QIAGEN Supplementary Protocol

Using the EGFR PCR Kit on the Rotor-Gene® Q Instrument

This protocol is designed for using the EGFR PCR Kit for detection of specific mutations in the EGFR gene by real-time PCR on Rotor-Gene Q instruments.

IMPORTANT: This product has not been validated on this platform. This protocol has been developed using the EGFR PCR Kit, version 1. The following information is for guidance only. Please read the *EGFR PCR Kit Handbook*, paying careful attention to the Safety Information section, before beginning this procedure. 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. Analytical and performance characteristics have not been established.

Equipment and reagents

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 "Specimen Material" in the *EGFR PCR Kit Handbook*)
- EGFR PCR Kit, version 1 (cat. no. 871101 or 871105)
- 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*
- Rotor-Gene Q software, version 2.0.2 or higher
- 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 instruments have been checked and calibrated according to the manufacturer's recommendations.

Important points before starting

- Before beginning the procedure, read "Warnings and Precautions" in the *EGFR PCR Kit Handbook*.
- Take time to familiarize yourself with the Rotor-Gene instrument before starting the protocol. See the instrument user manual.
- Do not vortex *Taq* DNA polymerase, or any reaction mixes that contain *Taq* DNA polymerase, as this may cause inactivation of the enzyme.
- To use the EGFR PCR Kit efficiently, samples must be grouped into a batch size of 7 (to fill one 72-well rotor). Smaller batch sizes will mean that fewer samples can be tested with the EGFR 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.
- All software 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.

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 (*Taq*) 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 from the EGFR PCR Kit, at room temperature (15–25°C). Once thawed, mix each solution by inverting the tube 10 times to avoid localized concentrations of salts. Prepare sufficient mixes for the DNA samples, 1 positive control, and 1 no template control (NTC), plus an excess of 2 reactions per mix, as shown in Table 1.**

Table 1. EGFR master mix volumes

	1 reaction			Per rotor x 11 reactions		
Assay	**Reaction Mix (µl)**	***Taq* (µl)**	**Water (µl)**	**Reaction Mix (µl)**	***Taq* (µl)**	**Water (µl)**
Control	16	0.2	3.8	176	2.2	41.8
Deletions	16	0.6	3.4	176	6.6	37.4
L858R	16	0.6	3.4	176	6.6	37.4
L861Q	16	0.8	3.2	176	8.8	35.2
G719X	16	0.6	3.4	176	6.6	37.4
S768I	16	0.8	3.2	176	8.8	35.2
Insertions	16	0.8	3.2	176	8.8	35.2

2. **Mix the master mixes by gently pipetting up and down. Proceed immediately to step 3.**
3. **Add 20 µl of the master mixes to the reaction wells. Proceed immediately to step 4.**
4. **Add 5 µl of sample, standard, or water (for the NTCs) to the reaction wells. Each DNA sample must be tested with both the control and mutation assays. The rotor layout is given in Table 2.**

Table 2. Rotor layout

	Controls		Sample number						
Assay	**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
Insertions	8	16	24	32	40	48	56	64	72

5. Create a temperature profile according to the following steps:

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

To summarize, the cycling parameters (Table 3) are as follows:

Table 3. Cycling parameters

Cycles	**Temperature**	**Time**	**Data acquisition**
1	95°C	10 min	None
40	95°C	30 s	None
	61°C	60 s	Green and yellow

6. **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.**
7. **To create a new template, select "Empty Run" and then click "New" to enter the "New Run Wizard".**
8. **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 1).**

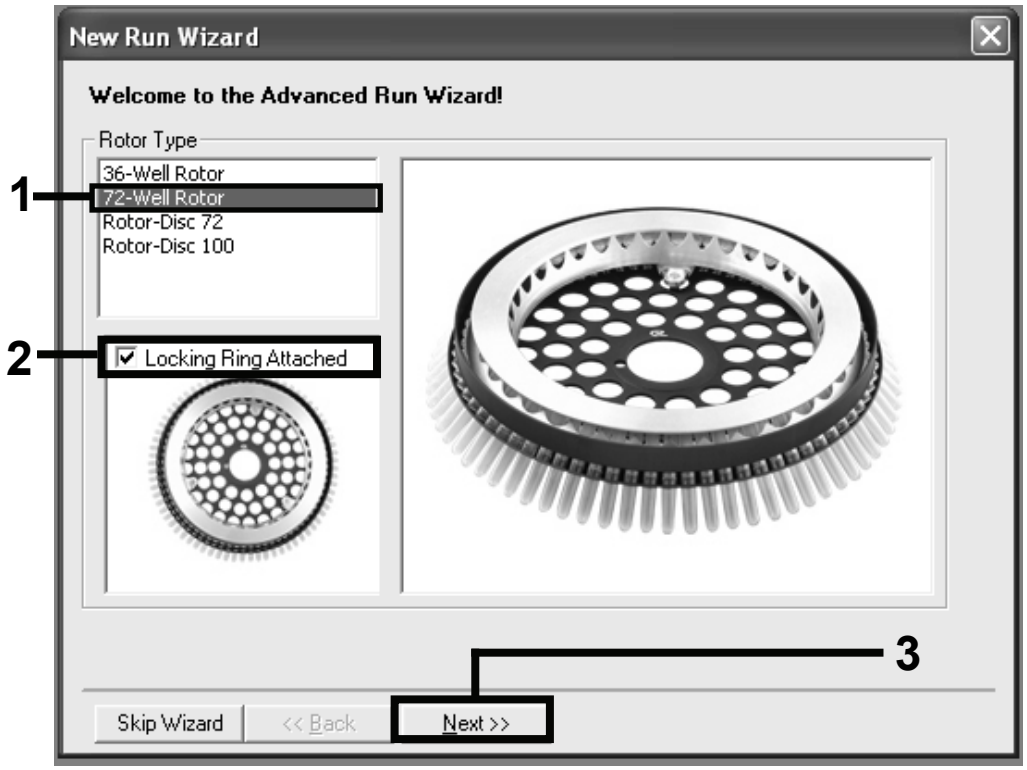


Figure 1. The "New Run Wizard" dialog box.

9. **Enter the name of the operator. Add any notes and enter the reaction volume as *25*. Ensure that "Sample Layout" reads "1,2,3..." and "Apply Ambient Air Correction" is selected. Click "Next" (Figure 2).**

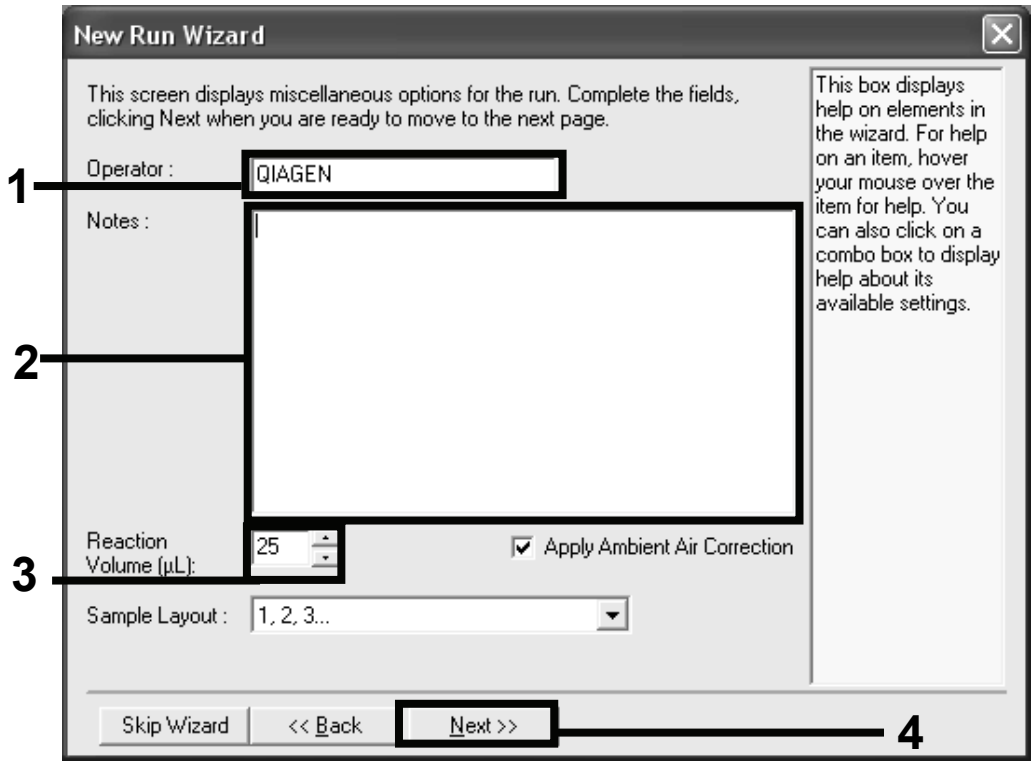


Figure 2. Setting the general assay parameters.

10. **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.**

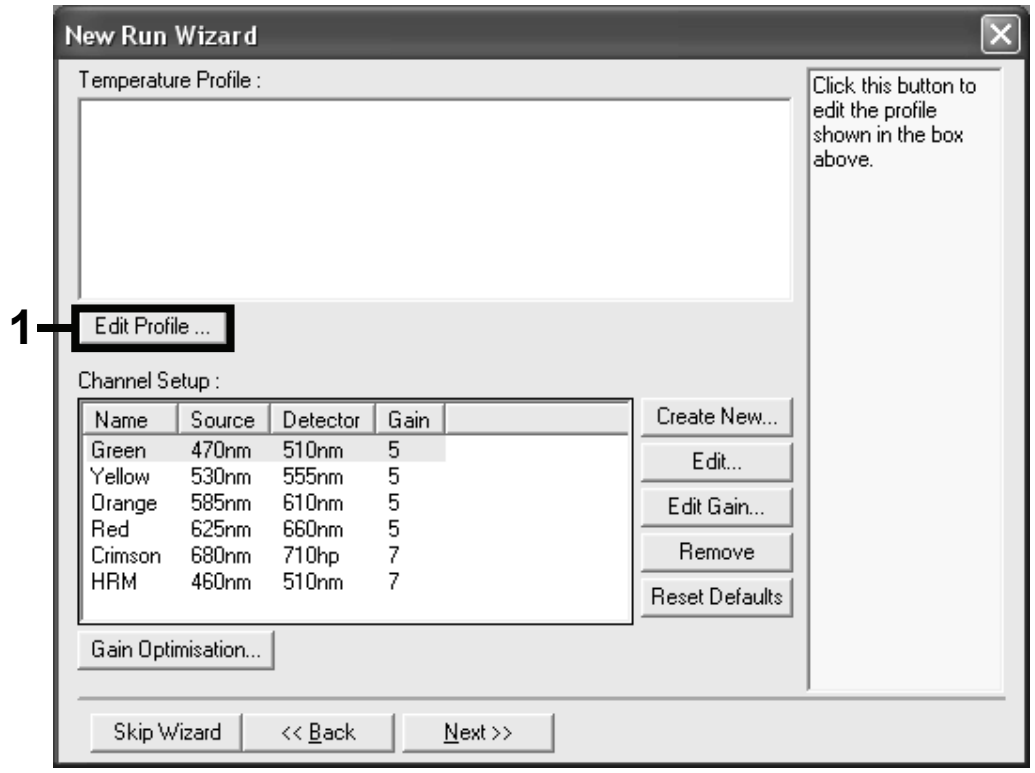


Figure 3. Editing the profile.

11. **Click the "Insert after" button and select *New Hold at Temperature* (Figure 4).**

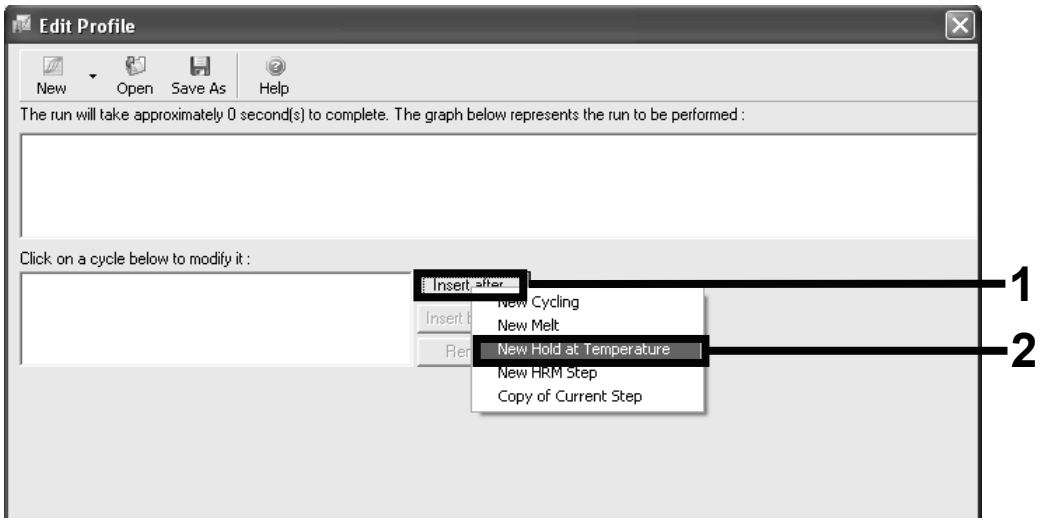


Figure 4. Initial incubation step at 95°C.

12. **Change the "Hold Temperature" to *95°C*, and the "Hold Time" to *10 mins*. Click the "Insert After" button and then select *New Cycling* (Figure 5).**

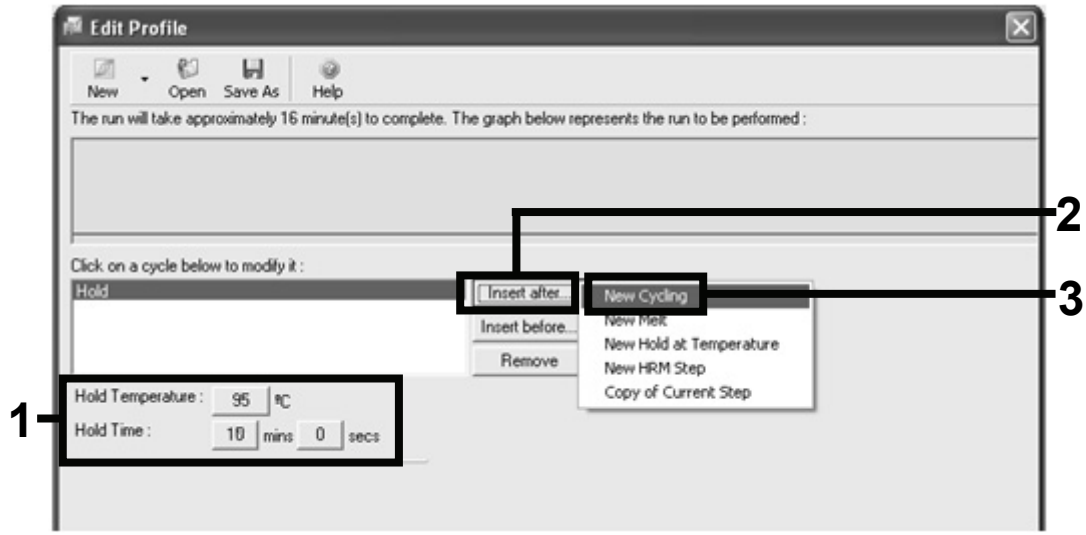


Figure 5. Initial incubation step at 95°C.

13. **Change the number of cycle repeats to *40*. Select the first step, and set to *95°C for 30 seconds* (Figure 6).**

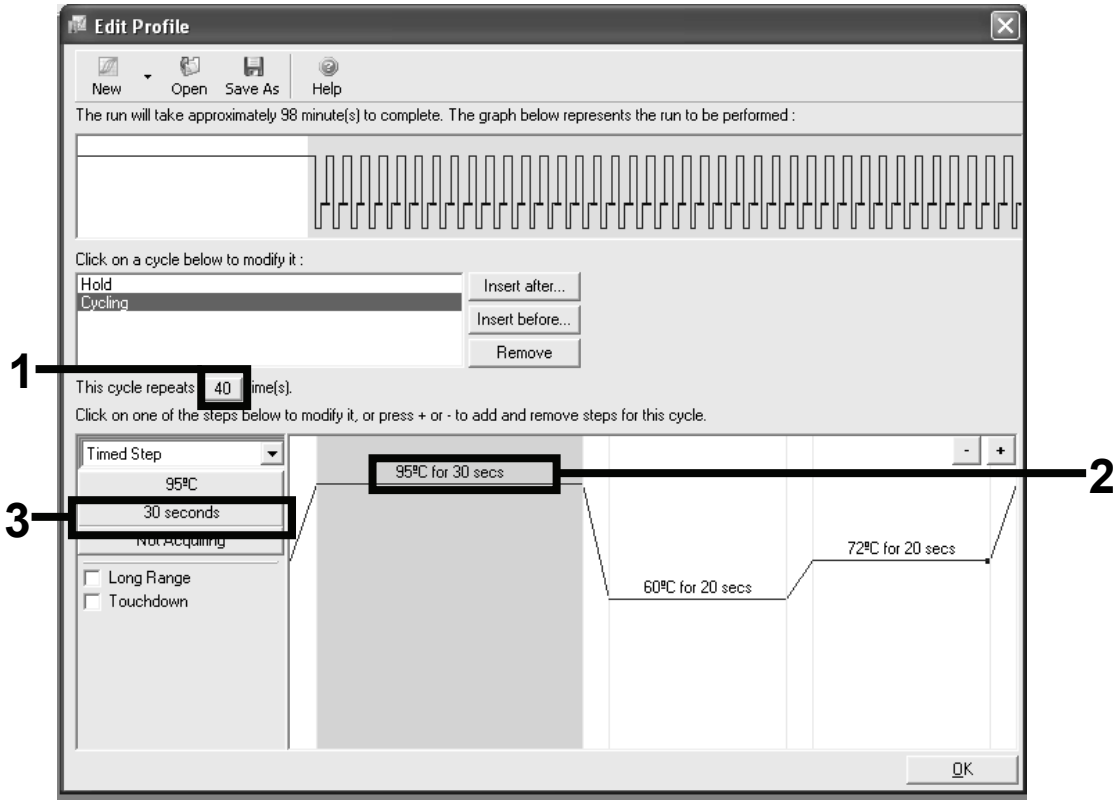


Figure 6. Cycling step at 95°C.

14. **Highlight the second step, and set to *61°C for 60 seconds*. Enable data acquisition during this step by selecting the "Not Acquiring" button (Figure 7).**

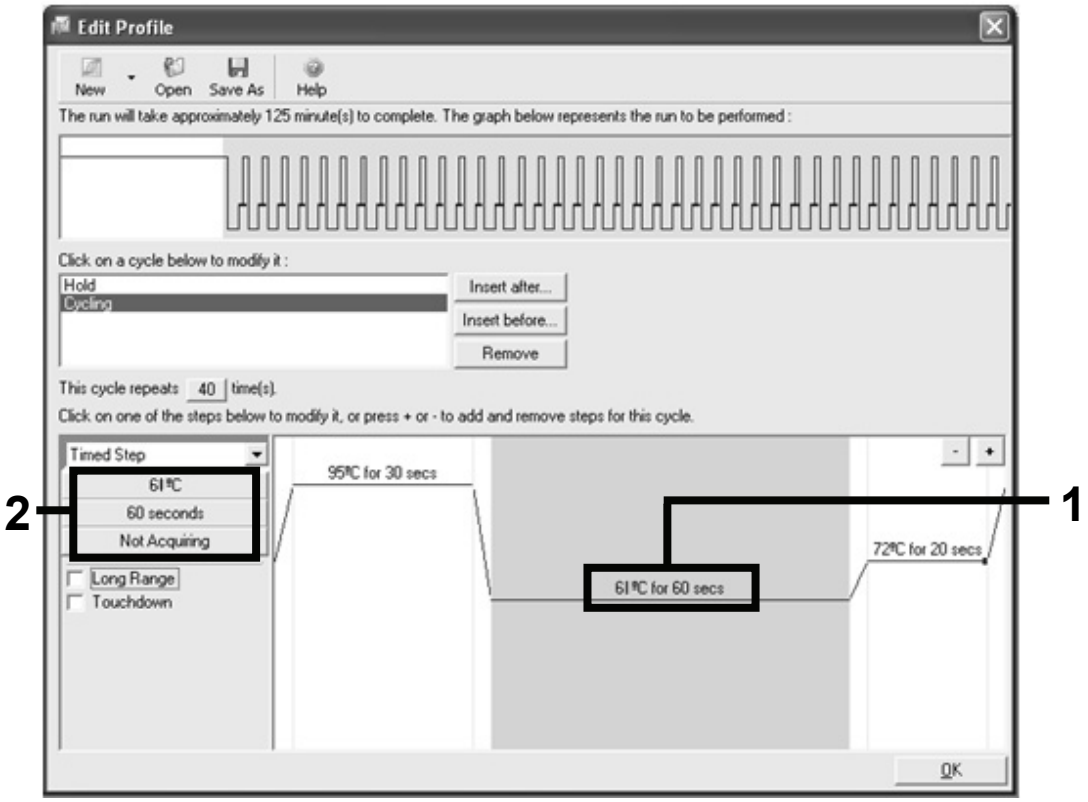
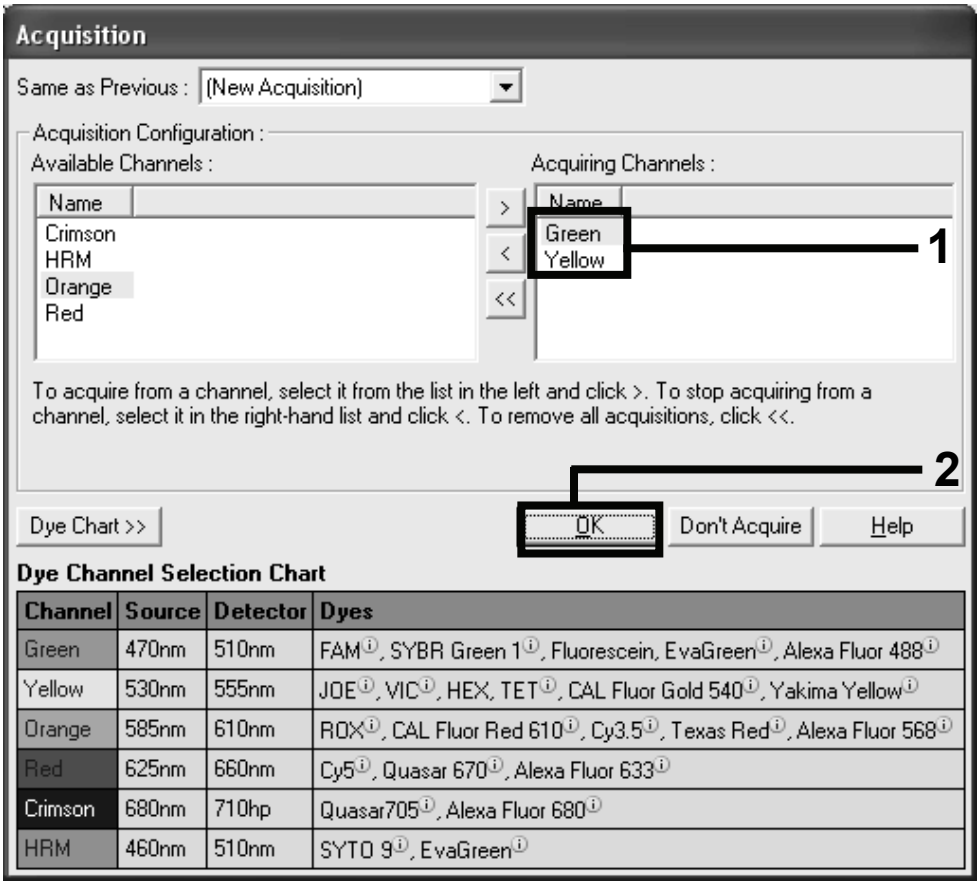


Figure 7. Cycling step at 61°C.

15. **Set *Green* and *Yellow* as acquiring channels by selecting the ">" button to transfer these from the "Available Channels" list. Click "OK" (Figure 8).**



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
HRM	460nm	510nm	SYTO 9, EvaGreen

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

16. Highlight the third step and delete by clicking the "-" button. Click "OK" (Figure 9).

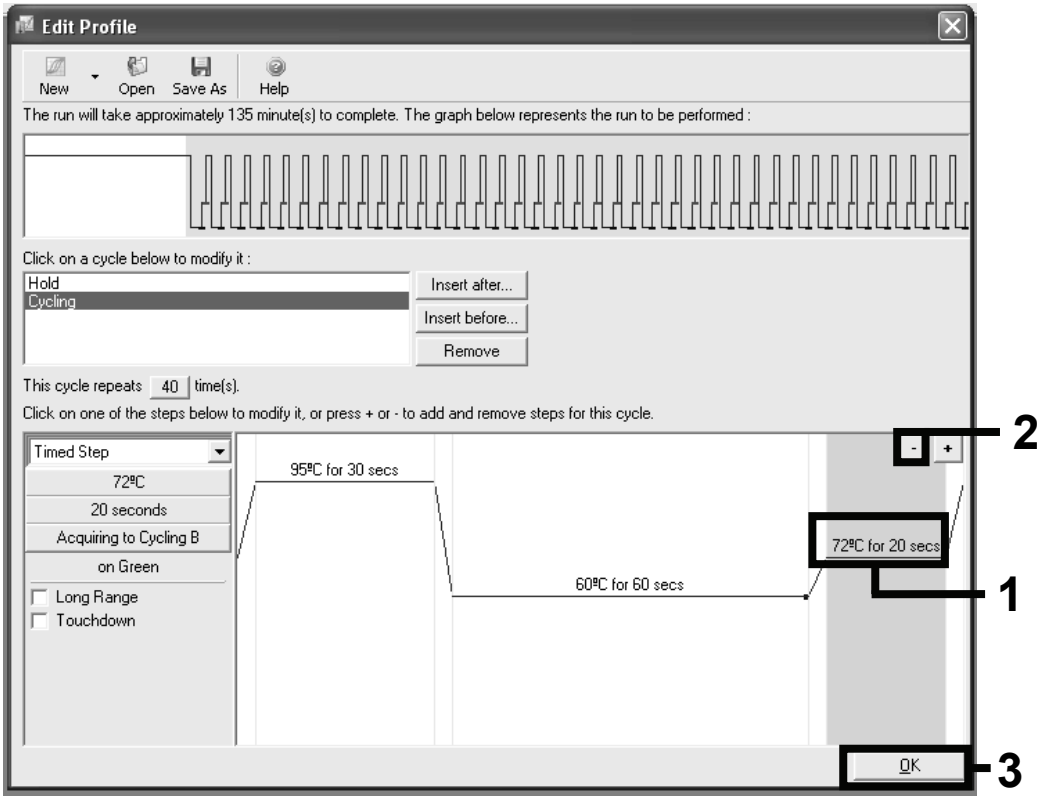


Figure 9. Removal of extension step.

17. In the next dialog box, click the "Gain Optimisation" button (Figure 10).

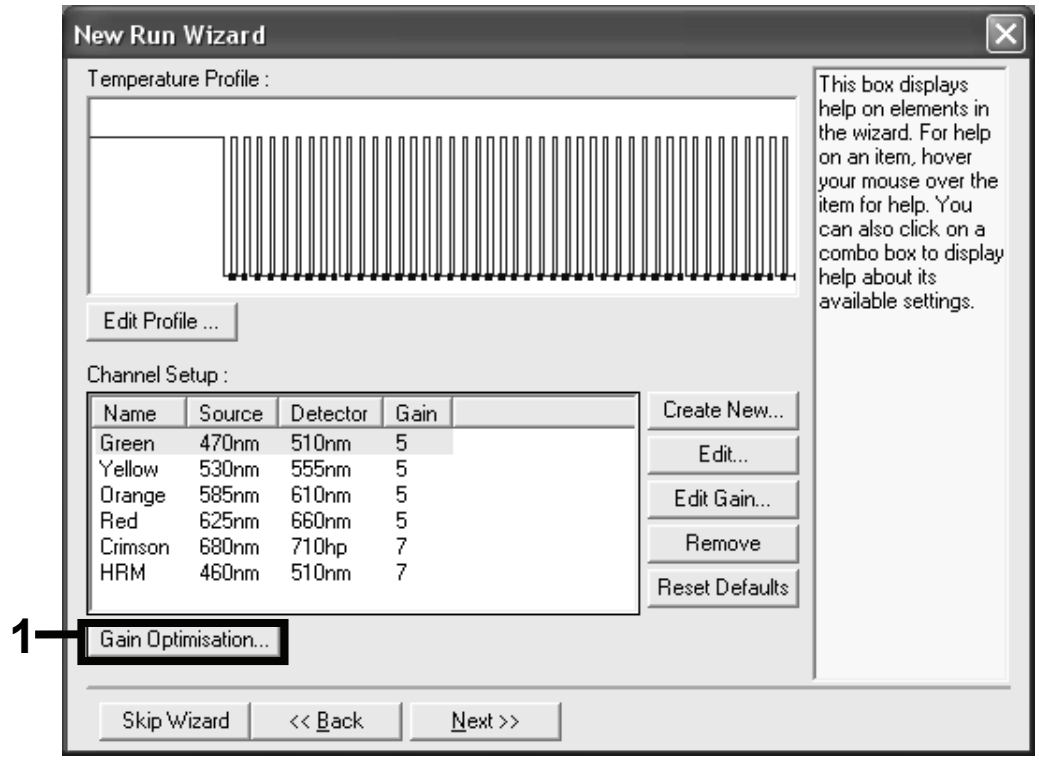


Figure 10. Gain optimization.

18. **Click the "Optimise Acquiring" button. Channel settings are then displayed for each channel. Accept these default values by clicking "OK" for both channels (Figure 11).**

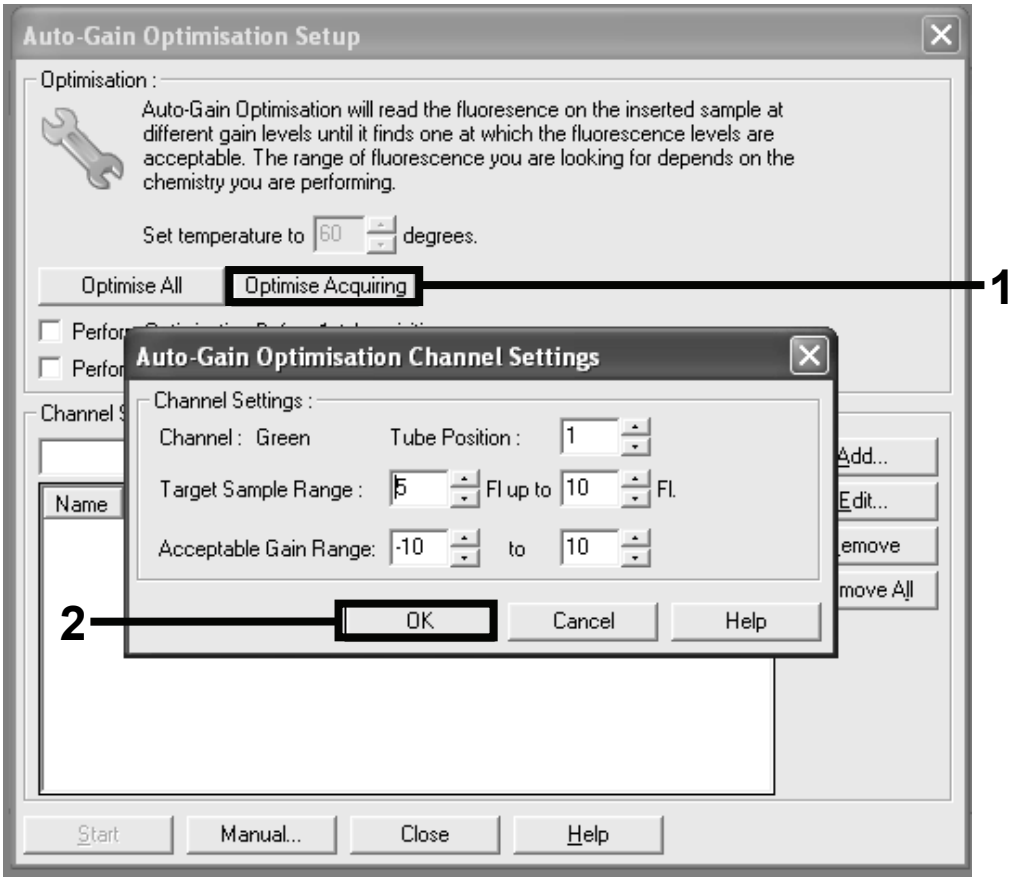


Figure 11. Auto-gain optimization for the green channel.

19. **Check the "Perform Optimisation before 1st Acquisition" box, then click the "Close" button to return to the wizard (Figure 12).**

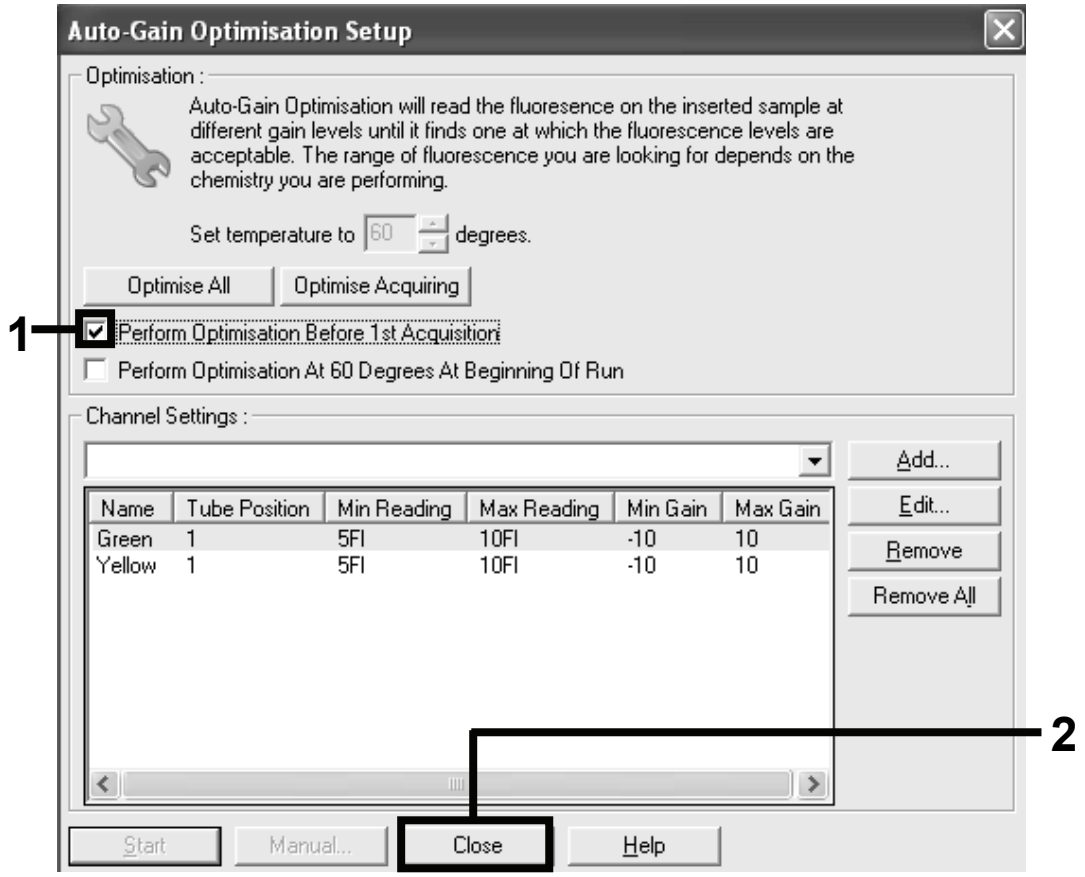


Figure 12. Selection of green and yellow channels.

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

21. **Check the summary and click "Start Run" to save the run file and start the run (Figure 13).**

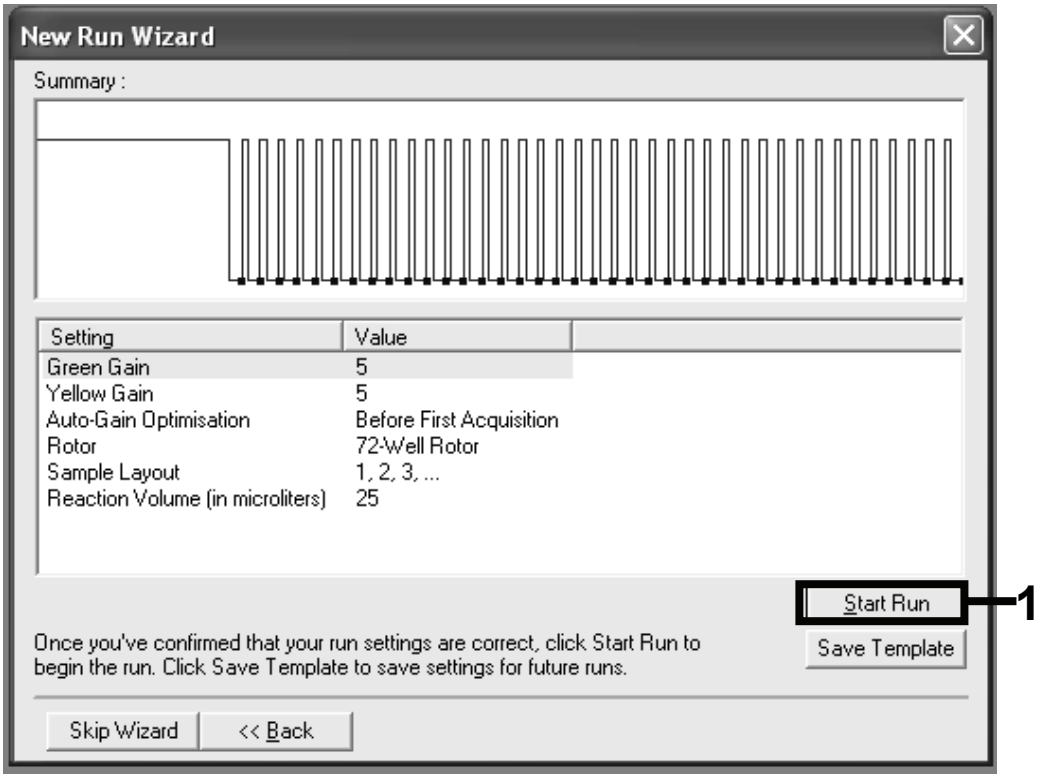


Figure 13. Starting the run.

22. **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.**

After the run is finished, analyze the data as follows.

Software analysis settings

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

8. **On the analysis page, click "Cycling A (from 15), Green" to check the FAM® channel.**
9. **The dynamic tube should be highlighted. Click "Slope correct" and "Linear scale".**
10. **Set the threshold at 0.075 and check the C_T values.**
11. **For data interpretation, see the *EGFR PCR Kit Handbook*.**

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

Material safety data sheets (MSDS) for any QIAGEN product can be downloaded from www.qiagen.com/Support/MSDS.aspx.

PCR118 February-11

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