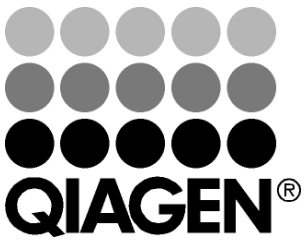
July 2010

EGFR RGQ PCR Kit Handbook

For qualitative measurement of 29 somatic mutations in the EGFR oncogene, for use with the Rotor-Gene® Q 5plex HRM® Instrument

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



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QIAGEN Sample and Assay Technologies

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- Nucleic acid and protein assays
- microRNA research and RNAi
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Contents

Kit Contents

EGFR RGQ PCR Kit		**(24)**
Catalog no.		**870101**
Number of reactions		**24***
Red	Control Reaction Mix	1200 µl
Purple	T790M Reaction Mix	600 µl
Orange	Deletions Reaction Mix	600 µl
Pink	L858R Reaction Mix	600 µl
Green	L861Q Reaction Mix	600 µl
Yellow	G719X Reaction Mix	600 µl
Gray	S768I Reaction Mix	600 µl
Blue	Insertions Reaction Mix	600 µl
Brown	EGFR Positive Control	300 µl
Turquoise	*Taq* DNA Polymerase	140 µl
White	Nuclease-Free Water	1.9 ml
	Handbook	1

Shipping and Storage

The EGFR RGQ PCR Kit is shipped on dry ice and must still be frozen on arrival. If the EGFR RGQ PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, 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).

The EGFR RGQ PCR Kit should be stored immediately upon receipt at –15°C to –25°C in a constant-temperature freezer and protected from light.

Make sure to protect the EGFR RGQ PCR Kit from sunlight.

When stored under the recommended storage conditions in the original packaging, the kit is stable until the expiration date stated on the label. Repeated thawing and freezing should be avoided. We recommend a maximum of 7 freeze-thaw cycles.

To ensure optimal activity and performance, Scorpions® (as with all fluorescently labeled molecules) must be protected from light to avoid photo bleaching.

Product Use Limitations

The EGFR 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.

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

Product Warranty and Satisfaction Guarantee

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

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

Quality Control

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

Technical Assistance

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

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

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

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles.

All chemicals and biological material must be considered as potentially hazardous. Specimens are potentially infectious and must be treated accordingly.

Discard sample and assay waste in accordance with your local safety regulations.

24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

The EGFR RGQ PCR Kit constitutes a ready-to-use kit for the detection of 29 somatic mutations in the EGFR oncogene using polymerase chain reaction (PCR) on the Rotor-Gene Q instrument. Using Scorpions and ARMS® technologies, the EGFR RGQ PCR Kit enables detection of the following mutations against a background of wild-type genomic DNA.

- 19 deletions in exon 19 (detects the presence of any of 19 deletions but does not distinguish between them)
- T790M
- L858R
- L861Q
- G719X (detects the presence of G719S, G719A, or G719C but does not distinguish between them)
- S768I
- 3 insertions in exon 20 (detects the presence of any of 3 insertions but does not distinguish between them)

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

Principle

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

ARMS

Allele- or mutation-specific amplification is achieved by ARMS (Amplification Refractory Mutation System). *Taq* DNA polymerase is effective at distinguishing between a match and a mismatch at the 3' end of a PCR primer. Specific mutated sequences are selectively amplified, even in samples where the majority of the sequences do not carry the mutation. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low-level background amplification occurs.

Scorpions

Detection of amplification is performed using Scorpions. Scorpions are bi-functional 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. During PCR, when the probe binds to the

amplicon, the fluorophore and quencher become separated. This leads to an increase in fluorescence from the reaction tube.

Procedure

The EGFR RGQ PCR Kit comprises a two-step procedure. The first step is performance of the control assay to assess the total DNA in a sample. The second step is to complete the mutation assay for the presence or absence of mutated DNA.

Equipment and Reagents to Be Supplied by User

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

- DNA isolation kit (see "DNA isolation", page 11)
- Dedicated pipets (adjustable) for PCR master mix preparation*
- Dedicated pipets (adjustable) for dispensing of template DNA*
- Sterile pipet tips with filters
- Benchtop centrifuge* with rotor for 2 ml reaction tubes
- Rotor-Gene Q 5plex HRM Instrument* with fluorescence channels for Cycling Green and Cycling Yellow
- Rotor-Gene Q software, version 2.0.2
- Strip Tubes and Caps, 0.1 ml, for use with 72-well rotor (cat. no. 981103 or 981106)
- Sterile microcentrifuge tubes for preparing master mixes

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

Important Notes

General precautions

The user should always pay attention to the following:

- Use sterile pipet tips with filters and make sure that pipets have been calibrated according to the manufacturer's instructions.
- 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.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by inverting each tube 10 times) and centrifuge briefly.

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.

Kit format

Eight assays are supplied in the EGFR RGQ PCR Kit:

- One control assay
- Seven mutation assays

All reaction mixes contain an internal control assay labeled with HEX™. This controls for the presence of inhibitors that may lead to false negative results.

Reagents for the EGFR 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 EGFR RGQ PCR Kit are formulated specifically for use with the stated tests. All reagents supplied in the EGFR RGQ PCR Kit are intended to be used solely with the other reagents in the same EGFR RGQ PCR Kit. Substitutions to the reagents in the kit must not be made if optimal performance is to be maintained.

Only use the *Taq* DNA polymerase that is 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.

Assays

Control assay

The control assay, labeled with FAM™, is used to assess the total DNA in a sample. The control assay amplifies a region of exon 2 of the EGFR gene. The primer and probe have been designed to avoid any known EGFR polymorphisms.

Mutation assays

Each mutation assay, labeled with FAM, contains one Scorpion probe plus primers for discrimination between the wild-type DNA and the mutant DNA detected by a real-time PCR assay.

Sample material

All samples must be treated as potentially infectious material.

Sample material must be human genomic DNA, extracted from formalin-fixed paraffin-embedded non-small cell lung tumor samples. Specimens must be transported according to standard pathology methodology to ensure specimen quality.

Tumor samples are non-homogeneous 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 the EGFR mutations detected by the EGFR RGQ PCR Kit.

DNA isolation

We recommend using the QIAamp® DNA FFPE Tissue Kit (QIAGEN, cat. no. 56404) for genomic DNA purification from formalin-fixed paraffin-embedded non-small cell lung cancer samples. Carry out the DNA purification according to the instructions in the *QIAamp DNA FFPE Tissue Kit Handbook* with the following changes:

- Collect FFPE sections on glass slides.
- Scrape excess paraffin away from around the tissue sections using a fresh, sterile scalpel.
- Scrape tissue sections into microcentrifuge tubes using a fresh scalpel for each sample to be extracted.
- Proteinase K digestion should be performed for 1 hour.
- Purified genomic DNA must be eluted in 200 µl of Buffer ATE (provided in the QIAamp DNA FFPE Tissue Kit).

- Store purified genomic DNA at –15°C to –25°C.

DNA assessment should be based on PCR and may differ from quantification based on absorbance readings. Additional control reaction mix is supplied to enable assessment of quality and quantity of the DNA in samples before analysis with the EGFR RGQ PCR Kit.

All assays in the EGFR RGQ PCR Kit generate short PCR products. However, the EGFR RGQ PCR Kit will not work with heavily fragmented DNA.

Controls

Internal control

The assays contain an internal control in addition to the reaction of interest (see "Assays", page 11). If both assays have failed, the data should automatically be discarded as there may be inhibitors present that could lead to false negative results. Diluting the sample may reduce the effect of inhibitors but it should be noted that this would also dilute the DNA.

All experimental runs must contain controls.

Sample assessment

We strongly recommend using the extra control assay mix supplied with the EGFR RGQ PCR Kit to assess the total DNA in a sample. The control assay amplifies a region of exon 2 of the EGFR gene. We recommend setting samples up with only the control assay using the EGFR positive control as a positive control and water as the no template control (NTC). Note that to obtain optimal use of the reagents in the EGFR RGQ PCR Kit, samples should be batched. If samples are tested individually this will use up more reagents and reduce the number of samples that can be tested with the EGFR RGQ PCR Kit.

Data analysis

Scorpions real-time assays use the number of PCR cycles necessary to detect a fluorescent signal above a background signal as a measure of the target molecules present at the beginning of the reaction. The point at which the signal is detected above background fluorescence is called the cycle threshold (C_T).

Sample ΔC_T values are calculated as the difference between the mutation assay C_T and control assay C_T from the same sample. Samples are classed as mutation positive if they give a ΔC_T less than the cut-off ΔC_T value for that assay.

Above this value, the sample may either contain less than the percentage of mutation able to be detected by the kit (beyond the limit of the assays), or the sample is mutation negative.

Mutation C_T values of 40 or above will be scored as negative or beyond the limits of the kit.

When using ARMS primers, some inefficient priming may occur, giving a very late background C_T from DNA not containing a mutation. All ΔC_T values calculated from background amplification will be greater than the cut-off ΔC_T values and the sample will be classed as mutation negative.

The EGFR RGQ PCR Kit is for use on the QIAGEN Rotor-Gene Q real-time PCR cycler, 72-well format.

Protocol 1: Sample Assessment

This protocol is to assess the total DNA in samples.

Important points before starting

- Before beginning the procedure, read "Important Notes", pages 10–13.
- Take time to familiarize yourself with the Rotor-Gene Q before starting the protocol. See the instrument user manual.

Things to do before starting

- Before each use, all reagents need to be thawed completely, mixed (by inverting 10 times), and centrifuged briefly.
- Ensure that *Taq* DNA polymerase is at room temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

Procedure

1. **Thaw the control reaction mix and EGFR positive control at room temperature (15–25°C). When the control reaction mix and EGFR positive control have thawed, mix them by inverting each tube 10 times to avoid localized concentrations of salts.**
2. **Prepare sufficient master mixes (control reaction mix plus *Taq* DNA polymerase) for the DNA samples, one positive control reaction, and one no template control reaction according to the volumes given in Table 1. Include reagents for 2 extra samples.**

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

   Do not vortex *Taq* DNA polymerase as this may inactivate the enzyme.

   Make sure that *Taq* DNA polymerase is at room temperature before use. Briefly centrifuge the vial to ensure that all enzyme is at the bottom of the tube. Pipet *Taq* DNA polymerase by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.

   Make sure to set up reactions correctly.

Table 1. Preparation of control master mix

	Master mix	
Assay	**Control reaction mix***	***Taq* DNA polymerase***
Control assay	19.5 µl	0.5 µl

* When preparing the master mix, prepare enough for 2 extra samples.

3. **Mix the master mix by gently pipetting up and down. Immediately add 20 µl master mix to each Rotor-Gene tube.**
4. **Immediately add 5 µl sample, EGFR positive control, or nuclease-free water (for the NTC) to each Rotor-Gene tube.**
5. **Close the PCR tubes and place them into the appropriate positions in the Rotor-Disc™. If the rotor is not evenly loaded, balance with additional empty Rotor-Gene Q tubes.**
6. **Immediately place the Rotor-Disc 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.**
7. **For sample assessment, create a temperature profile according to the following steps.**

Setting the general assay parameters	**Figures 1, 2, 3**
Initial activation of the hot-start enzyme	**Figures 4, 5**
Amplification of the DNA	**Figures 6, 7, 8, 9**
Adjusting the fluorescence channels	**Figures 10, 11, 12, 13**
Starting the run	**Figure 14**

All specifications refer to the Rotor-Gene Q software version 2.0.2. Please find further information on programming Rotor-Gene Q Instruments in the instrument user manual. In the illustrations, these settings are framed in bold black. Illustrations are included for Rotor-Gene Q Instruments.

8. **Double-click the Rotor-Gene Q Series Software 2.0.2 software icon on the desktop of the PC connected to the Rotor-Gene Q Instrument. Select the "Advanced" tab in the "New Run" dialog box that appears.**
9. **To create a new template, select "Empty Run" and then click "New" to enter the "New Run Wizard".**
10. **Select *72-Well Rotor* as the rotor type. Check that the locking ring is attached. Check the "Locking Ring Attached" box and click "Next" (Figure 1).**

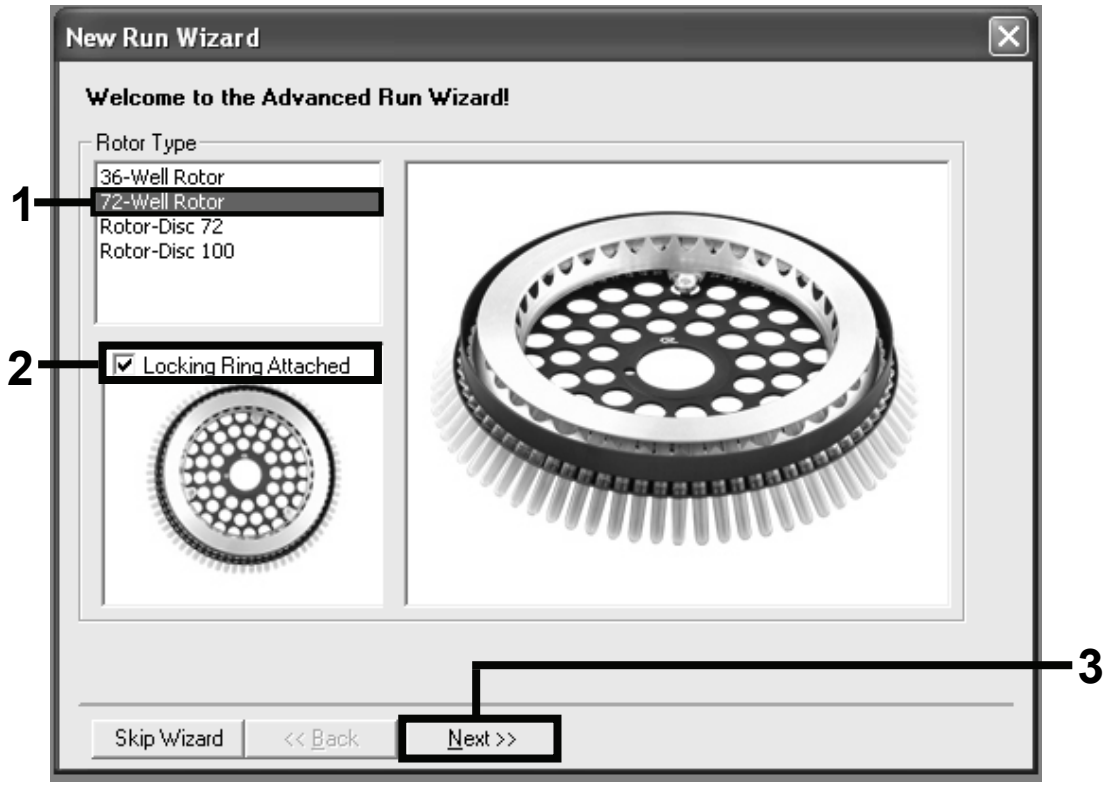


Figure 1. The "New Run Wizard" dialog box.

11. **Enter the name of the operator, enter *25 µl* for the reaction volume, and add any additional notes. Click "Next" (Figure 2).**

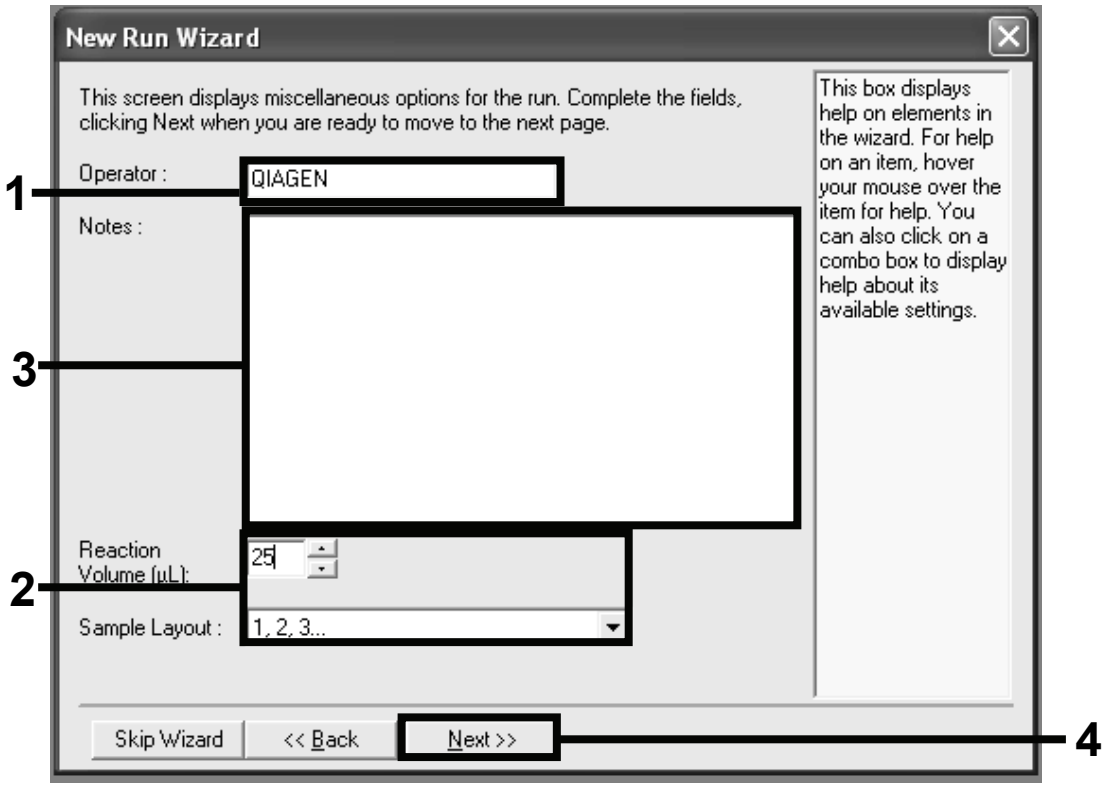


Figure 2. Setting the general assay parameters.

12. Click the "Edit Profile" button in the next "New Run Wizard" dialog box (Figure 3), and program the temperature profile according to the information in the following steps.

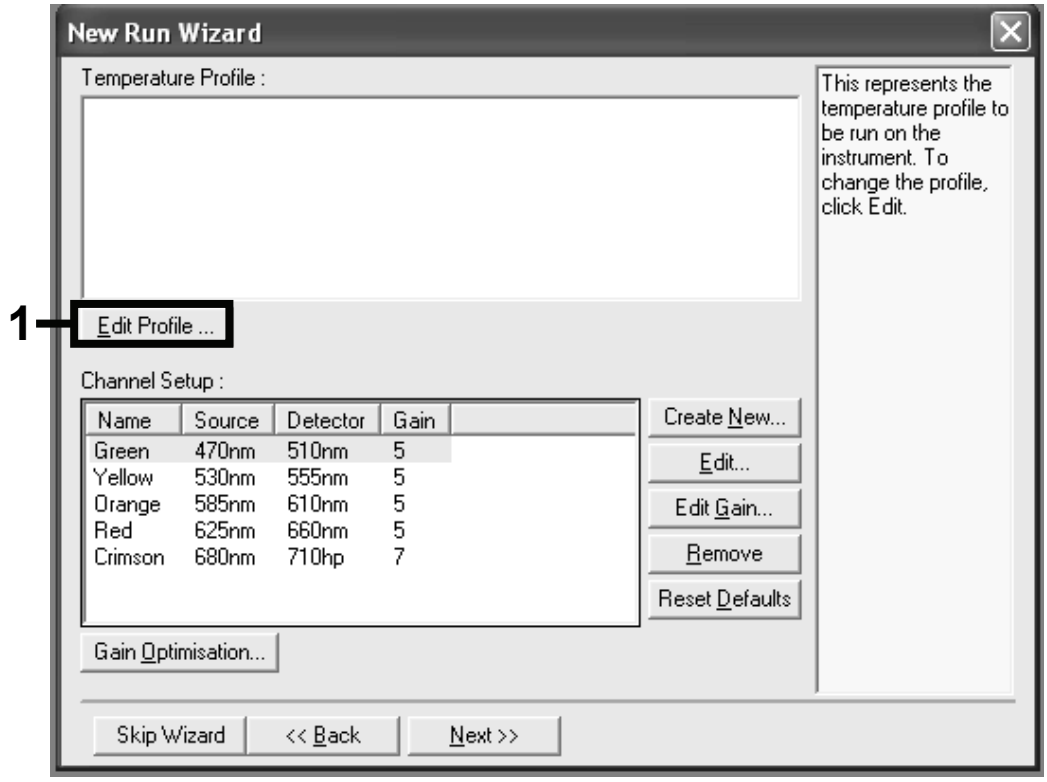


Figure 3. Editing the profile.

13. **Click the "Insert after" button and select "New Hold at Temperature" (Figure 4). Click "60" to change the temperature to 95°C.**

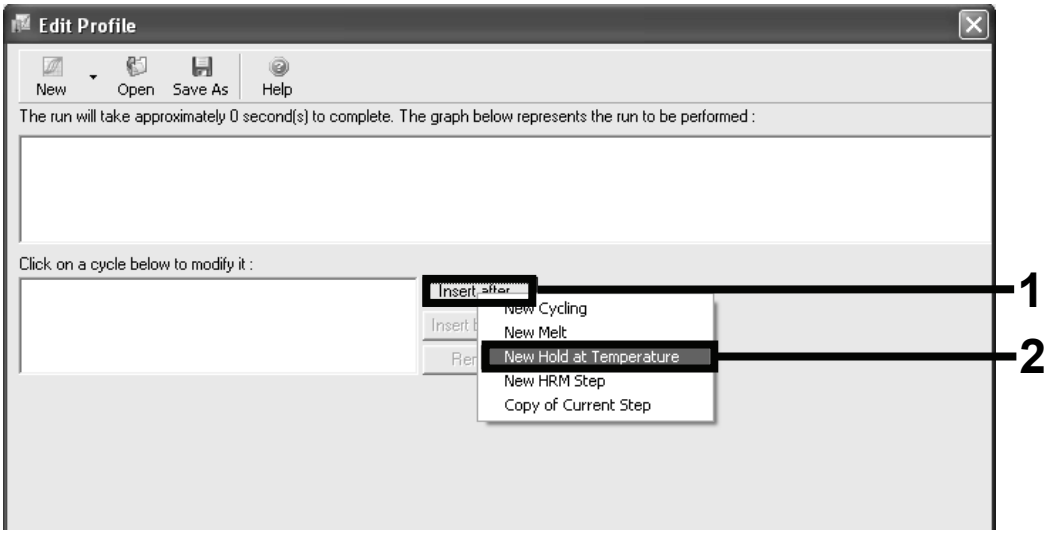


Figure 4. Initial incubation step at 95°C.

14. **Click "60°C" and change the "Hold Temperature" to 95°C, click "1" to change the "Hold Time" to 15 mins. Click the "Insert After" button and then select "New Cycling" (Figure 5).**

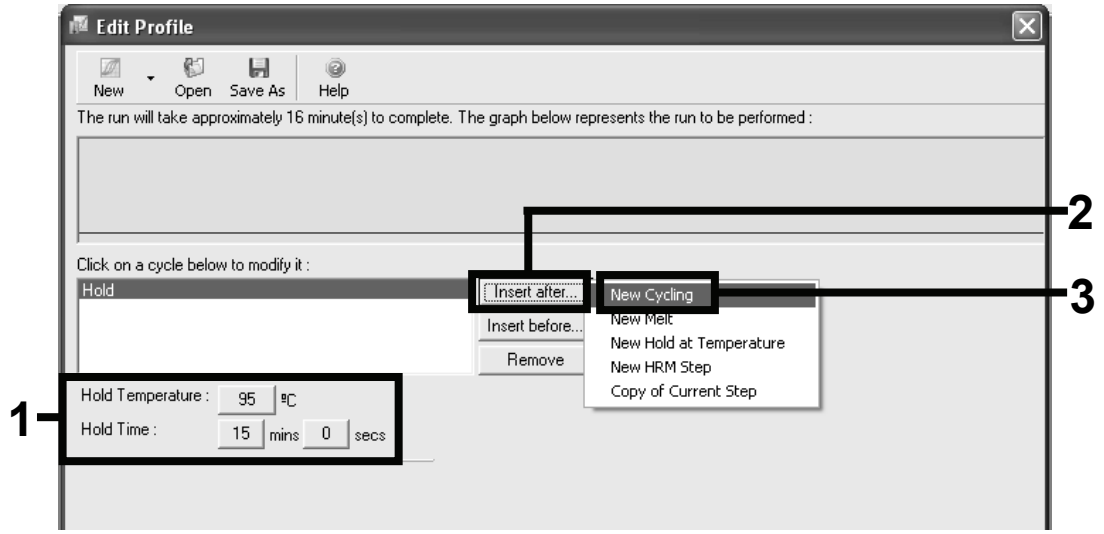


Figure 5. Initial incubation step at 95°C.

15. **Set the cycle number to 40 by clicking the number. Select "95°C for 20 seconds". Set the time to "30 secs" (Figure 6).**

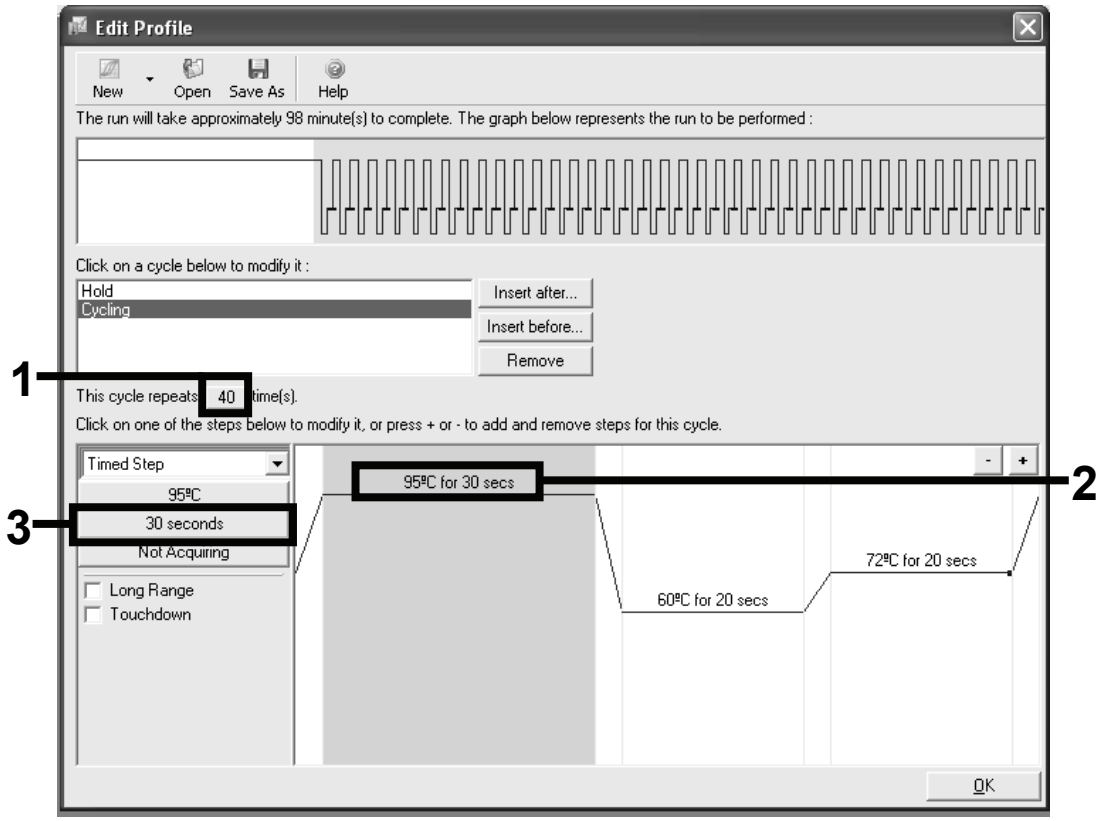


Figure 6. Cycling step at 95°C.

16. Highlight "60°C for 20 secs". Set the time to 60 seconds. Select the "Not Acquiring" button (Figure 7).

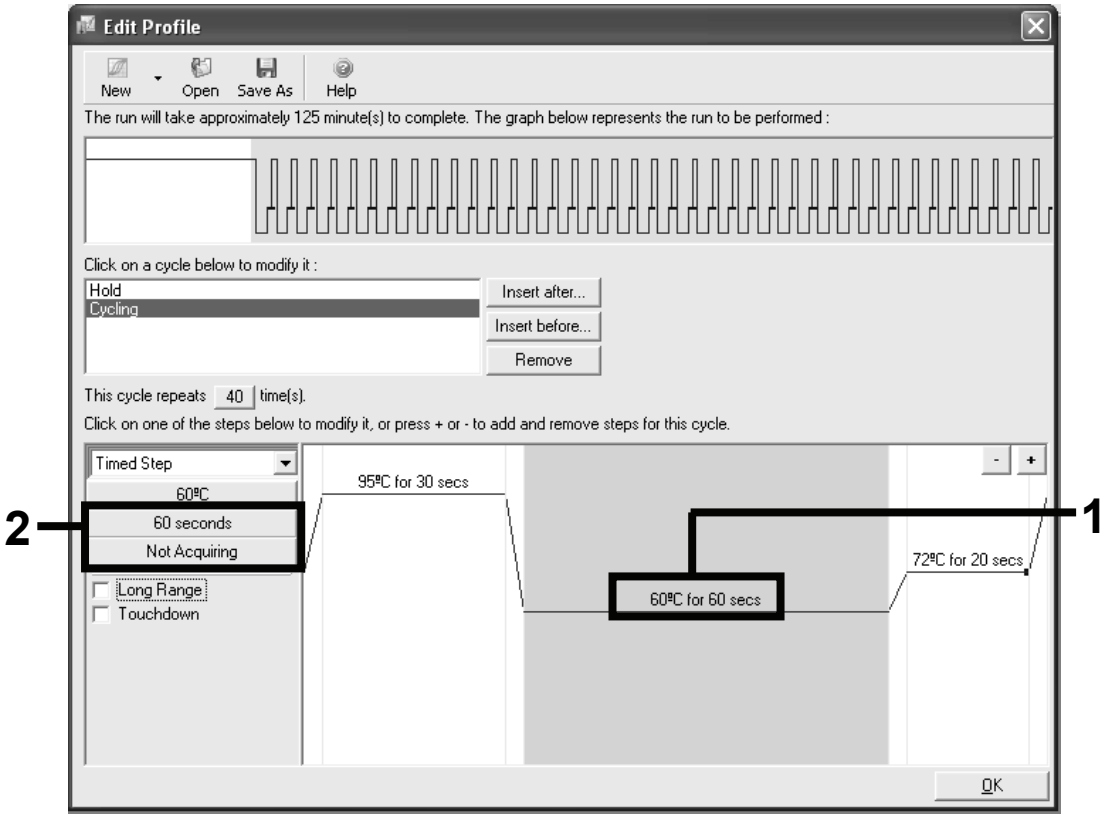


Figure 7. Cycling step at 60°C.

17. **In the "Available Channels" panel, select the "Yellow" and "Green" channels to acquire by highlighting them and then click ">" to transfer them to the "Acquiring Channels" panel (Figure 8).**

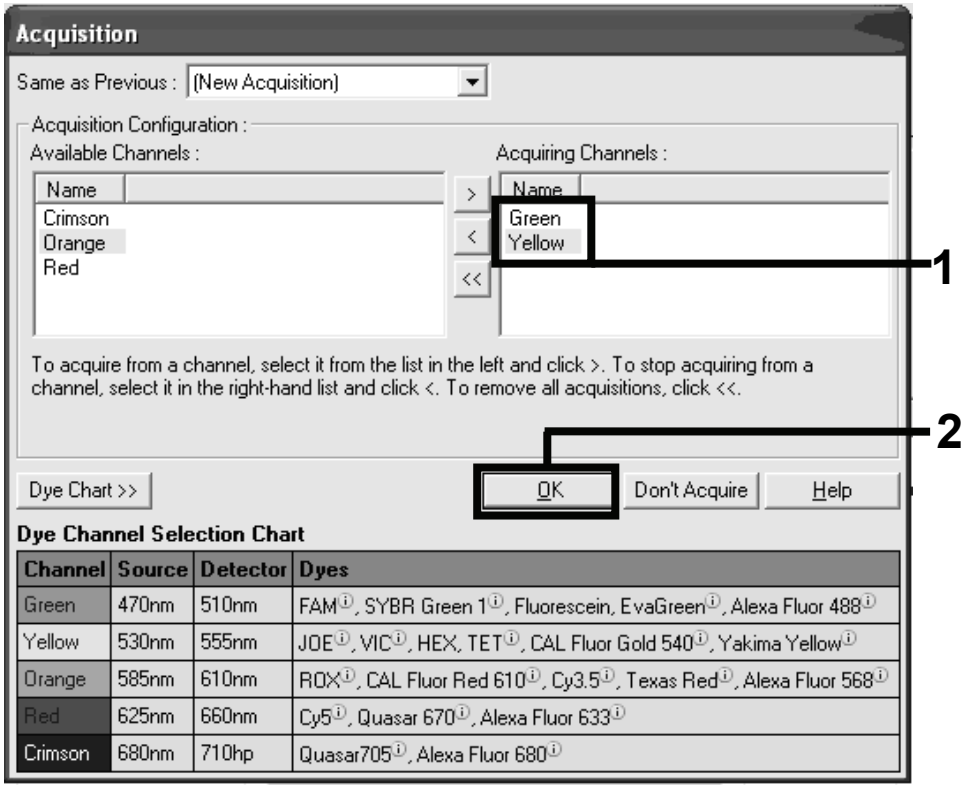


Figure 8. Acquiring at cycling step of 60°C.

18. **Highlight "72°C for 20 secs" and delete this section, then click "OK" (Figure 9).**

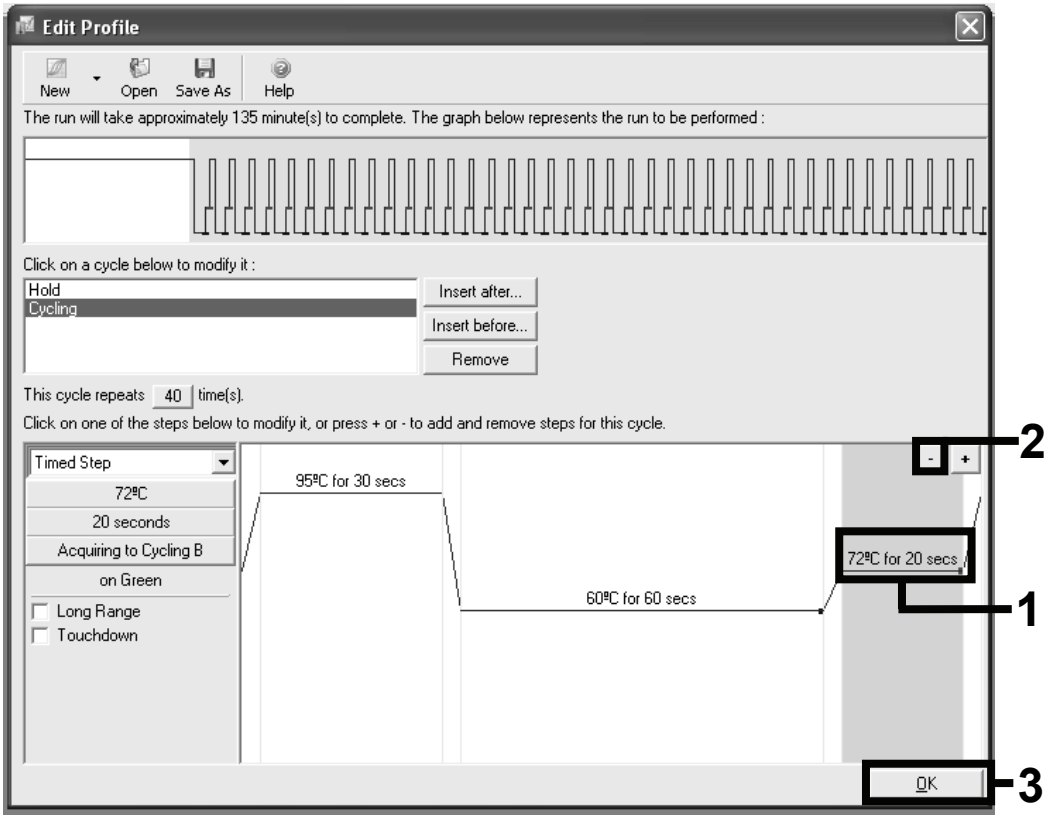


Figure 9. Removal of extension step.

19. Click the "Gain Optimisation" button (Figure 10).

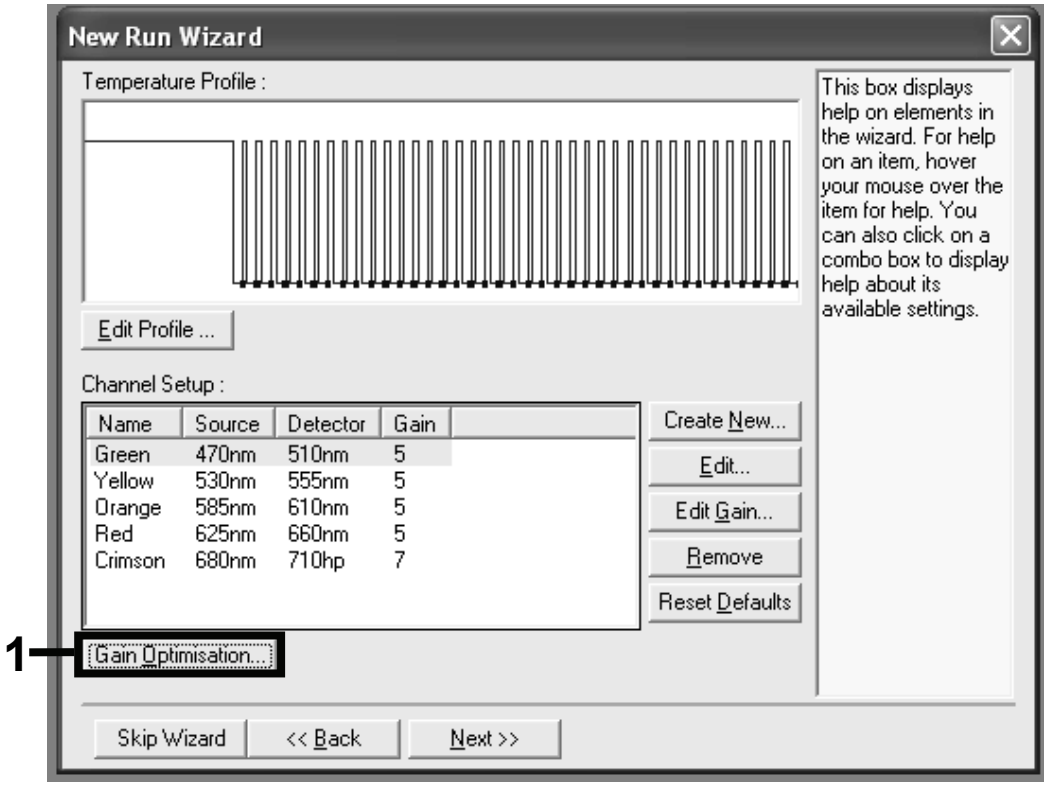


Figure 10. Gain optimisation.

20. Select "Optimise Acquiring" button, and then click "OK for both the green and yellow channels (Figures 11 and 12).

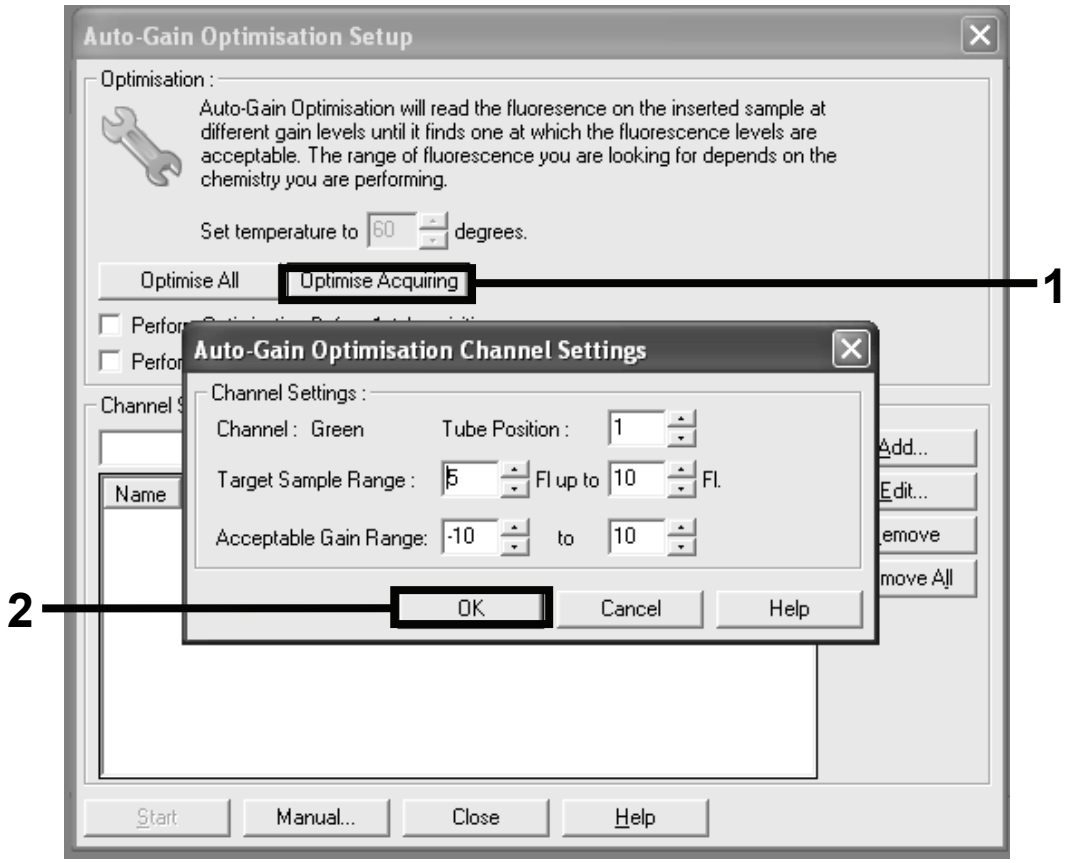


Figure 11. Auto-Gain optimisation for the green channel.

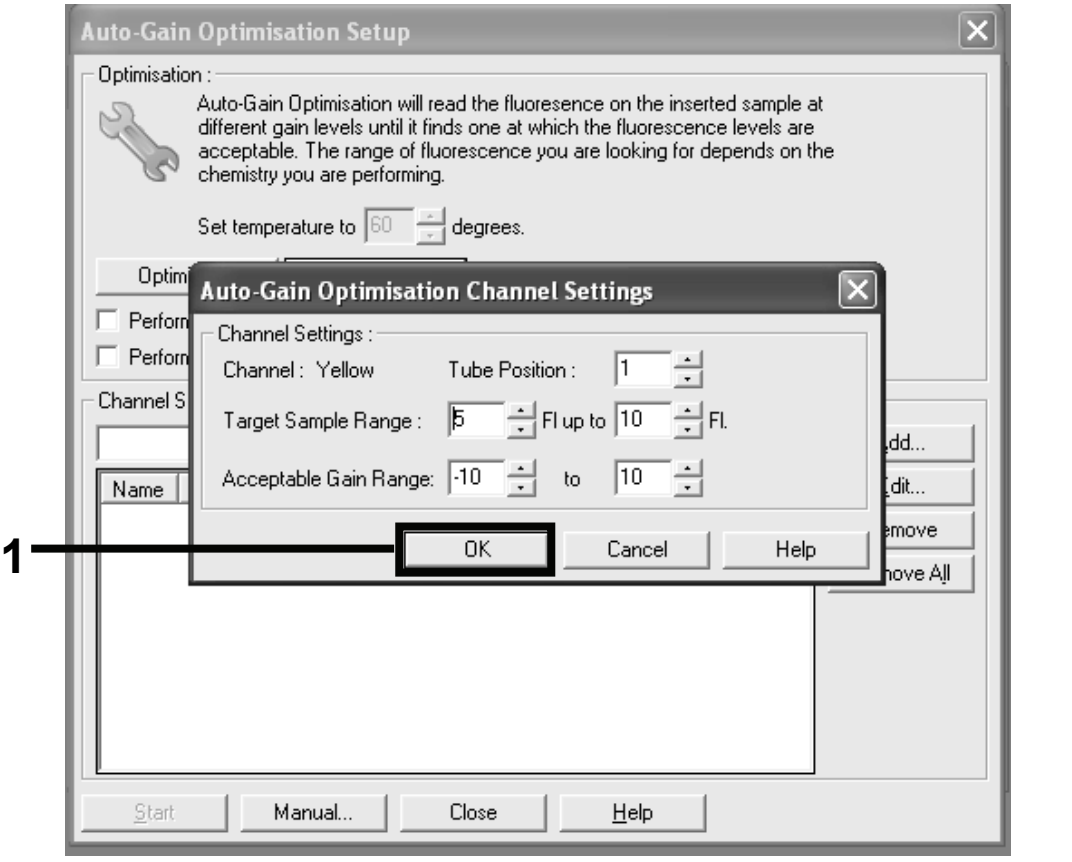


Figure 12. Auto-Gain optimisation for the yellow channel.

21. **Check the box for "Perform Optimisation before 1st Acquisition", and then click the "Close" button to return to the wizard (Figure 13).**

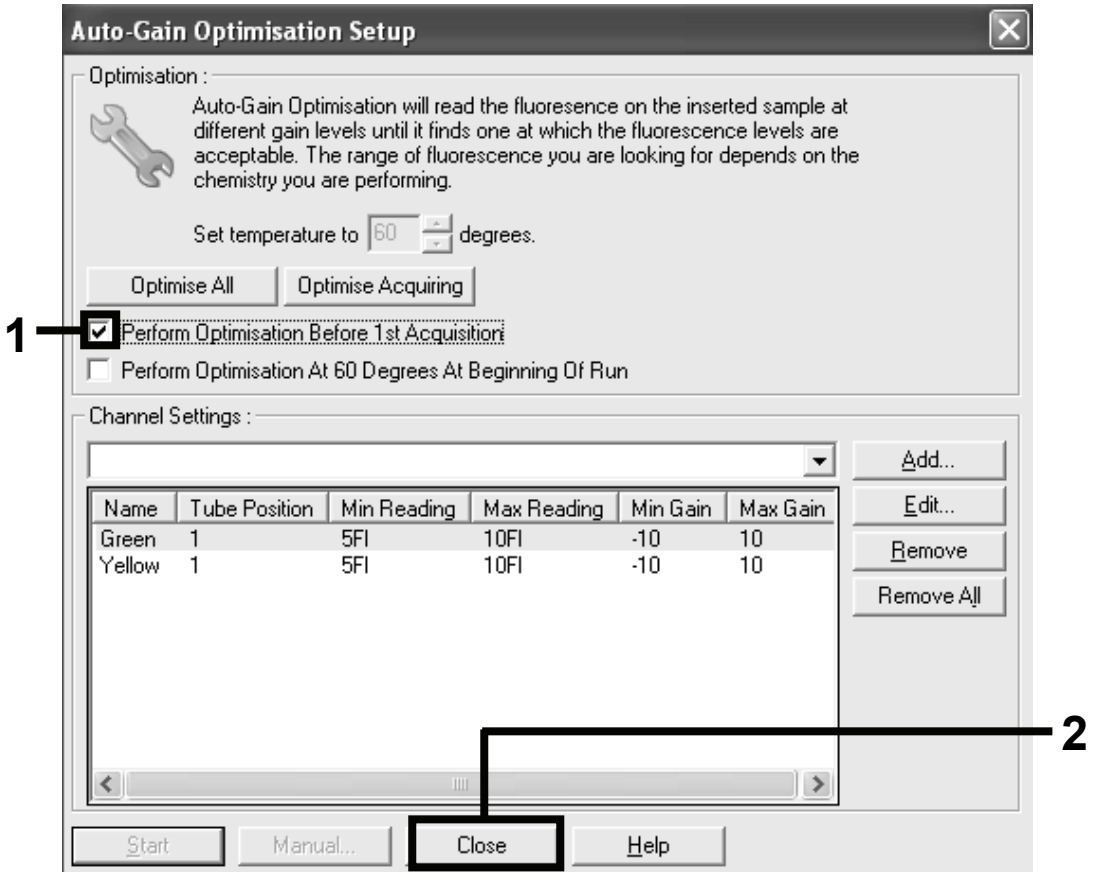


Figure 13. Selection of green and yellow channels.

22. **Click "Next" to save the template, select "Save Template", and then save the template in the templates folder.**
23. **Check the summary and then click "Start Run" to save the run file and start the run (Figure 14).**

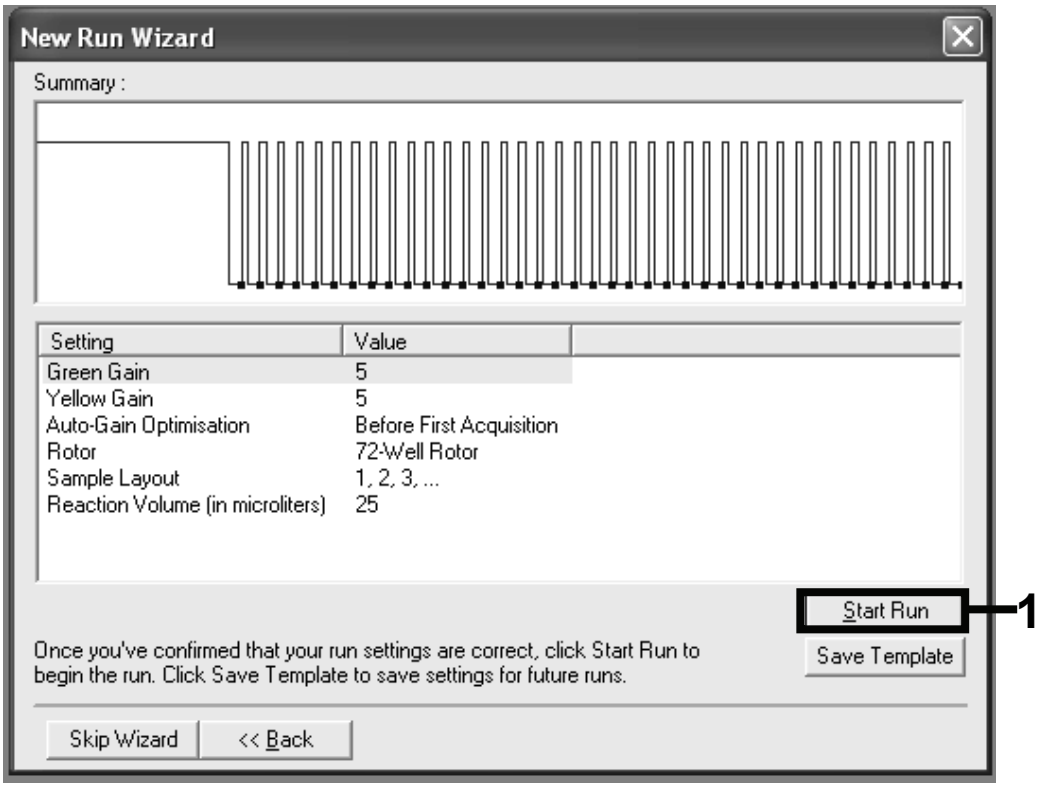


Figure 14. Starting the run.

24. **After the run starts a new window appears in which you can either enter sample names now or click "Finish" and enter them later.**
25. **Save all data to the appropriate folder.**
26. **After the run is finished, analyze the data.**
27. **Assess the NTC C_T values to ensure that there is no contamination giving positive amplification in the FAM channel (C_T less than 40) or a failed internal control in the HEX channel (no C_T), indicating a setup problem. The EGFR positive control (PC) must give a control assay C_T (FAM channel) between 26.26–30.95.**

    See Appendix A for more information about data analysis.

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

    Sample control assay C_T of 30.69–37.00: Interpret with caution as very low level mutations may not be detected.

    Sample control assay C_T of 37.00–40.00: Only a few amplifiable copies of DNA are present in such samples and mutations are only likely to be seen if most copies are mutated.

    Note that if a sample gives a late control assay C_T, the sample internal control C_T must be compared with the internal control of the NTC. If the internal control of the sample is delayed or negative, compared to the NTC, an inhibitor may be present. It is possible to reduce the effect of an inhibitor by diluting the sample, although this will also dilute the DNA.

Sample dilution: A control C_T of <23.00 will overload the mutation assays. Samples with a control C_T of <23.00 must be diluted. To see each mutation at a low level, the concentrated samples must be diluted to fall within the range >23.00 but <30.69, on the basis that diluting by half will increase the C_T by 1.

Protocol 2: Detection of EGFR Mutations and Data Analysis

This protocol is for detection of EGFR mutations and data analysis.

Important points before starting

- Before beginning the procedure, read "Important Notes", pages 10–13.
- Take time to familiarize yourself with the Rotor-Gene Q before starting the protocol. See the instrument user manual.
- For efficient use of the EGFR RGQ PCR Kit, samples must be grouped into batches of 7 (to fill the 72-well rotor). Smaller batch sizes will mean that fewer samples can be tested with the EGFR RGQ PCR Kit.
- For each DNA sample, the control and mutation assays must be analyzed in the same PCR run to avoid run-to-run variations.

Things to do before starting

- Before each use, all reagents need to be thawed completely, mixed (by inverting 10 times), and centrifuged briefly.
- Ensure that *Taq* DNA polymerase is at room temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

Procedure

1. **Thaw the reaction mixes and EGFR positive control at room temperature (15–25°C). When the reaction mixes and EGFR positive control have thawed, mix them by inverting each tube 10 times to avoid localized concentrations of salts.**
2. **Prepare sufficient master mixes (control reaction mix plus *Taq* DNA polymerase) for the DNA samples, one positive control reaction, and one no template control reaction according to the volumes given in Table 2. Include reagents for 2 extra samples.**

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

   Do not vortex *Taq* DNA polymerase as this may inactivate the enzyme.

Make sure that *Taq* DNA polymerase is at room temperature before use. Briefly centrifuge the vial to ensure that all enzyme is at the bottom of the tube. Pipet *Taq* DNA polymerase (by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme).

Make sure to set up reactions correctly.

Table 2. Preparation of control master mix

	Master mix	
Assay	**Control reaction mix***	***Taq* DNA polymerase***
Control assay	19.5 µl	0.5 µl
Mutation assay	19.5 µl	0.5 µl

* When preparing the master mix, prepare enough for 2 extra samples.

3. **Mix the master mix by gently pipetting up and down. Immediately add 20 µl master mix to each Rotor-Gene tube.**
4. **Immediately add 5 µl sample, EGFR positive control, or nuclease-free water (for the NTC) to each Rotor-Gene tube. Each DNA sample must be tested with both the control and all mutation assays. The layout for this is shown in Table 3.**

Table 3. Layout of control and mutation assays

	Controls		Sample number						
	PC	**NTC**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Ctrl	1	9	17	25	33	41	49	57	65
T790M	2	10	18	26	34	42	50	58	66
Deletions	3	11	19	27	35	43	51	59	67
L858R	4	12	20	28	36	44	52	60	68
L861Q	5	13	21	29	37	45	53	61	69
G719X	6	14	22	30	38	46	54	62	70
S768I	7	15	23	31	39	47	55	63	71
Ins	8	16	24	32	40	48	56	64	72

5. **Close the Rotor-Gene Q tubes and place them into the appropriate positions in the Rotor-Disc. If the rotor is not evenly loaded, balance with additional empty Rotor-Gene Q tubes.**
6. **Immediately place the Rotor-Disc 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.**
7. **For sample assessment, create a temperature profile according to the following steps.**

Setting the general assay parameters	**Figures 15, 16, 17**
Initial activation of the hot-start enzyme	**Figure 18, 19**
Amplification of the DNA	**Figure 20, 21, 22, 23**
Adjusting the fluorescence channels	**Figures 24, 25, 26, 27**
Starting the run	**Figure 28**

All specifications refer to the Rotor-Gene Q software version 2.0.2. 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.

8. **Double-click the Rotor-Gene Q Series Software 2.0.2 software icon on the desktop of the PC connected to the Rotor-Gene Q Instrument. Select the "Advanced" tab in the "New Run" dialog box that appears.**
9. **To create a new template, select "Empty Run" and then click "New" to enter the "New Run Wizard".**
10. **Select *72-Well Rotor* as the rotor type. Check that the locking ring is attached. Check the "Locking Ring Attached" box and click "Next" (Figure 15).**

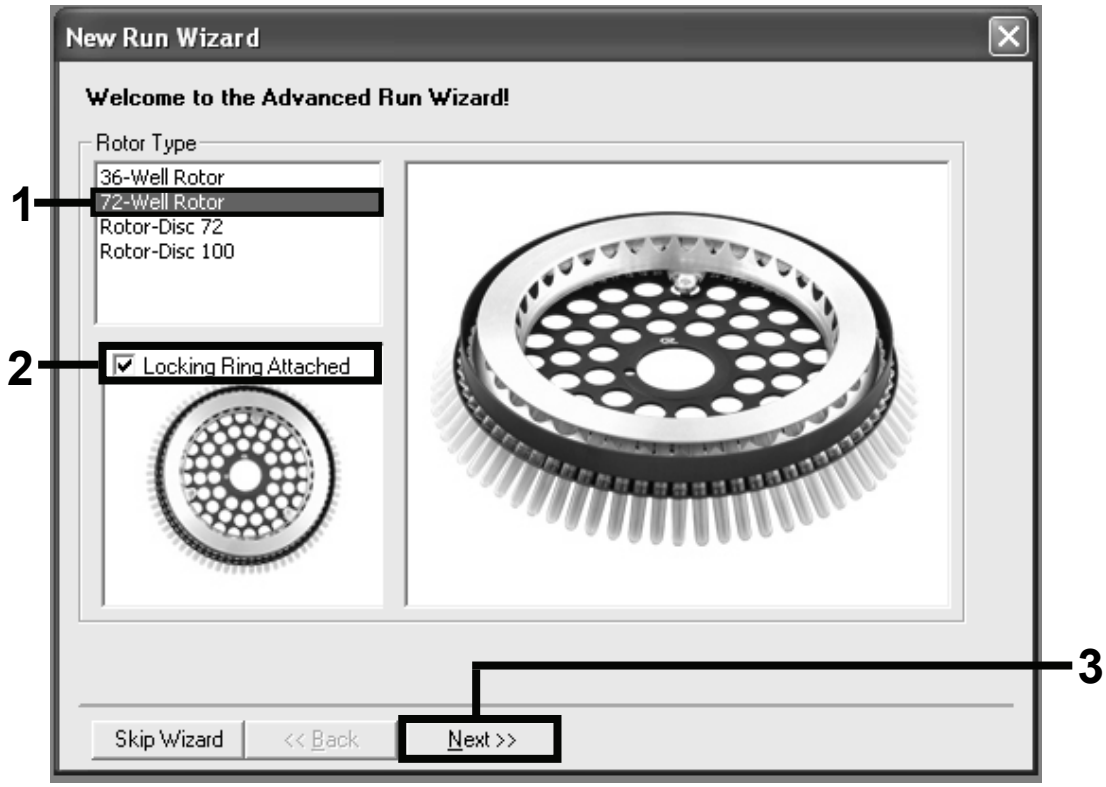


Figure 15. The "New Run Wizard" dialog box.

11. Enter the name of the operator, enter *25 µl* for the reaction volume, and add any additional notes. Click "Next" (Figure 16).

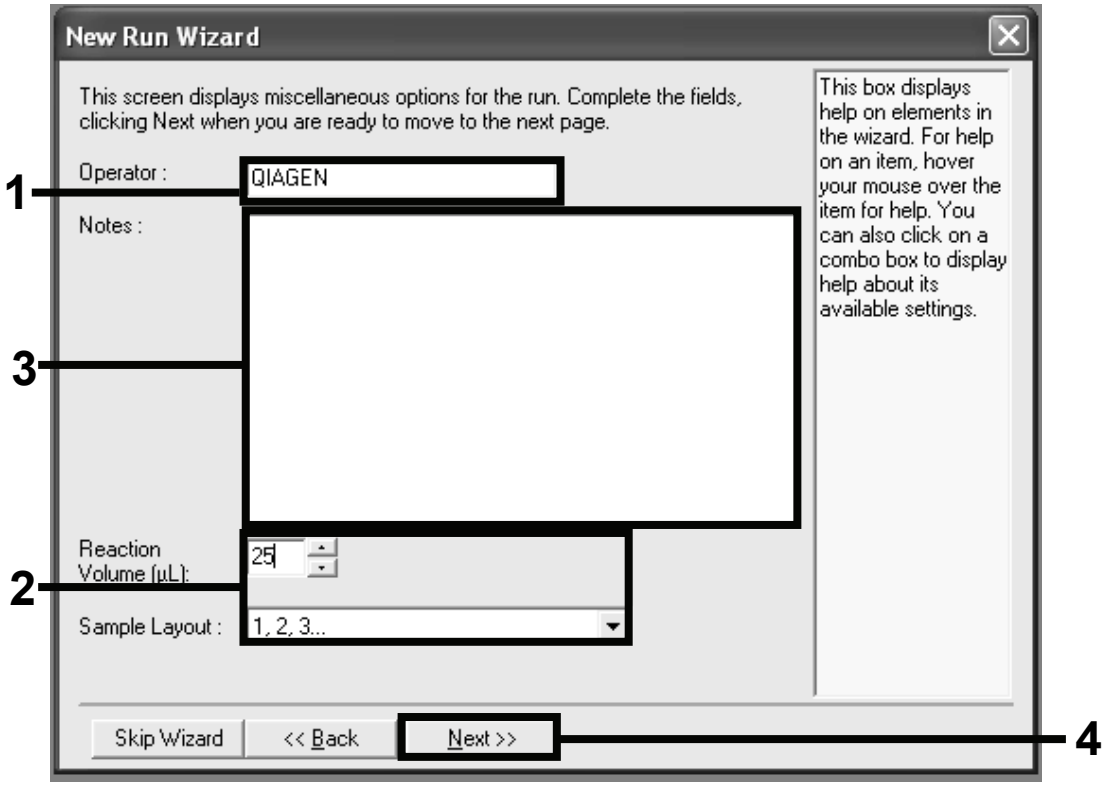


Figure 16. Setting the general assay parameters.

12. **Click the "Edit Profile" button in the next "New Run Wizard" dialog box (Figure 17), and program the temperature profile according to the information in the following steps.**

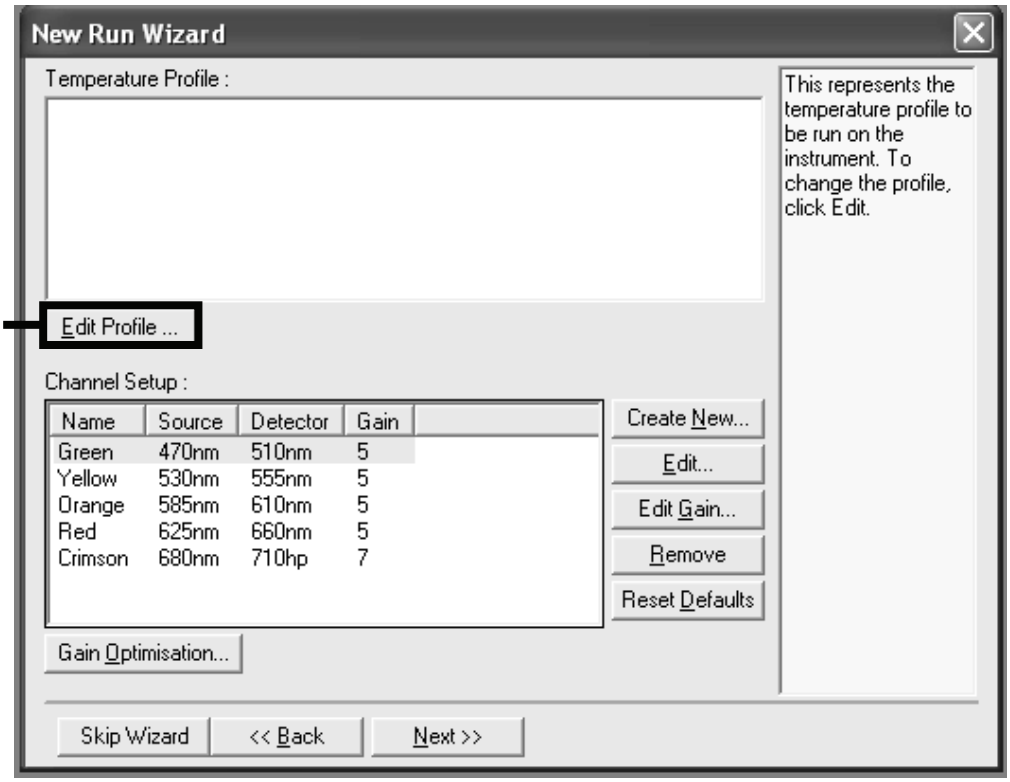


Figure 17. Editing the profile.

13. **Click the "Insert after" button and select "New Hold at Temperature" (Figure 18). Click "60" to change the temperature to 95°C.**

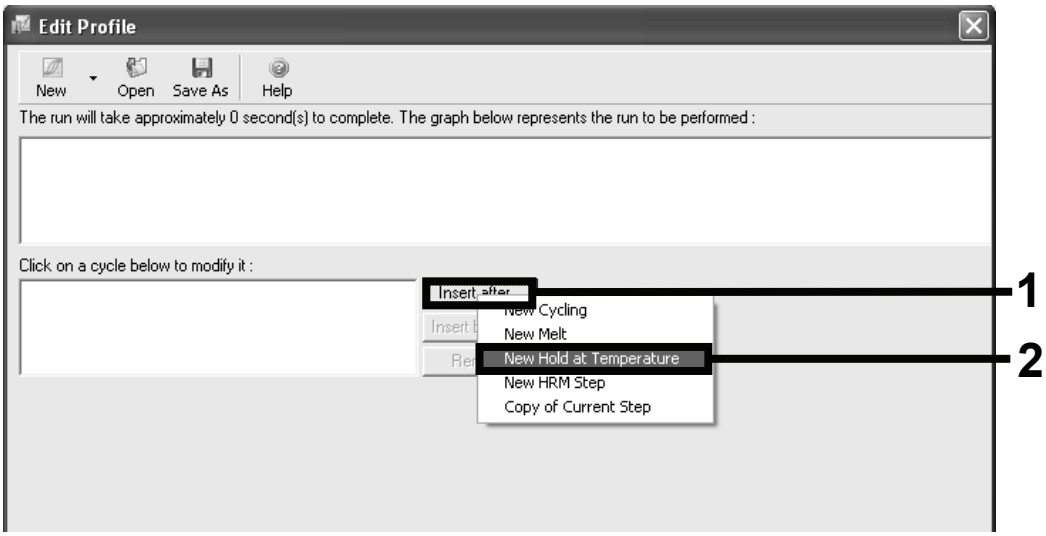


Figure 18. Initial incubation step at 95°C.

14. **Click "60°C" and change the "Hold Temperature" to 95°C, then click "1" to change the "Hold Time" to 15 mins. Click the "Insert After" button and then select "New Cycling" (Figure 19).**

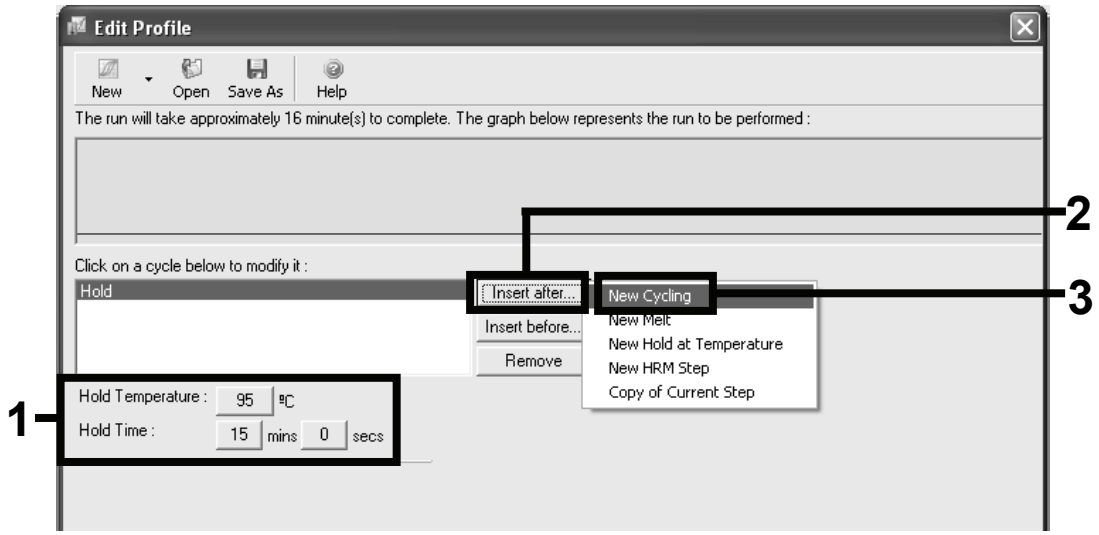


Figure 19. Initial incubation step at 95°C.

15. **Set the cycle number to 40 by clicking the number. Highlight "95°C for 20 secs". Set the time to *30 seconds* (Figure 20).**

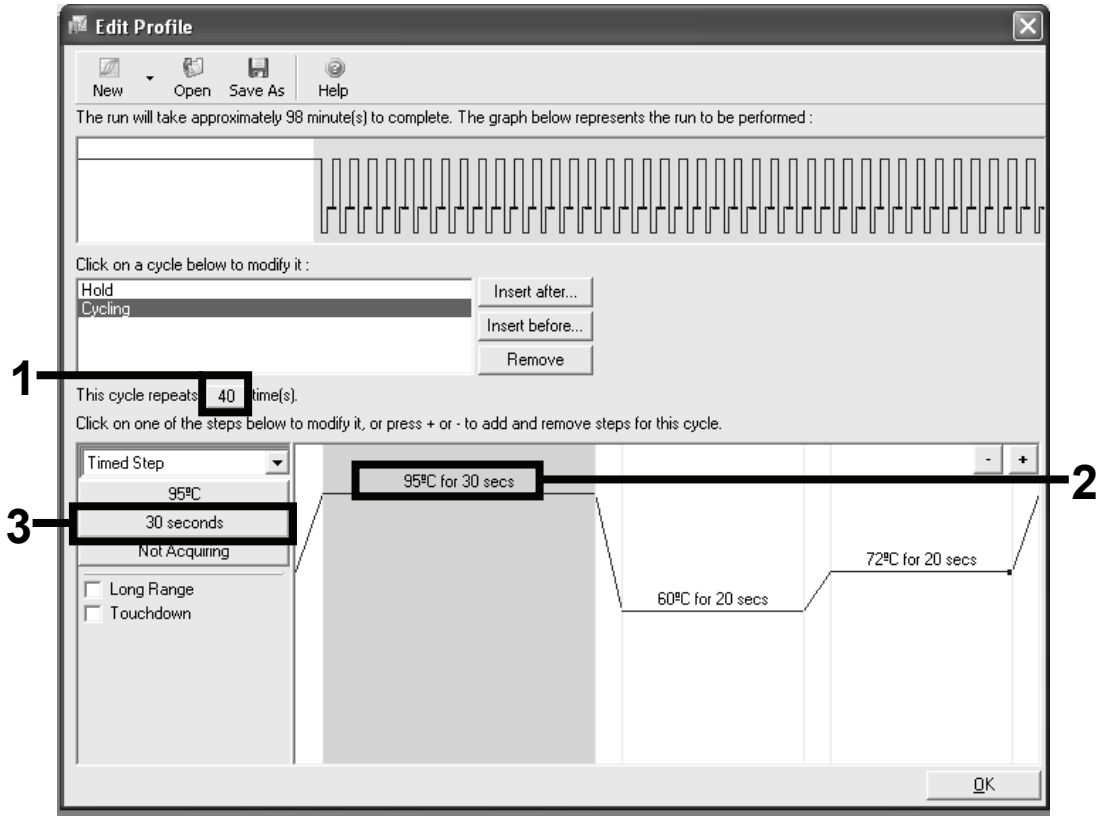


Figure 20. Cycling step at 95°C.

16. **Highlight "60°C for 20 secs". Set the time to *60 seconds*. Select the "Not Acquiring" button (Figure 21).**

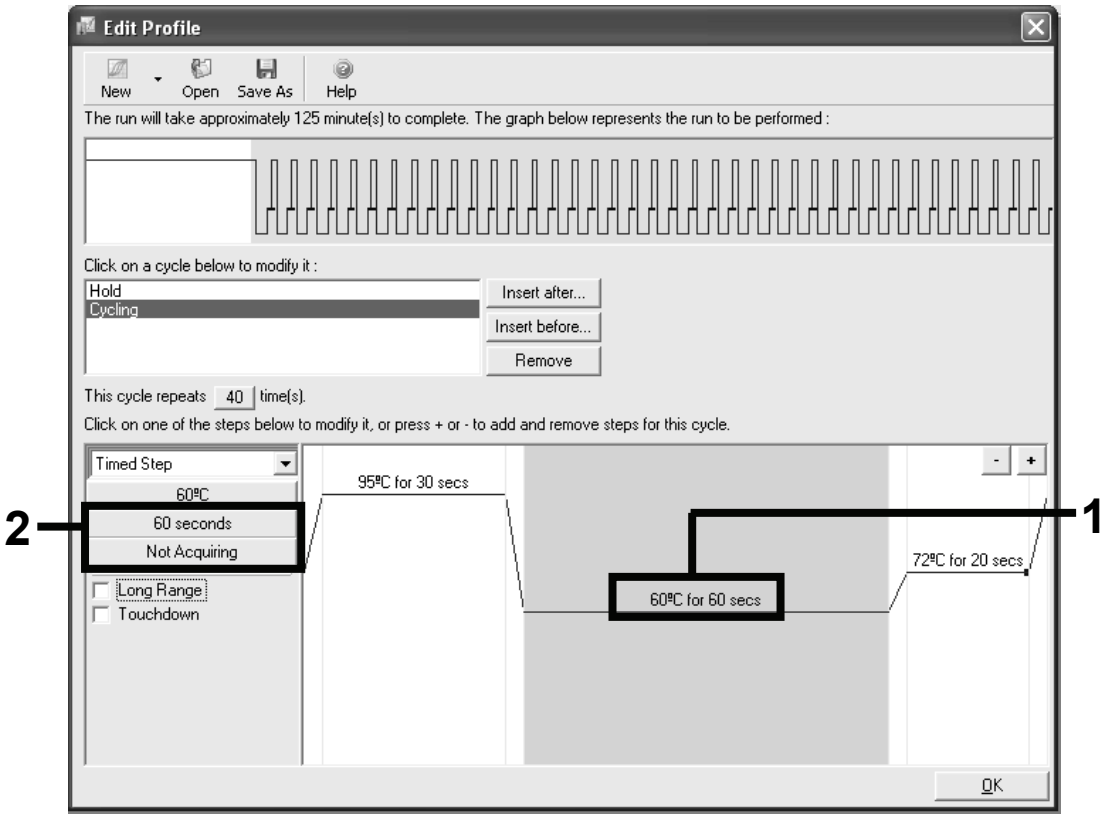
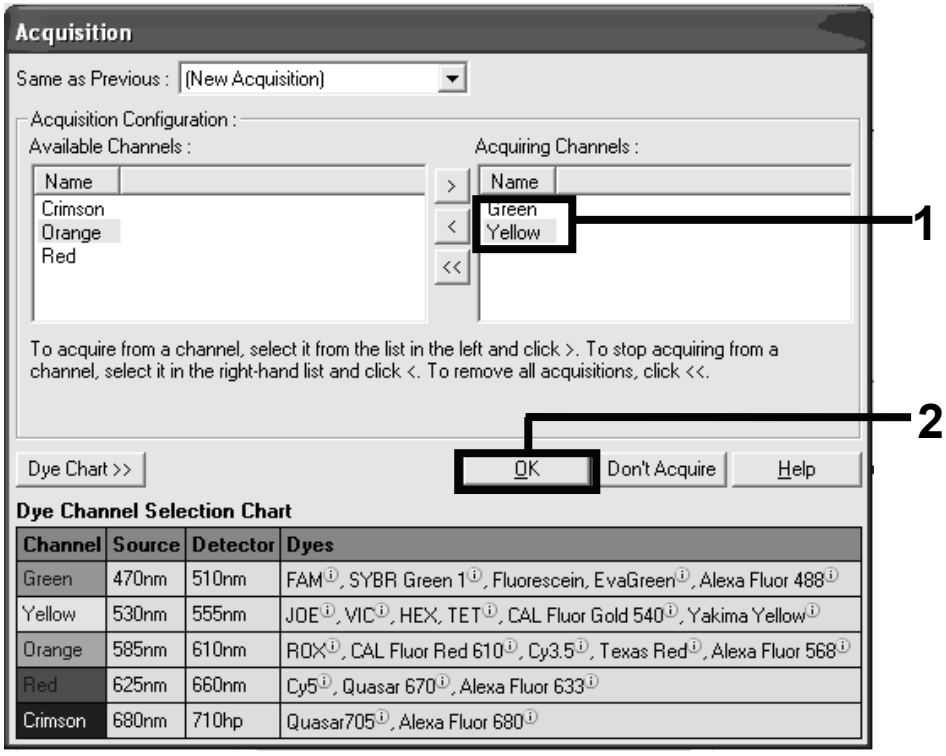


Figure 21. Cycling step at 60°C.

17. In the "Available Channels" panel, select the "Yellow" and "Green" channels to acquire by highlighting them and then click ">" to transfer them to the "Acquiring Channels" panel (Figure 22).



Channel	Source	Detector	Dyes
Green	470nm	510nm	FAM, SYBR Green 1, Fluorescein, EvaGreen, Alexa Fluor 488
Yellow	530nm	555nm	JOE, VIC, HEX, TET, CAL Fluor Gold 540, Yakima Yellow
Orange	585nm	610nm	ROX, CAL Fluor Red 610, Cy3.5, Texas Red, Alexa Fluor 568
Red	625nm	660nm	Cy5, Quasar 670, Alexa Fluor 633
Crimson	680nm	710hp	Quasar705, Alexa Fluor 680

Figure 22. Acquiring at cycling step of 60°C.

18. **Highlight "72°C for 20 secs" and delete this section, then click "OK" (Figure 23).**

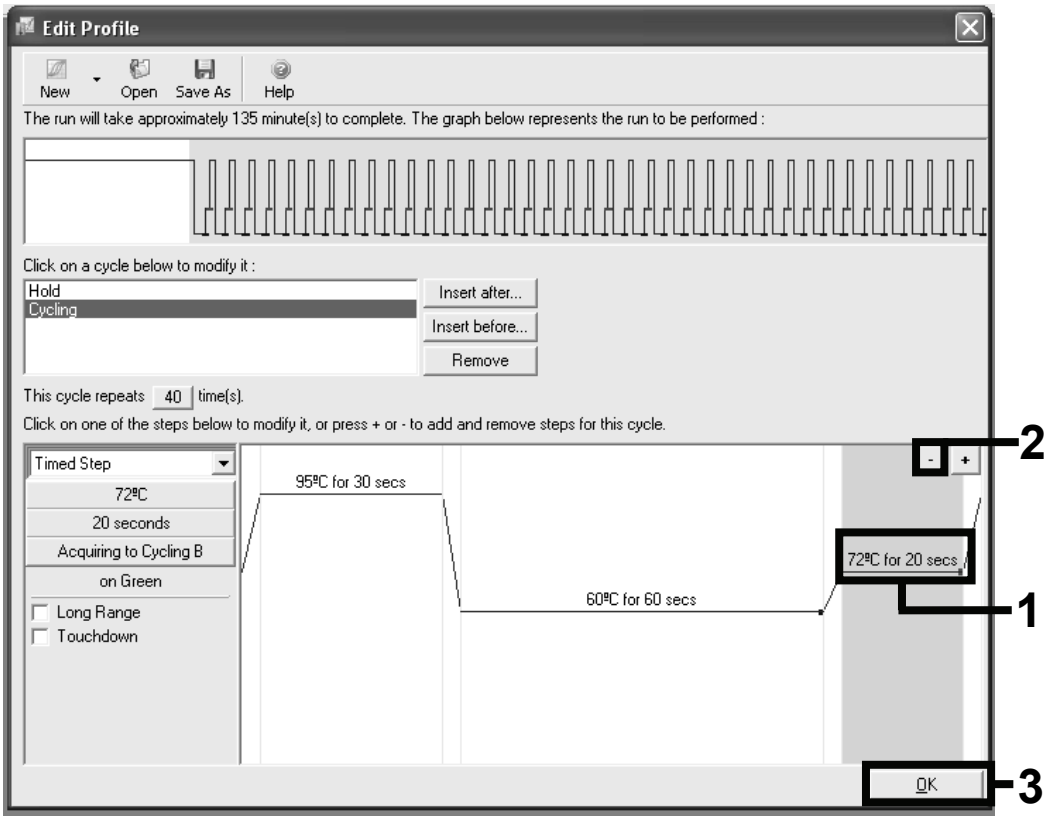


Figure 23. Removal of extension step.

19. **Click the "Gain Optimisation" button (Figure 24).**

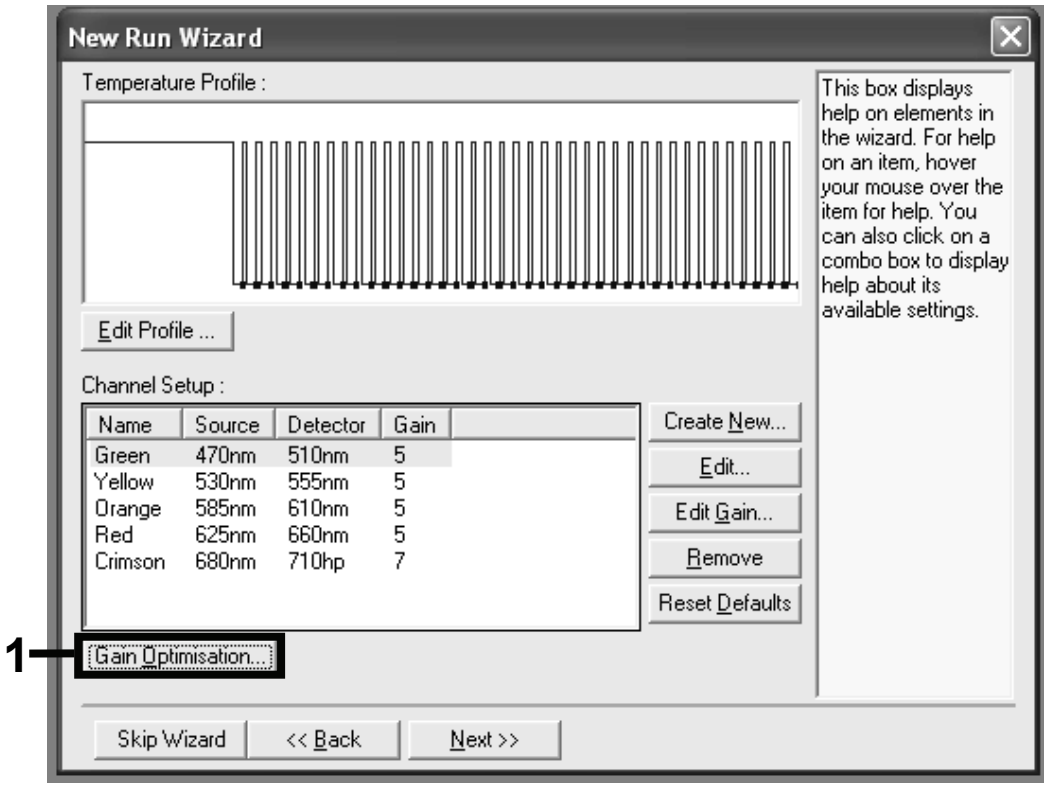


Figure 24. Gain optimisation.

20. Click "Optimise Acquiring" button, click "OK for both the green and yellow channels (Figures 25 and 26).

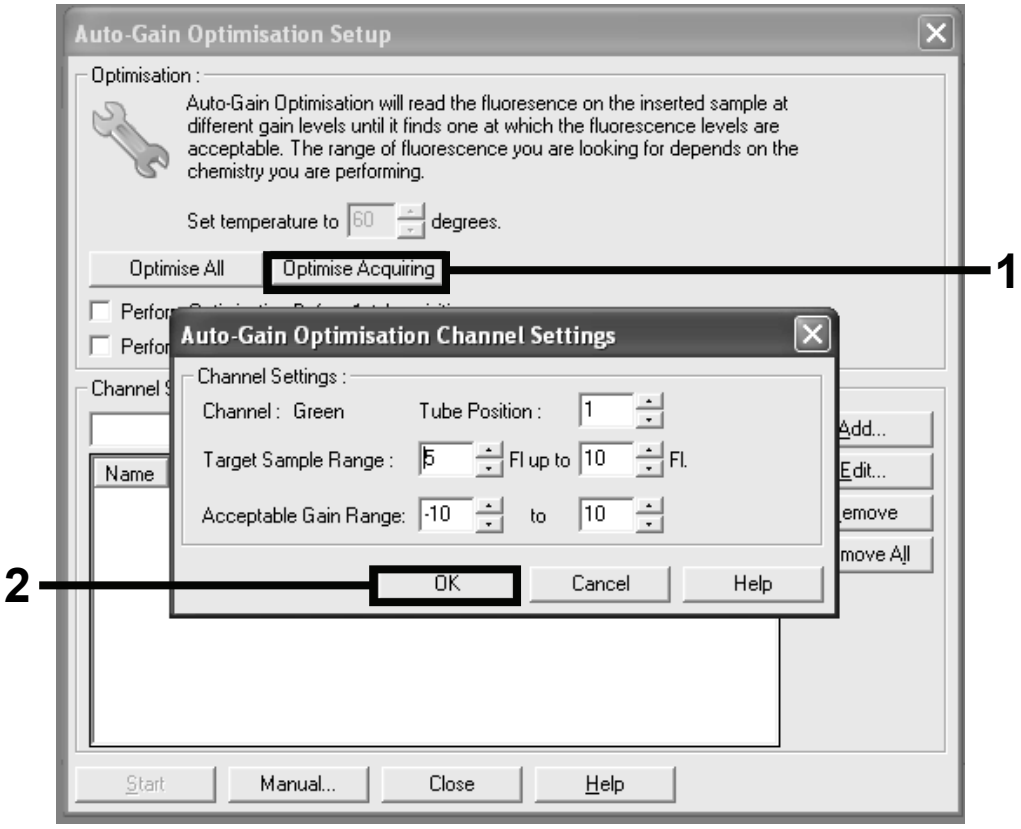


Figure 25. Auto-Gain optimisation for the green channel.

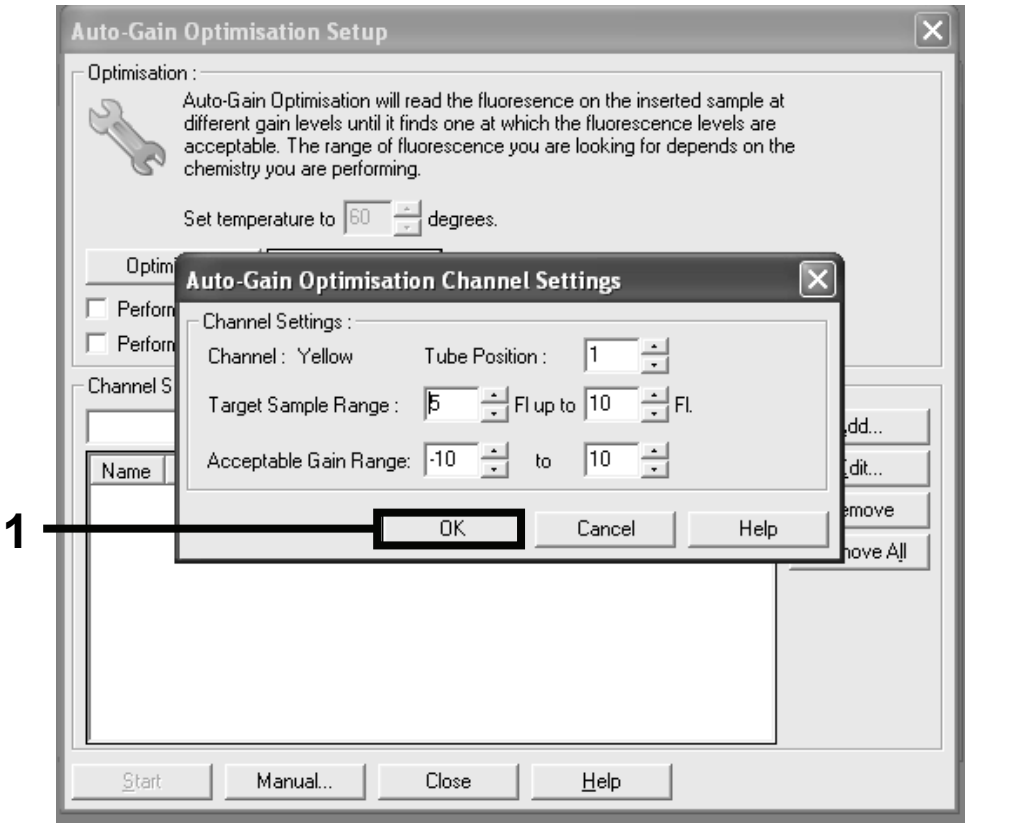


Figure 26. Auto-Gain optimisation for the yellow channel.

21. **Check the box for "Perform Optimisation before 1st Acquisition", and then click the "Close" button to return to the wizard (Figure 27).**

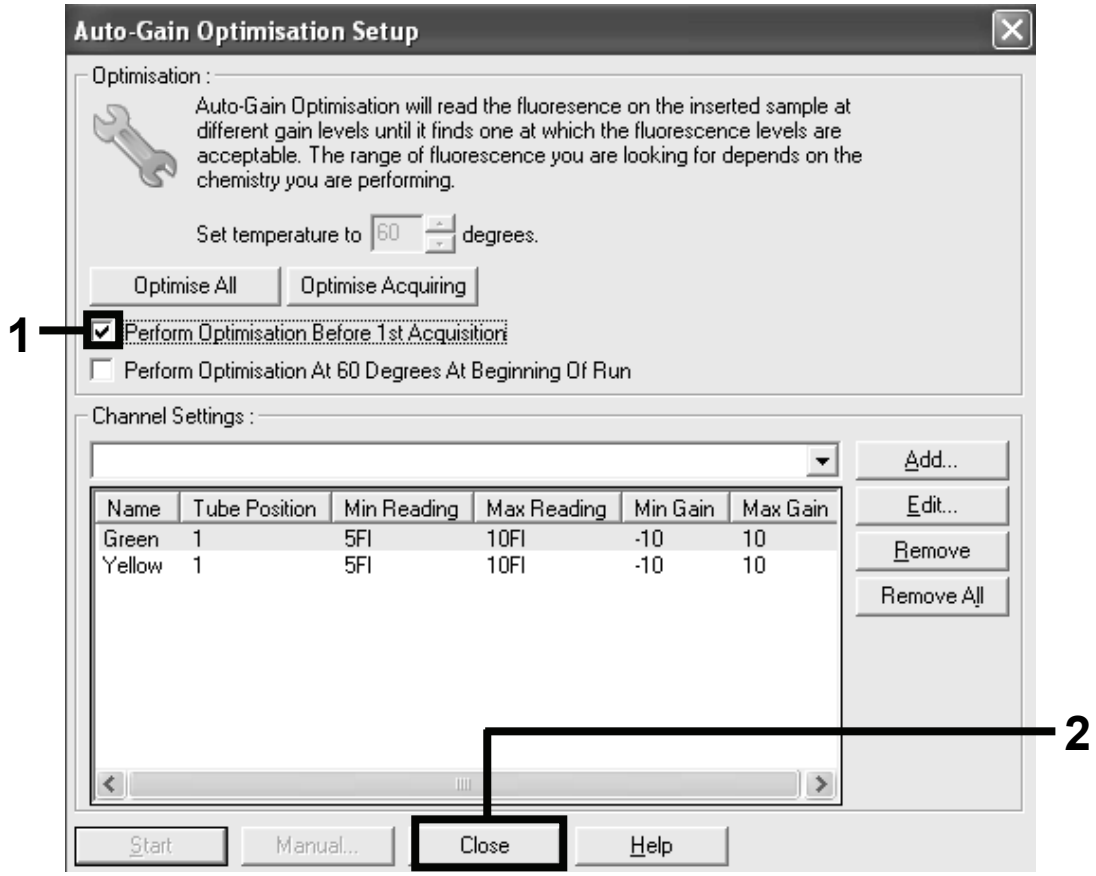


Figure 27. Selection of green and yellow channels.

22. **Click "Next" to save the template, select "Save Template", and then save the template in the templates folder.**
23. **Check the summary and then click "Start Run" to save the run file and start the run (Figure 28).**

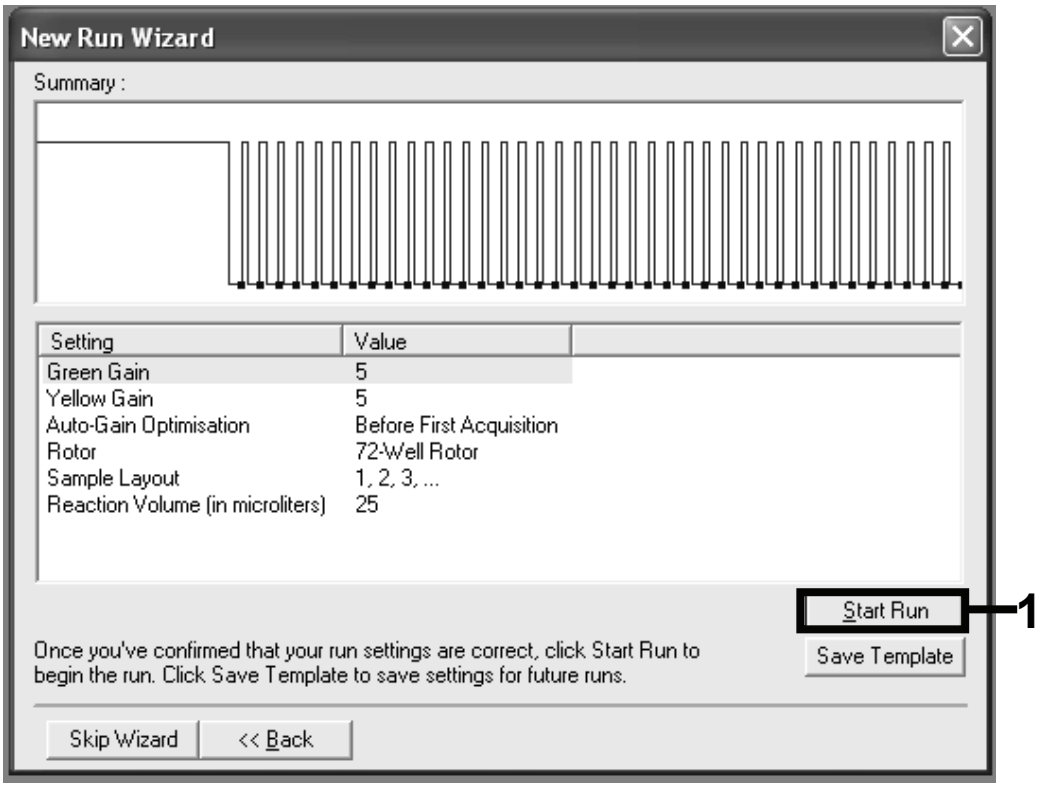


Figure 28. Starting the run.

24. **After the run starts a new window appears in which you can either enter sample names now or click "Finish" and enter them later.**
25. **Save all data to the appropriate folder.**
26. **After the run is finished, analyze the data.**

    See Appendix A for information about data analysis.

Troubleshooting Guide

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

	Comments and suggestions
No signal with EGFR positive control 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 EGFR PCR and the fluorescence channel Cycling Yellow for the internal control PCR.
b) Incorrect programming of the temperature profile of the Rotor-Gene Instrument	Compare the temperature profile with the protocol.
c) Incorrect configuration of the PCR	Check your work steps by means of the 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 "Shipping and Storage" (page 4)	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
e) The EGFR 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.

	Comments and suggestions
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.
b) Contamination occurred during extraction	Repeat the extraction and PCR of the sample to be tested using new reagents. Make sure that work space and instruments are decontaminated at regular intervals.

Appendix A: Data Analysis on the Rotor-Gene Q Instrument

Rotor-Gene Q plots from all reactions should be checked. Occasionally, an increase in fluorescence signal is seen in the no template control (NTC) and negative samples. If this is the case and a C_T value is obtained, the user is required to distinguish between a true amplification event, which would indicate contamination in the NTC and true amplification, and a linear increase in fluorescence, which may have arisen due to a fluorescence artifact.

Analysis of the NTC

Figures 29 and 30 show two examples of the behavior of NTCs with instructions indicating how the data from these types of plots should be handled. In Figure 29, non-linear, i.e. true amplification due to sample contamination is seen: this run should be discarded and the samples should be retested. In Figure 30, linear amplification in an NTC is seen. Under these circumstances, the raw fluorescence should be examined: the corresponding raw fluorescence plot is shown in Figure 31, indicating a linear increase in fluorescence, rather than a true amplification event. The data from this run can be used, providing the positive and internal controls have passed. For comparison with Figure 31, Figure 32 shows raw fluorescence data where true amplification has taken place. Under these circumstances, the data should be discarded and the samples retested, as this indicates contamination is present.

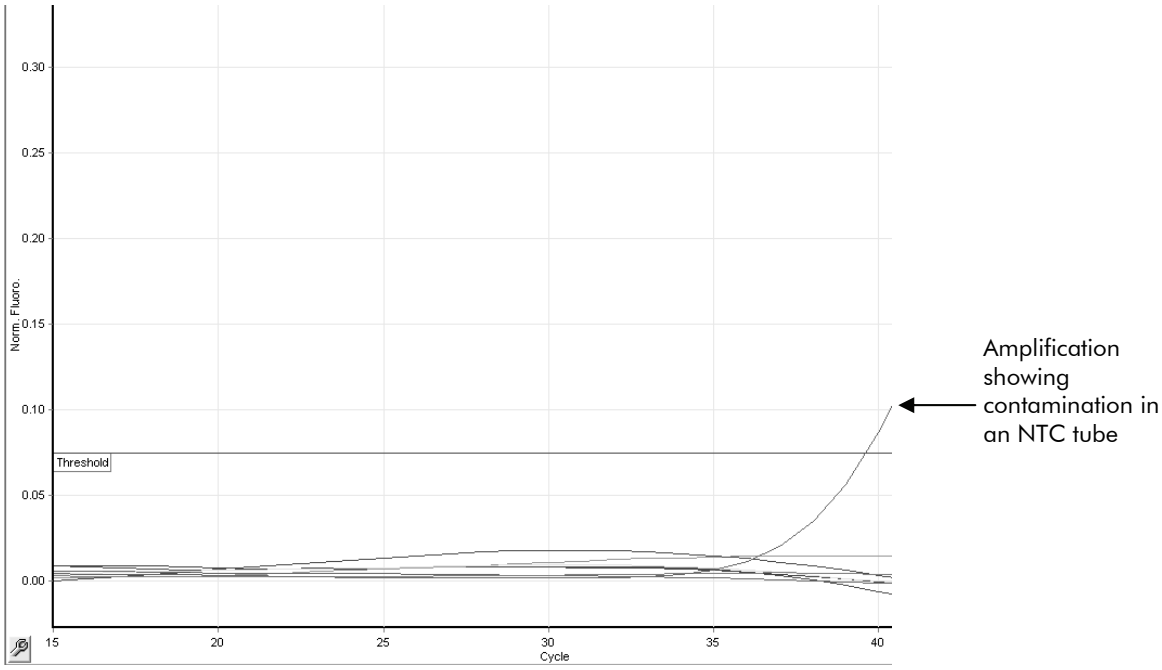


Figure 29. Contamination in an NTC of an assay in an analyzed run.

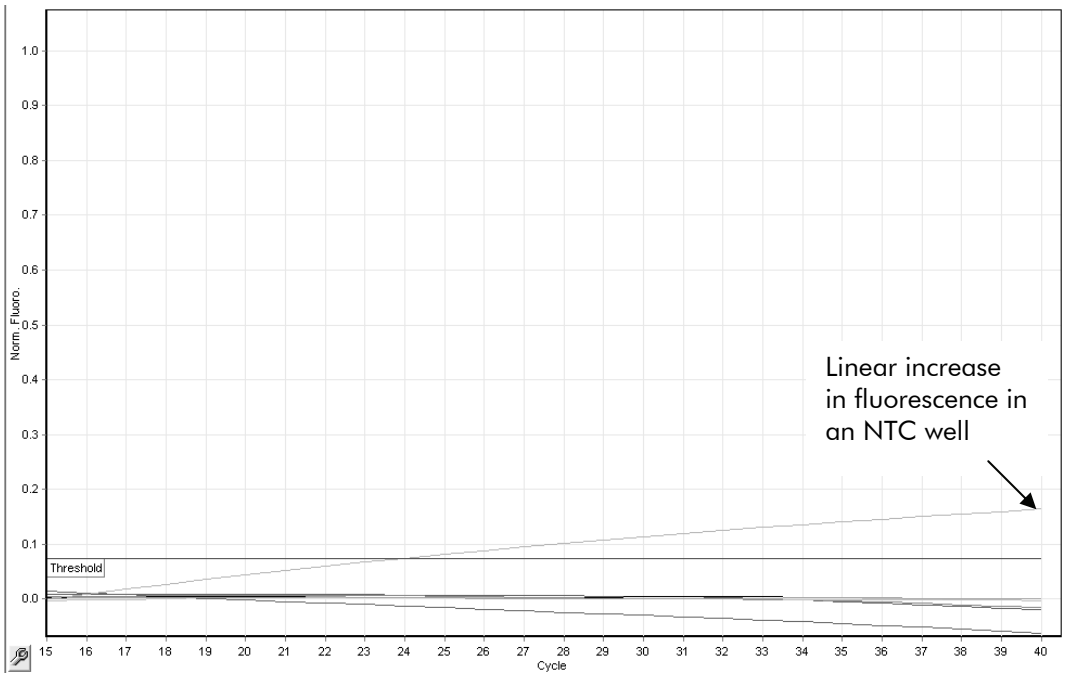


Figure 30. Example of a linear increase in fluorescence in an NTC well.

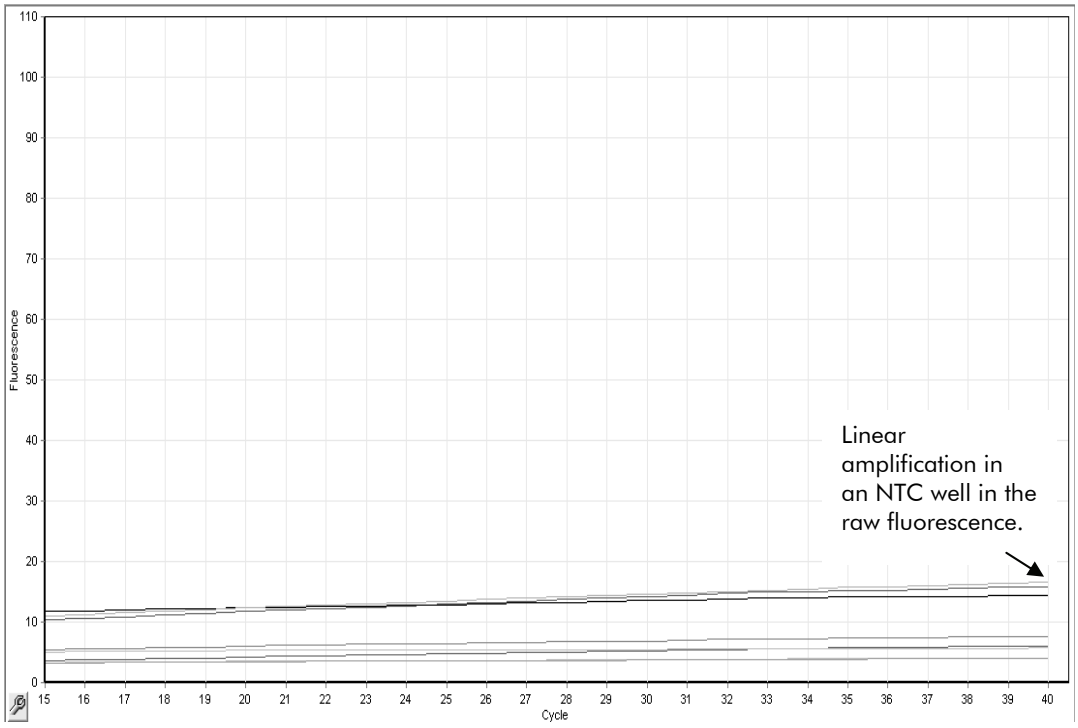


Figure 31. Raw fluorescence of Figure 30.

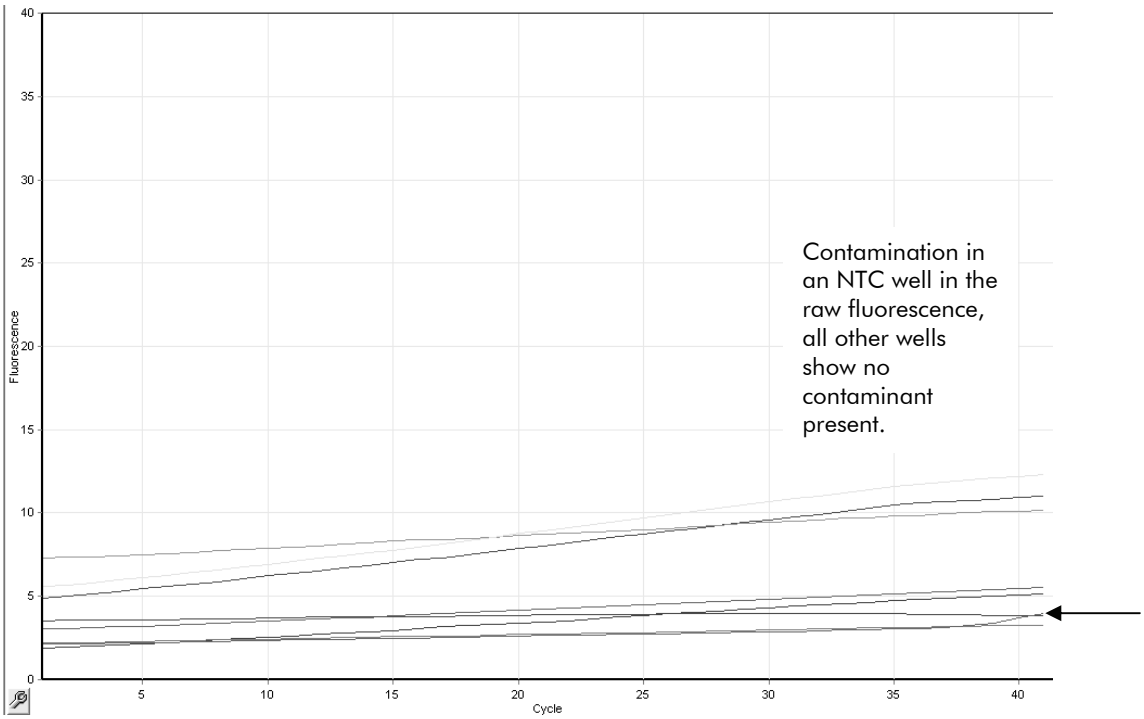


Figure 32. Raw fluorescence data showing an NTC well with a true amplification event.

Analysis of samples and positive control

Figures 33 and 34 show two examples of amplification in sample reactions, with instructions indicating how the data from these type of plots should be handled. In Figure 33, true amplification in a sample well in an analyzed run is seen. If a run shows this type of sigmoidal amplification curve then this is true amplification and the data from this run can be used, providing the positive and internal controls have passed. Figure 34 shows linear amplification in a sample reaction. Under these circumstances, the raw fluorescence data should be examined: the corresponding raw fluorescence plot is shown in Figure 35, indicating that the linear increase in Figure 33 corresponds to a linear increase in the raw fluorescence. Providing the positive and internal control checks have passed, sample results can be obtained for these runs.

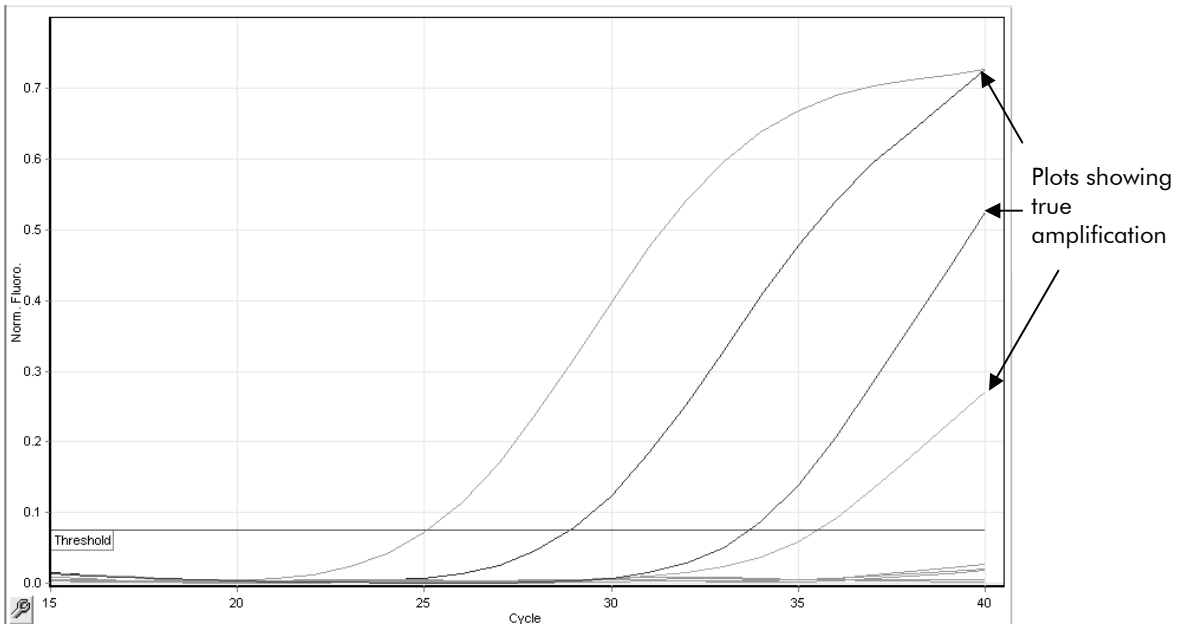


Figure 33. True amplification in a sample well in an analyzed run.

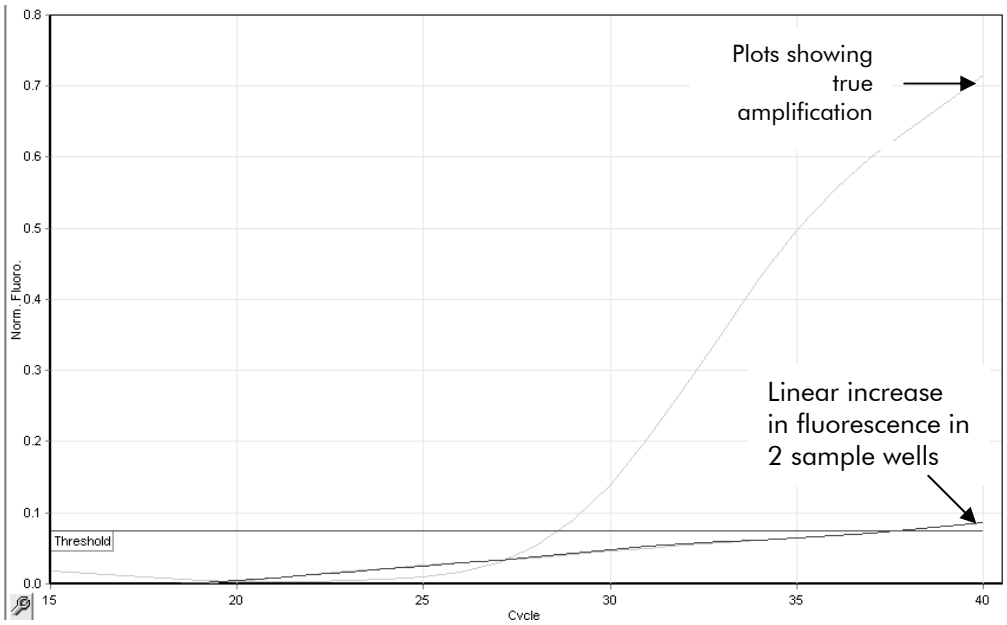


Figure 34. Example of a linear increase in fluorescence in two sample wells.

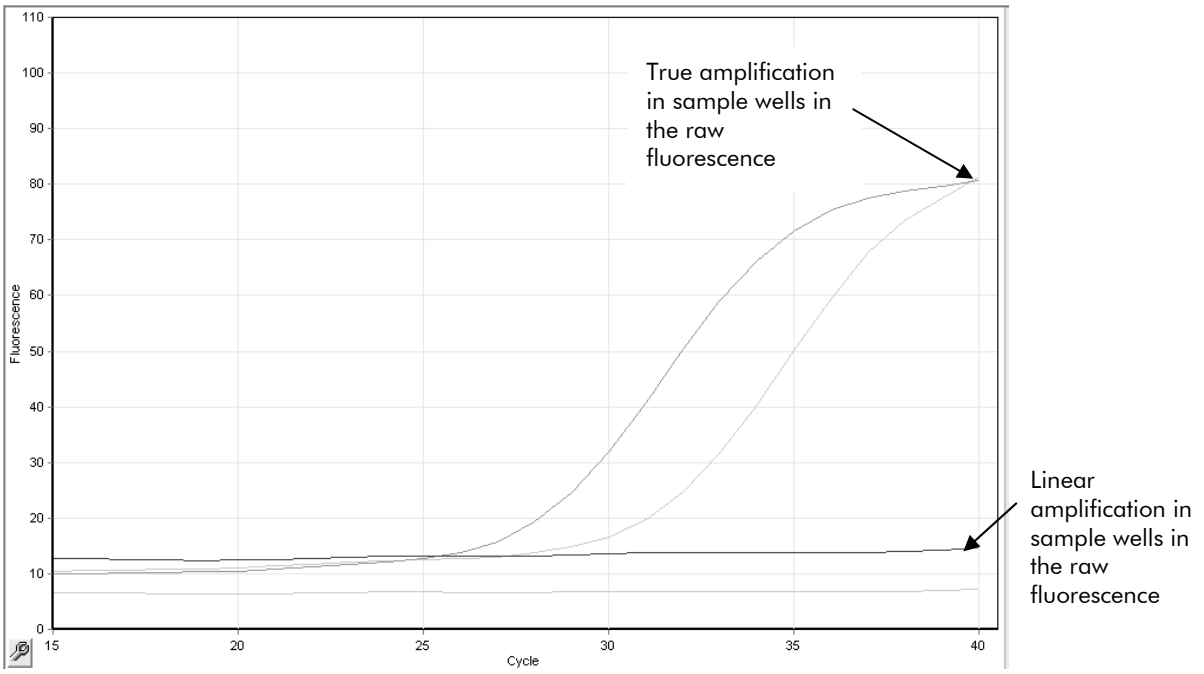


Figure 35. Raw fluorescence of Figure 34.

Rotor-Gene Q Instrument sample analysis

- Open the appropriate file using the Rotor-Gene Q series software (2.0.2).
- Label samples.
- When on raw channel page for each detector/channel, click "Options" and enter "Crop start cycles". On the page with "Remove data before cycle", enter *15* and click "OK".
- Click "Analyse". On the analysis page click "Cycling A (from 15), Yellow" to check the HEX channel.
- The dynamic tube should be highlighted. Click "Slope correct" and "Linear scale".
- Set the threshold at *0.02* and check the C_T values.
- On the analysis page click "Cycling A (from 15), Green" to check the FAM channel.
- The dynamic tube should be highlighted. Click "Slope correct" and "Linear scale".
- Set the threshold at *0.075* and check the C_T values.

Sample analysis: NTC and internal control

1. **Assess the NTC C_T values to ensure that there is no contamination giving a positive amplification in the FAM signal (C_T less than 40). Refer to pages 44–46 for guidance. In the NTC wells, the HEX signal from the internal control assay must give a positive result in all 8 wells ($C_T \leq 37.00$). If there is positive amplification in the FAM channel and amplification >37.00 in the HEX signal, the sample result must be discarded.**
2. **For all other wells, check that each well gives a HEX signal from the internal control. There are 3 possible outcomes:**

2a. **If the internal control assay gives a positive result ($C_T \leq 37.00$), continue with the analysis.**

2b. **If the internal control has failed ($C_T > 37.00$) but the FAM reaction has worked well, continue with the analysis as the FAM reaction has out-competed the internal control reaction.**

2c. **If both the FAM and internal control reactions have failed, the data must be discarded as there may be inhibitors present, which could lead to false negative results. Diluting the sample may reduce the effect of inhibitors but it should be noted that this would also dilute the DNA.**

3. **The control C_T value must be above 23.00 to avoid overloading the assay.**

Sample analysis: positive control

1. **The EGFR positive control must give a control assay C_T (FAM channel) between 26.26 – 30.95.**
2. **Calculate the ΔC_T value as follows, ensuring that the mutation and control C_T values are from the same sample:**

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

3. **The EGFR positive control ΔC_T values should fall within the values given in Table 4.**

Table 4. Rotor-Gene Q software (2.0.2) expected positive control ΔC_T values

Assay	Positive control ΔC_T value
T790M	–2.88 to 3.01
Deletions	–6.71 to 4.16
L858R	–2.41 to 0.90
L861Q	–4.61 to 1.48
G719X	–2.89 to 1.03
S768I	–3.37 to 2.31
Insertions	–2.93 to 1.28

Sample analysis:

a. Calculate the ΔC_T value as follows, ensuring that the mutation and control C_T values are from the same sample. Refer to pages 46–48 for guidance.

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

b. Compare the ΔC_T value for the sample with the cut-off point for the assay in question (Table 5), ensuring that the correct cut-off point is applied to each assay. The cut-off point is the point above which a positive signal could be due to background signal of the ARMS primer on wild-type DNA. If the sample ΔC_T value is higher than the cut-off point, it is classed as negative or beyond the limits of detection of the kit. If the sample value is lower than the cut-off point, the sample is classed as positive for a mutation detected by that assay. Refer to pages 46–48 for guidance.

Table 5. Rotor-Gene Q software (2.0.2) cut-off values

Assay	Cut-off ΔC_T value
T790M	6.38
Deletions	9.06
L858R	8.58
L861Q	9.26
G719X	9.31
S768I	9.26
Insertions	7.91

Appendix B: Mutation Details

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

Table 6. List of mutations and COSMIC IDs

Mutation	Exon	Base change	COSMIC ID
T790M	20	2369C>T	6240
L858R	21	2573T>G	6224
L861Q	21	2582T>A	6213
S768I	20	2303G>T	6241
G719A	18	2156G>C	6239
G719S	18	2155G>A	6252
G719C	18	2155G>T	6253
Insertions	20	2307_2308ins9	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
Deletions	19	2235_2249del15	6223
		2235_2252>AAT (complex)	13551
		2236_2253del18	12728
		2237_2251del15	12678
		2237_2254del18	12367
		2237_2255>T (complex)	12384
		2236_2250del15	6225
		2238_2255del18	6220
		2238_2248>GC (complex)	12422
		2238_2252>GCA (complex)	12419
		2239_2247del9	6218
		2239_2253del15	6254

Table continued on next page.

Table 6. List of mutations and COSMIC IDs (continued)

Mutation	Exon	Base change	COSMIC ID
Deletions	19	2239_2256del18	6255
		2239_2248TTAAGAGAAG>C (complex)	12382
		2239_2258>CA (complex)	12387
		2240_2251del12	6210
		2240_2257del18	12370
		2240_2254del15	12369
		2239_2251>C (complex)	12383

References

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

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Cited references

a. Paez, J.G. et. al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science **304**, 1497.

b. Lynch, T.J. et. al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. **350**, 2129.

c. Pao, W. (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PloS Medicine **2**, 1.

d. Newton, C.R. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS) Nucleic Acids Res. **17** 2503.

e. Whitcombe, D., Theaker J., Guy, S.P., Brown, T., Little, S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotech **17**, 804.

f. Thelwell, N., Millington, S., Solinas, A., Booth, J. and Brown, T. (2000). Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Res. **28**, 3752.

Ordering Information

Product	Contents	Cat. no.
EGFR RGQ PCR Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, *Taq* DNA Polymerase	870101
Rotor-Gene Q and accessories		
Rotor-Gene Q, 5plex HRM	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor	Inquire
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet 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

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

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Finland ■ Orders 0800-914416 ■ Fax 0800-914415 ■ Technical 0800-914413

France ■ Orders 01-60-920-926 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930 ■ Offers 01-60-920-928

Germany ■ Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

Hong Kong ■ Orders 800 933 965 ■ Fax 800 930 439 ■ Technical 800 930 425

Ireland ■ Orders 1800 555 049 ■ Fax 1800 555 048 ■ Technical 1800 555 061

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

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

Korea (South) ■ Orders 080-000-7146 ■ Fax 02-2626-5703 ■ Technical 080-000-7145

Luxembourg ■ Orders 8002-2076 ■ Fax 8002-2073 ■ Technical 8002-2067

Mexico ■ Orders 01-800-7742-639 ■ Fax 01-800-1122-330 ■ Technical 01-800-7742-436

The Netherlands ■ Orders 0800-0229592 ■ Fax 0800-0229593 ■ Technical 0800-0229602

Norway ■ Orders 800-18859 ■ Fax 800-18817 ■ Technical 800-18712

Singapore ■ Orders 1800-742-4362 ■ Fax 65-6854-8184 ■ Technical 1800-742-4368

Spain ■ Orders 91-630-7050 ■ Fax 91-630-5145 ■ Technical 91-630-7050

Sweden ■ Orders 020-790282 ■ Fax 020-790582 ■ Technical 020-798328

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

UK ■ Orders 01293-422-911 ■ Fax 01293-422-922 ■ Technical 01293-422-999

USA ■ Orders 800-426-8157 ■ Fax 800-718-2056 ■ Technical 800-DNA-PREP (800-362-7737)

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