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# KRAS RGQ PCR Kit

## Instructions for Use (Handbook)

For qualitative measurement of 7 somatic mutations in the KRAS oncogene. For use with the Rotor-Gene® Q 5plex HRM Instrument.

For research use only. Not for use in diagnostic procedures. Version 4



**REF**

870001



QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden,  
GERMANY

**R4**

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## Intended Use

The KRAS RGQ PCR Kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

The KRAS RGQ PCR Kit is intended for the detection of seven somatic mutations in the KRAS oncogene and will provide a qualitative assessment of mutation status. The KRAS RGQ PCR Kit is to be used with DNA samples extracted from formalin-fixed paraffin-embedded (FFPE) colorectal tissue and non-small cell lung cancer tissue.

## Summary and Explanation

The KRAS RGQ PCR Kit is a ready-to-use kit for the detection of seven somatic mutations in the KRAS oncogene using real-time polymerase chain reaction (PCR) on the Rotor-Gene Q instrument.

Using Scorpions® and ARMS® technologies, the KRAS RGQ PCR Kit enables detection of mutations in codons 12 and 13 of the KRAS oncogene against a background of wild-type genomic DNA (Table 1).

**Table 1. List of mutations and COSMIC IDs**

<b>Mutation</b>	<b>Base change</b>	<b>COSMIC ID</b>
GLY12ALA (G12A)	GGT>GCT	522
GLY12ASP (G12D)	GGT>GAT	521
GLY12ARG (G12R)	GGT>CGT	518
GLY12CYS (G12C)	GGT>TGT	516
GLY12SER (G12S)	GGT>AGT	517
GLY12VAL (G12V)	GGT>GTT	520
GLY13ASP (G13D)	GGC>GAC	532

COSMIC IDs are taken from the *Catalogue of Somatic Mutations in Cancer* ([www.sanger.ac.uk/genetics/CGP/cosmic](http://www.sanger.ac.uk/genetics/CGP/cosmic)).

The methods used are highly selective and, depending on the total amount of DNA present, enable detection of a low percentage of mutants in a background of wild-type genomic DNA. These selectivity and detection limits are superior to technologies such as dye terminator sequencing.

## Principle of the Procedure

The KRAS RGQ PCR Kit utilizes two technologies — ARMS and Scorpions — for detection of mutations in real-time PCR.

### Mutation reaction mixes

Each reaction mix uses a mutation-specific ARMS primer to selectively amplify the mutated DNA and then a Scorpions primer to detect the amplification product.

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## ARMS

Allele-specific amplification is achieved by ARMS, which exploits the ability of *Taq* DNA polymerase to distinguish between a matched and a mismatched base at the 3' end of a PCR primer. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low-level background amplification may occur. Therefore, a mutated sequence is selectively amplified even in samples where the majority of the DNA does not carry the mutation.

## Scorpions

Detection of amplification is performed using Scorpions. Scorpions are bifunctional molecules containing a PCR primer covalently linked to a probe. The fluorophore in this probe interacts with a quencher also incorporated into the probe, which reduces fluorescence. When the probe binds to the amplicon during PCR, the fluorophore and quencher become separated. This leads to an increase in fluorescence from the reaction tube.

## Kit format

The KRAS RGQ PCR Kit contains eight assays:

- One control assay (Control Reaction Mix; CTRL)
- Seven mutation assays (mutant reaction mixes; 12ALA, 12ASP, 12ARG, 12CYS, 12SER, 12VAL, and 13ASP)

All reaction mixes are duplex and contain reagents to detect targets, which are labeled with FAM™, and internal control, which is labeled with HEX™. The internal control assay controls for the presence of inhibitors that may lead to false negative results.

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## Procedure

### **Assays**

The KRAS RGQ PCR Kit comprises a two-step procedure. In the first step, the control assay is performed to assess the total amplifiable DNA in a sample. In the second step, both the mutation and control assays are performed to determine the presence or absence of mutated DNA.

#### **Control reaction**

The Control Reaction Mix (CTRL) uses a Scorpions primer and an unlabeled primer to amplify a short sequence of exon 4 of the KRAS gene. The control reaction is used to determine if an appropriate level of amplifiable DNA is present in the sample and is a factor used in the analytical calculations that are used to determine mutation status.

#### **Control assay**

The control assay, labeled with FAM, is used to assess the total amplifiable KRAS DNA in a sample. The control assay amplifies a region of exon 4 of the KRAS gene. The primer and Scorpion probe have been designed to amplify independently of any known KRAS polymorphisms.

#### **Mutation assays**

Each mutation assay contains a FAM-labeled Scorpion probe and an ARMS primer, for discrimination between wild-type DNA and a specific mutant DNA.

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## Controls

**Note:** All experimental runs must contain positive and negative controls.

### Positive control

Each run must contain a positive control in tubes 1–8. The KRAS RGQ PCR Kit contains KRAS Positive Control (PC) to be used as the template in the positive control reaction. The positive control results will be assessed to ensure that the kit performs within the stated acceptance criteria.

### Negative control

Each run must contain a negative control (“No Template Control”) in tubes 9–16. The KRAS RGQ PCR Kit contains Water for NTC (NTC) to be used as the “template” for the No Template Control. The No Template Control is used to assess any potential contamination during the run setup and to assess the performance of the internal control reaction.

### Internal control

Each reaction mix contains an internal control in addition to the target reaction. A failure indicates that either there may be inhibitors present that could lead to an inaccurate result or an operator setup error has occurred for that tube. If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of the inhibitors. However, it should be noted that this also dilutes the target DNA. A tube of water for sample dilution (Dil.) is provided with the kit. Sample dilution must be performed using the water for sample dilution (Dil.).

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## Sample assessment

The Control Reaction Mix (CTRL) is supplied with the KRAS RGQ PCR Kit to assess the total amplifiable DNA in a sample. The control assay amplifies a region of exon 4 of the KRAS gene. We strongly recommend setting samples up with only the control assay using the KRAS Positive Control (PC) as a positive control and Water for NTC (NTC) as the No Template Control.

To obtain optimal use of the reagents in the KRAS RGQ PCR Kit, samples should be batched. If samples are tested individually, this will use more reagents and reduce the number of samples that can be tested with the KRAS RGQ PCR Kit.

# Materials Provided

## Kit contents

<b>KRAS RGQ PCR Kit</b>				<b>(24)</b>
<b>Catalog number</b>			<b>870001</b>	
<b>Number of preps</b>			<b>24</b>	
<b>Color</b>	<b>Identity</b>	<b>Tube ID</b>	<b>Volume</b>	
Red	Control Reaction Mix	1 CTRL	2 x 600 µl	
Purple	12ALA Reaction Mix	2 12ALA	600 µl	
Orange	12ASP Reaction Mix	3 12ASP	600 µl	
Pink	12ARG Reaction Mix	4 12ARG	600 µl	
Green	12CYS Reaction Mix	5 12CYS	600 µl	
Yellow	12SER Reaction Mix	6 12SER	600 µl	
Gray	12VAL Reaction Mix	7 12VAL	600 µl	
Blue	13ASP Reaction Mix	8 13ASP	600 µl	
Beige	KRAS Positive Control	9 PC	250 µl	
Mint	<i>Taq</i> DNA Polymerase	<i>Taq</i>	80 µl	
White	Water for NTC	NTC	1.9 ml	
White	Water for Sample Dilution	Dil.	1.9 ml	

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# 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

- QIAamp® DNA FFPE Tissue Kit (QIAGEN, cat. no. 56404; see “DNA extraction,” page 16)
- Xylene
- Ethanol (96–100%)\*

## Consumables

- Sterile pipet tips with filters (to avoid cross-contamination, we recommend pipet tips with aerosol barriers)
- Sterile microcentrifuge tubes for preparing master mixes
- 0.1 ml Strip Tubes and Caps, for use with 72-well rotor (QIAGEN, cat. no. 981103 or 981106)

## Equipment

- Rotor-Gene Q with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and HEX, respectively)
- Rotor-Gene Q software version 2.3
- Thermomixer†, heated orbital incubator, heating block or water bath capable of incubation at 56°C and 90°C

\* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

† Ensure that instruments have been checked and calibrated according to the manufacturer’s recommendations.

- Benchtop centrifuge\* with rotor for 1.5 ml tube
- Benchtop vortex\*
- Dedicated pipets (adjustable) for sample preparation\*
- Dedicated pipets (adjustable) for PCR master mix preparation\*
- Dedicated pipets (adjustable) for dispensing of template DNA\*

## Warnings and Precautions

### Safety information

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

### General precautions

The user should always pay attention to the following.

- 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.
- Use extreme caution to prevent contamination of PCRs with synthetic control material. We recommend using separate, dedicated pipets for setting up reaction mixes and adding DNA template. Preparation and dispensing of reaction mixes must be carried out in a separate area to the addition of template. Rotor-Gene Q tubes must not be opened after the PCR run has finished. This is to prevent laboratory contamination with PCR products.

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

- Reagents for the KRAS RGQ PCR Kit have been optimally diluted. We do not recommend further dilution of reagents as this may result in a loss of performance. We do not recommend using reaction volumes of less than 25 µl since this will increase the risk of false negatives.
- All reagents in the KRAS RGQ PCR Kit are formulated specifically for optimal performance. All reagents supplied in the KRAS RGQ PCR Kit are intended to be used solely with the other reagents in the same KRAS RGQ PCR kit. Substitutions to the reagents in the kit must not be made if optimal performance is to be maintained.
- Only use *Taq* DNA polymerase (*Taq*) provided in the kit. Do not substitute with *Taq* DNA polymerase from other kits of the same or any other type, or with *Taq* DNA polymerase from another supplier.

## Reagent Storage and Handling

The KRAS RGQ PCR Kit is shipped on dry ice and must still be frozen on arrival. If any component of the KRAS RGQ PCR Kit is not frozen on arrival; the outer packaging has been opened during transit; or the shipment does not contain a packing note, handbook, or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit [www.qiagen.com](http://www.qiagen.com)).

The KRAS RGQ PCR Kit should be stored immediately upon receipt at  $-30$  to  $-15^{\circ}\text{C}$  in a constant-temperature freezer and protected from light. As with all fluorescently labeled molecules, Scorpions must be protected from light to avoid photo bleaching and loss of performance.

When stored under the recommended storage conditions in the original packaging, the kit is stable until expiration date. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 6 freeze-thaw cycles.

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# Specimen Collection, Preparation for Analysis and Storage

**Note:** All samples must be treated as potentially infectious material.

Sample material must be human genomic DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue. Specimens must be transported according to standard pathology methodology to ensure specimen quality.

Tumor samples are heterogeneous and data from a sample of tumor may not be concordant with other sections from the same tumor. Tumor samples may also contain non-tumor tissue. DNA from non-tumor tissue would not be expected to contain mutations detected by the KRAS RGQ PCR Kit.

## Preparation of tissue samples

**Note:** Use dry scalpels. Do not perform this step in a laminar flow or fume hood.

- Scrape the tumor tissue from the sections into labeled microcentrifuge tubes using a fresh scalpel for each sample.
- Using standard materials and methods, fix the tissue specimen in 10% neutral buffered formalin (NBF), and embed the tissue specimen in paraffin. Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides.
- A trained individual (e.g., a pathologist) must assess a Hematoxylin & Eosin (H&E)-stained section for tumor content and area determination. Mark the stained slide to distinguish tumor from normal tissue. Use serial sections for DNA extraction.
- Use sections with >20% tumor content by area for processing without macrodissection.
- For sections that are <20% tumor content by area, macrodissect one or more sections. Discard the non-tumor tissue.

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- For sections that are  $<4 \text{ mm}^2$  in area, process two or more sections to increase the total tumor area to at least  $4 \text{ mm}^2$  (applies to samples both with and without macrodissection). Discard the non-tumor tissue.
  - Scrape excess paraffin away from the tissue using a fresh, sterile scalpel.

## Storage

Store FFPE blocks and slides at room temperature. Slides may be stored at ambient temperature for up to 4 weeks prior to DNA extraction.

Genomic DNA may be stored at  $2\text{--}8^\circ\text{C}$  for 1 week post extraction, then at  $-15$  to  $-25^\circ\text{C}$  for up to 8 weeks before use.

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# Procedure

## DNA extraction

The performance characteristics for the KRAS RGQ PCR Kit were generated using DNA extracted with the QIAamp DNA FFPE Tissue Kit (cat. no. 56404). If using the QIAamp DNA FFPE Tissue Kit, carry out the DNA extraction according to instructions in the handbook, noting the following.

### DNA extraction (CRC samples)

- The QIAamp DNA FFPE Tissue Kit must be used manually only.
- Do not use the RNase step described in the *QIAamp DNA FFPE Tissue Kit Handbook*.
- Do not use QIAGEN deparaffinization solution. Use only the xylene/ethanol method for deparaffinization, as described in the *QIAamp DNA FFPE Tissue Handbook*.
- Proteinase K digestion (step 11 in the *QIAamp DNA FFPE Tissue Kit Handbook*) must be performed for 1 hour.
- The samples must be eluted using 200 µl elution buffer (Buffer ATE) from the QIAamp DNA FFPE Tissue Kit.

### DNA extraction (NSCLC samples)

- Use 2 x 5 µm sections per extraction.
- The QIAamp DNA FFPE Tissue Kit must be used manually only.
- Do not use the RNase step in the *QIAamp DNA FFPE Tissue Kit Handbook*.
- Do not use the QIAGEN deparaffinization solution. Use only the xylene/ethanol method for deparaffinization described in the *QIAamp DNA FFPE Tissue Kit Handbook*.
- Proteinase K digestion (step 11 in the *QIAamp DNA FFPE Tissue Kit Handbook*) must be performed for 1 hour.

- Add 60 µl elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit and incubate for 2.5 minutes at room temperature.
- Centrifuge at full speed for 1 minute.
- Add a further 60 µl elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit and incubate for 2.5 minutes at room temperature.
- Centrifuge at full speed for 1 minute.

## Protocol: Sample assessment

This protocol is used to assess the total DNA in samples.

### Important points before starting

- Up to 24 samples can be assessed using the Control Reaction Mix available.
- Use the Control Reaction Mix (tube CTRL) to assess the DNA prior to testing.  
**Note:** It is important to use the Control Reaction Mix (CTRL) as described below for this assessment and not spectrophotometry or other alternative methods. Heavily degraded DNA may not amplify even though the primers generate short DNA fragments.
- For efficient use of the reagents in the KRAS RGQ PCR Kit, batch DNA samples as far as possible to create full runs. Testing samples individually or in smaller numbers uses up more reagents and reduces the overall number of samples that can be tested with a single KRAS RGQ PCR Kit.

### Procedure

1. Completely thaw the Control Reaction Mix (tube CTRL), nuclease-free water for no template control (tube NTC) and KRAS Positive Control (tube PC) at room temperature (15–25°C) for a minimum of 1 hour.

**Note:** Bring *Taq* DNA polymerase (*Taq*) to room temperature (15–30°C) at the same time as the other reagents (see Reagent Storage and Handling page 13). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

The times for thawing reagents, PCR setup, and storage before starting the run are indicated in Table 2.

**Note:** Perform PCR setup at room temperature.

**Table 2. Thawing time, PCR setup times, and storage temperatures**

Thaw time			
Minimum	Maximum	Storage* temperature after PCR setup	Maximum PCR setup and storage time
1 hour	4.5 hours	Room temperature (15–25°C)	7 hours
1 hour	4.5 hours	2–8°C	18 hours

\* "Storage" refers to the time between completion of PCR setup and start of the PCR run on the Rotor-Gene Q instrument.

- Mix the thawed reagents by inverting each tube 10 times to avoid localized concentrations of salts, then centrifuge briefly to collect the contents at the bottom of the tube.

**Note:** Do not vortex the *Taq* DNA polymerase (*Taq*) or any mix containing *Taq*, as this may inactivate the enzyme.

- Prepare sufficient master mixes (Control Reaction Mix [CTRL] plus *Taq* DNA polymerase [*Taq*]) according to the volumes in Table 3 for the following:
  - All the DNA samples
  - 1 KRAS Positive Control (PC) reaction
  - 1 nuclease-free water for No Template Control (NTC) reaction
  - 1 extra sample to allow sufficient coverage for the PCR setup

The master mix contains all of the components needed for the PCR, except the sample.

**Table 3. Preparation of control assay master mix**

Component of control assay master mix	Volume required for master mix
Control Reaction Mix (tube CTRL)	19.76 $\mu\text{l}$ x (n + 1)*
<i>Taq</i> DNA Polymerase (tube <i>Taq</i> )	0.24 $\mu\text{l}$ x (n + 1)
<b>Total volume</b>	<b>20 <math>\mu\text{l}</math> per reaction</b>

\* n = number of reactions (samples plus controls).

Prepare enough master mix for an extra sample (n+1) to allow for sufficient coverage for the PCR setup.

The value n should not exceed 24 (plus controls) as 24 is the maximum number of samples that can fit on a run.

**Note:** When preparing the master mix, the required volume of the Control Reaction Mix (CTRL) is added to the relevant tube first and the *Taq* DNA polymerase (*Taq*) is added last.

**Note:** Pipet *Taq* DNA polymerase by carefully placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.

- Place the appropriate number of PCR 4-strip tubes (each strip has 4 tubes) in the loading block according to the layout in Table 4. Numbers denote positions in the loading block and indicate final rotor position. Do not cap the tubes.

**Note:** Leave the caps in the plastic container until required.

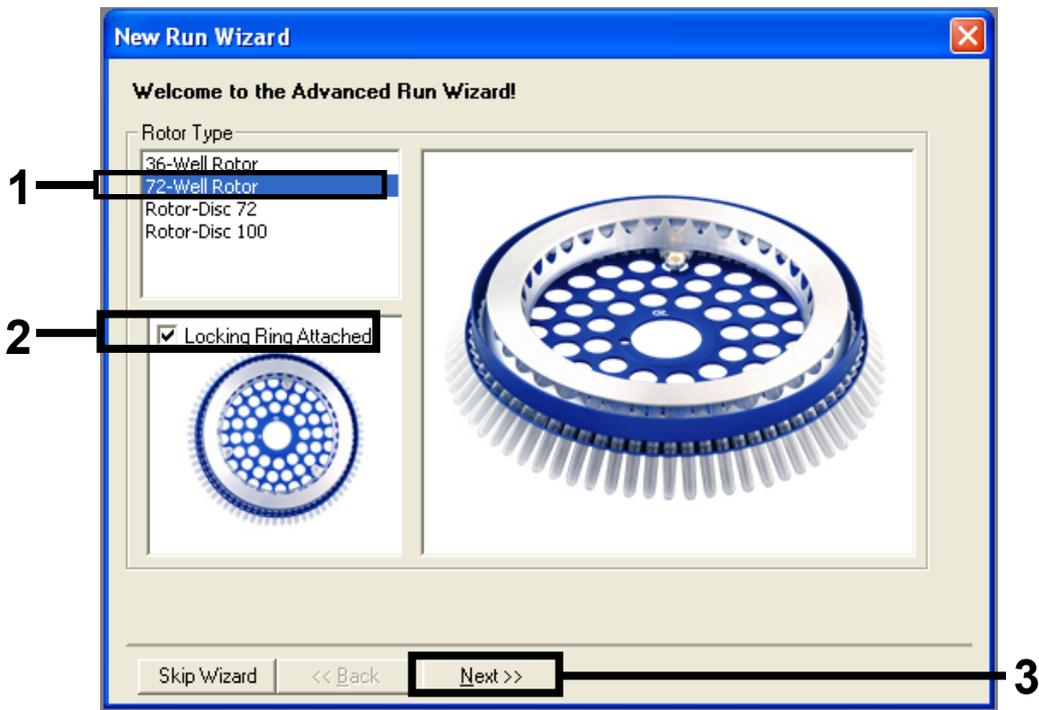
**Table 4. Run layout in the loading block for DNA sample assessment**

Assay									
Control	1[PC]	9	17	25	–	–	–	–	–
Control	2[NTC]	10	18	26	–	–	–	–	–
Control	3	11	19	–	–	–	–	–	–
Control	4	12	20	–	–	–	–	–	–
Control	5	13	21	–	–	–	–	–	–
Control	6	14	22	–	–	–	–	–	–
Control	7	15	23	–	–	–	–	–	–
Control	8	16	24	–	–	–	–	–	–

- Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the control assay master mix by fully aspirating up and down 10 times.
- Immediately add 20 µl master mix to each PCR strip tube.  
**Note:** See Table 4 for the tube layout. For DNA sample assessment, Control assay master mix should be added to one PC tube, one NTC tube, and one tube for each DNA sample.
- Immediately add 5 µl of nuclease-free water for No Template Control (NTC) to the NTC tube (tube position 2) and cap the tube.
- Add 5 µl of each DNA sample to the sample tubes (tube positions 3–26) and cap the tubes.
- Add 5 µl of KRAS Positive Control (PC) to the PC tube (tube position 1) and cap the tube.

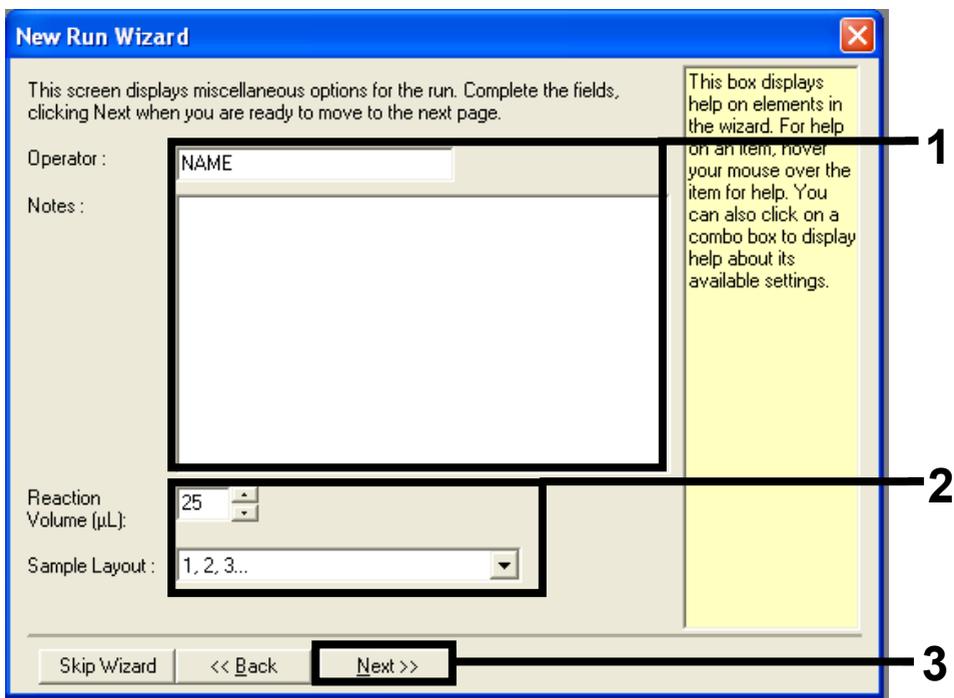
Each tube should contain a total reaction volume of 25 µl (20 µl master mix prepared in Table 2, plus 5 µl of NTC/sample/PC).

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10. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q instrument.
  11. Invert capped tubes 4 times to mix the sample and reaction mix.
  12. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 3) using the marks for orientation.  
**Note:** If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q instrument is maintained.
  13. Place the 72-well rotor into the Rotor-Gene Q instrument. Make sure the locking ring (supplied with the Rotor-Gene Q instrument) is placed on top of the rotor to secure the tubes during the run.
  14. Open the Rotor-Gene Q series software 2.3 and the appropriate created temperature profile.  
Create the temperature profile according to “Protocol: Creating a temperature profile,” page 32.
  15. Ensure the correct rotor is selected and check the box to confirm the locking ring is attached. Click “Next” (Figure 1).



**Figure 1.** The “New Run Wizard” dialog box and welcome screen. 1 = Rotor type, 2 = “Locking Ring Attached box,” 3 = “Next.”

**16.** Enter the name of the operator. Add any notes and check that the reaction volume is set to 25 and the “Sample Layout” box reads “1, 2, 3...”. Click “Next” (Figure 2).



**Figure 2. The “New Run Wizard” dialog box.** 1 = “Operator” and “Notes” fields, 2 = “Reaction Volume” and “Sample Layout” fields, 3 = “Next.”

17. This next window allows editing of the temperature profile. No editing is required as the temperature profile has been created according to the instructions in “Protocol: Creating a temperature profile,” page 32. Click “Next” (Figure 3).

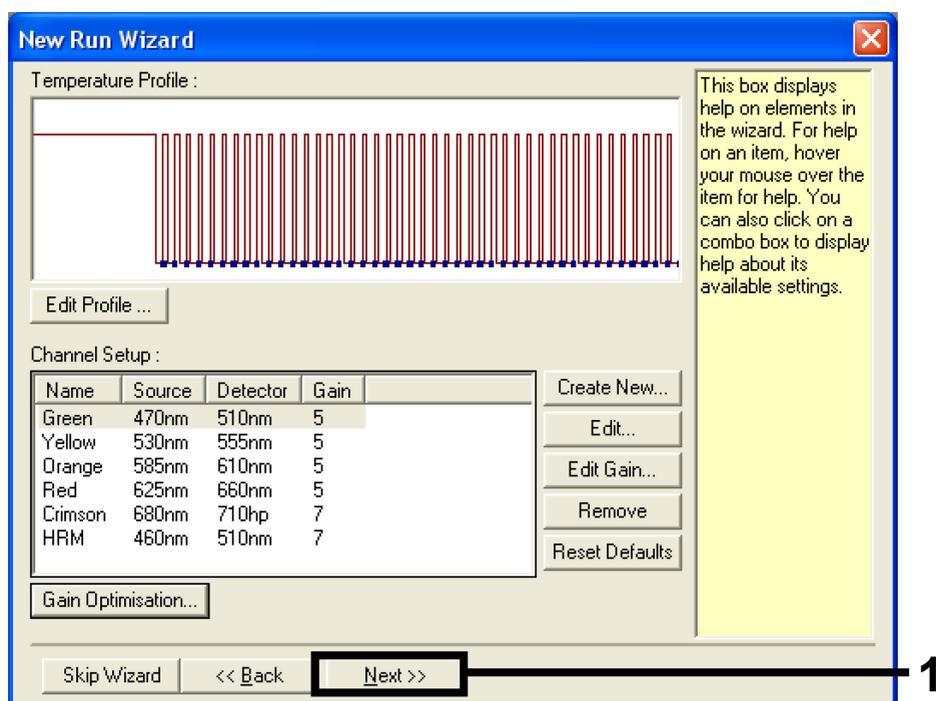


Figure 3. The “New Run Wizard” dialog box and temperature editing screen. 1 = “Next.”

18. Check the summary, and click “Start Run” to save the run file and start the run (Figure 4).

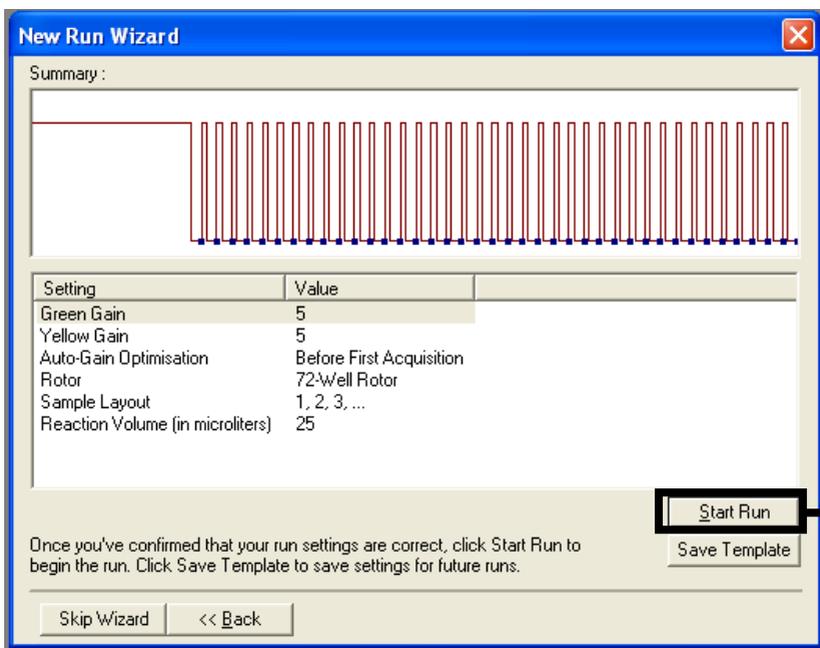


Figure 4. The “New Run Wizard” dialog box. 1 = “Start Run.”

19. After the run starts, a new window appears in which you can either enter sample names now or click “Finish” and enter them later by selecting the “Sample” button during the run, or once the run is complete.

Clicking “Finish and Lock Samples” will prevent you from editing the sample names. The user should take particular care when entering sample names to ensure correct sample testing and analysis.

**Note:** When naming samples, empty wells should be left blank in the “Name” column.

20. After the run is complete, analyze data according to sections “Sample assessment data analysis,” page 45.

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## Protocol: Detection of KRAS mutations

This protocol is for the detection of KRAS mutations.

### Important points before starting

- A sample can be tested using the KRAS mutation assays once it has passed the sample assessment.

### Procedure

1. Mix the thawed reagents by inverting each tube 10 times to avoid localized concentrations of salts. Centrifuge briefly to collect the contents at the bottom of the tube.
2. Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the master mixes by fully aspirating up and down 10 times.
3. Immediately add 20  $\mu$ l master mix to each appropriate PCR strip tube.

**Note:** See Table 5 for the tube layout while setting up the reaction mixes. For detection of KRAS mutations, master mixes should be added to 8 PC tubes, 8 NTC tubes and 8 tubes for each DNA sample.

**Table 5. Run layout in the loading block for detection of KRAS mutations**

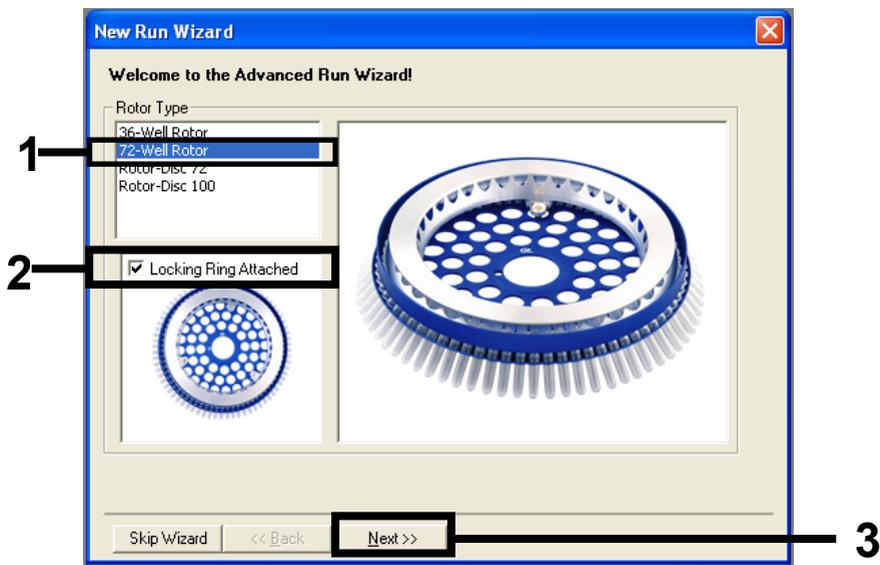
Assay	Controls		Sample number						
	PC	NTC	1	2	3	4	5	6	7
Control	1*	9	17	25	33	41	49	57	65
12ALA	2	10	18	26	34	42	50	58	66
12ASP	3	11	19	27	35	43	51	59	67
12ARG	4	12	20	28	36	44	52	60	68
12CYS	5	13	21	29	37	45	53	61	69
12SER	6	14	22	30	38	46	54	62	70
12VAL	7	15	23	31	39	47	55	63	71
12ASP	8	16	24	32	40	48	56	64	72

\* Numbers denote positions in the loading block and indicate final rotor position.

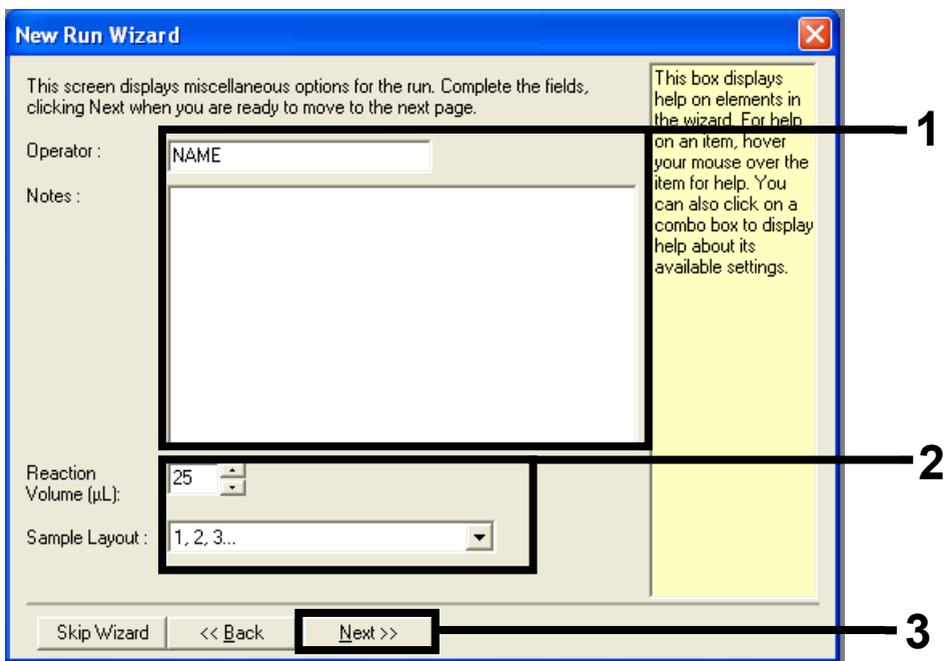
4. Immediately add 5  $\mu$ l of nuclease-free water for No Template Control (NTC) to the NTC tubes (tube positions 9–16) and cap the tubes.
5. Add 5  $\mu$ l of each DNA sample to the sample tubes (tube positions 17–72) and cap the tubes.
6. Add 5  $\mu$ l of KRAS Positive Control (PC) to the PC tubes (tube positions 1–8) and cap the tubes.
7. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g. positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q instrument.
8. Invert the capped tubes four times to mix the sample and reaction mix.
9. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 5) using the marks for orientation.

**Note:** A maximum of 7 samples can be included in each PCR run. If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q instrument is maintained.

10. Place the 72-well rotor into the Rotor-Gene Q instrument. Ensure the locking ring (supplied with the Rotor-Gene Q instrument) is placed on top of the rotor to secure the tubes during the run.
11. Open the Rotor-Gene Q series software 2.3 and the appropriately created temperature profile.  
Create the temperature profile according to the "Protocol: Creating a temperature profile," page 32.
12. Ensure the correct rotor is selected and check the box to confirm the locking ring is attached. Click "Next" (Figure 5).



**Figure 5. The "New Run Wizard" dialog box and welcome screen.** 1 = Rotor type, 2 = "Locking Ring Attached box," 3 = "Next." Enter the name of the operator. Add any notes and check that the reaction volume is set to 25 and the "Sample Layout" box reads "1, 2, 3...". Click "Next" (Figure 6).



**Figure 6.** The “New Run Wizard” dialog box. 1 = “Operator” and “Notes” fields, 2 = “Reaction Volume” and “Sample Layout” fields, 3 = “Next.”

13. This next window allows editing of the temperature profile. No editing is required as the temperature profile has been created according to the instructions in the “Protocol: Creating a temperature profile,” page 32. Click “Next” (Figure 7).

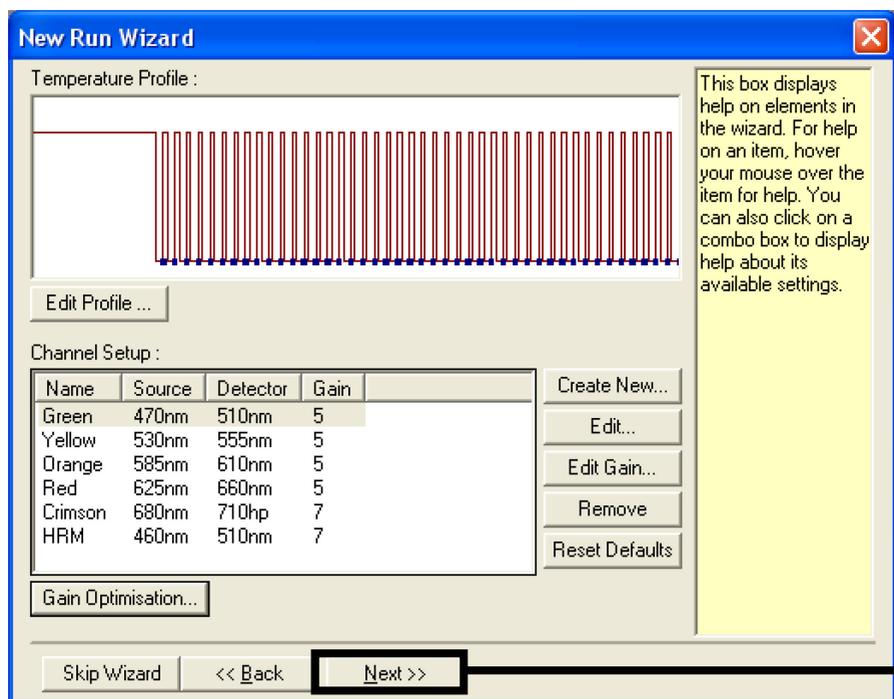


Figure 7. The “New Run Wizard” dialog box and temperature editing screen. 1 = “Next.”

14. Check the summary, and click “Start Run” to save the run file and start the run (Figure 8).

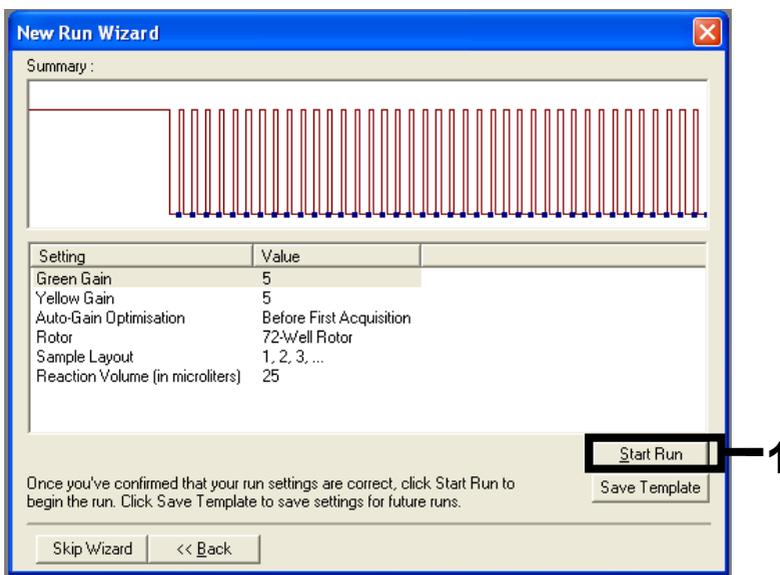


Figure 8. The “New Run Wizard” dialog box. 1 = “Start Run.”

15. After the run starts, a new window appears in which you can either enter sample names now or click “Finish” and enter them later by selecting the “Sample” button during the run, or once the run is complete.

**Note:** When naming samples, empty wells should be left blank in the “Name” column.

16. After the run is complete, analyze data according to “KRAS mutation detection data analysis,” page 46.
17. If quantification reports are required, click the “Reports” icon of the toolbar in the Rotor-Gene Q run file.

## Protocol: Creating a temperature profile

This protocol is referenced within "Protocol: Sample assessment" (page 17) and Protocol: Detection of KRAS mutations" (page 26). Before starting, create a temperature profile for the KRAS analysis. The cycling parameters are the same for both Sample Assessment and Mutation Assessment.

### Procedure

The cycling parameters are shown in Table 6.

**Table 6. Cycling parameters**

<b>Cycles</b>	<b>Temperatures</b>	<b>Time</b>	<b>Time</b>
1	95°C	15 minutes	None
40	95°C	30 seconds	None
	60°C	60 seconds	Green and yellow

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.

1. Double-click the Rotor-Gene Q Series Software 2.3 software icon on the desktop of the laptop connected to the Rotor-Gene Q Instrument. Select the "Advanced" tab in the "New Run" dialog box that appears.
2. To create a new template, select "Empty Run" and then click "New" to enter the "New Run Wizard."
3. Select 72-Well Rotor as the rotor type. Confirm that the locking ring is attached and check the "Locking Ring Attached" box. Click "Next" (Figure 9).

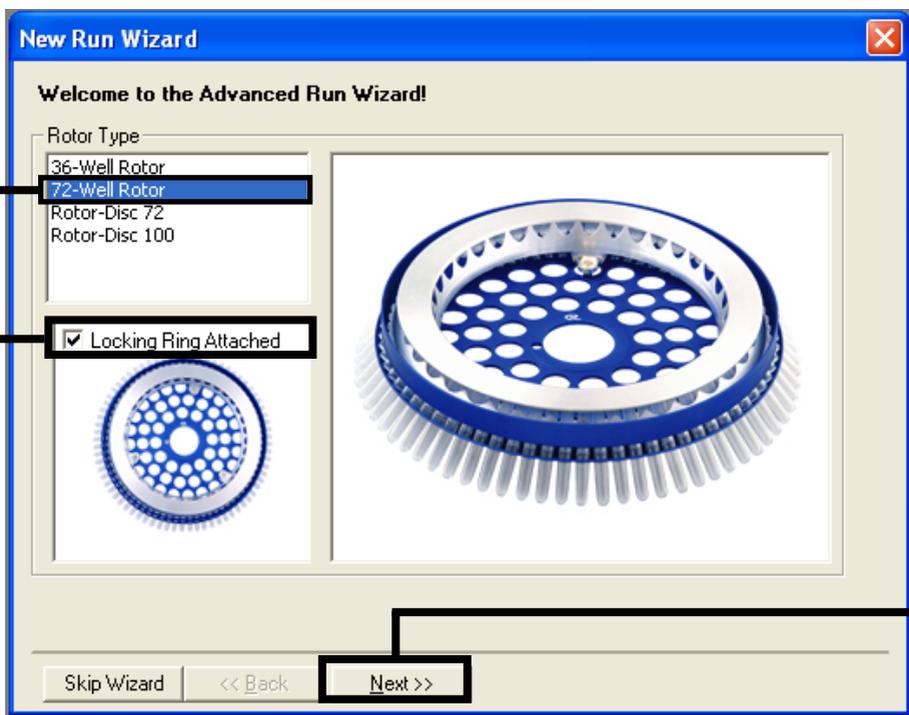
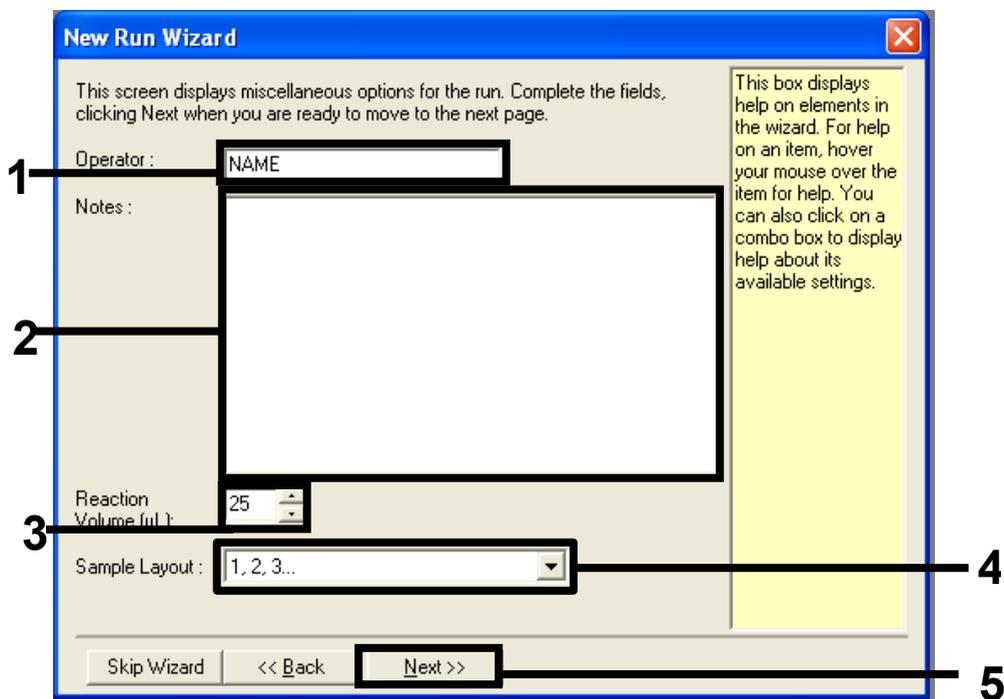


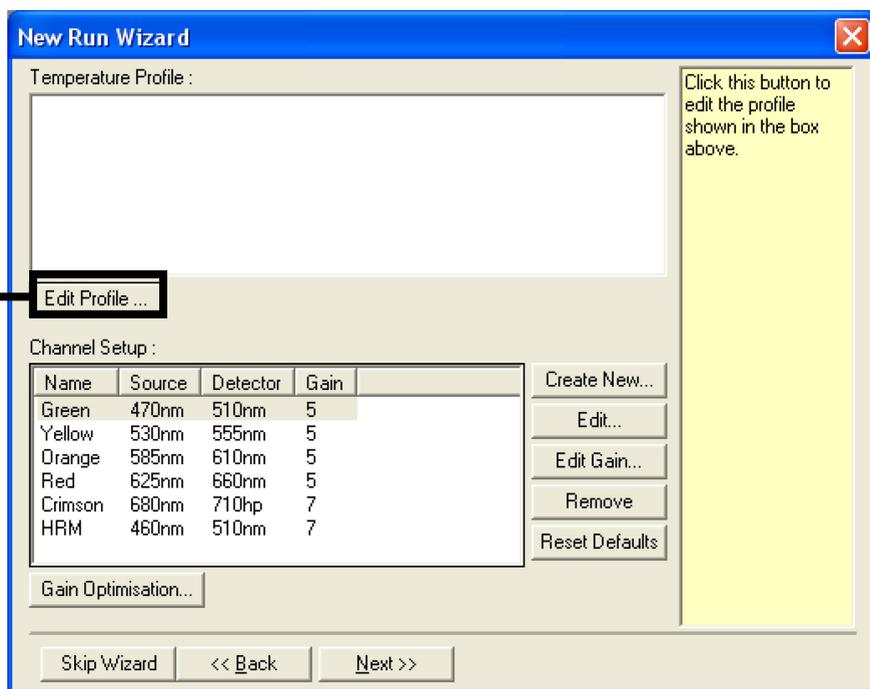
Figure 9. The “New Run Wizard” dialog box. 1 = “Rotor type, 2 = “Locking Ring Attached box,” 3 = “Next.”

4. Enter the name of the operator. Add any notes and enter the reaction volume as 25. Ensure that “Sample Layout” reads “1, 2, 3...”. Click “Next” (Figure 10).



**Figure 10. Entering operator name and reaction volumes.** 1 = "Operator" dialog field, 2 = "Notes" dialog field, 3 = "Reaction Volume" field, 4 = "Sample Layout," 5 = "Next."

5. Click "Edit Profile" in the "New Run Wizard" dialog box (Figure 11) and program the temperature profile according to the information in the following steps.



**Figure 11. Editing the profile.**

6. Click "Insert after" and select New Hold at Temperature (Figure 12).

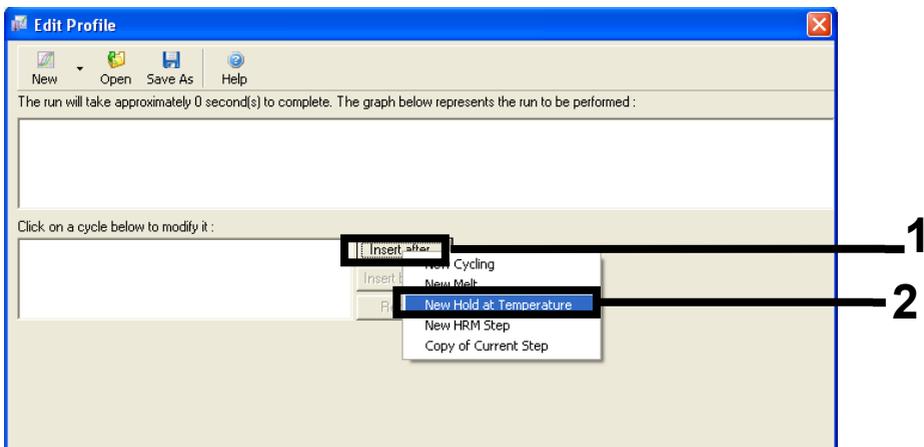


Figure 12. Inserting an initial incubation step. 1 = "Insert after," 2 = "New Hold at Temperature."

7. Change "Hold Temperature" to 95°C and "Hold Time" to 15 minutes 0 second. Click "Insert After" and then select "New Cycling" (Figure 13).

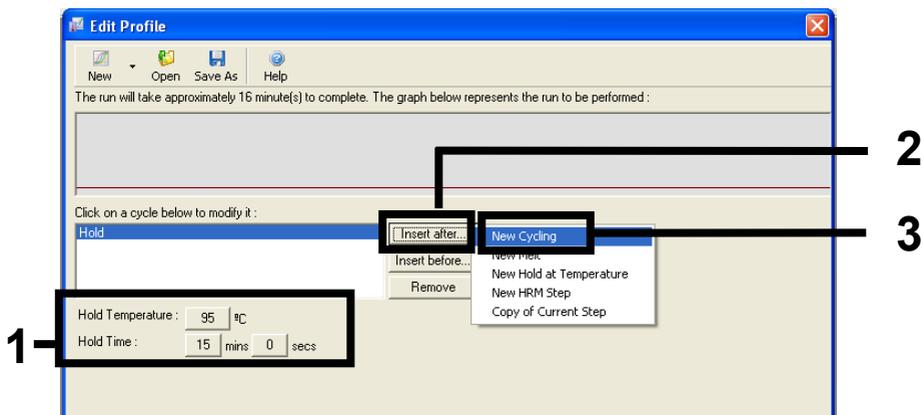


Figure 13. Initial incubation step at 95°C. 1 = "Hold Temperature and Hold Time," 2 = "Insert after," 3 = "New Cycling."

8. Change the number of cycle repeats to 40. Select the first step and set to “95°C for 30 secs” (Figure 14).

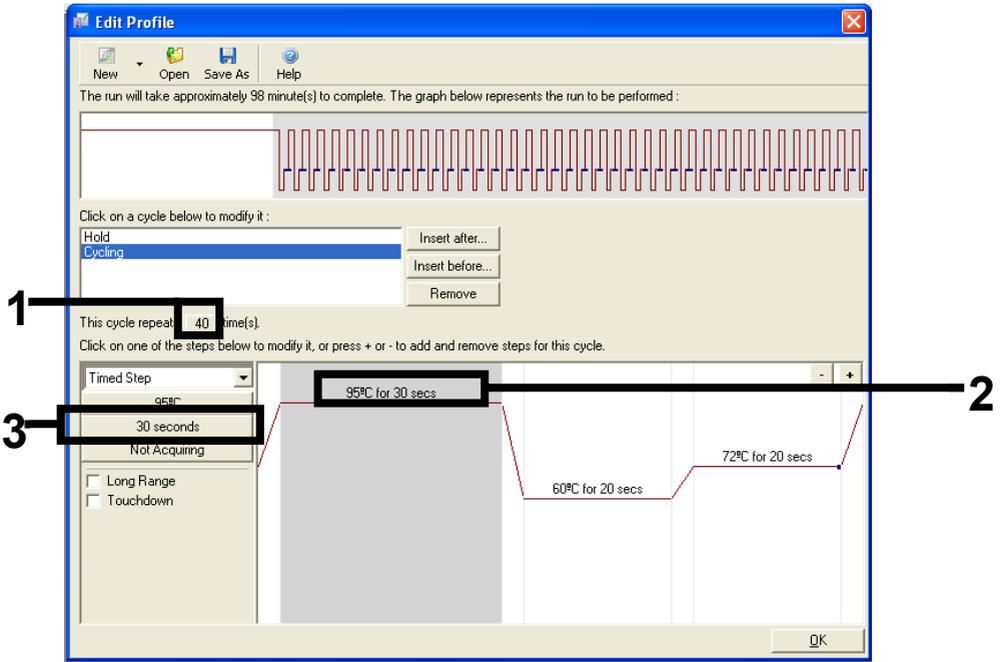


Figure 14. Cycling step at 95°C. 1 = “Cycle repeats” box, 2 = “step one temperature” setting, 3 = step one time setting.

9. Highlight the second step and set to “60°C for 60 seconds.” Enable data acquisition during this step by selecting “Not Acquiring” (Figure 15).

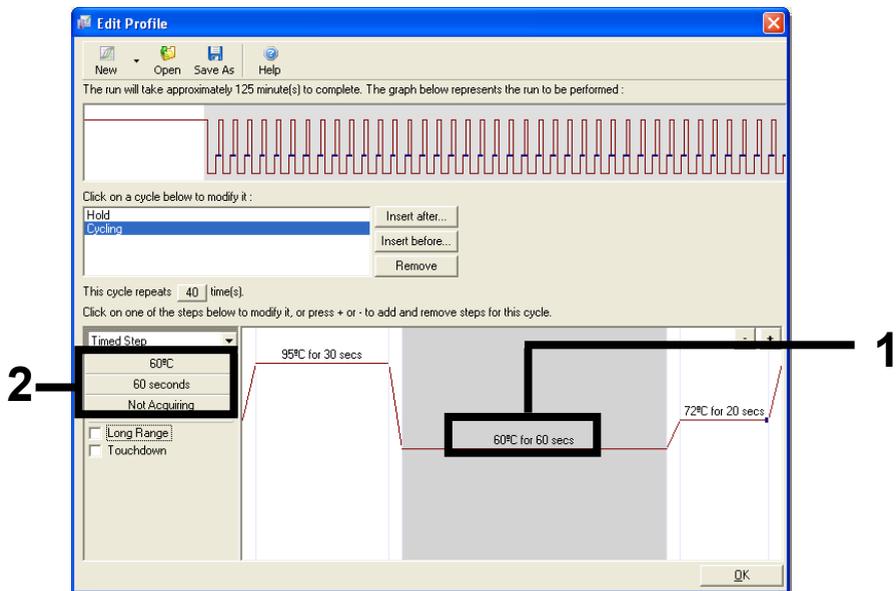


Figure 15. Cycling step at 60°C. 1 = second step temperature and time setting, 2 = Not Acquiring.

10. Set "Green" and "Yellow" as acquiring channels by selecting the ">" button to transfer them from the "Available Channels" list. Click "OK" (Figure 16).

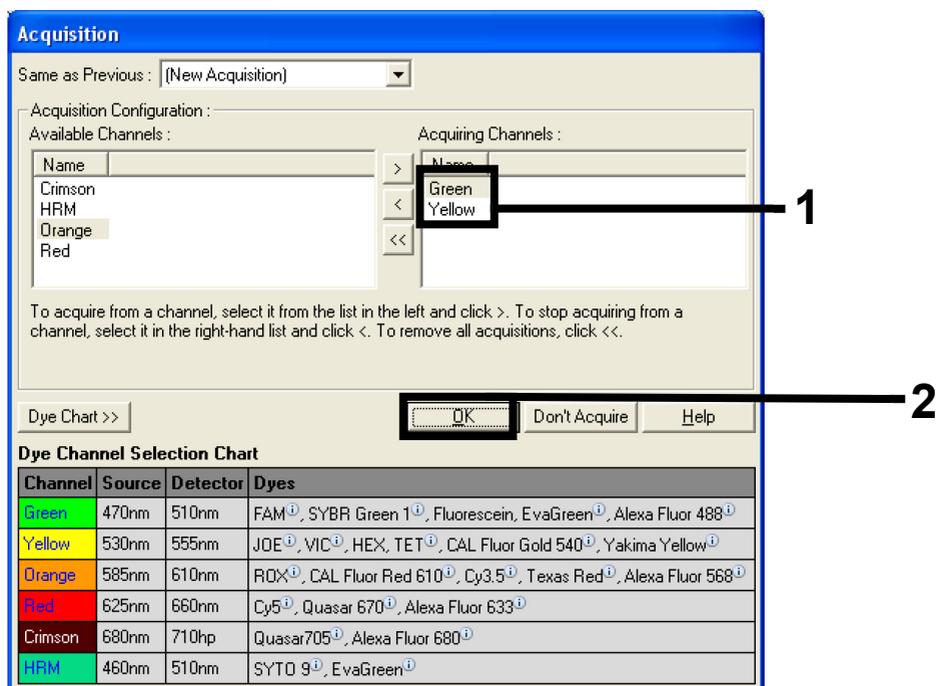


Figure 16. Acquiring at cycling step of 60°C.

11. Highlight the third step and delete by clicking “-”. Click “OK” (Figure 17).

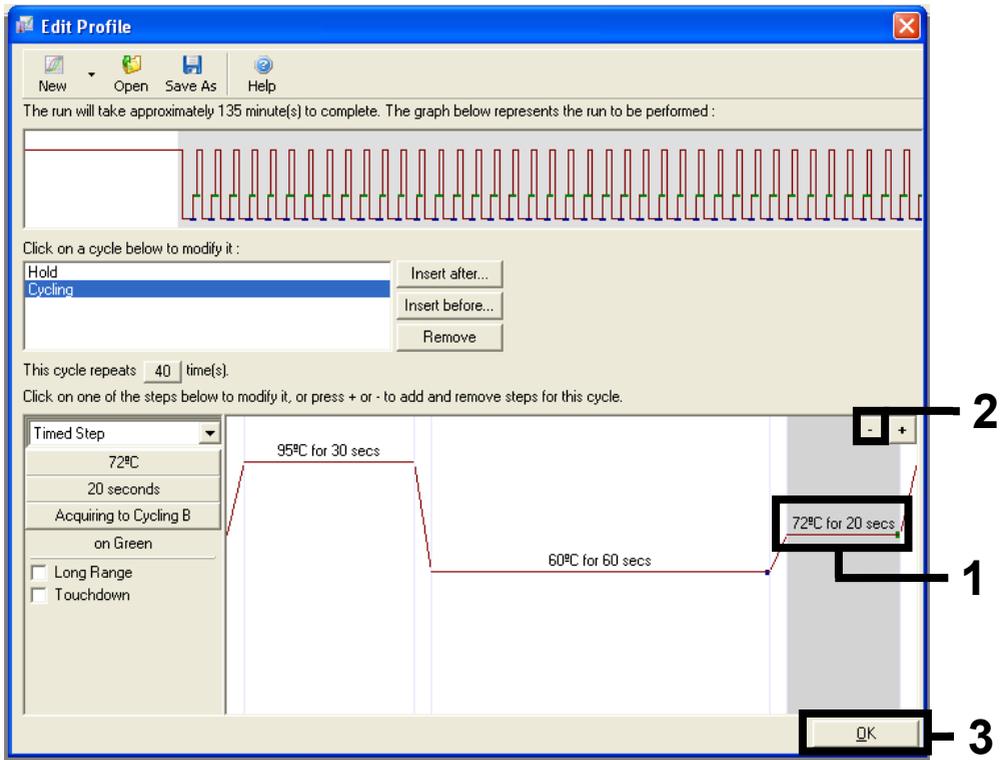


Figure 17. Removal of extension step.

12. In the next dialog box, click "Gain Optimization" (Figure 18).

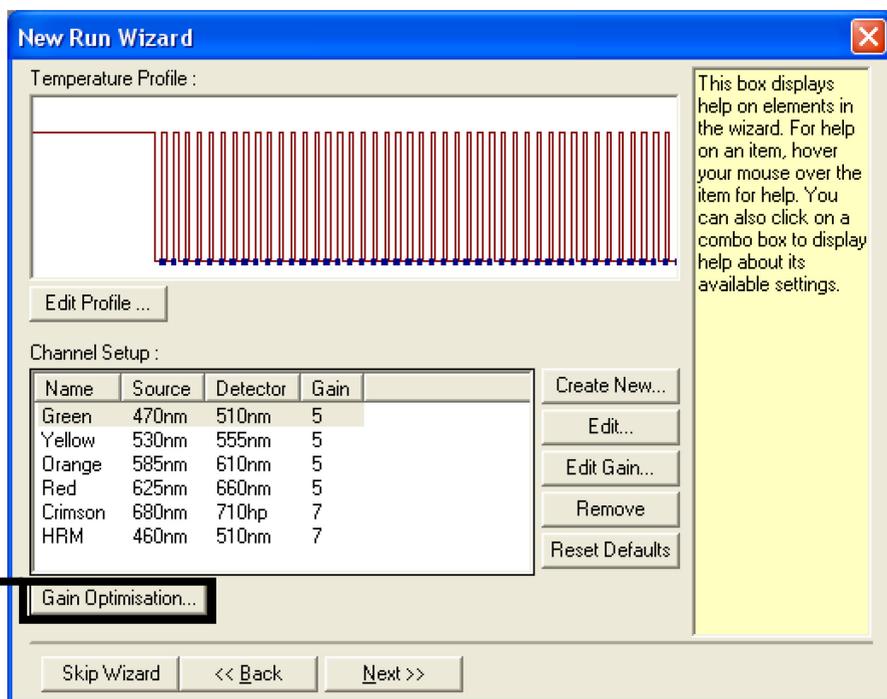


Figure 18. "Gain Optimization."

13. Click "Optimize Acquiring." Channel settings are displayed for each channel. Accept these default values by clicking "OK" for both channels (Figure 19).

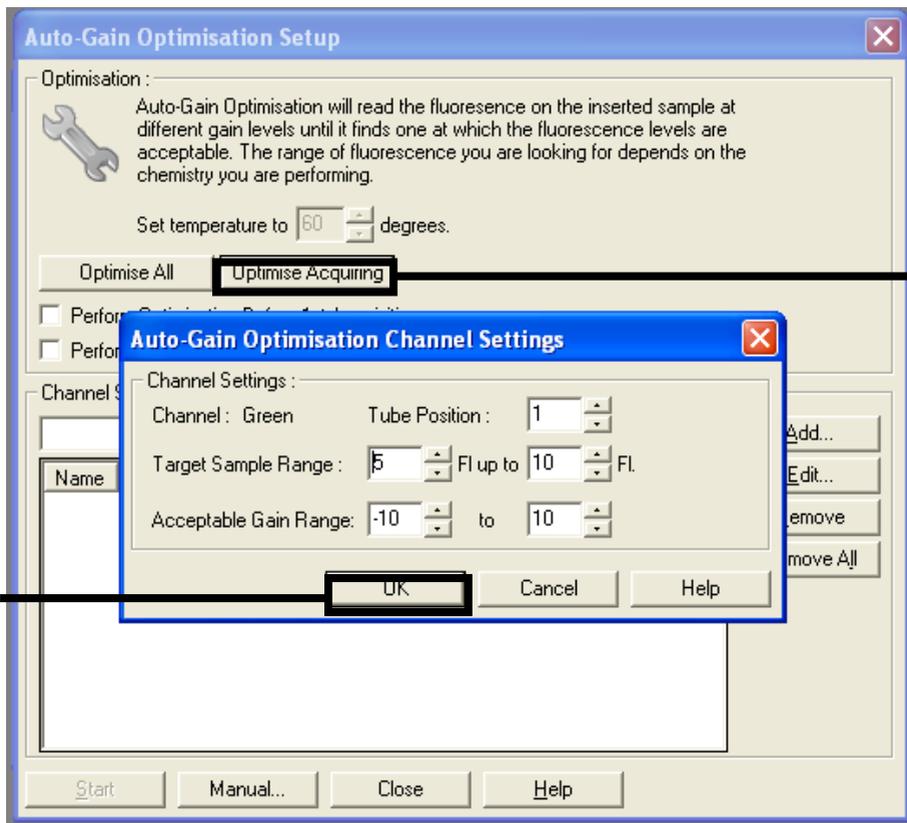


Figure 19. "Auto-gain Optimization" for the green channel.

14. Check the "Perform Optimization before 1st Acquisition" box and then click "Close" to return to the wizard (Figure 20).

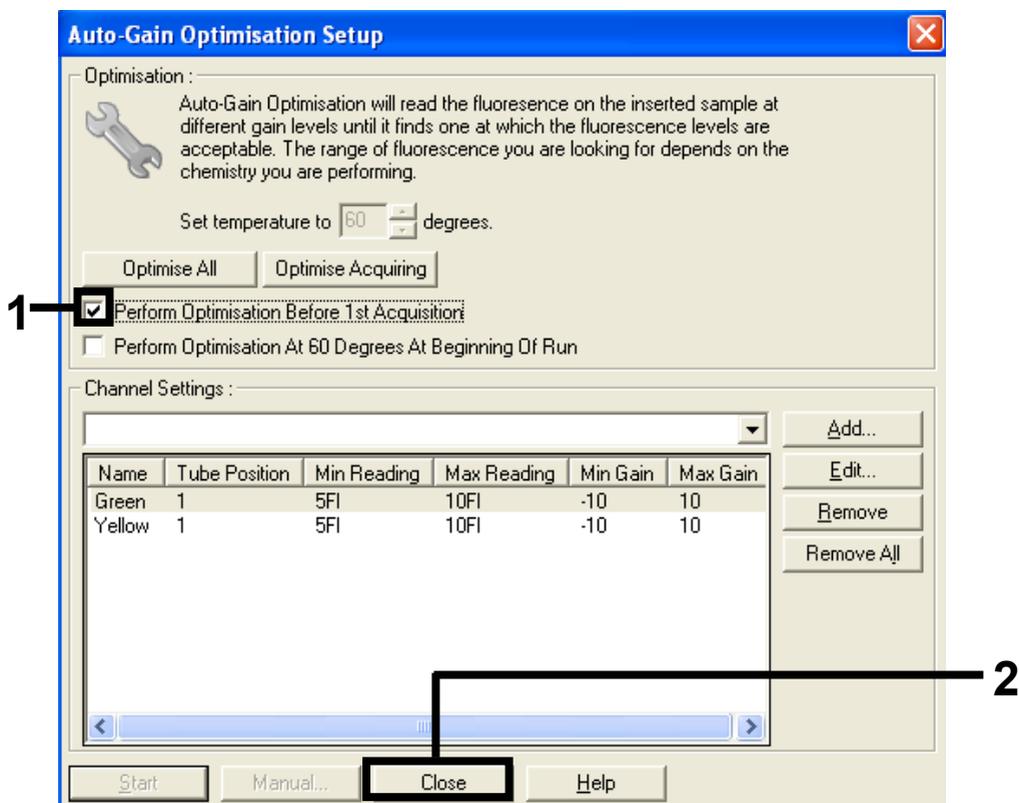


Figure 20. Selection of green and yellow channels.

15. Click "Next" to save the template in an appropriate location by selecting "Save Template."

---

# Interpretation of Results

After the run is complete, analyze the data according to the following procedure.

## Software analysis settings

1. Open the appropriate file using the Rotor-Gene Q series software 2.3.
2. If you have not already named your samples prior to performing the run, click "Edit Samples."
3. Insert the names of your samples in the "Name" column.
4. Click "Analysis." On the analysis page, click "Cycling A. Yellow" to view the HEX channel.
5. Click "Named On."  
**Note:** This ensures that empty wells do not feature in the analysis.
6. Select "Dynamic Tube."
7. Select "Linear Scale."
8. Click "Outlier Removal" and enter "10%" for the "NTC Threshold."
9. Set the threshold at 0.05 and check the HEX  $C_T$  values.
10. On the analysis page, click "Cycling A. Green" to view the FAM channel.
11. Check that "Dynamic Tube" is highlighted. Select "Linear Scale."
12. Click "Outlier Removal" and enter "10%" for the "NTC Threshold."
13. Set the threshold at 0.05 and check the FAM  $C_T$  values.

---

## Sample assessment data analysis

### Run control analysis

Refer to the “Run control analysis” flowchart in Figure 21, page 47.

- **Negative control:** To ensure there is no contamination of the reaction mix, the No Template Control must not generate a  $C_T$  value in the green channel below 40. To ensure that the plate was set up correctly, the NTC must show amplification of 31.91–35.16 in the yellow channel. The values specified are within and including those values.
- **Positive control:** The KRAS Positive Control (PC) must give a control assay  $C_T$  (FAM channel) of 23.5–29.5. The values specified are within and including those values. A value outside this range indicates an assay setup problem.

**Note:** Sample data must not be used if either of these 2 run controls has failed.

Provided that both run controls are valid, each sample  $C_T$  value must be within the range of 21.92–32.00 in the green channel. If the sample is out of this range, the following guidance is provided.

### Sample analysis — control assay

- **Sample control assay  $C_T < 21.92$ :** Samples with a control  $C_T$  of  $< 21.92$  will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range on the basis that diluting by half will increase the  $C_T$  by 1. Samples should be diluted using the water provided in the kit (Nuclease-Free Water for Dilution [Dil.]).
- **Sample control assay  $C_T > 32$ :** Re-extraction of the sample is recommended as insufficient starting DNA template will be present to detect all mutations at the stated cutoff values for the assay.

---

## KRAS mutation detection data analysis

- **Negative control:** To ensure there is no contamination of the reaction mix, the No Template Control must not generate a  $C_T$  value in the green channel below 40. To ensure that the plate was set up correctly, the NTC must show amplification of 31.91–35.16 in the yellow channel. The values specified are within and including those values.
- **Positive control:** The KRAS Positive Control (PC) must give a control assay  $C_T$  (FAM channel) of 23.5–29.5. The values specified are within and including those values. A value outside this range indicates an assay setup problem.

**Note:** Sample data must not be used if either of these 2 run controls has failed.

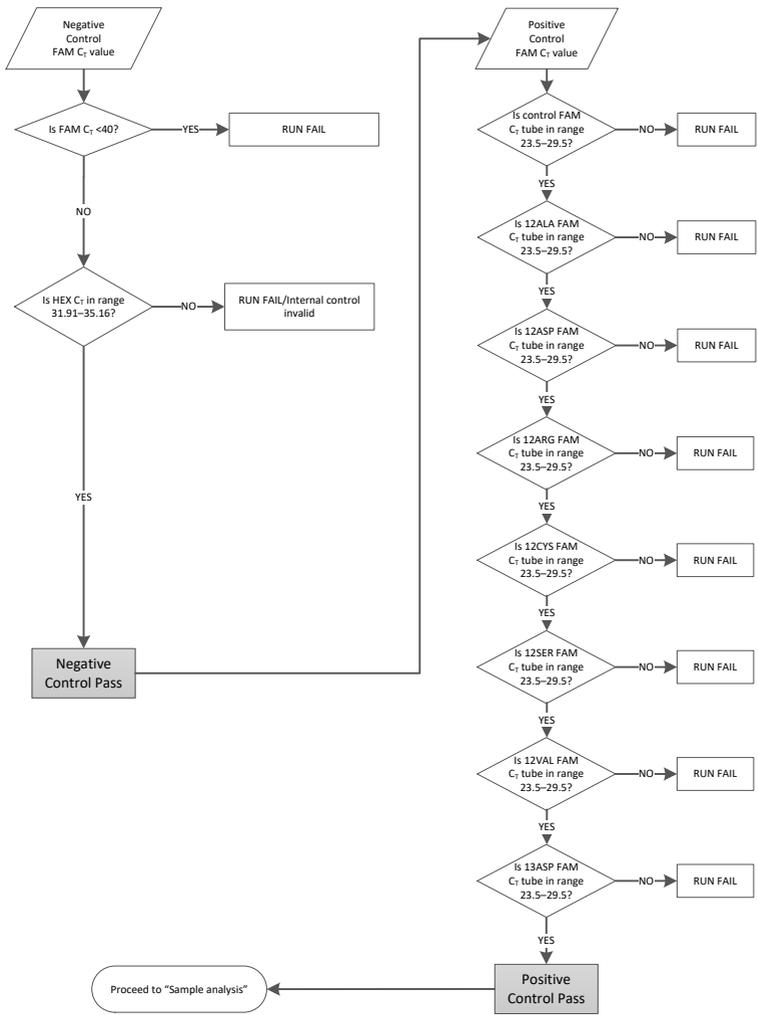


Figure 21. Run control analysis flowchart.

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## Sample analysis

Refer to the “Sample analysis” flowchart in Figure 22, page 49.

### Sample control FAM $C_T$ value

Provided that both run controls are valid for the control assay, each sample control  $C_T$  value must be within the range of 21.92–32.00 in the green channel.

If the sample is out of this range, the following guidance is provided.

- **Sample control assay  $C_T < 21.92$ :** Samples with a control  $C_T$  of  $< 21.92$  will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range on the basis that diluting by half will increase the  $C_T$  by 1. Samples should be diluted using the water provided in the kit (Nuclease-Free Water for Dilution; Dil.)
- **Sample control assay  $C_T > 32$ :** Interpret with caution as very low level mutations may not be detected.



Figure 22. Sample analysis flowchart.

---

## Sample internal control mutation assays HEX C<sub>T</sub> value

Refer to the “Sample analysis” flowchart in Figure 22, page 49.

All wells of each sample must be analyzed. Check that each well generates a HEX signal from the internal control. There are 3 possible outcomes.

- If the internal control C<sub>T</sub> falls within the specified range (31.91–35.16), it is HEX amplification positive.
- If the internal control C<sub>T</sub> is above the specified range (>35.16), it is HEX amplification negative.
- If the internal control C<sub>T</sub> is below the specified range (<31.91), it is invalid.
- If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of inhibitors, but it should be noted that this would also dilute the target DNA. A tube of Water for Sample Dilution (Dil.) is included with the kit.

## Sample mutation assays FAM C<sub>T</sub> value

The FAM values for all 7 reaction mixes should be checked against the values listed in Table 7.

**Table 7. Acceptable sample mutation reaction values (FAM)\***

Assay	Acceptable C <sub>T</sub> range	ΔC <sub>T</sub> cutoff
12ALA	0.00–40.00	≤8.00
12ASP	0.00–40.00	≤6.60
12ARG	0.00–40.00	≤8.00
12CYS	0.00–40.00	≤8.00
12SER	0.00–40.00	≤8.00
12VAL	0.00–40.00	≤7.50
13ASP	0.00–40.00	≤7.50

\*Acceptable values are within and including the values shown.

- If the FAM C<sub>T</sub> falls within the specified range, it is amplification positive.
- If the FAM C<sub>T</sub> is above the specified range or there is no amplification, it is amplification negative.

Calculate the ΔC<sub>T</sub> value for each mutation tube that is FAM amplification positive as follows, ensuring that the mutation and control C<sub>T</sub> values are from the same sample.

$$\Delta C_T = \text{mutation } C_T - \text{control } C_T$$

Compare the ΔC<sub>T</sub> value for the sample with the cutoff point for the assay in question (Table 7), ensuring that the correct cutoff point is applied to each assay.

The cutoff point is the point above which a positive signal could potentially be due to background signal of the ARMS primer on wild-type DNA. If the sample ΔC<sub>T</sub> value is higher than the cutoff point, it is classed as negative or beyond the limits of detection of the kit.

---

For every sample, each mutation reaction will be given a status of “mutation detected,” “mutation not detected,” or “invalid” using the following criteria.

Mutation detected:

- FAM amplification positive and the  $\Delta C_T$  are at or below the cutoff value. If multiple mutations are detected, the mutation reported should be the one with the smallest  $\Delta C_T$  value

Mutation not detected:

- FAM amplification positive and the  $\Delta C_T$  are above the cutoff value
- FAM amplification negative and HEX (internal control) amplification positive

Invalid:

- HEX (internal control) is invalid.
- FAM amplification negative and HEX amplification negative.

If a sample is HEX amplification negative in a tube but FAM amplification positive in a different tube, then a “mutation detected” result in the different tube can still be considered valid, but the particular mutation identified may not be reliably assigned.

- If a sample is HEX amplification negative and FAM amplification positive in the same tube, then the “mutation detected” result should be considered valid.
- If a tube is HEX (internal control) invalid, the result of that tube must not be used.

---

## Assigning sample mutation status

Once all mutation reaction tubes are assessed, the mutation status of the sample is determined as follows:

- **Mutation detected:** One or more of the 7 mutation reactions are positive. If multiple mutations are detected, the mutation reported should be the one with the smallest  $\Delta C_T$  value.
- **Mutation not detected:** All 7 mutation reactions are negative.
- **Invalid:** No mutation reactions are positive, and one or more mutation reactions are invalid.

**Note:** The KRAS RGQ PCR Kit is intended to detect mutations in the KRAS gene in a DNA sample. When a sample is called KRAS mutation detected, only one specific mutation should be reported. If multiple mutations are detected, the mutation reported should be the one with the smallest  $\Delta C_T$  value.

Some cross-reactivity may occur between mutation reactions. For example, if a high level 12ALA mutation is observed, some of the other mutation reactions may also give a positive result. This is due to the ARMS primers detecting other mutations of a similar sequence to one another. If a second mutation assay gives a positive result, this is likely to be cross-reactivity. Double mutants have been observed, although these are rare.

If one or more of the mutation reactions are invalid but one or more are positive, the sample can still be called KRAS mutation detected as a mutation is present. However, the specific mutation reported may not be accurate and may be a result of cross-reactivity. Therefore, the sample should only be called KRAS mutation detected.

## 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](http://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](http://www.qiagen.com)).

### Comments and suggestions

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#### No signal with KRAS Positive Control (PC) in fluorescence channel Cycling Green

- |   |  |
|---|--|
| a) The selected fluorescence channel for PCR data analysis does not comply with the protocol  | For data analysis, select the fluorescence channel Cycling Green for the analytical KRAS PCR and the fluorescence channel Cycling Yellow for the internal control PCR. |
| b) Incorrect programming of the temperature profile of the Rotor-Gene Q Instrument  | Compare the temperature profile with the protocol and if incorrect, repeat the run.  |
| c) Incorrect configuration of the PCR   | Check your pipetting scheme and repeat the PCR, if necessary.  |
| d) The storage conditions for one or more kit components did not comply with the instructions given in "Reagent Storage and Handling" (page 13) | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.  |

## Comments and suggestions

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- e) The KRAS RGQ PCR Kit has expired      Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

### Signals with the negative controls in fluorescence channel Cycling Green of the analytical PCR

- a) Contamination occurred during preparation of the PCR      Repeat the PCR with new reagents in replicates.

If possible, close the PCR tubes directly after addition of the sample to be tested.

Make sure that work space and instruments are decontaminated at regular intervals.

## Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the KRAS RGQ PCR Kit is tested against predetermined specifications to ensure consistent product quality.

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## Limitations

For research use only. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

The product is to be used only by personnel trained in general molecular biology laboratory skills.

We recommend the use of DNA extracted using the QIAGEN QIAamp DNA FFPE Tissue Kit.

The product is intended for use only on the Rotor-Gene Q real-time PCR cyclyer, 5plex HRM series.

Strict compliance with the *KRAS RGQ PCR Kit Handbook* is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended and will result in a loss of performance.

It is important that the amount and quality of DNA in the sample is assessed prior to performing sample analysis using the KRAS RGQ PCR Kit. See page 17.

Additional Control Reaction Mix (CTRL) is provided to determine if the samples are acceptable for the assay. Absorbance readings must not be used for this purpose as they do not correlate with PCR performance in fragmented DNA samples.

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

# Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Manufacturer
	Material number
	Lot number
	Global Trade Item Number
Rn	R is for revision of the Handbook and n is the revision number
	Use by
	Contains reagents sufficient for N reactions
	Temperature limitation
	Consult instructions for use
	Keep away from sunlight

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**REF**

Catalog number

## Contact Information

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

# Ordering Information

Product	Contents	Cat. no.
KRAS RGQ PCR Kit (24)	For 24 reactions: 2 Control Assay tubes, 7 Mutation Assays, Positive Control, <i>Taq</i> DNA Polymerase, Water for NTC, and Water for Sample Dilution	870001
<b>Rotor-Gene Q and other accessories</b>		
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt (HRM) analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9001580
Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and HRM analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9001650
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipette in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106

Product	Contents	Cat. no.
<b>QIAamp DNA FFPE Tissue Kit – for purification of genomic DNA from paraffin-embedded tissues</b>	QIAamp DNA FFPE Tissue Kit (50) For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404

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](http://www.qiagen.com) or can be requested from QIAGEN Technical Services or your local distributor.

## Revision History

Document Revision History	
R4 01/2019	Updated address of legal manufacturer (front cover).

Applicable Countries:

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Not for use with urine samples.

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## Notes

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## Notes

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