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EGFR RGQ PCR Kit Handbook



Version 1

For use with plasma or FFPE tissue

For the detection of 28 mutations in the EGFR gene.

For Research Use Only. Not for use in diagnostic procedures



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Store at -30 to -15°C



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Intended Use

The EGFR RGQ PCR Kit is intended for the detection of 28 somatic mutations in the EGFR gene. It provides a qualitative assessment of the mutation status of DNA samples.

The EGFR RGQ PCR Kit will test DNA samples extracted from plasma samples or formalin-fixed, paraffin embedded (FFPE) tumor tissue, and run on a Rotor-Gene® Q instrument. It is to be used by trained personnel in a professional laboratory environment.

Summary and Explanation

The EGFR RGQ PCR Kit is a ready-to-use kit for the detection of 28 mutations in the EGFR cancer-related gene using the polymerase chain reaction (PCR) on a Rotor-Gene Q instrument.

Using Scorpions® (1) and ARMS (Amplification Refractory Mutation System) (2) technologies, the EGFR RGQ PCR Kit enables detection of 28 mutations in exons 18, 19, 20, and 21 of the EGFR oncogene against a background of wild type genomic DNA (Table 1). In summary:

- 18 deletions in exon 19 (detects the presence of any of 18 deletions but does not distinguish between them)
- 3 insertions in exon 20 (detects the presence of any of 3 insertions but does not distinguish between them)
- G719X (detects the presence of G719S, G719A, or G719C but does not distinguish between them)
- S768I
- T790M
- L858R
- L861Q

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

Table 1. List of mutations and COSMIC identities

Exon	Mutation	COSMIC* ID	Base change
18	G719A	6239	2156G>C
	G719S	6252	2155G>A
	G719C	6253	2155G>T
19	Deletions	12384	2237_2255>T
		12387	2239_2258>CA
		12419	2238_2252>GCA
		12422	2238_2248>GC
		13551	2235_2252>AAT
		12678	2237_2251del15
		6218	2239_2247del9
		12728	2236_2253del18
		12367	2237_2254del18
		6210	2240_2251del12
		6220	2238_2255del18
		6223	2235_2249del15
		6225	2236_2250del15
		6255	2239_2256del18
		12369	2240_2254del15
		12370	2240_2257del18
		12382	2239_2248TTAAGAGAAG>C
12383	2239_2251>C		
20	S768I	6241	2303G>T
	Insertions	12376	2307_2308insGCCAGCGTG
		12378	2310_2311insGGT
		12377	2319_2320insCAC
T790M	6240	2369C>T	
21	L858R	6224	2573T>G
	L861Q	6213	2582T>A

* COSMIC: Catalogue of somatic mutations in cancer: <http://cancer.sanger.ac.uk/>.

Principle of the Procedure

The EGFR RGQ PCR Kit comprises 8 separate PCR amplification reaction mixes: 7 mutation-specific reactions in exons 18, 19, 20, and 21 of the EGFR oncogene, and a wild-type control in exon 2. The principal components of the kit are explained below.

ARMS

Allele- or mutation-specific amplification is achieved using ARMS. *Taq* DNA polymerase (*Taq*) 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 bifunctional molecules containing a PCR primer covalently linked to a probe. The fluorophore in the 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 leading to a detectable increase in fluorescence.

Kit format

Eight assays are supplied in the EGFR RGQ PCR Kit:

- One control assay (CTRL)
- Three mutation assays for the plasma setup and seven mutation assays for the FFPE tissue setup

All reaction mixes contain reagents to detect targets that are labeled with carboxyfluorescein (FAM™), and an internal control assay labeled with hexachlorofluorescein (HEX™). The internal control assay can detect the presence of inhibitors that may lead to false negative results. FAM amplification can out-compete the internal control amplification and the purpose of the internal control is simply to show that where there is no FAM amplification, this is a true negative result and not a failed PCR reaction.

Note: For the plasma workflow, we recommend to only use the 3 mutation assays for detection of T790M, L858R, and deletions, as sample material can be limited.

Assays

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

Control assay

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

Mutation assays

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

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 EGFR RGQ PCR Kit contains EGFR 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”: NTC) in tubes 9–16. The EGFR RGQ PCR Kit contains water for the 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 reaction assessment

Each reaction mix contains an internal control (IC) 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. The IC employs a non-EGFR related oligonucleotide target sequence, an unlabeled primer, and a Scorpions primer labeled with HEX in order to distinguish it from the FAM-labeled Scorpions in the control and mutation reaction mixes. FAM amplification can out-compete the IC amplification so that the IC C_T (HEX) value generated may fall outside the specified range. The FAM results are still valid for these samples.

Platform and software

The EGFR RGQ PCR Kit is specifically designed to be used with Rotor-Gene Q instruments with Rotor-Gene Q software version 2.3 in the open mode.

Materials Provided

Kit contents

EGFR RGG PCR Kit				(24)
Catalog number				874101
Number of reactions				24
Color	Identity	Tube ID		Volume
Red	Control Reaction Mix	1	CTRL	2 x 600 µl
Purple	T790M Reaction Mix	2	T790M	600 µl
Orange	Deletions Reaction Mix	3	Del	600 µl
Pink	L858R Reaction Mix	4	L858R	600 µl
*Green	L861Q Reaction Mix	5	L861Q	600 µl
*Yellow	G719X Reaction Mix	6	G719X	600 µl
*Gray	S768I Reaction Mix	7	S768I	600 µl
*Blue	Insertions Reaction Mix	8	Ins	600 µl
Beige	EGFR Positive Control	9	PC	300 µl
Mint	Taq DNA Polymerase	Taq		2 x 80 µl
White	Nuclease-free water for No Template Control	NTC		1.9 ml
White	Nuclease-free water for Dilution	Dil.		1.9 ml
EGFR RGG PCR Kit Handbook (English)				1

* For use in the FFPE tissue setup only.

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

- DNA extraction kit (see “DNA extraction and preparation,” page 18)

Consumables and general laboratory equipment

- Dedicated pipets* (adjustable) for sample preparation
- Dedicated pipets* (adjustable) for PCR master mix preparation
- Dedicated pipets* (adjustable) for dispensing of template DNA
- DNase, RNase, and DNA-free pipet tips with filters (to avoid cross contamination, pipet tips with aerosol barriers are recommended)
- Strip Tubes and Caps, 0.1 ml, for use with 72-well rotor (cat. no. 981103 or 981106)
- DNase, RNase, and DNA-free micro centrifuge tubes for preparing master mixes
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction setup with a single-channel pipet (cat. no. 9018901)
- Thermomixer, heated orbital incubator, heating block or water bath capable of incubation at 90°C*
- Benchtop centrifuge* with rotor for 2 ml reaction tubes
- Vortex mixer*

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

In addition, when extracting DNA from plasma:

- Water bath or similar device capable of holding 50 ml centrifuge tubes at 60°C
- Heating block or similar device capable of incubation at 56°C *
- Crushed ice
- QIAvac 24 Plus vacuum manifold (cat. no. 19413), Vacuum Pump (cat. no. 84010 [USA and Canada], 84000 [Japan] or 84020 [rest of world]), or equivalent pump capable of producing a vacuum of –800 to –900 mbar
- QIAvac Connecting System (cat. no. 19419)
- Cooling centrifuge

Equipment for PCR

- Rotor-Gene Q instrument with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and HEX, respectively)*
- Rotor-Gene Q software version 2.3

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

Warnings and Precautions

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

For safety information regarding the Rotor-Gene Q instrument, see the user manual supplied with the instrument.

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

General precautions

Always pay attention to the following:

- The test is for use with plasma samples or FFPE NSCLC tissue specimens.
- Store and extract positive materials (specimens and positive controls) separately from all other reagents and add them in 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 post-PCR products.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.

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- Reagents for the EGFR RGQ PCR Kit have been optimally diluted. Do not dilute reagents further as this may result in a loss of performance. Do not use reaction volumes (reaction mix plus sample) of less than 25 μ l since this will increase the risk of a false negative.
 - All reagents supplied in the EGFR RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same EGFR RGQ PCR Kit. Do not substitute the reagents in the EGFR RGQ PCR Kit or between EGFR RGQ PCR Kits, as this may affect performance.
 - Only use the *Taq* DNA polymerase (tube *Taq*) that is provided in the EGFR RGQ PCR 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.
 - Do not use expired or incorrectly stored components.

Note: Caution must be observed to ensure correct sample testing with emphasis on eliminating wrong sample entry, loading errors, and pipetting errors.

Note: The reagents are validated for manual setup. If an automated method is used, this may reduce the number of possible reactions due to reagent required to fill “dead volumes” on these instruments.

Reagent Storage and Handling

The EGFR RGQ PCR Kit is shipped on dry ice and must be frozen on arrival. If the EGFR RGQ PCR Kit is not frozen on arrival, if the outer packaging has been opened during transit, or if the shipment does not contain a packing note, handbook, or the reagents, please contact QIAGEN Technical Services or your local distributor (visit www.qiagen.com).

The EGFR RGQ PCR Kit should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. Scorpions (as with all fluorescently labeled molecules) 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 the expiration date stated on the label. Repeated freezing and thawing should be avoided. A maximum of 8 freeze–thaw cycles is recommended.

The reagents must be thawed at ambient temperature (15 – 25°C) for a minimum of 1 hour and a maximum of 4.5 hours. Once the reagents are ready to use, the PCR reactions can be set up. The Rotor-Gene Q tubes, containing the master mixes and the DNA sample, can be loaded onto a Rotor-Gene Q instrument immediately. The total time prior to run once the PCR reactions are set up should not exceed:

- 7 hours if stored at ambient temperature. This time includes both the PCR setup and storage.
- 18 hours if stored in the refrigerator (2 – 8°C). This time includes both the PCR setup and storage.

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

Note: 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 more reagents and reduce the number of samples that can be tested with the EGFR RGQ PCR Kit.

Specimen Handling and Storage

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

Sample material must be human genomic DNA extracted from plasma or FFPE tissue. Specimens must be transported according to standard pathology methodology to ensure specimen quality.

FFPE tissue 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 mutations detected by the EGFR RGQ PCR Kit.

To prepare tissue samples for DNA extraction:

- 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.
- Use a trained individual (e.g., a pathologist) to assess a Hematoxylin & Eosin (H&E)-stained section to confirm that there is tumor present.
- The stained sections must not be used for DNA extraction.
- Store all FFPE blocks and slides at room temperature (15–25°C). Slides may be stored at ambient temperature for up to 1 month prior to DNA extraction.

Procedure

Protocol: DNA extraction and preparation from plasma

For DNA extraction from plasma, use the QIAamp® Circulating Nucleic Acid Kit (cat. no. 55114). Carry out the DNA extraction according to the instructions in the handbook noting the following:

- The starting volume of plasma is 2 ml.
- Prior to DNA extraction, 2 ml plasma should be centrifuged at 3000 rpm for 2 minutes and the supernatant transferred to a clean tube.
- Proteinase K volume should be 250 µl.
- Proteinase K digestion should be performed for 1 hour at 60°C.
- Purified genomic DNA must be eluted in 55 µl Buffer AVE (provided in the QIAamp Circulating Nucleic Acid Kit).
- Purified genomic DNA should be stored at –30 to –15°C.

Protocol: DNA extraction and preparation from FFPE tissue

For DNA extraction from FFPE tissue, use the QIAamp DNA FFPE Tissue Kit (cat. no. 56404). Carry out the DNA extraction according to the instructions in the handbook noting the following:

- Make sure to use molecular biology grade ethanol* for all required steps.
- Scrape the entire tissue area from 2 sections into a labeled microcentrifuge tube using a fresh scalpel for each sample.
- Proteinase K digestion (step 11 in the *QIAamp DNA FFPE Tissue Kit Handbook*) must be performed for 1 hour ± 5 minutes at 56°C ± 3°C.

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

- Proteinase K digestion (step 12 in the *QIAamp DNA FFPE Tissue Kit Handbook*) must be performed for 1 hour ± 5 minutes at 90°C ± 3°C.
- Do not use the RNase step described in the *QIAamp DNA FFPE Tissue Kit Handbook*.
- The samples must be eluted with 120 µl of elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit (step 20 in the *QIAamp DNA FFPE Tissue Kit Handbook*).
- Genomic DNA may be stored at 2–8°C for 1 week post extraction, or at –15 to –25°C for up to 8 weeks before use.

Note: 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.

Protocol: EGFR mutation detection run setup

- Prepare samples as described in section “Protocol: DNA extraction and preparation from plasma.”
- Set up a temperature profile as described in “Creating a temperature profile.”

Creating a temperature profile

A summary of cycling parameters is shown in Table 2. Ensure that the temperature profile is changed accordingly and any pre-settings are deleted (e.g., delete preset 72°C step).

Table 2. Temperature profile

Cycles	Temperature	Time	Data acquisition
1	95°C	15 minutes	None
40	95°C	30 seconds	None
	60°C	60 seconds	Green and Yellow

Procedure

1. Double-click the Rotor-Gene Q Series Software 2.3 icon on the desktop of the computer connected to the Rotor-Gene Q instrument.
2. To create a new template, select **Empty Run**, then click **New**.

The New Run Wizard dialog box appears.

3. Select **72-well rotor** in the Rotor Type list. Confirm that the locking ring is attached and check the **Locking Ring Attached** box. Click **Next** (Figure 1).

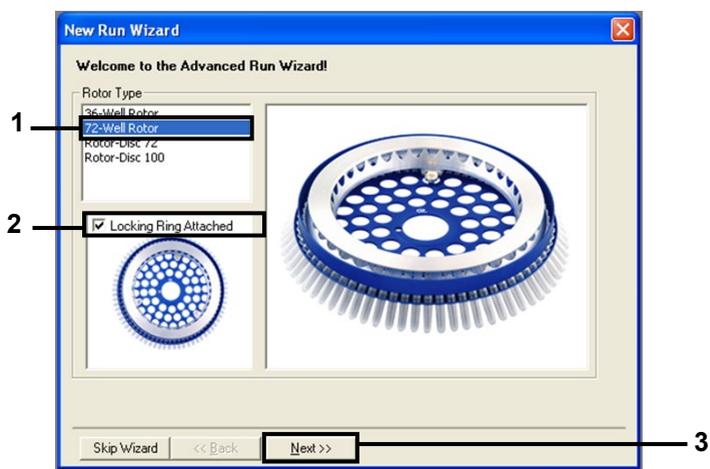


Figure 1. The “New Run Wizard” dialog box. (1 = “Rotor type”; 2 = “Locking Ring Attached” box; 3 = “Next.”)

4. Perform these steps in the next window (Figure 2).
 - 4a. Enter the name of the operator in the **Operator** field.
 - 4b. In the Notes field, enter any description you want regarding the template.
 - 4c. Enter 25 in the **Reaction Volume (µl)** field.
 - 4d. Ensure that **1, 2, 3...** is specified in the **Sample Layout** field.
 - 4e. Click **Next**.

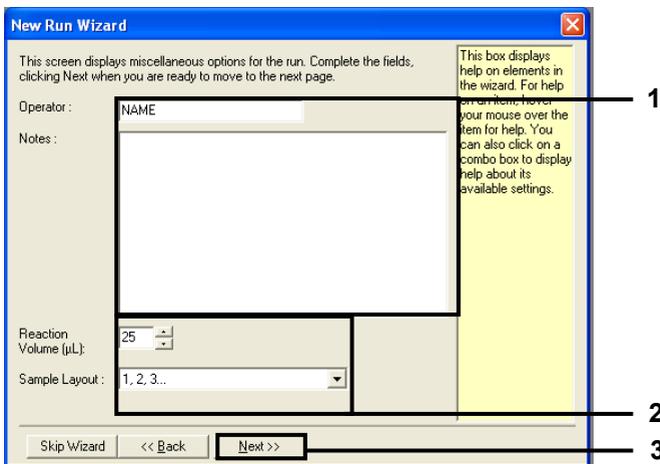


Figure 2. Entering operator name and reaction volumes. (1 = "Operator" and "Notes" dialog fields; 2 = "Reaction Volume" field and "Sample Layout" field; 3 = "Next.")

5. Click **Edit Profile** (Figure 3). Check the run parameters according to the following steps.



Figure 3. "Edit Profile" in the "New Run Wizard."

6. Click **Insert after** and select **New Hold at Temperature** (Figure 4).

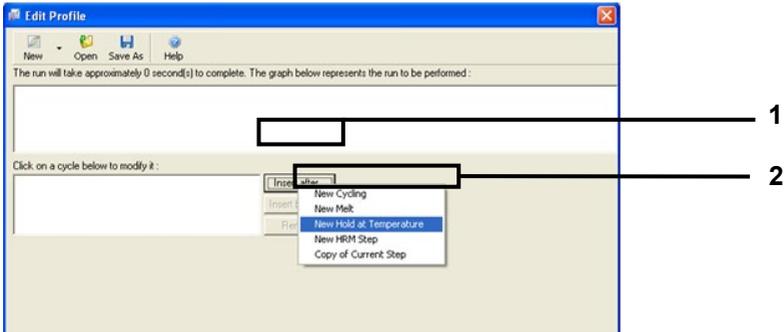


Figure 4. Inserting an initial incubation step (1 = "Insert after"; 2 = "New Hold at Temperature").

7. Set the value in the **Hold Temperature** field to to **95°C** and the value in the **Hold Time** field to **15 minutes 0 second**. Click **Insert After**, then select **New Cycling** (Figure 5).

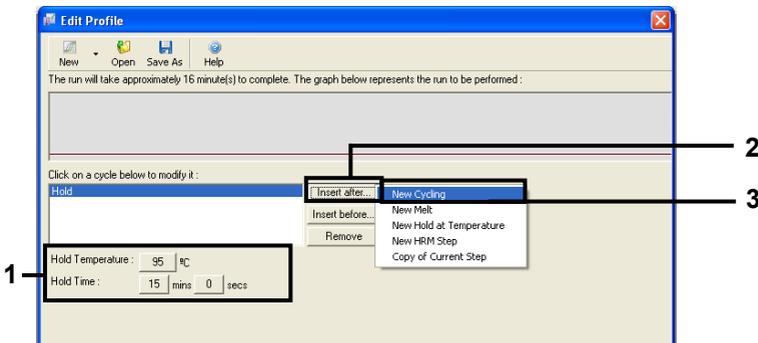


Figure 5. Initial incubation step at 95°C. (1 = "Hold Temperature and Hold Time"; 2 = "Insert after"; 3 = "New Cycling.")

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

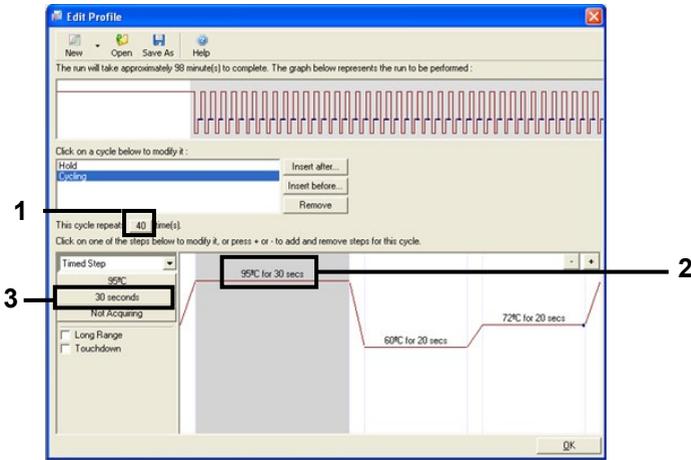


Figure 6. Cycling step at 95°C. (1 = "Cycle repeats" box; 2 = Step one: temperature setting; 3 = Step one: time setting.)

9. Highlight the second step and set to **60°C for 60 seconds**. Click **Not Acquiring** to enable data acquisition during this step (Figure 7).

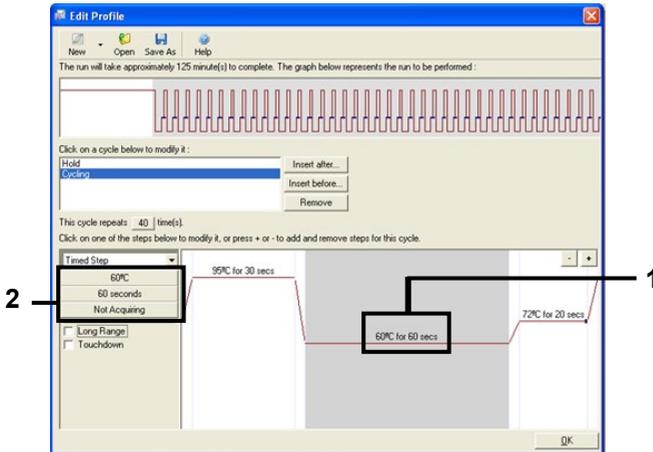


Figure 7. Cycling step at 60°C. (1 = Step two: temperature and time setting; 2 = "Not Acquiring.")

10. Select **Green** and **Yellow** from the Available Channels list, then click > to transfer these channels to the Acquiring Channels list. Click **OK** (Figure 8).

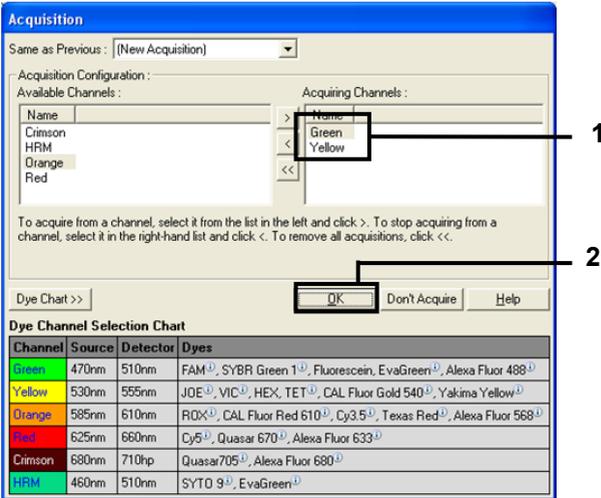


Figure 8. Acquiring at cycling step of 60°C. (1 = Selected channels; 2 = "OK")

11. Highlight the third step and delete and click - to delete. Click **OK** (Figure 9).

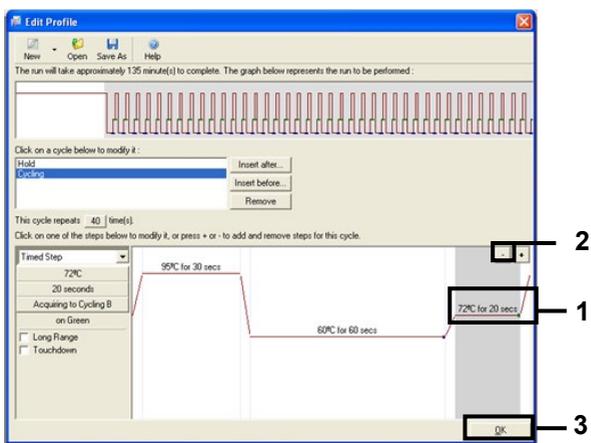


Figure 9. Removal of the extension step. (1 = Third step; 2 = "Delete"; 3 = "OK")

12. In the next dialog box, click **Gain Optimization** (Figure 10).

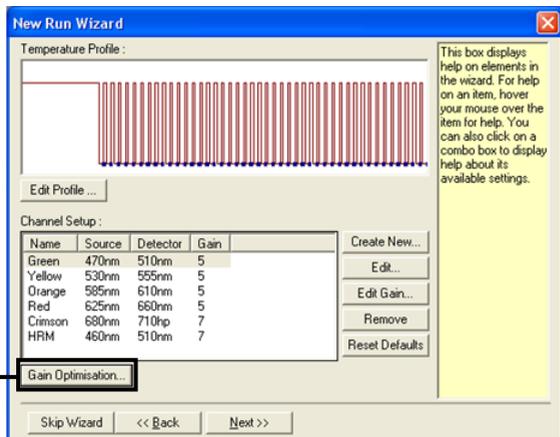


Figure 10. Gain optimization (1).

13. Click **Optimize Acquiring**. Channel settings are displayed for each channel. Click **OK** to accept these default values for both channels (Figure 11).

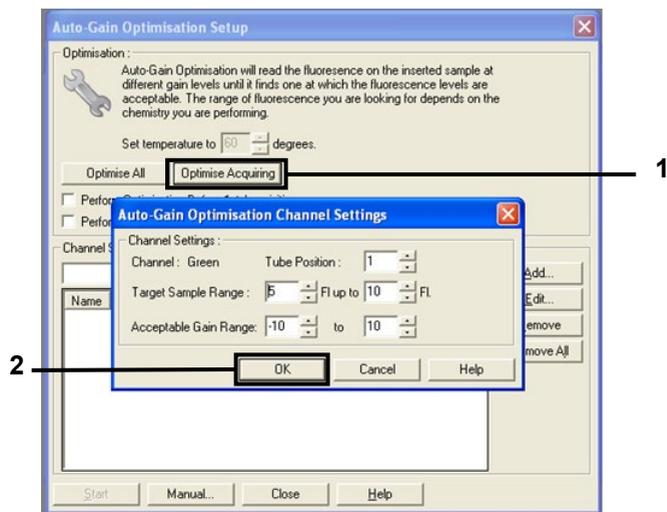


Figure 11. Auto-gain optimization for the Green channel. (1 = "Optimize Acquiring"; 2 = "OK")

14. Check the **Perform Optimization before 1st Acquisition** box, then click **Close** to return to the New Run Wizard dialog box (Figure 12).

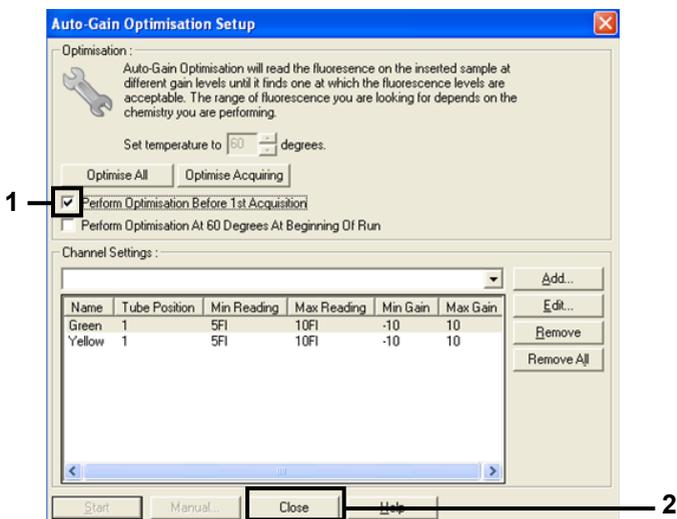


Figure 12. Selection of Green and Yellow channels. (1 = "Perform Optimization Before 1st Acquisition" check box; 2 = "Close")

15. Click **Next** (Figure 13) to save the EGFR RGQ PCR Kit template (*.ret file) in an appropriate location by selecting **Save Template**.

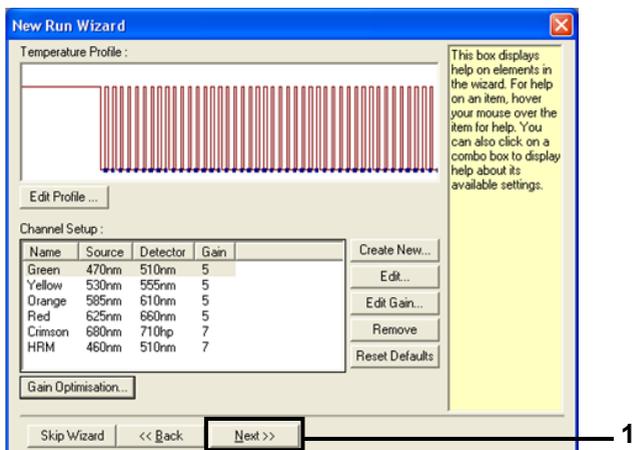


Figure 13. Saving the EGFR RGQ PCR Kit template. (1 = "Next")

Protocol: Sample assessment

This protocol is used to assess the total amplifiable DNA in samples extracted from FFPE tissue. **For DNA extracted from plasma, skip sample assessment** and go directly to “Protocol: EGFR mutation detection run setup,” page 19.

Important points before starting

- Before beginning the procedure, read “General precautions,” page 14.
- Take time to familiarize yourself with the Rotor-Gene Q instrument before starting the protocol. See the instrument user manual.
- Do not vortex *Taq* or any mix containing *Taq*, as this may inactivate the enzyme.
- Pipet *Taq* by placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.
- Up to 24 samples can be assessed using the control reaction mix available.

Things to do before starting

- Before each use, all reagents must be thawed completely for a minimum of 1 hour and a maximum of 4.5 hours at room temperature (15–25°C), **mixed by inverting 10 times**, and centrifuged briefly to collect the contents at the bottom of the tube.
- Mix all samples by inverting 10 times, and centrifuge briefly to collect the contents at the bottom of the tube.
- Ensure that *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 Control Reaction Mix (CTRL), nuclease-free water for No Template Control (NTC), and EGFR Positive Control (PC) at ambient temperature (15–25°C) for a minimum of 1 hour and a maximum of 4.5 hours.

The times for thawing reagents, PCR setup, and storage before starting the run are indicated in Table 3.

Table 3. Thawing times, PCR setup times, and storage temperatures

Thawing time		Storage temperature after PCR setup	Maximum PCR setup and storage time
Minimum	Maximum		
1 h	4.5 h	Ambient temperature (15–25°C)	7 h
1 h	7 h	2–8°C	18 h

Note: PCR setup is performed at ambient temperature (15–25°C). The term “storage” refers to the time between completion of PCR setup and start of the PCR run on the Rotor-Gene Q instrument.

Note: Bring *Taq* to ambient temperature (15–25°C) at the same time as the other reagents (see “Reagent Storage and Handling,” page 16). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

- When the reagents have thawed, **mix by inverting each tube 10 times** to avoid localized concentrations of salts, and then centrifuge briefly to collect the contents at the bottom of the tube.
- Prepare sufficient Control master mix (Control Reaction Mix [CTRL] plus *Taq*) for the DNA samples, an EGFR PC reaction, and an NTC reaction according to the volumes in Table 4. Include reagents for one extra sample to allow sufficient overage for the PCR setup.

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

Table 4. Preparation of Control assay master mix

Component	Volume
Control Reaction Mix (CTRL)	19.5 μl x (n + 1)*
<i>Taq</i> DNA polymerase (<i>Taq</i>)	0.5 μl x (n + 1)
Total volume	20 μl/reaction

* n = number of reactions (samples plus controls). Prepare enough master mix for one extra sample (n + 1) to allow for sufficient coverage for the PCR setup. The value n should not exceed 26 (24 samples, plus 2 controls).

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

4. Thoroughly mix the master mix by gently pipetting up and down 10 times. Place the appropriate number of strip tubes in the loading block according to the layout in Table 5. Immediately add 20 μl of master mix to each PCR strip tube.

Note: Caps remain in the plastic container until required. For DNA sample assessment, Control assay master mix is added to one PC tube, one NTC tube, and one tube for each sample.

Table 5. Layout of DNA sample assessment assays in the loading block*

Assay	Position								
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	-	-	-	-	-	-

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

5. Immediately add 5 μl water for NTC to the tube in position 2 and cap the tube.

6. Add 5 μ l of each sample to the sample tubes (tube positions 3–26) and cap the tubes.
7. Add 5 μ l of EGFR PC to the tube in position 1 and cap the tube.

Note: Take care to avoid loading or pipetting error to ensure correct addition of NTC, samples, and PC to the appropriate tubes. Mark the lids of the tubes to show the direction to load the tubes into the Rotor Gene Q instrument.

8. After all PCR tubes are capped, perform a visual check on fill levels of sample tubes to ensure sample has been added to all tubes.
9. Invert all PCR tubes 4 times to mix samples and reaction mixes.
10. Place the PCR strip tubes into the appropriate positions in the 72-well rotor according to the layout in Table 5.

If the rotor is not fully occupied, fill all empty positions on the rotor with capped, empty tubes.

11. Immediately place the 72-well rotor into the Rotor-Gene Q instrument. Ensure that the locking ring (accessory of the Rotor-Gene Q instrument) is placed on top of the rotor to secure the tubes during the run.
12. Open the Rotor-Gene Q series software version 2.3 and open the appropriate EGFR Kit temperature profile (*.ret file).

Note: For instructions on creating the temperature profile and checking the run parameters, see “Creating a temperature profile,” page 19.

13. Ensure that the correct rotor is selected, and check the box to confirm that the locking ring is attached. Click **Next** (Figure 14).

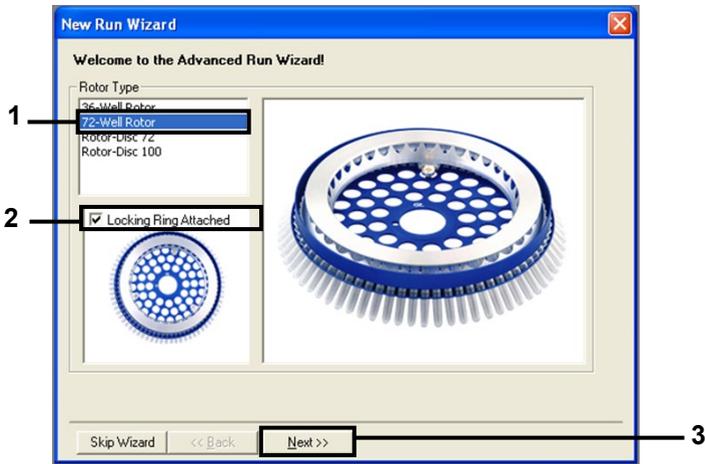


Figure 14. The “New Run Wizard” dialog box. (1 = “Rotor type”; 2 = “Locking Ring Attached” box; 3 = “Next”)

14. Perform these steps in the next window (Figure 15).

14a. Enter the name of the operator in the **Operator** field.

14b. In the Notes field, enter any description you want regarding the template.

14c. Enter 25 in the **Reaction Volume (µl)** field.

14d. Ensure that **1, 2, 3...** is specified in the **Sample Layout** field.

14e. Click **Next**.

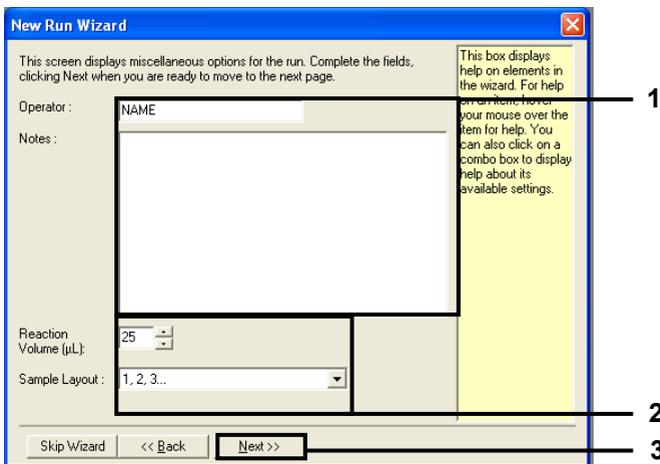


Figure 15. Entering operator name and reaction volumes. (1 = "Operator" and "Notes" dialog fields; 2 = "Reaction Volume" field and "Sample Layout" field; 3 = "Next")

15. The next window allows editing of the temperature profile. (No editing is required as the temperature profile was created according to the instructions in "Creating a temperature profile," page 19). Click **Next** (Figure 16).

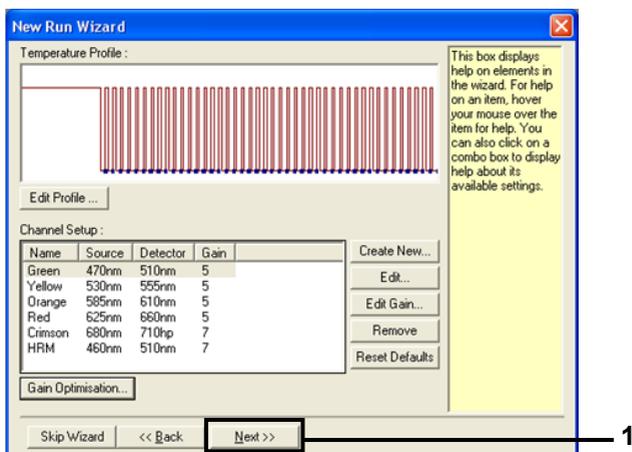


Figure 16. The "New Run Wizard" dialog box and temperature editing screen. (1 = "Next")

16. Check the summary and click **Start Run** to save the run file and start the run (Figure 17).

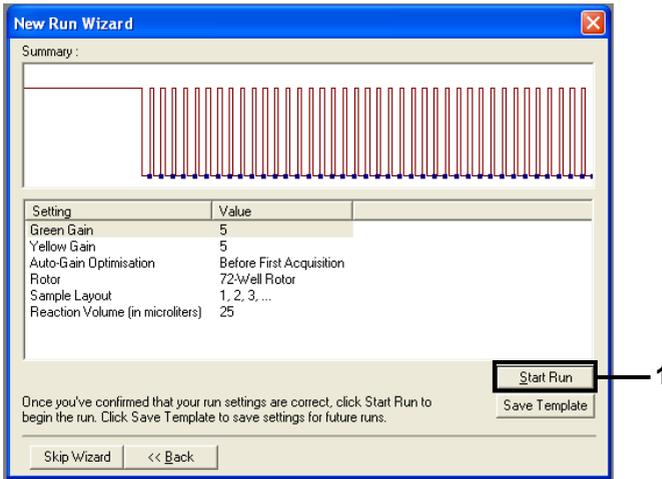


Figure 17. The “New Run Wizard” dialog box and summary screen. (1 = “Start Run”)

17. After the run starts, a new window opens. You can enter sample names in this window, or click **Finish**. You can enter the sample names at a later time by clicking **Sample** during the run, or once the run is complete.

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

Note: When naming samples, fields for empty tubes should be left blank in the Name column.

18. After the run is complete, analyze the data according to “EGFR mutation detection data analysis,” page 47, as appropriate.

19. If quantitation reports are required, click the Reports icon on the toolbar in the Rotor-Gene Q run file. In the report browser, click **Cycling A Green (page 1)** in the **Report**

Categories section (Figure 18).

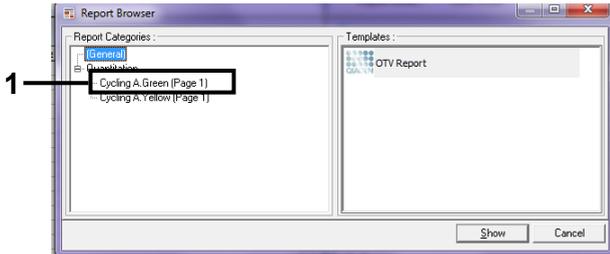


Figure 18. Report browser. (1 = "Cycling A. Green [Page 1]").

20. Select **Quantitation (Full Report)** in the **Templates** section (Figure 19).

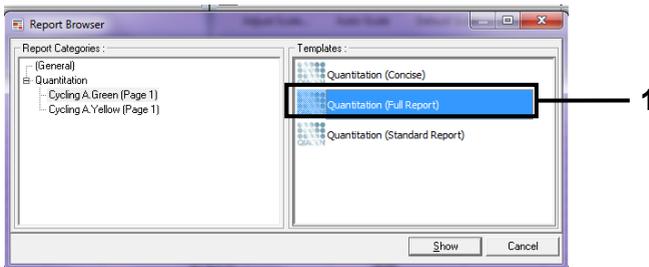


Figure 19. Quantitation report (Full Report) (1).

21. Click **Show** to generate the report.
22. Click **Save As** to save an electronic version.
23. Repeat for **Cycling A Yellow (Page 1)**.

Protocol: EGFR mutation detection

For DNA extracted from plasma, the sample assessment step can be skipped and samples can be tested directly. For DNA extracted from FFPE tissue, only samples that successfully passed sample assessment can be tested.

Important points before starting

- Before beginning the procedure, read “General precautions,” page 14.
- Familiarize yourself with the Rotor-Gene Q instrument before starting the protocol. See the instrument user manual.
- For efficient use of the EGFR RGQ PCR Kit, samples must be grouped as suggested in the layouts for plasma (16 samples) or FFPE tissue (7 samples). See Table 8 on page 39. Smaller batch sizes mean that fewer samples can be tested with the EGFR RGQ PCR Kit.
- Do not vortex the *Taq* or any mix containing *Taq*, as this may inactivate the enzyme.
- Pipet *Taq* by carefully placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.

Things to do before starting

- Before each use, all reagents must be thawed completely for a minimum of 1 hour and a maximum of 4.5 hours at ambient temperature (15–25°C), mixed by inverting 10 times, and centrifuged briefly to collect the contents at the bottom of the tube.
- Mix all samples by inverting 10 times, and centrifuge briefly to collect the contents at the bottom of the tube.
- Ensure that *Taq* is at ambient temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

Procedure

1. Thaw all reaction mix tubes, water for NTC, and EGFR PC at ambient temperature (15–25°C) for a minimum of 1 hour and a maximum of 4.5 hours.

The times for thawing reagents, PCR setup, and storage before starting the run are indicated in Table 6.

Table 6. Thawing times, PCR setup times, and storage temperatures

Thawing time		Storage temperature after PCR setup	Maximum PCR setup and storage time
Minimum	Maximum		
1 h	4.5 h	Ambient temperature (15–25°C)	7 h
1 h	7 h	2–8°C	18 h

Note: PCR setup is performed at ambient temperature (15–25°C). The term “storage” refers to the time between completion of PCR setup and start of the PCR run on the Rotor-Gene Q instrument.

Note: Bring *Taq* (tube *Taq*) to ambient temperature (15–25°C) at the same time as the other reagents (see “Reagent Storage and Handling,” page 16). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

2. When the reagents have thawed, mix by inverting each tube 10 times to avoid localized concentrations of salts, and then centrifuge briefly to collect the contents at the bottom of the tube.
3. Prepare sufficient assay master mixes (assay reaction mix plus *Taq*) for the DNA samples, an EGFR PC, and a NTC reaction according to the volumes in Table 7. Include reagents for one extra sample to allow sufficient coverage for the PCR setup. The master mixes contain all of the components needed for the PCR, except the sample.

Table 7. Preparation of assay master mixes

Assay	Reaction mix tube	Volume of reaction mix	Volume of Taq DNA polymerase (tube Taq)
Control	CTRL	19.5 μ l x (n+1)*	0.5 μ l x (n+1)*
T790M	T790M	19.5 μ l x (n+1)	0.5 μ l x (n+1)
Deletions	Del	19.5 μ l x (n+1)	0.5 μ l x (n+1)
L858R	L85R	19.5 μ l x (n+1)	0.5 μ l x (n+1)
In addition for FFPE tissue workflow prepare:			
L861Q	L861Q	19.5 μ l x (n+1)	0.5 μ l x (n+1)
G719X	G719X	19.5 μ l x (n+1)	0.5 μ l x (n+1)
S768I	S768I	19.5 μ l x (n+1)	0.5 μ l x (n+1)
Insertions	Ins	19.5 μ l x (n+1)	0.5 μ l x (n+1)

* n = number of reactions (samples plus controls). Prepare enough master mix for one extra sample (n + 1) to allow for sufficient coverage for the PCR setup. The value n should not exceed 7 (plus controls) as 7 is the maximum number of samples that can fit on a run.

4. Thoroughly mix the assay master mixes by gently pipetting up and down 10 times. Place the appropriate number of strip tubes in the loading block according to the layout in Table 8. Immediately add 20 μ l of the appropriate assay master mix to each PCR strip tube.

Caps remain in the plastic container until required.

7. Add 5 μ l of EGFR PC to the tubes in position 1–8 and cap the tubes.

Note: Take care to avoid loading or pipetting error to ensure correct addition of NTC, samples, and EGFR PC to the appropriate tubes.

Important: Each tube should contain a total reaction volume of 25 μ l (20 μ l of assay master mix prepared in step 3 (Table 6), plus 5 μ l of NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position.

Mark the lids of the tubes to show the direction to load the tubes into the Rotor Gene Q instrument.

8. After all PCR tubes are capped, perform a visual check on fill levels of sample tubes to ensure sample has been added to all tubes.

9. Invert all PCR tubes 4 times to mix samples and reaction mixes.

10. Place the PCR strip tubes into the appropriate positions in the 72-well rotor according to the layout in Table 7.

A maximum of 16 samples in the plasma layout or 7 samples in the FFPE tissue layout can be included in each PCR run. If the rotor is not fully occupied, fill all empty positions on the rotor with capped, empty tubes.

11. Immediately place the 72-well rotor into the Rotor-Gene Q instrument. Ensure that the locking ring (accessory of the Rotor-Gene Q instrument) is placed on top of the rotor to secure the tubes during the run.

12. Open the Rotor-Gene Q series software version 2.3 and open the appropriate EGFR Kit temperature profile (*.ret file).

For instructions on creating the temperature profile and checking the run parameters, see “Creating a temperature profile,” page 19.

13. Ensure that the correct rotor is selected, and check the **Locking Ring Attached** box to confirm that the locking ring is attached. Click **Next** (Figure 20).

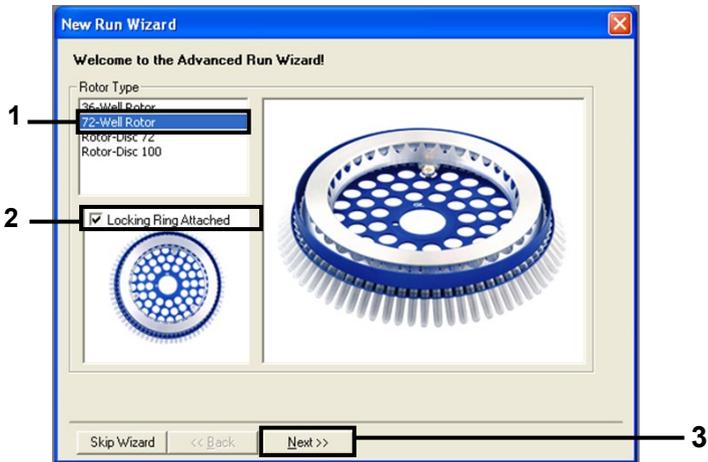


Figure 20. The “New Run Wizard” dialog box. (1 = “Rotor type”; 2 = “Locking Ring Attached” box; 3 = “Next”)

14. Perform these steps in the next window (Figure 21).

14a. Enter the name of the operator in the **Operator** field.

14b. In the Notes field, enter any description you want regarding the template.

14c. Enter 25 in the **Reaction Volume (µl)** field.

14d. Ensure that **1, 2, 3...** is specified in the **Sample Layout** field.

14e. Click **Next**.

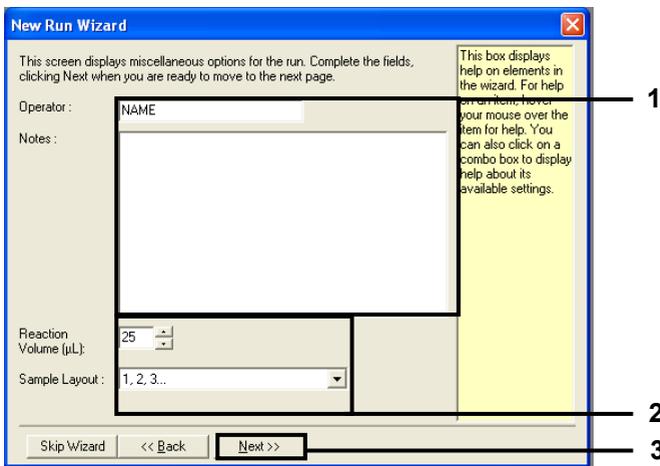


Figure 21. Entering operator name and reaction volumes. (1 = "Operator" and "Notes" dialog fields; 2 = "Reaction Volume" field and "Sample Layout" field; 3 = "Next")

15. The next window allows editing of the temperature profile. (No editing is required as the temperature profile was created according to the instructions in "Creating a temperature profile," page 19). Click **Next** (Figure 22).

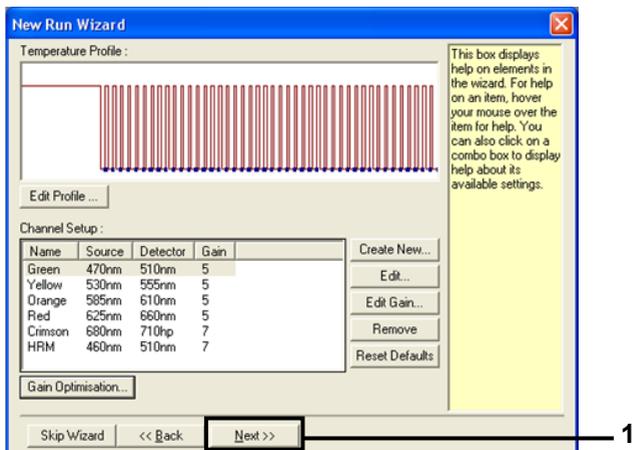


Figure 22. The "New Run Wizard" dialog box and temperature editing screen. (1 = "Next")

16. Check the summary and click **Start Run** to save the run file and start the run (Figure 23).

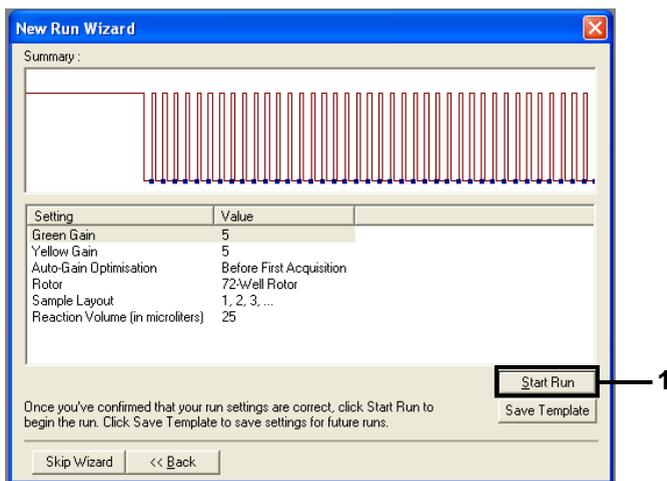


Figure 23. The "New Run Wizard" dialog box and summary screen. (1 = "Start Run")

17. After the run starts, a new window opens. You can enter sample names in this window, or click **Finish**. You can enter the sample names at a later time by clicking **Sample** 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, fields for empty tubes should be left blank in the Name column.

18. After the run is complete, analyze the data according to sections "EGFR mutation detection data analysis," page 47, as appropriate.
19. If quantitation reports are required, click the Reports icon on the toolbar in the Rotor-Gene Q run file.

20. In the report browser, click **Cycling A Green** in the **Report Categories** section (Figure 24).

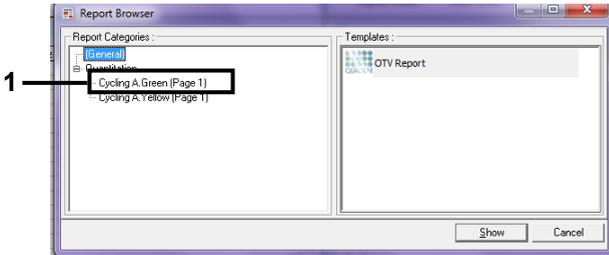


Figure 24. Report browser. (1 = "Cycling A. Green [Page 1]")

21. Select **Quantitation (Full Report)** in the **Templates** section (Figure 25).

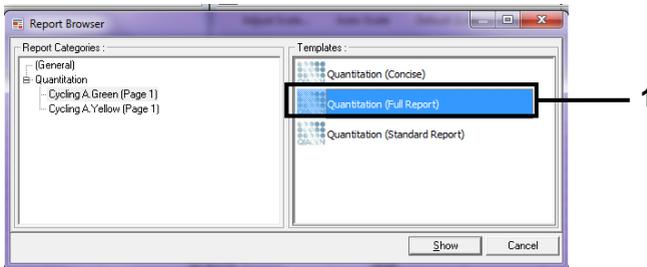


Figure 25. Quantitation report (Full Report) (1).

22. Click **Show** to generate the report.

23. Click **Save As** to save an electronic version.

24. Repeat for **Cycling A Yellow (Page 1)**.

Interpretation of Results

After the EGFR RGQ PCR Kit run (for DNA sample assessment or EGFR mutation analysis) is complete, analyze the data according to the following procedures:

- Software settings for analysis
- DNA sample assessment analysis

Note: See Table 8, page 39 for tube layout

- EGFR mutation detection analysis

Note: See Table 8, page 39 for tube layout

Software analysis settings

1. Open the appropriate run file (*.rex) using the Rotor-Gene Q series software version 2.3.
2. If samples are not named prior to performing the run, click **Edit Samples**.
3. Insert the sample names in the Name column.
Note: Leave the names of any empty tubes blank.
4. Click **Analysis**. On the analysis page, click **Cycling A Yellow** to check the Yellow (HEX) channel.
5. Click **Named On**.
Note: This ensures that empty tubes do not feature in the analysis.
6. Select **Dynamic tube**.
7. Select **Slope correct**.
8. Select **Linear scale**.

9. Select **Take Off Adj** and enter the values **15.01** in the top box (“If take off point was calculated before cycle”) and 20.01 in the bottom box (“then use the following cycle and take off point”).
10. Set the threshold to **0.02** and check the Yellow (HEX) channel C_T values.
11. On the analysis page, click **Cycling A Green** to view the Green (FAM) channel.
12. Select **Named On**.
13. Select **Dynamic tube**.
14. Select **Slope correct**.
15. Select **Linear scale**.
16. Select **Take Off Adj** and enter the values **15.01** in the top box (“If take off point was calculated before cycle”) and **20.01** in the bottom box (“then use the following cycle and take off point”).
17. Set the threshold to **0.075** and check the Green (FAM) channel C_T values.

Sample assessment data analysis

After the DNA sample assessment run is finished, refer to “Software analysis settings,” page 45, and analyze the data as follows. (See Table 8, page 39, for tube layout.)

Run control analysis

Negative control

To ensure that no contamination of the template is present, the NTC must not generate a C_T value below 40 in the Green (FAM) channel.

To ensure that the run was set up correctly, the NTC must show amplification in the range of 29.85 to 35.84 in the Yellow (HEX) channel. The values specified are within and including these values.

Positive control

The EGFR PC must give a C_T value in the Green (FAM) channel within the range of 28.13 to 34.59. A value outside this range indicates an assay setup problem. The run has failed.

Note: Sample data must not be used if either the negative or positive control has failed.

Sample analysis

If the DNA sample assessment run controls are valid, the analysis may proceed. The control C_T value for a sample must be within the range of 23.70 to 31.10 in the Green (FAM) channel. If the sample C_T is out of this range, the following guidance is provided.

- Sample control assay $C_T < 23.70$

Samples with a control C_T of < 23.70 (high DNA concentration) will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples are diluted to fall within the C_T range of 23.70 to 31.10. Diluting sample DNA increases the C_T (dilution of 1:1 increases the C_T value by approximately 1.0). Dilute samples using the water provided in the kit (Water for Dilution [Dil.]).

- Sample control assay $C_T > 31.10$

Re-extraction of samples with a control $C_T > 31.10$ in the Green (FAM) channel is recommended. Insufficient starting DNA template is present to detect all EGFR mutations at the stated cutoff values for the assay.

EGFR mutation detection data analysis

After the EGFR mutation detection run is finished, refer to “Software analysis settings,” page 45, and analyze the data as follows. (See Table 8, page 39 for tube layout.)

Analysis steps are the same for detection from plasma and tissue. However, we recommend only analyzing the most frequent mutations (exon 19 deletions, L858R, T790M) with plasma samples due to limited starting material.

Run control analysis

Refer to the run control analysis flowchart in Figure 26.

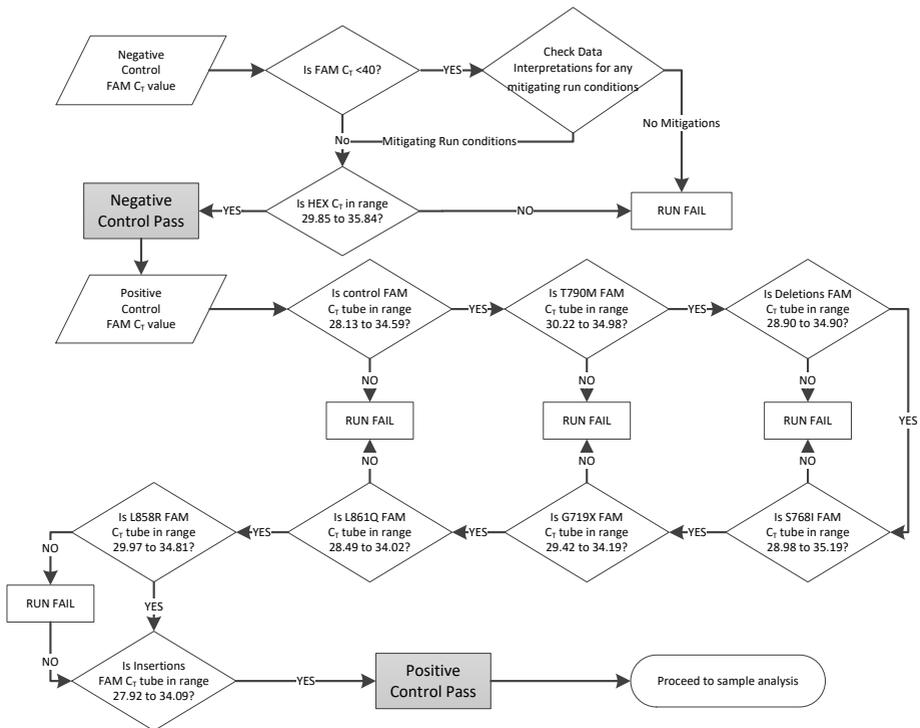


Figure 26. Run control analysis flowchart for EGFR mutation detection.

Negative control

To ensure that no contamination of the template is present, the NTC for each EGFR mutation assay must not generate a C_T value below 40 in the Green (FAM) channel.

To ensure that the run was set up correctly, the NTC must show amplification in the range of 29.85 to 35.84 in the Yellow (HEX) channel. The values specified are within and including these values.

Positive control

For each EGFR mutation assay, the EGFR PC must give a C_T value in the Green (FAM) channel within the range as shown in Table 9. A value outside this range indicates an assay setup problem. The run has failed.

Note: Sample data must not be used if either the negative or positive run control has failed.

Table 9. Acceptable C_T ranges for reaction positive controls (EGFR mutation detection assay)

Reaction mix	Sample	Channel	C_T range
Control	PC	Green	28.13 to 34.59
T790M	PC	Green	30.22 to 34.98
Deletions	PC	Green	28.90 to 34.90
L858R	PC	Green	29.97 to 34.81
In addition for FFPE tissue workflow prepare:			
L861Q	PC	Green	28.49 to 34.02
G719X	PC	Green	29.42 to 34.19
S768I	PC	Green	28.98 to 35.19
Insertions	PC	Green	27.92 to 34.09

Sample analysis: Sample control Green (FAM) channel C_T value

If the positive and negative controls for the EGFR mutation detection run are valid, then EGFR mutation detection in samples may proceed.

The control C_T value for a sample in the Green (FAM) channel must be within the range of 23.70 to 31.10. (See Table 8, page 39 for tube layout.)

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

- Sample control assay $C_T < 23.70$

Samples with a control C_T of < 23.70 (high DNA concentration) will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples are diluted to fall within the C_T range of 23.70 to 31.10. Diluting sample DNA increases the C_T (dilution of 1:1 increases the C_T value by approximately 1.0). Dilute samples using the water provided in the kit (Water for Dilution [Dil.]).

- Sample control assay $C_T > 31.10$

Re-extraction of samples with a control $C_T > 31.10$ in the green (FAM) channel is recommended. Insufficient starting DNA template is present to detect all EGFR mutations at the stated cutoff values for the assay.

Refer to the sample analysis flowchart for EGFR mutation detection in Figure 27.

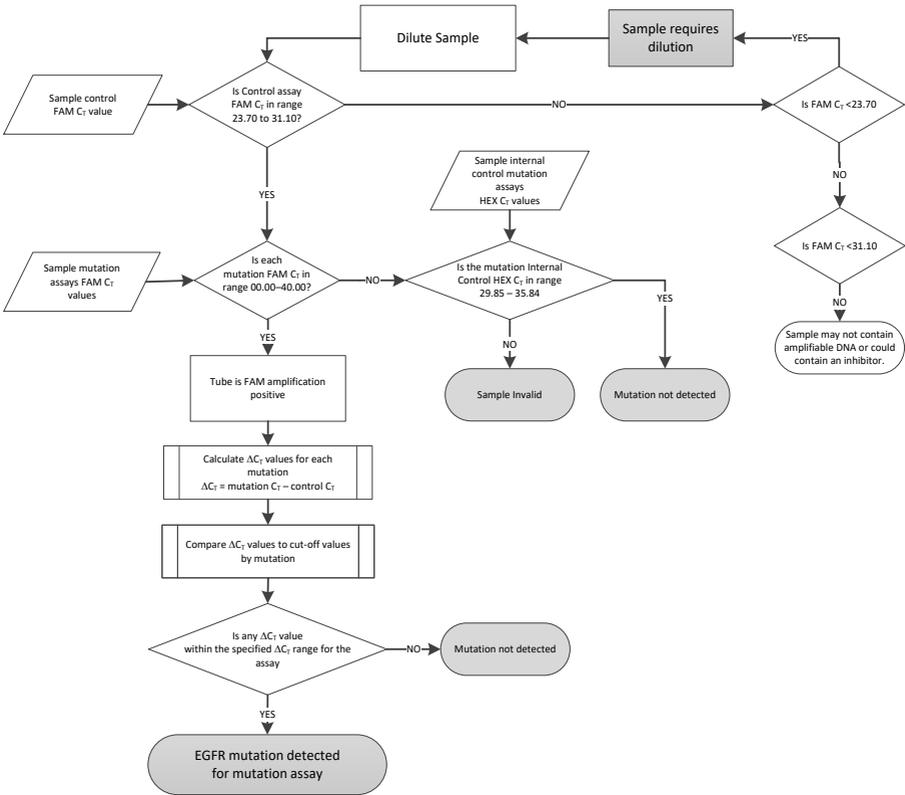


Figure 27. Sample analysis flowchart for EGFR mutation detection.

Sample analysis: Sample internal control Yellow (HEX) channel C_T value

Refer to the sample analysis flowchart for EGFR mutation detection in Figure 27.

All tubes of each sample must be analyzed. Check that each tube generates a HEX signal in the range 29.85 to 35.84 from the internal control in the Yellow (HEX) channel. There are two possible outcomes.

- If the internal control C_T falls within the specified range (29.85 to 35.84), the result is positive for Yellow (HEX) channel amplification.

The Yellow (HEX) channel amplification for that tube is valid.

- If the internal control C_T is above the specified range (>35.84), the result is negative for Yellow (HEX) channel amplification.

Note: If there is amplification in the Green (FAM) channel for that tube, the Yellow (HEX) channel amplification is valid. If there is no amplification in the Green (FAM) channel for the tube, the Yellow (HEX) channel amplification is invalid.

The internal control amplification in the Yellow (HEX) channel may fail due to PCR inhibition. Diluting the sample may reduce the effect of inhibitors. It should be noted that this action also dilutes target DNA in the sample. Dilute samples using the water provided in the kit (Water for Dilution [Dil.]).

Sample analysis: Sample mutation assays Green (FAM) channel C_T value

The Green (FAM) channel values for all 7 EGFR mutation reaction mixes should be checked against the values listed in Table 10. The values specified are within and including the values shown. (See Table 8, page 39 for tube layout.)

Table 10. Acceptable values for sample EGFR mutation reactions in the Green (FAM) channel (EGFR mutation detection assay)

Assay	C_T range	Cutoff (ΔC_T)*
T790M	0.00 to 40.00	-10.00 to 7.40
Deletions	0.00 to 40.00	-10.00 to 8.00
L858R	0.00 to 40.00	-10.00 to 8.90
In addition for use with FFPE tissue samples:		
L861Q	0.00 to 40.00	-10.00 to 8.90
G719X	0.00 to 40.00	-10.00 to 8.90
S768I	0.00 to 40.00	-10.00 to 8.90
Insertions	0.00 to 40.00	-10.00 to 8.00

* -10.00 lower bound introduced to remove possible PCR artefact

- If the Green (FAM) channel C_T for the sample falls within the specified range, it is FAM amplification positive.
- If the Green (FAM) channel C_T for the sample is above the specified range, or there is no amplification, it is FAM amplification negative.

Calculate the ΔC_T value for each EGFR mutation detection tube that is FAM amplification positive as follows, ensuring that the mutation and control C_T values are from the same sample. (See Table 8, page 39 for tube layout.)

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

Compare the ΔC_T value for the sample with the cutoff range for the assay in question (Table 10). Ensure that the correct cutoff range is applied.

The upper cutoff value is the point above which a positive signal for an assay could potentially be due to background signal of the ARMS primer on wild-type DNA. If the sample ΔC_T value is higher than the upper cutoff value for an assay. If the sample ΔC_T value is lower than the lower cutoff value for an assay, the sample is classed as invalid due to a PCR artefact. Retest the sample adhering to the required mixing steps during the assay setup.

The status of each mutation reaction for every sample may be one of the following:

- Mutation detected
- Mutation not detected
- Invalid

Mutation detected

Green (FAM) channel amplification is positive and the ΔC_T value is within the cutoff range. If multiple mutations are detected for a sample, all can be reported.

Mutation not detected

Green (FAM) channel amplification is positive and the ΔC_T value is above the cutoff value.

Green (FAM) channel amplification is negative and Yellow (HEX) channel amplification (internal control) is positive.

Invalid

Yellow (HEX) channel amplification (internal control) is invalid.

Green (FAM) channel amplification is negative and Yellow (HEX) channel amplification (internal control) is negative.

ΔC_T value lower than -10.00

Note: A sample may be Yellow (HEX) channel amplification negative in one tube but Green (FAM) channel amplification positive in a second tube. In that case, a “mutation detected” result in the second tube can be considered valid but the particular mutation identified may not be the only possible mutation in that sample.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/support (for contact information, visit www.qiagen.com)

Comments and suggestions

NTC samples show positive results in the Green FAM channel

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.
--	---

No signal with the EGFR positive control

- | | |
|--|--|
| 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 Q instrument | Compare the temperature profile with the protocol. If incorrect, repeat the run. |
| 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 Reagent Storage and Handling, page 16 | 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. |

Limitations

- The product is intended for use on a Rotor-Gene Q real-time PCR cycler.
- Strict compliance with the *EGFR 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 EGFR RGQ PCR Kit. Additional Control Reaction Mix is provided to determine that the C_T value is acceptable for the assay. Absorbance readings must not be used as they do not correlate with the C_T values 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.

Quality Control

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

References

1. Whitcombe, D. et al. (1999) Detection of PCR products using self-probing amplicons and fluorescence. *Nature Biotech.* **17**, 804.
2. Thelwell, N. et al. (2000) Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res.* **28**, 3752.

Symbols

The symbols in the following table are used in this Instructions for Use.

Symbol	Symbol definition
 Σ <N>	Contains sufficient for N tests
	Use by
	Catalog number
	Lot number
	Material number
	Keep away from sunlight

Symbol

Symbol definition



Global Trade Item Number

Rn

R is for revision of the Handbook and n is the revision number



Temperature limitation



Manufacturer



Consult instructions for use



Caution

Ordering Information

Product	Contents	Cat. no.
EGFR RGQ PCR Kit (24)	For 24 reactions: Control Assay, 7 Mutation Assays, Positive Control, Taq DNA Polymerase, Water for NTC, and Water for Sample Dilution	874101
Extraction kits		
QIAamp Circulating Nucleic Acid Kit	For 50 preps: QIAamp Mini Columns, Tube Extenders (20 ml), QIAGEN Proteinase K, Carrier RNA, Buffers, VacConnectors, and Collection Tubes (1.5 ml and 2 ml)	55114
QIAamp DNA FFPE Tissue Kit (50)	For 50 preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, and Collection Tubes (2 ml)	56404
Rotor-Gene Q		
Rotor-Gene Q MDx 5plex System	Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9002023

Rotor-Gene Q 5plex Platform	Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9002022
Rotor-Gene Q accessories		
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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Document Revision History

Date	Changes
R2, January 2019	Updated address of legal manufacturer (front cover)
R3, February 2020	Revised cover page to change 29 mutations to 28 mutations Updated Table 1 to remove COSMIC ID 6254 due to mutation with 12369 Updated the EGFR mutation detection data analysis section to describe the change from a cutoff point to a cutoff range Updated Table 10 to include data for cutoff range

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