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therascreen[®] EGFR Plasma RGQ PCR Kit Handbook



Version 1



For in vitro diagnostic use

For use with Rotor-Gene[®] Q MDx 5plex HRM instruments



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Intended Use

The *therascreen* EGFR Plasma RGQ PCR Kit is an in vitro diagnostic test for the detection of exon 19 deletions, exon 20 and 21 substitutions (T790M and L858R respectively) in the epidermal growth factor receptor (EGFR) gene, and will provide a qualitative assessment of the mutation status. Results are intended to aid the clinician in identifying patients with NSCLC who may benefit from treatment with IRESSA® (gefitinib) when a tissue sample cannot be evaluated.

The *therascreen* EGFR Plasma RGQ PCR Kit is to be used by trained personnel in a professional laboratory environment with DNA samples extracted from plasma obtained from non-small cell lung cancer (NSCLC) patient blood.

The *therascreen* EGFR Plasma RGQ PCR Kit is intended for in vitro diagnostic use.

Summary and Explanation

The *therascreen* EGFR Plasma RGQ PCR Kit is a ready-to-use kit for the detection of mutations in the EGFR cancer-related gene using polymerase chain reaction (PCR) on Rotor-Gene Q MDx 5plex HRM instruments.

Using Scorpions® and ARMS technologies, the *therascreen* EGFR Plasma RGQ PCR Kit enables detection of the following EGFR gene mutations against a background of wild-type genomic DNA.

- Deletions in exon 19
- T790M
- L858R

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

Principle of the Procedure

The *therascreen* EGFR Plasma RGQ PCR Kit utilizes two technologies – ARMS and Scorpions – for detection of mutations during real-time PCR.

ARMS

Allele- or mutation-specific amplification is achieved using ARMS (Amplification Refractory Mutation System). *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, 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 results in an increase in fluorescence from the reaction tube.

Kit format

Four assays are supplied in the *therascreen* EGFR Plasma RGQ PCR Kit:

- One control assay (Ctrl)
- Three mutation assays

All reaction mixes contain reagents to detect targets which are labeled with FAM™, and an internal control assay labeled with 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.

Assays

Control assay

The control assay, labeled with FAM, is used to assess the total DNA in a sample. This 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 contains a FAM-labeled Scorpion probe and an ARMS primer for discrimination between wild-type DNA and a specific mutant DNA.

Controls

All experimental runs must contain the following controls:

Positive control

Each run must contain a positive control in tubes 1–4. The *therascreen* EGFR Plasma 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 9–12. The NTC consists of Nuclease-Free Water (H₂O) 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 in addition to the target reaction. A failure indicates that either there may be inhibitors present that could lead to false negative results, or that an operator setup error has occurred for that tube.

If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of inhibitors, but it should be noted that this would also dilute the target DNA. FAM amplification can out-compete the internal control 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.

Materials Provided

Kit contents

<i>therascreen</i> EGFR Plasma RGQ PCR Kit			(24)
Catalog no.			870311
Number of reactions			24
Red	Control Reaction Mix	Ctrl	2 x 600 µl
Purple	T790M Reaction Mix	T790M	600 µl
Orange	Deletions Reaction Mix	Del	600 µl
Pink	L858R Reaction Mix	L858R	600 µl
Beige	EGFR Positive Control	PC	300 µl
Mint	<i>Taq</i> DNA Polymerase	<i>Taq</i>	2 x 80 µl
White	Nuclease-Free Water for No Template Control	NTC	1 x 1.9 ml
White	Nuclease-Free Water for Dilution	Dil	1 x 1.9 ml
<i>therascreen</i> EGFR Plasma RGQ PCR Kit Instructions for Use (Handbook)			1

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.

- DNA extraction kit (see “Procedure,” page 17)
- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR master mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA
- DNase, RNase and DNA-free pipette tips with filters (to avoid cross-contamination, we recommend pipette tips with aerosol barriers)
- 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
- Bench top centrifuge* with rotor for 2 ml reaction tubes
- Vortex
- Rotor-Gene Q MDx 5plex HRM instrument* † with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and HEX, respectively)
- Rotor-Gene Q software, version 2.3.5 or later
- Strip Tubes and Caps, 0.1 ml, for use with 72-well rotor (cat. no. 981103 or 981106)
- DNase, RNase, and DNA-free microcentrifuge tubes for preparing master mixes
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction setup with a single-channel pipette (QIAGEN, cat. no. 9018901)

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

† In some countries, if applicable the Rotor-Gene Q 5plex HRM instrument with a production date of May 2011, or later can be used. The production date can be obtained from the serial number on the back of the instrument. The serial number is in the format “mmyynnn” where “mm” indicates the production month in digits, “yy” indicates the last two digits of the production year, and “nnn” indicates the unique instrument identifier.

Warnings and Precautions

For In Vitro Diagnostic Use

For Professional Use

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in 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.

General precautions

The user should always pay attention to the following:

- Use DNase, RNase, and DNA-free pipette tips with filters and make sure that pipettes 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.

Note: Use extreme caution to prevent contamination of PCRs with synthetic control material. We recommend using separate, dedicated pipettes for setting up reaction mixes and adding DNA template. Preparation and dispensing of reaction mixes must be carried out in an area separate from the area used for 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.

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

Note: All reagents in the *therascreen* EGFR Plasma RGQ PCR Kit are formulated specifically for use with the stated tests. All reagents supplied in the *therascreen* EGFR Plasma RGQ PCR Kit are intended to be used solely with the other reagents in the same *therascreen* EGFR Plasma RGQ PCR Kit.

Substitutions to the reagents in the kit must not be made if optimal performance is to be maintained.

Note: Use only the *Taq* DNA polymerase (*Taq*) provided in the kit. Do not substitute with *Taq* DNA polymerase from other kits of the same or any other type, or with *Taq* DNA polymerase from another supplier.

Note: Reagents for the *therascreen* EGFR Plasma 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 μ l, since this will increase the risk of false negatives.

Reagent Storage and Handling

The *therascreen* EGFR Plasma RGQ PCR Kit is shipped on dry ice. If any component of the *therascreen* EGFR Plasma RGQ PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, Instructions for Use, or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (visit www.qiagen.com).

The *therascreen* EGFR Plasma RGQ PCR Kit should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. When stored under the specified storage conditions, the *therascreen* EGFR Plasma RGQ PCR Kit is stable until the stated expiration date.

Once opened, reagents can be stored in their original packaging at -30 to -15°C for 12 months or until the stated expiration date, whichever comes first. Repeated thawing and freezing should be avoided. Do not exceed a maximum of eight freeze–thaw cycles.

The reagents must be thawed at ambient temperature 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 and the Rotor-Gene Q tubes containing the master mixes and the DNA sample should be loaded onto the Rotor-Gene Q MDx 5plex HRM immediately. The total time from start of PCR setup to the start of the run should not exceed:

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

Note: Scorpions (as with all fluorescently labeled molecules) in the reaction mix reagents are light sensitive. Protect control and reaction mix reagents from light to avoid photo bleaching.

Reagents in the *therascreen* EGFR Plasma RGQ PCR Kit are diluted optimally and no further purification or treatment is required prior to their use in analysis as directed by the *therascreen EGFR Plasma RGQ PCR Kit Instructions for Use (Handbook)*.

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.

Specimen Storage and Handling

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

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

Procedure

DNA extraction

The performance characteristics for this kit have been generated using DNA extracted with the QIAamp® Circulating Nucleic Acid Kit (cat. no. 55114). When using the QIAamp Circulating Nucleic Acid Kit, 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).
- Store purified genomic DNA at –30 to –15°C.

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

Protocol: Detection of EGFR Mutations

Important points before starting

- To obtain correct results, ensure that the described mixing procedure is performed at each mixing step of the assay setup process.
- Up to 16 samples may be assessed on each run.
- Before beginning the procedure, read “General precautions,” page 11.
- Take time to familiarize yourself with the Rotor-Gene Q MDx 5plex HRM before starting the protocol. See the instrument user manual.
- Do not vortex the *Taq* DNA Polymerase (*Taq*) or any mix containing *Taq* DNA polymerase, as this may inactivate the enzyme.
- Pipet *Taq* by placing the pipette tip just under the liquid surface to avoid the tip being coated in excess enzyme.
- For each DNA sample, the control and mutation assays must be analyzed in the same PCR run to avoid run-to-run variations.
- For efficient use of the reagents in the *therascreen* EGFR RGQ PCR Kit, batch DNA samples as far as possible to create full runs. Testing samples individually or in smaller numbers uses up more reagents and reduces the overall number of samples that can be tested with a single *therascreen* EGFR RGQ PCR Kit.

Things to do before starting

- Before each use, all reagents need to 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.
- 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.
- **Mix all samples by inverting 10 times**, and centrifuge briefly to collect the contents at the bottom of the tube.

Procedure

1. Completely thaw all reaction mixes, Nuclease-Free Water for No Template Control (NTC), and EGFR Positive Control (PC) at room temperature (15–25°C) for a minimum of 1 hour (Table 1). When the reagents have thawed, **mix them 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.

Table 1. Thawing times, PCR set up times and storage temperatures

Minimum thaw time	Maximum thaw time	Storage temperature after PCR setup	Maximum PCR setup and storage time
1 hour	4.5 hours	Ambient temperature (15–25°C)	6 hours
1 hour	4.5 hours	2 – 8°C	18 hours

Note: PCR setup is to be performed at ambient temperature. Storage refers to the time between completion of PCR setup and start of the PCR run on the Rotor-Gene Q MDx 5plex HRM.

Note: Bring *Taq* DNA polymerase (tube *Taq*) to ambient temperature (15–25°C) at the same time as the other reagents (see “Reagent Storage and Handling,” page 13). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

2. Perform the following steps:
 - 2a. Label four microcentrifuge tubes (not provided) according to each corresponding reaction mix shown in Table 2.
 - 2b. Prepare sufficient master mixes (control or mutation reaction mix [tube CTRL, T790M, Deletions, L858R] plus *Taq* DNA polymerase [*Taq*]) for the DNA samples, one EGFR positive control (tube PC) reaction, and one Nuclease-Free Water for No Template Control (tube NTC) reaction according to the volumes in Table 2.
- Note:** Include reagents for one extra sample to allow sufficient overage for the PCR setup. The master mixes contain all of the components needed for PCR except the sample.

Table 2. Preparation of master mixes*

Assay	Reaction mix tube	Volume of reaction mix	Volume of <i>Taq</i> DNA polymerase (tube <i>Taq</i>)
Control	CTRL	19.50 $\mu\text{l} \times (n+1)$	0.50 $\mu\text{l} \times (n+1)$
T790M	T790M	19.50 $\mu\text{l} \times (n+1)$	0.50 $\mu\text{l} \times (n+1)$
Deletions	Del	19.50 $\mu\text{l} \times (n+1)$	0.50 $\mu\text{l} \times (n+1)$
L858R	L858R	19.50 $\mu\text{l} \times (n+1)$	0.50 $\mu\text{l} \times (n+1)$

* When preparing the master mix, prepare enough for one extra sample to allow for sufficient coverage for the PCR setup.

Note: When preparing the master mix, the required volume of the control or mutation reaction mix is added to the relevant tube first and the *Taq* DNA polymerase is added last.

- Place the appropriate number of PCR 4-strip tubes (each strip has 4 tubes) in the loading block according to the layout in Table 3. Do not cap the tubes.

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

- Cap the tube for the master mix and **invert 10 times to mix the master mix** followed by brief centrifugation to ensure the mix is at the bottom of the tube. Immediately add 20 μl master mix to each appropriate PCR strip tube.
- Immediately add 5 μl of Nuclease-Free Water (H_2O) to the no template control PCR strip tubes (PCR tube numbers 9–12) and cap the tubes.
- Add 5 μl of each sample to the sample tubes (PCR tubes 5–8, 13–16 and 17–72) and cap the tubes.
- Add 5 μl of EGFR Positive Control (PC) to the positive control tubes (PCR tube numbers 1–4). Each DNA sample must be tested with the control and all mutation assays. The layout is shown in Table 3.

Table 3. Layout of control and mutation assays

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
Sample number									
Assay	8	9	10	11	12	13	14	15	16
Ctrl	5	13	21	29	37	45	53	61	69
T790M	6	14	22	30	38	46	54	62	70
Deletions	7	15	23	31	39	47	55	63	71
L858R	8	16	24	32	40	48	56	64	72

8. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5, 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q MDx 5plex HRM.
9. **Invert capped tubes 4 times to mix the sample and reaction mix.**
10. Place all PCR 4-strip tubes into the appropriate positions in the 72-well rotor and visually check that all tubes contain an equal volume.

Note: Ensure that strips of tubes are not reversed when transferring them to the rotor.
11. If the rotor is not full, fill remaining spaces with capped empty tubes.
12. Immediately place the rotor into the Rotor-Gene Q MDx 5plex HRM. Make sure that the locking ring (Rotor-Gene Q MDx accessory) is placed on top of the rotor to secure the tubes during the run.
13. Refer to Rotor-Gene Q MDx 5plex HRM setup (see "Protocol: Rotor-Gene Q EGFR Setup," page 20) to create the temperature profile and start the run.

Protocol: Rotor-Gene Q EGFR Setup

The cycling parameters are shown in Table 4.

Table 4. Cycling parameters

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

1. Double-click the **Rotor-Gene Q Series Software 2.3** software icon on the desktop of the laptop connected to the Rotor-Gene Q MDx 5plex HRM. Select the “Advanced” tab in the “New Run” dialog box that appears.
2. To create a new template, select **Empty Run**, then click **New**.
The “New Run Wizard” window appears.
3. Select **72-Well Rotor** as the rotor type. Ensure that the locking ring is attached and check the **Locking Ring Attached** box. Click **Next** (Figure 1).

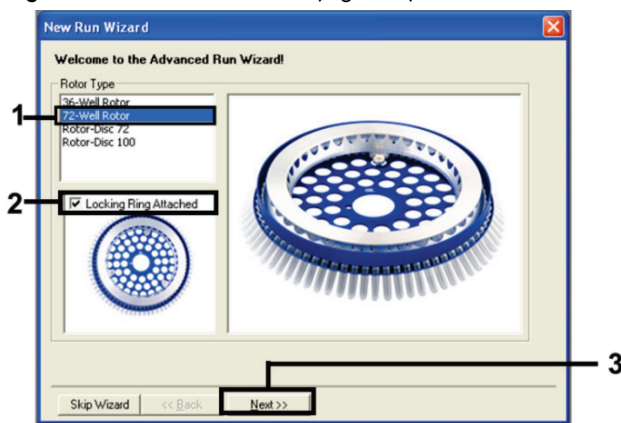


Figure 1. The “New Run Wizard” dialog box.

4. Enter the name of the operator in the **Operator** field. Add any notes and set the value in the **Reaction Volume** field as **25**. Ensure that the values in the **Sample Layout** field is set as **1, 2, 3...** Click **Next** (Figure 2).

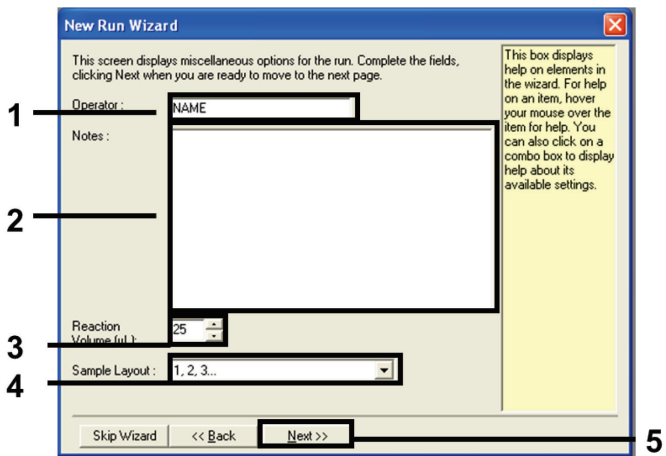


Figure 2. Entering operator name and reaction volumes.

5. Click **Edit Profile** in the “New Run Wizard” dialog box (Figure 3) and set the run parameters according to the following steps.

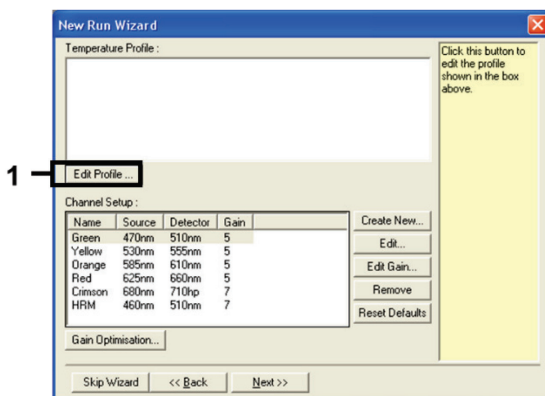


Figure 3. Editing the profile.

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

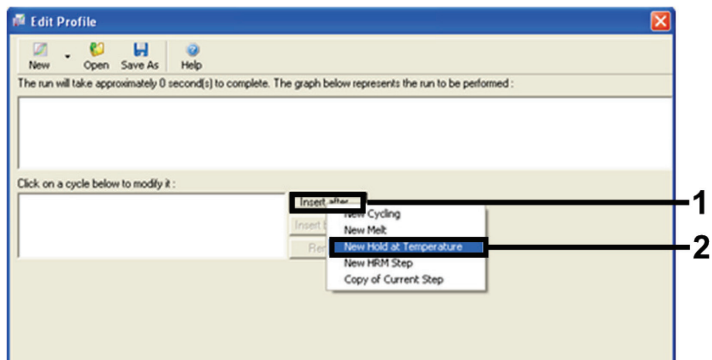


Figure 4. Inserting an initial incubation step.

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

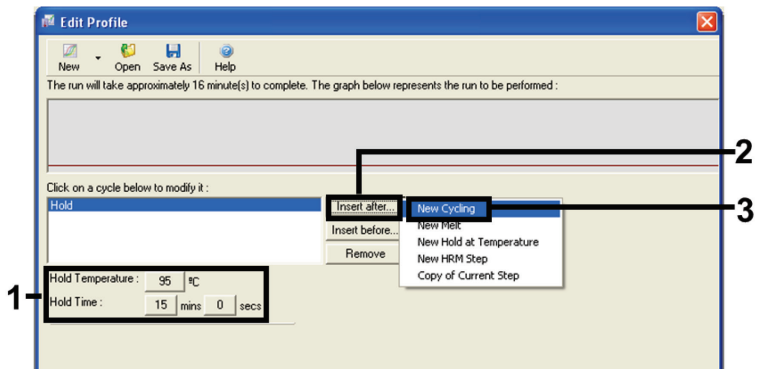


Figure 5. Initial incubation step at 95°C.

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

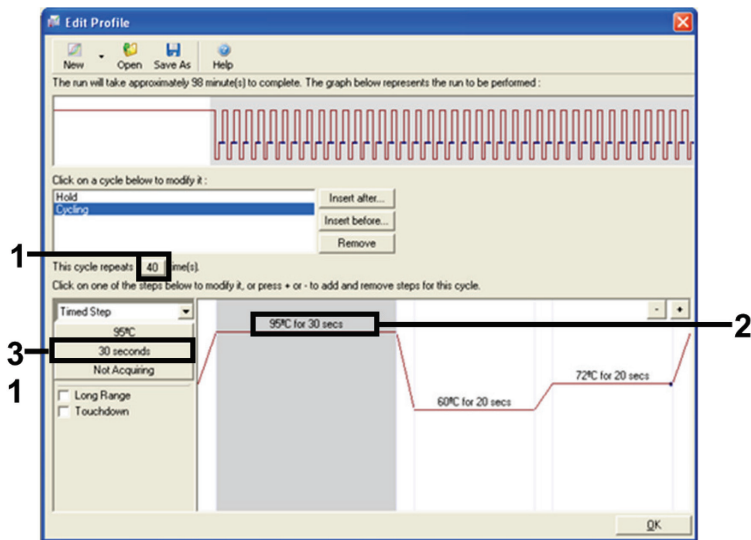


Figure 6. Cycling step at 95°C.

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).

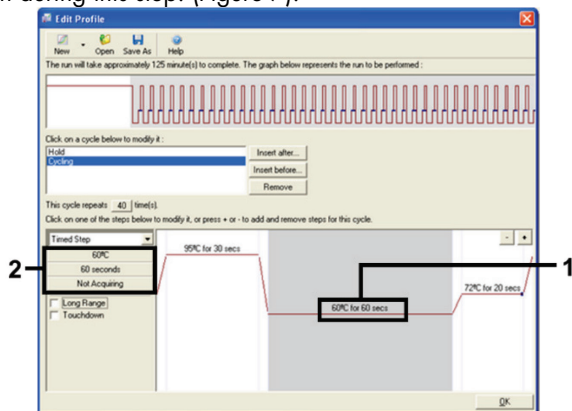


Figure 7. Cycling step at 60°C.

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

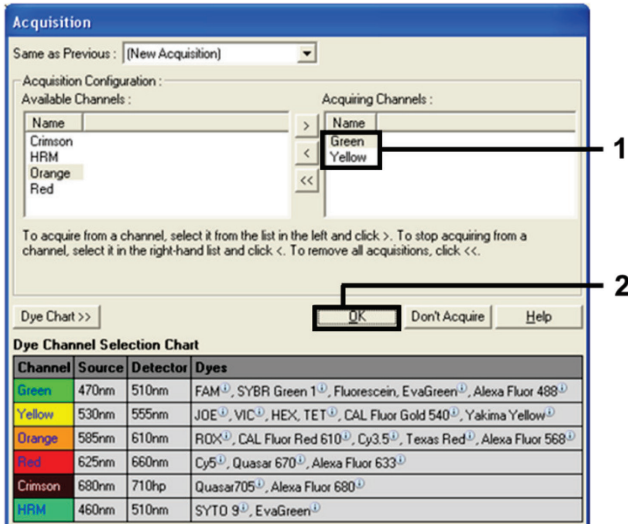


Figure 8. Acquiring at cycling step of 60°C.

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

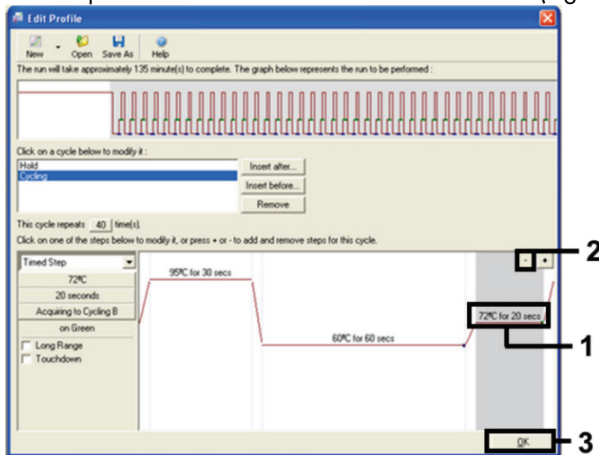


Figure 9. Removal of the extension step.

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

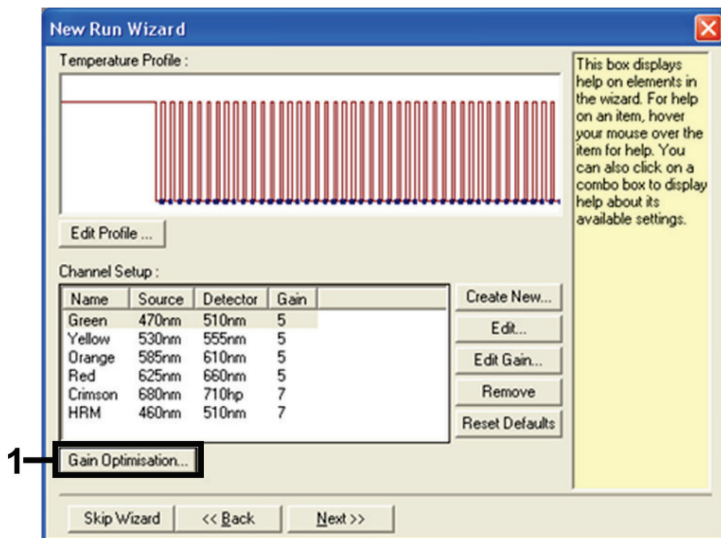


Figure 10. Gain optimization.

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

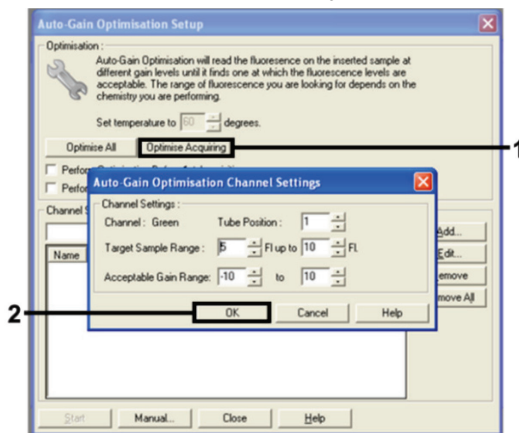


Figure 11. Auto-gain optimization for the Green channel.

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

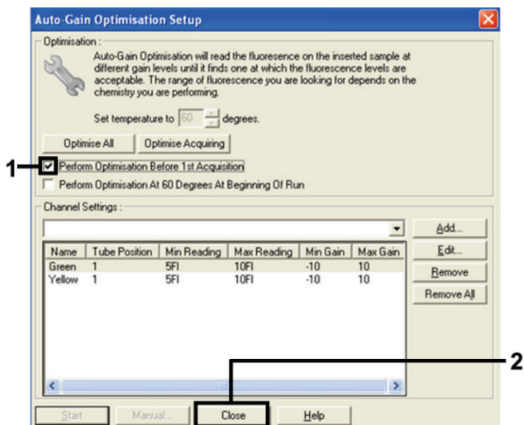


Figure 12. Selection of Green and yellow channels.

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

Mutation assessment data analysis

After the run is complete, analyze the data using the following procedure.

Setting up the software analysis

1. Open the appropriate file using the Rotor-Gene Q Series Software 2.3.5 or later.
2. If the samples are not named prior to performing the run, click **Edit Samples**.
3. Enter the sample names in the **Name** column.

Note: Leave the names of any empty wells blank.

4. Click **Analysis**. On the analysis page, click **Cycling A Yellow** to check the HEX channel.
5. Check that the **Dynamic Tube** option is highlighted. Click **Slope Correct** and **Linear scale**.
6. Click **Take Off Adj** and enter **15.01** and **20.01** as shown in Figure 13.

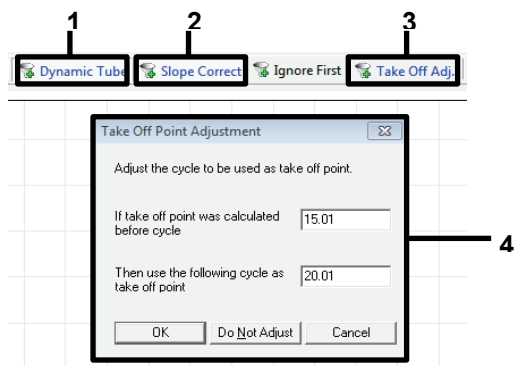


Figure 13. EGFR analysis normalization settings. 1 = "Dynamic Tube," 2 = "Slope Correct," 3 = "Take Off Adj," 4 = "Take Off Adj" dialog window with parameter values.

7. Set the threshold at **0.02** and check the HEX C_T values.
8. On the analysis page, click **Cycling A , Green** to view the FAM channel. Set parameters as in Figure 13 above.
The dynamic tube should be highlighted.
9. Click **Slope Correct** and **Linear scale**.

10. Set the threshold at **0.075** and check the FAM C_T values.

Run control analysis

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

- **Negative control:** To ensure that no template contamination is present, the NTC must not generate a C_T value in the green (FAM) channel below 40. To ensure that the run was set up correctly, the NTC must show amplification of 29.85 to 35.84 in the yellow (HEX) channel (Internal Control).

If there is positive amplification in the green channel and/or amplification is outside the range 29.85 to 35.84 in the yellow channel, the run is invalid.

- **Positive control:** The EGFR Positive Control (PC) must give a C_T for each reaction mix within and including the stated range in Table 5. A run with a positive control value outside this range indicates an assay setup problem and the run should be designated as a failure. If the positive control gives a C_T within range (FAM) but an internal control C_T (HEX) outside the range of 29.85 to 35.84, continue with the analysis.

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

Table 5. Acceptable C_T range for run controls

Reaction Control	Assay	Channel	C_T range
Positive control	Control	Green (FAM)	28.13–34.59
	T790M	Green (FAM)	30.22–34.98
	Deletions	Green (FAM)	28.90–34.90
	L858R	Green (FAM)	29.97–34.81
No-template control	All four reaction mixes	Green (FAM)	≥40.00
	All four reaction mixes	Yellow (HEX)	29.85–35.84

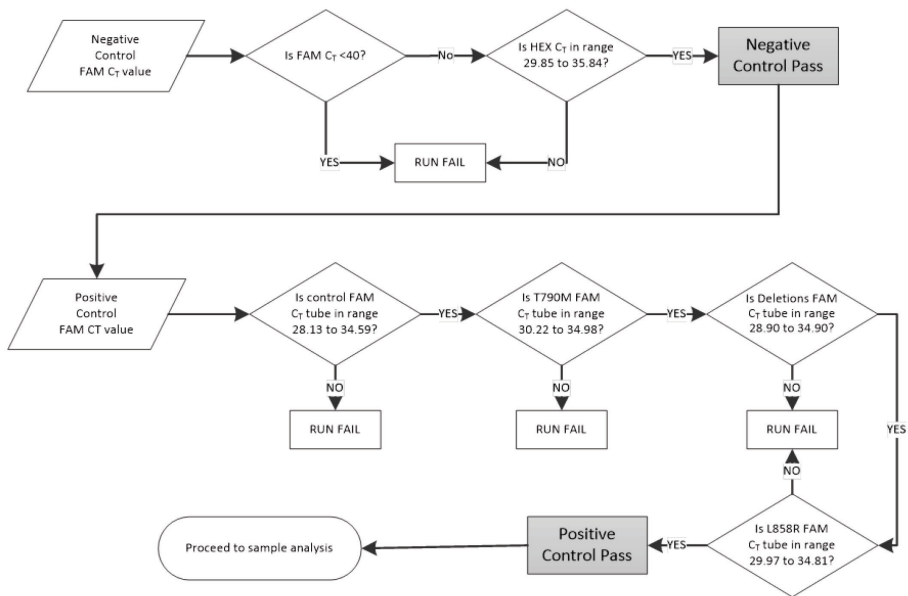


Figure 14. Run control analysis workflow.

Provided that both run controls are valid, each sample control assay C_T value must be within the range 23.70 to 31.10 in the green (FAM) channel (Table 6).

Table 6. Acceptable FAM C_T range for sample control reaction

Reaction Mix	Channel	Acceptable C_T Range
Control	Green (FAM)	23.70 – 31.10

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

- **Sample control assay C_T of <23.70**: Samples with a control C_T of <23.70 will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range on the basis that diluting by half will increase the C_T by 1.
- **Sample control assay C_T >31.10**: Sample does not contain sufficient DNA to permit analysis.

Provided that both run controls are valid and the control assay is within the range stated in Table 6, each sample mutation C_T value must be within the range detailed in Table 7 in the green (FAM) channel. If the sample is out of this range, the following guidance is provided.

Table 7. Acceptable sample mutation reaction values

Reaction	Reaction mix	Channel	C_T range
Mutation reaction	T790M	Green (FAM)	0.00–40.00
	Deletions	Green (FAM)	0.00–40.00
	L858R	Green (FAM)	0.00–40.00
	All three mutations	Yellow (HEX)	29.85–35.84

Note: If a sample does not generate a C_T (i.e., $C_T >40$), it may be due to the presence of an inhibitor, an error in assay setup, or there is no amplifiable EGFR DNA.

- **Internal control C_T value is within 29.85–35.84**: There is no amplifiable EGFR DNA.
- **Internal control C_T value is not within the range of 29.85–35.84**: This could indicate an assay setup error or the presence of an inhibitor. It is possible to reduce the effect of an inhibitor by diluting the sample, although this will also dilute the DNA.

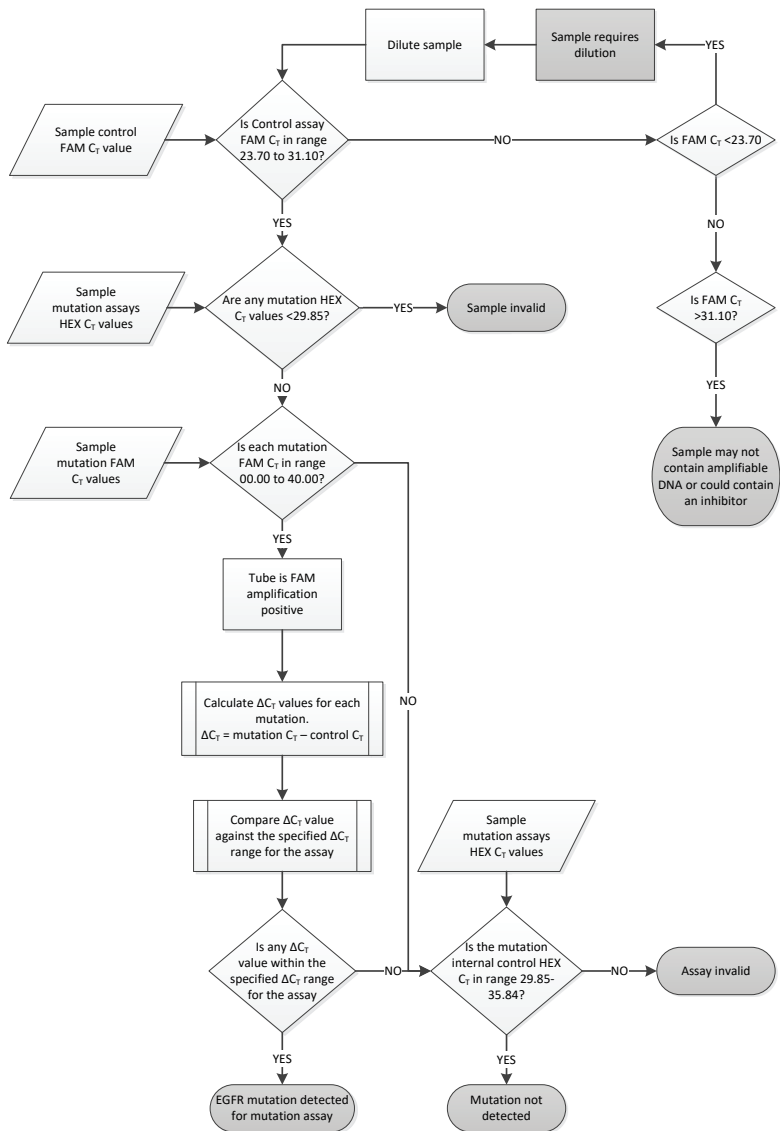


Figure 15. Mutation analysis flowchart.

Sample mutation assays FAM C_T value

The FAM values for all three mutation reaction mixes should be checked against the values listed in Table 8.

Calculate the ΔC_T cutoff value for each mutation sample showing positive amplification as follows, ensuring that the mutation and control C_T are from the same sample.

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

Compare the ΔC_T value for the sample with the ΔC_T cutoff range for the assay in question (Table 8), ensuring that the correct cutoff point is applied to each assay.

Table 8. Mutation assay ΔC_T cutoff range

Mutation assay	ΔC_T cutoff range
T790M	-10.00 \geq to \leq 7.40
Deletions	-10.00 \geq to \leq 8.00
L858R	-10.00 \geq to \leq 8.90

The upper limit of the ΔC_T cutoff range is the point above which a positive signal could potentially be due to background signal of the ARMS primer on wild type DNA. If the sample ΔC_T value is higher than the upper point of the ΔC_T cutoff range, it is classed as “Mutation not detected” or beyond the limits of detection of the kit. If the sample value is within the ΔC_T cutoff points, the sample is considered positive for a mutation detected by that assay. If the sample value is below the lower boundary of the ΔC_T cutoff range, then this could be potentially due to a fluorescence artefact.

Note: For samples showing no FAM mutation C_T, an evaluation of the internal control (HEX) C_T is required to determine whether the mutation is not detected or whether the assay is invalid. If the HEX C_T value is between 29.85 and 35.84, then the mutation is not detected. If the HEX ΔC_T cutoff value is outside of this range, then the sample is invalid.

In summary, for every sample, each mutation reaction will be given a status of mutation detected, mutation not detected, or invalid using the following criteria.

- **Mutation detected:** FAM amplification positive and the ΔC_T is within the ΔC_T cutoff range. If multiple mutations are detected, all can be reported.
- **Mutation not detected:**
 - FAM amplification positive and the ΔC_T cutoff value is above the ΔC_T cutoff range and HEX (internal control) is within 29.85–35.84.
 - FAM amplification negative and HEX (internal control) is within 29.85–35.84.
- **Invalid:** FAM amplification negative and HEX amplification outside specifications.
 - The calculated ΔC_T is below the ΔC_T cutoff range and HEX (internal control) is within the expected range. A ΔC_T value lower than -10.00 indicates that a fluorescence artefact may have occurred.

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 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 (PC) in fluorescence channel Cycling Green

- | | |
|---|--|
| a) The selected fluorescence channel for PCR data analysis does not comply with the protocol. | For data analysis, select the fluorescence channel Cycling Green for the analytical 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 MDx 5plex HRM instrument | Compare the temperature profile with the protocol and if incorrect, then 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 13) | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary. |
| e) The <i>therascreen</i> EGFR Plasma RGQ PCR Kit has expired | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary. |

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

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.

Multiple threshold crossing or ΔC_T value below cut off range

Incorrect mixing during assay setup

Repeat the PCR if this affects a control or retest failing sample.

Follow the Instructions for Use, paying close attention to the mixing steps.

Quality Control

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

Limitations

Results from the product must be interpreted within the context of all relevant clinical and laboratory findings and are not to be used alone for diagnosis.

The product is to be used only by personnel specially instructed and trained in in vitro diagnostics procedures and the Rotor-Gene Q MDx 5plex HRM instrument.

Analytical validation studies included human DNA extracted from plasma samples.

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

Strict compliance with the *therascreen EGFR Plasma 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.

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.

The primers in the EGFR Deletions Reaction mix have been designed to target multiple Exon 19 deletions, spanning nucleotides 55174772 to 55174795 (GRCh38 chr7), a range of 23 bp.

While the Exon 19 deletions assay has been analytically validated and demonstrated to detect specified deletions within Exon 19 (see Table 13 of this handbook), it is however, possible for additional mutations (including, but not limited to, additional Exon 19 deletions, Exon 19 insertions, and the L747P mutation) to be amplified by the Deletions Reaction Mix.

If present, such additional mutations will give rise to a “Deletions Detected” result for a given patient sample.

Additionally, it is possible for the L858Q mutation to be detected by the L858R Reaction Mix. Therefore, if present in a patient sample, the L858Q mutation can give rise to a “L858R Mutation Detected” result.

Performance Characteristics

Analytical sensitivity — limit of blank (LOB)

To assess performance of the *therascreen* EGFR Plasma RGQ PCR Kit in the absence of template and to ensure that a blank sample or a sample with wild-type DNA does not generate an analytical signal that may indicate a low concentration of mutation, NSCLC plasma EGFR wild-type DNA was evaluated from 59 different samples. The study acceptance criteria (at least 95% of wild-type samples must have a ΔC_T cutoff value above the respective cutoff) was met.

Limit of detection (LOD)

The LOD is the minimum percentage of mutant DNA that can be detected in a background of wild-type DNA when the total amplifiable DNA (within the input range), produced correct mutation calls at 95% for each mutation positive sample (C95). The DNA input working range for the assay is defined by the control C_T at the pre-specified range of 23.70 to 31.10.

The LOD was determined at low DNA input (control C_T approximately 30.10) levels using DNA derived from FFPE tissue for the *therascreen* EGFR RGQ PCR Kit. LOD was determined using both FFPE clinical specimens and FFPE cell lines at low DNA input levels for these EGFR mutations.

The LOD values established using FFPE tissue were verified for the *therascreen* EGFR Plasma RGQ PCR Kit with DNA derived from contrived mutant positive plasma samples.

The final LOD claims listed in Table 9, next page, indicate the mutation percentage that gave a predicted probability of correct calls, of 95% for each of the mutations.

Table 9. LODs for each of the EGFR mutation assays

Exon	Mutation	COSMIC ID*	% LOD claim
20	T790M	6240	17.5*
		6223	6.4*
		13551	4.24*
		12728	2.43†
		12419	16.87†
		12422	3.24†
		6218	9.83†
		6210	7.44†
		6254	10.2*
		12370	8.1*
		19	Deletions
12367	4.39†		
12384	7.54†		
6225	6.5*		
6220	2.7*		
6255	0.81*		
12382	1.45*		
12383	4.58*		
12387	4.91†		
21	L858R	6224	5.94*

* LOD claims verified in plasma as part of the *therascreen* EGFR Plasma RGQ PCR Kit LOD confirmation study.

† These mutations were not confirmed in plasma.

Analytical sensitivity – ΔC_T cutoffs and ΔC_T cutoff range

A risk-based approach was taken with regard to false-positive rates when setting the assay cutoffs, and estimated LOB values were used as one component in developing cutoff values.

The respective ΔC_T cutoff ranges established for each mutation assay in the *therascreen* EGFR Plasma RGQ PCR Kit are given in Table 10.

Table 10. *therascreen* EGFR Plasma RGQ PCR Kit ΔC_T cutoff ranges

Mutation assay	ΔC_T cutoff range
T790M	$-10.00 \geq$ to ≤ 7.40
Deletion	$-10.00 \geq$ to ≤ 8.00
L858R	$-10.00 \geq$ to ≤ 8.90

Repeatability and reproducibility

Repeatability and reproducibility was assessed by testing mutation level at 3xLOD in a background of wild-type genomic DNA at 3 testing sites using multiple kit batches, operators, and runs over different days, with 2 replicates of each sample. For all 3 mutation assays, 100% of mutant DNA samples tested mutation-positive. Wild-type samples tested mutation-negative in all assays at all sites.

Effect of DNA input on C_T values

DNA input level is defined as the total quantity of amplifiable EGFR DNA in a sample as determined by the C_T values from the control reaction. To demonstrate that the performance of the *therascreen* EGFR Plasma RGQ PCR Kit is consistent across the control reaction C_T range (23.70–31.10), all 3 EGFR mutation assays were tested against a six-point, 1-in-3 dilution series (DNA extracted from FFPE cell lines). The target C_T for dilution one, for each mutation, was approximately 24.70. The final dilution, which gave a C_T of approximately 32–33, was

outside of control reaction C_T range. Overall, the ΔC_T cutoff values measured at different total DNA input levels were consistent across the working range of the *therascreen* EGFR Plasma RGQ PCR Kit.

Interfering substances

Endogenous interfering substances

The potentially interfering substances were spiked into 3xLOD contrived mutant positive plasma samples. Samples were then tested with the *therascreen* EGFR Plasma RGQ PCR Kit. Samples containing the potential interfering substances were compared to 3xLOD contrived mutant positive plasma samples containing no spiked interfering substance. Each interfering substance was tested with 4 replicates.

A difference of >2x standard deviations (SD) (taken from precision study) between the “test” and the “control” ΔC_T (i.e., no interfering substance) was considered to indicate a potential interference. In these instances, the observed difference in ΔC_T is provided.

The test concentrations provided in Table 11 were selected based on guidance provided in the CLSI guideline EPO7-A2 and are representative of the maximum concentrations expected to be observed in a clinical sample.

Note: These endogenous compounds were spiked into contrived mutant positive plasma samples which comprised plasma from healthy donors. Therefore, these endogenous compounds would have been naturally present in the samples at unknown concentrations prior to spiking. The final concentration of each potential endogenous interfering substance tested would likely be greater than the test concentration.

Table 11. Potential interfering endogenous substances

Potential interfering substance (IS)	Test concentration
Bilirubin unconjugated	150 mg/dl
Hemoglobin (human)	0.2 g/dl
Triglycerides	3 g/dl

T790M assay

The following endogenous compounds at the concentrations stated in Table 11 were shown to have an effect $>2 \times \text{SD}$ ($0.40 \Delta C_T$) on the performance of the T790M assay:

- Triglycerides, difference of $1.37 \Delta C_T$

Deletions assay

The following endogenous compounds at the concentrations stated in Table 11 were shown to have an effect $>2 \times \text{SD}$ ($0.71 \Delta C_T$) on the performance of the deletions assay:

- Hemoglobin, difference of $0.80 \Delta C_T$

L858R assay

The following endogenous compounds at the concentrations stated in Table 11 were shown to have an effect $>2 \times \text{SD}$ ($0.56 \Delta C_T$) on the performance of the L858R assay:

- Bilirubin, difference of $1.13 \Delta C_T$
- Triglycerides, difference of $1.53 \Delta C_T$

Exogenous interfering substances

The potentially interfering substances were spiked into 3xLOD contrived mutant positive plasma samples. Samples were then tested with the *therascreen* EGFR Plasma RGQ PCR Kit. Samples containing the potential interfering substances were compared to 3xLOD contrived mutant positive plasma samples, containing no spiked interfering substance. Each interfering substance was tested with 4 replicates.

A difference of >2x standard deviations (taken from precision study) between the “test” ΔC_T and the “control” ΔC_T (i.e., no interfering substance) was considered to indicate a potential interference. In these instances, the observed difference in ΔC_T is provided.

The test concentrations provided in Table 12 were selected based on guidance provided in the CLSI guideline EP07-A2, and are in excess of the therapeutic concentration in all cases.

Table 12. Potential interfering endogenous substances

Potential interfering substance (IS)	Test concentration ($\mu\text{g/ml}$)
Citalopram hydrobromide	0.75
Paroxetine hydrochloride hemihydrate	1.14
Sertraline hydrochloride	0.67
Fluoxetine hydrochloride	3.87
Acetaminophen	200.7
K ₂ EDTA	3600

T790M assay

The following exogenous compounds at the concentrations stated in Table 12 were shown to have an effect >2xSD (0.40 ΔC_T) on the performance of the T790M assay:

- Citalopram hydrobromide, difference of 0.52 ΔC_T
- Sertraline hydrochloride, difference of 0.47 ΔC_T

- Fluoxetine hydrochloride, difference of 0.48 ΔC_T

Deletions assay

The following exogenous compounds at the concentrations stated in Table 12 were shown to have an effect $>2 \times SD$ (0.71 ΔC_T) on the performance of the deletions assay:

- Fluoxetine, difference of 0.73 ΔC_T

L858R assay

The following exogenous compounds at the concentrations stated in Table 12 were shown to have an effect $>2 \times SD$ (0.56 ΔC_T) on the performance of the L858R assay:

- Citalopram hydrobromide, difference of 0.72 ΔC_T
- Paroxetine hydrochloride hemihydrate, difference of 0.92 ΔC_T
- Sertraline hydrochloride, difference of 0.82 ΔC_T
- Fluoxetine hydrochloride, difference of 0.98 ΔC_T
- Acetaminophen, difference of 0.81 ΔC_T
- K₂ EDTA, difference of 0.57 ΔC_T

Clinical performance

The NCT01203917 clinical trial was a phase-IV, open-label, single-arm study to assess efficacy and safety/tolerability of first line gefitinib in Caucasian patients with stage IIIA/B/IV, EGFR mutation positive NSCLC.

The eligibility of patients for enrollment onto NCT01203917 clinical trial was determined by the presence of EGFR sensitizing mutations. The EGFR mutation status of NSCLC patients was assessed using the Clinical Trial Assay (CTA), with DNA from matched tissue and plasma samples. The study comprised a pre-planned biomarker exploratory objective to establish whether plasma samples could be considered for mutation analysis if tissue samples are unavailable. The results demonstrated high rates of concordance between matched tissue and plasma samples at 94.3%, with assay specificity of 99.8% and sensitivity of 65.7%.

Retrospective testing of plasma specimens from patients screened for the NCT01203917 clinical trial was performed using the *therascreen* EGFR Plasma RGQ PCR Kit. A bridging study was conducted to assess the concordance of the *therascreen* EGFR Plasma RGQ PCR Kit with the CTA used to select patients for the NCT01203917 clinical trial. Equivalence between the CTA and *therascreen* EGFR Plasma RGQ PCR Kit was demonstrated.

References















1. Douillard, J.Y., et al. (2014). First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study. *Br J Cancer* 110(1), 55.
2. Walsh, K., et. al. (2014) A cautionary lesson on the use of targeted methods for EGFR mutation analysis; a case report. *J. Clin. Pathol.* **67**, 734
3. Huang, J., Wang, Y., Zhai, Y., and Wang, J. (2018) Non-small cell lung cancer harboring a rare EGFR L747P mutation showing intrinsic resistance to both gefitinib and osimertinib (AZD9291): A case report. *Thorac. Cancer.* **9**, 745

Contact Information

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Symbols

The following symbols may appear on the packaging and labeling:

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

Appendix A: Mutation Details

Table 13 shows the COSMIC IDs taken from the Catalogue of Somatic Mutations in Cancer (www.sanger.ac.uk/genetics/CGP/cosmic).

Table 13. List of mutations and COSMIC IDs

Mutation	Exon	Base change	COSMIC ID
T790M	20	2369C>T	6240
L858R	21	2573T>G	6224
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
		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		

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> EGFR Plasma RGQ PCR Kit – for detection of mutations in the EGFR gene		
<i>therascreen</i> EGFR Plasma RGQ PCR Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, Taq DNA Polymerase	870311
Rotor-Gene Q MDx and accessories		
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9002033
Rotor-Gene Q MDx 5plex HRM Platform	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, installation and training	9002032
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipette in 72 x 0.1 ml tubes	9018901
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103

Product	Contents	Cat. no.
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Related products		
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

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.

Document Revision History

Document	Changes
R3, January 2019	Addition of Authorized Representative (front cover). "Symbols" section updated.
R4, October 2019	Changed legal manufacturer (cover page) Adaption of instrument name from Rotor-Gene Q MDx to Rotor-Gene Q MDx 5plex HRM to align with name on the instrument label Corrected reagent storage instructions from 90 days to 12 months or until expiration date Updated the Limitations section with information regarding the exon 19 deletions assay and the L858R assay Revised Table 9 to replace duplicated exon 21 L858R with exon 19 Deletions Removed EC + REP symbol from the cover page and Symbols section
R5, June 2020	Updated the references to RGQ software version from 2.3 to 2.3.5 or later Updated Tables 8 and 10 to implement the new ΔC_T cutoff range and adjusted all the relevant descriptions accordingly in the entire handbook Updated all Protocol chapters to include information about the importance of mixing in Important points before starting sections; highlighted the mixing details at all mixing steps Added a mixing step to the section "Protocol: Detection of EGFR Mutations" Updated the Troubleshooting section to add solution for multiple threshold crossing

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