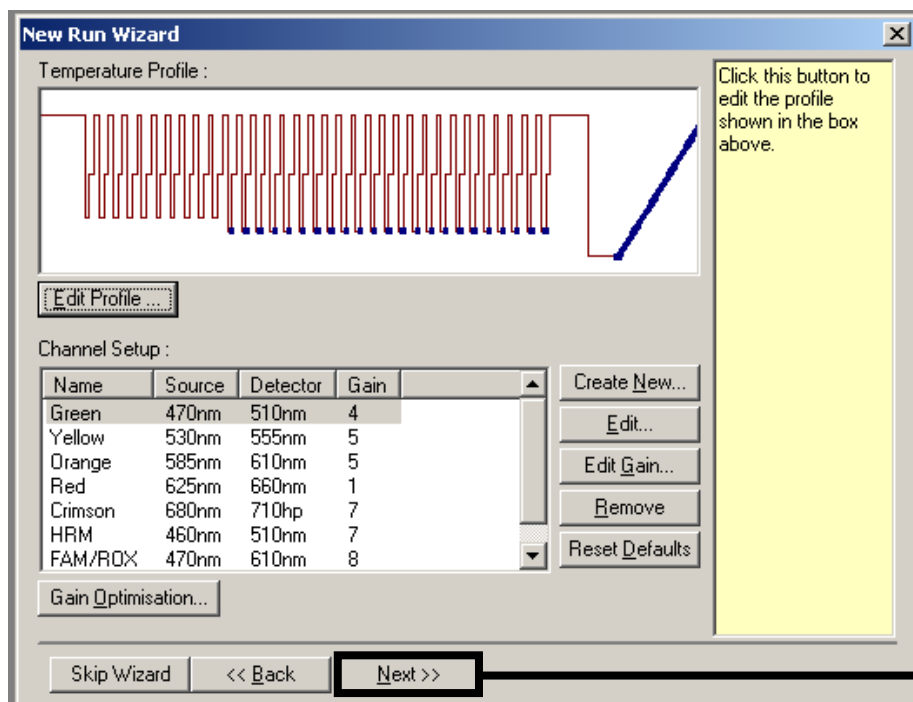


Figure 18. Summary of temperature profile.

12. Save the template for later usage (Figure 19).

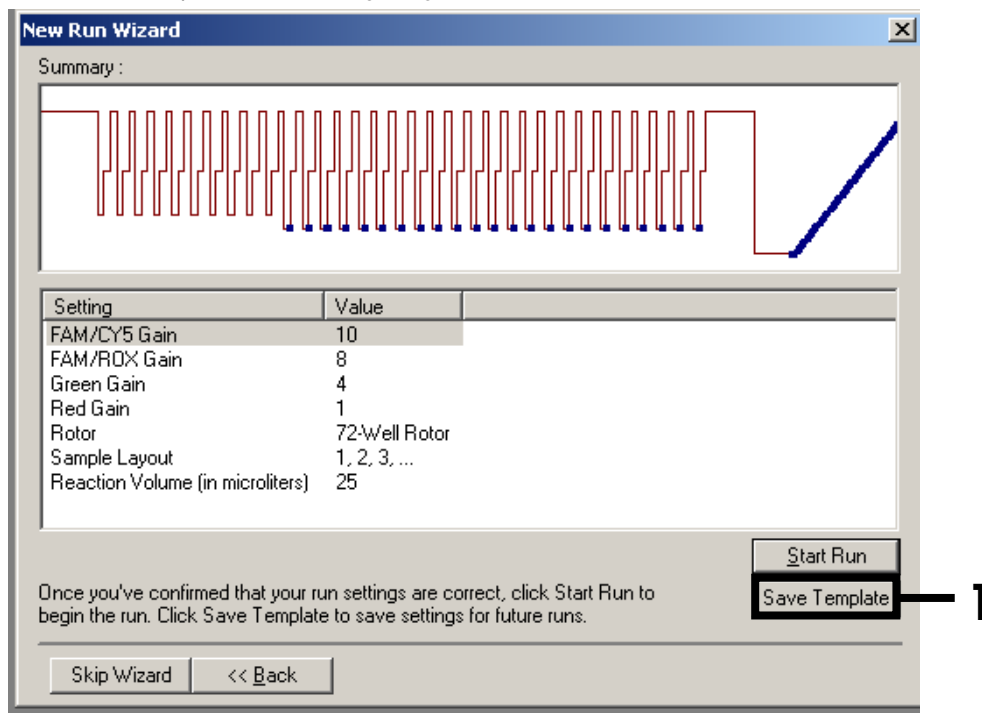


Figure 19. Save template for later usage.

13. Prepare the Step 2 mix 1 according to Table 11 and Step 2 mix 2 according to Table 12, and mix gently but thoroughly.

If Step 2 mixes were already prepared in step 1, wait until the pre-amplification program is finished, and then proceed to step 14.

Table 11. Preparation of Step 2 mix 1

Number of samples (cap color of reagent tube)	1	12
RespiFast buffer 1 (red)	19 μ l	228 μ l
RespiFast enzyme (orange)	1 μ l	12 μ l
Total volume of Step 2 mix 1	20 μl	240 μl

Table 12. Preparation of Step 2 mix 2

Number of samples (cap color of reagent tube)	1	12
RespiFast buffer 2 (blue)	19 μ l	228 μ l
RespiFast enzyme (orange)	1 μ l	12 μ l
Total volume of Step 2 mix 2	20 μl	240 μl

14. For each sample, pipet 20 μ l of Step 2 mix 1 into a PCR strip tube for Rotor-Gene, and pipet 20 μ l of Step 2 mix 2 into a PCR strip tube for Rotor-Gene.

15. After the pre-amplification program finishes (step 5), take the PCR tubes out of the thermal cycler, and centrifuge briefly. Pipet 5 μ l of each pre amplification reaction into each of the 2 PCR strip tubes — one with Step 2 mix 1 and one with Step 2 mix 2 (see Table 13).

Table 13. Preparation of the Step 2 reactions

Number of samples	1	12
Step 2 mix 1	20 μ l	20 μ l each
Pre-amplification reaction	5 μ l	5 μ l each
Total volume	25 μl	25 μl each
Step 2 mix 2	20 μ l	20 μ l each
Pre-amplification reaction	5 μ l	5 μ l each
Total volume	25 μl	25 μl each

16. Close the Strip Tubes with the appropriate caps and place them in the rotor of the Rotor-Gene Q instrument.

- Place the rotor into the Rotor-Gene Q instrument. Make sure that the locking ring (accessory of the Rotor-Gene Q instrument) is placed on top of the rotor to prevent accidental opening of the tubes during the run.
- Start the run (**Figure 20**) using the saved template (step 12).

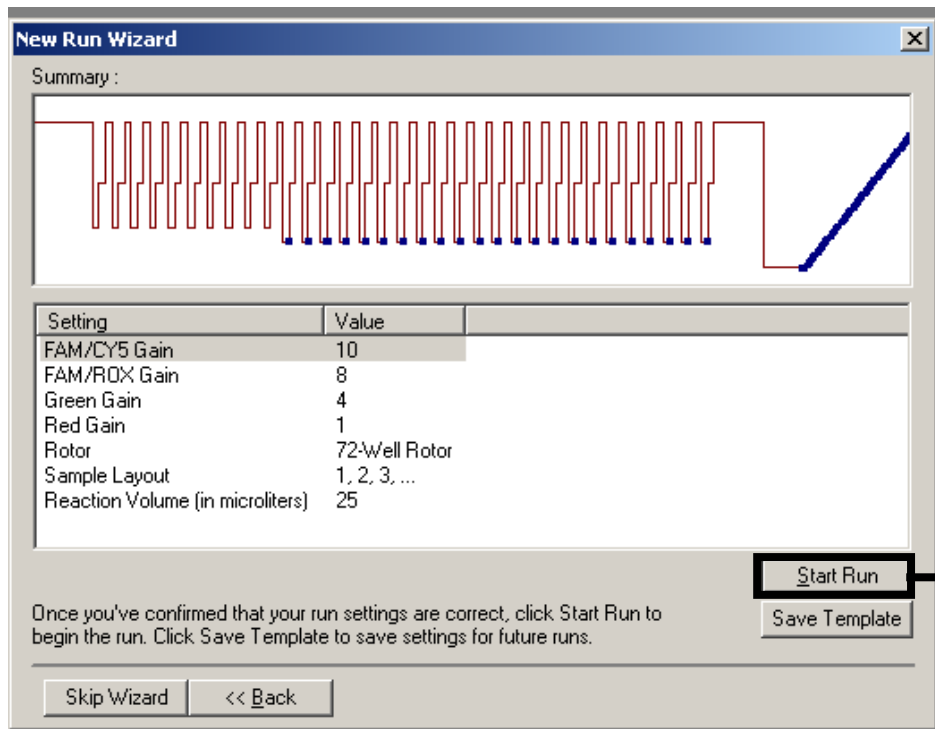


Figure 20. Starting the run.

Interpretation of Results

Normalization

On Rotor-Gene Q instruments, the raw data for the FAM/Cy5 channel must be normalized first to compensate for the crosstalk between the different channels.

For normalization of the Cy5 signals, open the raw channel Melt A.FAM/Cy5 (filter settings 470–660 nm). Click on the “Option” button, and select “Normalise to Melt A.FAM/ROX”. The newly generated raw data file **Melt A.FAM/Cy5/ Melt A.FAM/ROX** has to be normalized additionally for the Red channel (filter settings 625/660 nm). This is done by opening the new raw data file **Melt A.FAM/Cy5/ Melt A.FAM/ROX**, clicking on the “Option” button, and selecting “Normalise to Melt A.Red”. With this normalization, the temperature-dependent fluorescence of Cy5 is corrected, resulting in a more symmetrical melt peak.

Analysis

To analyze the raw data file, open the “Analysis” window and select the appropriate normalized raw data file for FAM/Cy5 under the tab “Melt”. Signals in the Green channel (IC) (filter settings 470–510 nm) and FAM/ROX channel (filter settings 470–610 nm) do not need to be normalized; corresponding raw data files for FAM/ROX and Green channels are selected as well. The different melting temperatures of the SMART probes are indicated in Table 14, page 46.

Threshold

Using the gain optimization in the Rotor-Gene Q software in the melt program of the RespiFast RG Panel Step 2 reaction as described in Table 10 (page 23) and **Figure 16** (page 38), the following threshold values should be set in the melt curve analysis plots to analyze sample results.

- FAM/ROX channel: Threshold = 1
- FAM/Cy5 channel (normalized): Threshold = 0
- Green channel (IC): Flip sign of dF/dT , and select all samples that revealed no positive melting peak in FAM/ROX and FAM/Cy5 channels with both mixes. Set threshold manually at the highest fluorescence level between 40 and 45 °C obtained with these negative samples. Samples which reveal melting peaks with both mixes within the T_m acceptance ranges of the internal control and amplification controls can now be called as true negative samples (see example in **Figure 21**). Samples that reveal only one or no positive internal control result or one or no amplification control result must be called invalid. Invalid samples must be repeated.

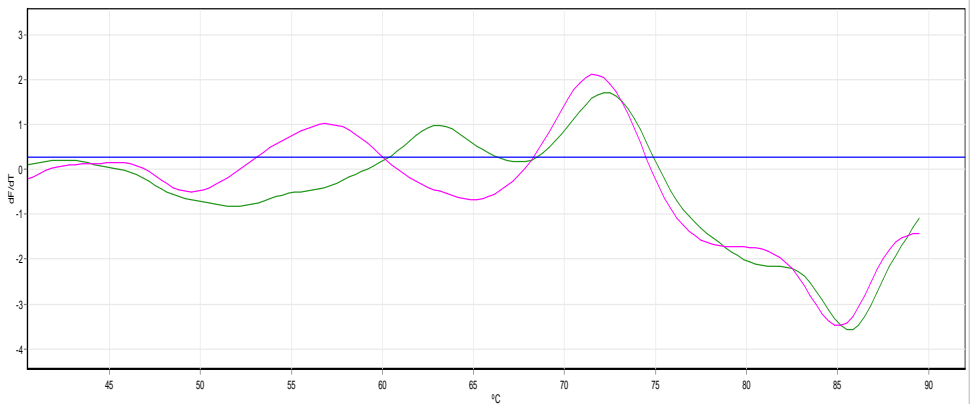


Figure 21. Example of a negative sample detected in the Green channel: In green mix 1 reaction, in pink mix 2 reaction. Threshold is set on highest fluorescence level between 40 and 45 °C.

Table 14. Targets and corresponding T_m values of SMART probes

Label	SMART probe	T_m (°C) acceptance range	Pathogen code (mix 1)	Pathogen code (mix 2)
Cy5	Cy5 probe 1	50.5–53.5*	L. pneu	OC43
	Cy5 probe 2a	55-58	B. pert	–
	Cy5 probe 2b	52.5-55.	–	HKU1/NL63
	Cy5 probe 3	60.5–63.5 58.5-61.5	Rhino/Entero	229E
	Cy5 probe 4	66.5–69.5	C. pneu	–
	Cy5 probe 5	70.5-73.5	M. pneu	–
	Cy5 probe 6	76–79	–	H1N1
ROX	ROX probe 1	53.5–56.5	RSVA	PIV1
	ROX probe 2	58-61	Adeno	PIV2
	ROX probe 3	62.5–65.5	hMPV	PIV3
	ROX probe 4	66.5–69.5	RSVB	PIV4
	ROX probe 5	72.5–75.5	InfA	Boca
	ROX probe 6	76.5–79.5	InfB	–
BHQ1	BHQ1 probe 1	70.5-73.5	IC	IC
	BHQ1 probe AC1	61-64	AC1	–
	BHQ1 probe AC	55.5-58.5	–	AC2

* The Cy5 probe 1 peaks sometimes appears a double peak. The T_m value of the second peak is the correct one.

An overview of melting curve results is given in Table 15.

Table 15. RespiFast RG Panel melting curve results

Sample reaction RespiFast								Sample result
Mix 1				Mix 2				
FAM				FAM				
ROX	Cy5	IC	AC1	ROX	Cy5	IC	AC2	
+	-	+/-	+/-	-	-	+/-	+	Positive ROX 1 target*
-	+	+/-	+/-	-	-	+/-	+	Positive Cy5 1 target*
-	-	+/-	+	+	-	+/-	+/-	Positive ROX 2 target*
-	-	+/-	+	-	+	+/-	+/-	Positive Cy5 2 target*
-	-	+	+	-	-	+	+	Negative
+	+	+/-	+/-	-	-	+/-	+	Positive ROX1 and Cy5 1 targets*
-	-	+/-	+	+	+	+/-	+/-	Positive ROX 2 and Cy5 2 targets*
+	-	+/-	+/-	+	-	+/-	+/-	Positive ROX1 and ROX 2 targets*†
-	+	+/-	+/-	-	+	+/-	+/-	Positive Cy5 1 and Cy5 2 targets*†
+	-	+/-	+/-	-	+	+/-	+/-	Positive ROX1 and Cy5 2 targets*†
-	+	+/-	+/-	+	-	+/-	+/-	Positive Cy5 1 and ROX 2 targets*†
+	-	+/-	+/-	-	-	-	-	Invalid due to no Mix 2 reaction
-	+	+/-	+/-	-	-	-	-	Invalid due to no Mix 2 reaction
-	-	-	-	+	-	+/-	+/-	Invalid due to no Mix 1 reaction
-	-	-	-	-	+	+/-	+/-	Invalid due to no Mix 1 reaction
-	-	-	-	-	-	-	-	Invalid
-	-	-	+	-	-	-	+	Invalid incorrect extraction/no sample added in 2SMART reaction

* The corresponding pathogen(s) can be found in Table 14.

† Any combination of these results is possible, resulting in a positive result for multiple targets detected with Mix 1 and Mix 2 in both ROX and Cy5 detection channels.

Note: a RespiFast RG Panel mix reaction is validated by the presence of either:

- Positive pathogen melting peak
- Positive AC melting peak and a positive IC peak
- Positive AC melting peak (IC is out-competed due to strong pathogen infection detected with the corresponding RespiFast step 2 mix).

Invalid reactions must be repeated. When only one of the two RespiFast Step 2 reactions of a sample reveals an invalid result, the invalid RespiFast RG Panel reaction must be repeated, starting with the pre-amplification reaction (using available nucleic acid extract).

When a sample reveals an invalid sample result due to absence of pathogen, IC and AC or due to absence of pathogen and IC the RespiFast RG Panel reaction must be repeated starting from nucleic acid extraction of the original sample.

Troubleshooting Guide

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

Comments and suggestions

No specific melting peaks visible and IC melting peak not visible

- | | |
|--------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| a) Programming error(s) in thermal cycling program | Program the thermal cycler according to "Protocol: PCR Amplification and Melt Analysis", page 20, and repeat the assay. |
| b) Pipeting error or missing reagent | Repeat the RespiFast assay, following "Protocol: PCR Amplification and Melt Analysis", page 20. |
| c) IC not added or IC added before lysis buffer is added to sample | It is important to add the IC and carrier RNA to the lysis buffer, preferably after it is added to the sample, regardless of the extraction protocol used (see "Using an internal control and carrier RNA", page 15). Otherwise the IC could degrade, due to nucleases in the sample. Repeat the nucleic acid extraction and the RespiFast assay. |
| d) PCR inhibitors present in the sample | Repeat the assay using a fivefold dilution of the isolated RNA/DNA. |

No IC melting peak visible in the presence of pathogen-specific melting peaks

- | | |
|--------------------------------------------|-----------------------------------------------------------------------|
| Strong infection and/or multiple infection | The IC has been out competed in the assay. The result is still valid. |
|--------------------------------------------|-----------------------------------------------------------------------|

Spurious melting peaks are visible

- | | |
|---------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cross-contamination | Make sure the reaction steps are performed in separate rooms to prevent cross-contamination.
Repeat the RespiFast assay.
Check the cycling program and make sure that all handling steps are performed as described in "Protocol: PCR Amplification and Melt Analysis", page 20. Make sure to work on ice where indicated in the protocol.
Repeat the RespiFast assay. |
|---------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

Quality Control

The RespiFast RG Panel is manufactured by PathoFinder BV in Maastricht, The Netherlands, within quality systems accredited to EN ISO 13485:2012.

Limitations

The RespiFast RG Panel aids in the diagnosis of respiratory tract infection when used in conjunction with other clinical and laboratory findings. Negative results do not necessarily indicate absence of viral or bacterial respiratory tract infection. Negative results should not be used as the sole basis for diagnosis, therapy, or other treatment decisions. Positive results do not exclude co-infection with other pathogens. The pathogen(s) detected may not be the definite cause of disease.

Other laboratory testing and assessment of clinical presentation must be included in the final diagnosis.













The product is for use by laboratory professionals only.

Performance Characteristics

See www.qiagen.com/p/RespiFast-RG-Panel-CE for performance characteristics of the RespiFast RG Panel.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Consult instructions for use
	Caution
	Keep away from sunlight

Contact Information

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Ordering Information

Product	Contents	Cat. no.
RespiFast RG Panel	CE IVD, For 25 reactions: Pre-amplification Master Mix, Internal Control, Pre-amplification Primermix, RespiFast buffers, RespiFast enzyme, Positive control	4693163
QIAamp MinElute Virus Spin Kit — for simultaneous purification of viral DNA and RNA from nasopharyngeal swabs		
QIAamp MinElute Virus Spin Kit (50)	For 50 minipreps: 50 QIAamp MinElute Columns, QIAGEN Protease, carrier RNA, Buffers, Collection Tubes (2 ml)	57704
QIAasymphony DSP Virus/Pathogen Mini Kit — for simultaneous automated purification of viral DNA and RNA from nasopharyngeal swabs for use in combination with the QIAasymphony SP instrument		
QIAasymphony Virus/Pathogen Mini Kit	DSP For 192 preps (200 µl each): includes 2 reagent cartridges and enzyme racks and accessories	937036
QIAasymphony SP		
QIAasymphony sample prep module, 1-year warranty on parts and labor		9001297

Rotor-Gene Q MDx — for IVD-validated real-time PCR analysis in clinical applications

Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9002032
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Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9002033
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Rotor-Gene Q — for outstanding performance in real-time PCR

Rotor-Gene Q 5plex HRM Platform	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9001580
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Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9001650
Rotor-Gene Q accessories		
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions of 10–50 µl	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions of 10–50 µl	981106

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