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therascreen® EGFR RGQ PCR Kit Handbook

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Version 2

IVD

For in vitro diagnostic use

For use with Rotor-Gene® Q MDx instruments

CE

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Contents

Intended Use

The *therascreen* EGFR RGQ PCR Kit is an in vitro diagnostic test for the detection of 29 somatic mutations in the EGFR gene. It provides a qualitative assessment of the mutation status in tumor samples from non-small cell lung cancer (NSCLC) patients.

Results are intended to aid the clinician in identifying patients with NSCLC who may benefit from treatment with EGFR tyrosine kinase inhibitors.

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

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

Summary and Explanation

Mutations in the EGFR oncogene are found in human cancers (1, 2). The presence of these mutations correlates with response to certain tyrosine kinase inhibitor (TKI) therapies in patients with non-small cell lung cancer (NSCLC) (3–8). Such mutations in the EGFR oncogene are present in the general population of patients with NSCLC at a frequency of approximately 10% in patients from the USA, Europe, or Australia and up to 30% in patients from Japan and Taiwan (1, 2, 9).

The *therascreen* EGFR RGQ PCR Kit is a ready-to-use kit for the detection of 29 mutations in the EGFR cancer-related gene using the polymerase chain reaction (PCR) on a Rotor-Gene Q MDx instrument.

Using Scorpions® (10) and ARMS (Amplification Refractory Mutation System) (11) technologies, the *therascreen* EGFR RGQ PCR Kit enables detection of 29 mutations in exons 18, 19, 20, and 21 of the EGFR oncogene against a background of wild type genomic DNA (Table 1). In summary,

- 19 deletions in exon 19 (detects the presence of any of 19 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
		6254	2239_2253del15
		6255	2239_2256del18

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

Table continued on next page

Table 1. Continued

Exon	Mutation	COSMIC* ID	Base change
19	Deletions	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 *therascreen* 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 principle 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 *therascreen* EGFR RGQ PCR Kit:

- 1 control assay (CTRL)
- 7 mutation assays

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.

Assays

The *therascreen* 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 Scorpion 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 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 *therascreen* 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 *therascreen* 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.

Sample assessment

It is strongly recommended to use the Control Reaction Mix (tube CTRL) supplied with the *therascreen* EGFR RGQ PCR Kit to assess the total amplifiable EGFR DNA in a sample. The control assay amplifies a region of exon 2 of the EGFR gene. It is recommended to set up samples with only the control assay using the EGFR PC as a positive control and water for the "template" as the no template control.

Note: DNA assessment should be based on PCR and may differ from quantification based on absorbance readings. Additional Control Reaction Mix (tube CTRL) is supplied to enable assessment of quality and quantity of the DNA in samples before analysis with the *therascreen* EGFR RGQ PCR Kit.

Platform and software

The *therascreen* EGFR RGQ PCR Kit is specifically designed to be used with Rotor-Gene Q MDx instruments. The Rotor-Gene Q MDx instrument is programmed for different cycle parameters, or "runs", by the *therascreen* EGFR CE Assay Package.

The *therascreen* EGFR CE Assay Package consists of two templates: the "therascreen EGFR CE Control Run Locked Template" (for sample assessment) and the "therascreen EGFR CE Locked Template" (for detection of EGFR mutations). These templates contain the PCR run parameters and calculate the results.

It is also possible to use the *therascreen* EGFR RGQ PCR Kit with Rotor-Gene Q software version 2.3 in the open mode (i.e., without using the Rotor-Gene Q *therascreen* EGFR CE Assay Package). For details, see "Appendix A: *therascreen* EGFR RGQ PCR Kit Manual Protocol", page 57.

Materials Provided

Kit contents

***therascreen* EGFR RGQ PCR Kit**				**(24)**
Catalog no.				**874111**
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
therascreen *EGFR RGQ PCR Kit Handbook* (English)				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.

Reagents

- DNA extraction kit (see "DNA extraction and preparation", page 16)

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 2ml reaction tubes
- Vortex mixer*

Equipment for PCR

- Rotor-Gene Q MDx instrument with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and HEX, respectively)*†
- Rotor-Gene Q software version 2.3
- Rotor-Gene Q *therascreen* EGFR CE Assay Package CD, version 3.0.5 (cat. no. 9023537)

  Note: Rotor-Gene Q *therascreen* EGFR CE Assay Package software requires Rotor-Gene Q software version 2.3.

* Ensure that instruments and equipment 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

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.

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 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.
- Reagents for the *therascreen* 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 µl since this will increase the risk of a false negative.
- All reagents supplied in the *therascreen* EGFR RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same *therascreen* EGFR RGQ PCR Kit. Do not substitute the reagents in the *therascreen* EGFR

RGQ PCR Kit or between *therascreen* EGFR RGQ PCR Kits, as this may affect performance.

- Only use the *Taq* DNA polymerase (tube *Taq*) that is provided in the *therascreen* 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 *therascreen* EGFR RGQ PCR Kit is shipped on dry ice and must be frozen on arrival. If the *therascreen* 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 (see back cover or visit www.qiagen.com.

The *therascreen* EGFR RGQ PCR Kit should be stored immediately upon receipt at –15 to –30°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 MDx 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

  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: 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 *therascreen* 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 *therascreen* 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 FFPE tissue. Specimens must be transported according to standard pathology methodology to ensure specimen quality.

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

DNA extraction and preparation

The performance characteristics for this kit have been generated using DNA extracted with the QIAamp® DSP DNA FFPE Tissue Kit. If using the functionally equivalent QIAamp DNA FFPE Tissue Kit (cat. no. 56404), carry out the DNA extraction according to the instructions in the handbook **noting the following**:

- Do not use the QIAGEN Deparaffinization Solution. Use only the xylene/ethanol method for deparaffinization described in the *QIAamp DNA FFPE Tissue Kit Handbook*.
- 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**.
- 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 *therascreen* EGFR RGQ PCR Kit generate short PCR products. However, the *therascreen* EGFR RGQ PCR Kit will not work with heavily fragmented DNA.

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

Protocol: Sample assessment

This protocol is used to assess the total amplifiable DNA in samples using the "therascreen EGFR CE Control Run Locked Template" of the Rotor-Gene Q *therascreen* EGFR CE Assay Package for automated sample assessment.

Note: For manual DNA sample assessment refer to "Appendix A: *therascreen* EGFR RGQ PCR Kit Manual Protocol", page 57.

Important points before starting

- Before beginning the procedure read "General precautions", page 13.
- Take time to familiarize yourself with the Rotor-Gene Q MDx 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

- Ensure that the *therascreen* EGFR CE Assay Package software is installed before first use of the Rotor-Gene Q MDx instrument (see "Appendix B: Installation of the *therascreen* EGFR CE Assay Package", page 78).
- 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 2.

Table 2. 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	4.5 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 MDx instrument.

Note: Bring *Taq* to ambient temperature (15–25°C) at the same time as the other reagents (see "Reagent Storage and Handling", page 14). 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 Control master mix (Control Reaction Mix [CTRL] plus *Taq*) for the DNA samples, an EGFR PC reaction, and a NTC reaction according to the volumes in Table 3. 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 3. Preparation of Control assay master mix

Component	Volume
Control Reaction Mix (CTRL)	19.5 µl x (n + 1)*
Taq DNA polymerase (*Taq*)	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 4. Immediately add 20 µl of master mix to each PCR strip tube.**

   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 4. Layout of DNA sample assessment assays in the loading block. Numbers denote positions in the loading block and indicate final rotor position.

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	–	–	–	–	–	–

5. **Immediately add 5 µl water for NTC to the tube in position 2 and cap the tube.**
6. **Add 5 µl of each sample to the sample tubes (tube positions 3–26) and cap the tubes.**
7. **Add 5 µl of EGFR PC to the tube in position 1 and cap the tube.**

   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 MDx 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 4.**

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 MDx instrument. Make sure that the locking ring (accessory of the Rotor-Gene Q MDx instrument) is placed on top of the rotor to secure the tubes during the run.**

    Note: If using manual sample assessment, refer to "Appendix A: *therascreen* EGFR RGQ PCR Kit Manual Protocol", page 57.

12. **Start the Rotor-Gene Q software by double-clicking the "therascreen EGFR CE Control Run Locked Template" icon on the desktop of the computer connected to the Rotor-Gene Q MDx instrument (Figure 1).**

Figure 1. EGFR CE Locked Template icon for control run (sample assessment).

13. **The "Setup" tab opens as the default (Figure 2). Ensure that the locking ring is properly attached then check the "Locking Ring Attached" box. Close the lid of the Rotor-Gene Q MDx instrument.**

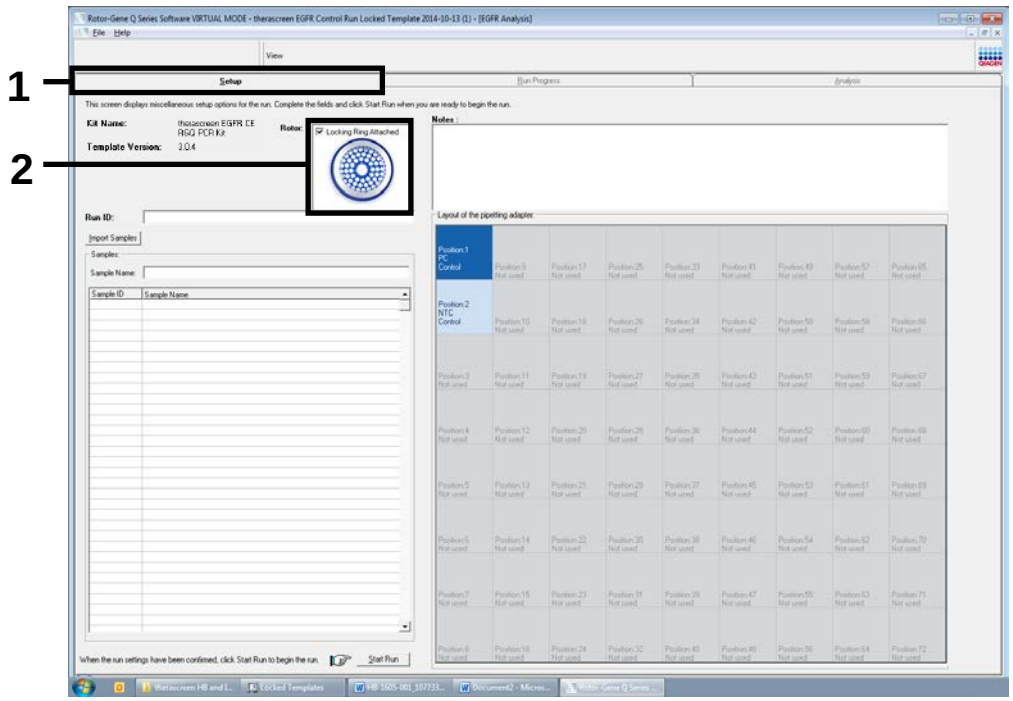

Figure 2. The "Setup" tab (1) and "Locking Ring Attached" box (2).

14. **Enter the run ID in the "Run ID" dialog field according to your local naming convention. Enter the sample name in the "Sample Name" dialog field according to your local naming convention and press the return key.**

This adds the sample name to the sample list below and assigns the sample a "Sample ID" (1, 2, 3, etc.). In addition, the "Layout of the pipetting adaptor" panel on the right side updates to include the sample name (Figure 3).

Note: Alternatively, sample names stored in ***.smp** (Rotor-Gene Q sample file) or ***.csv** (comma separated values) formats can be imported using the "Import Samples" button. Sample names are populated automatically using this method.

Note: In the "Layout of the pipetting adaptor" panel, check that addition of the sample name is highlighted by a change in color and the sample name is in the sample position (Figure 3).

Note: Sample names with more than 8 characters may not be completely displayed in the "Layout of the pipetting adaptor" panel.

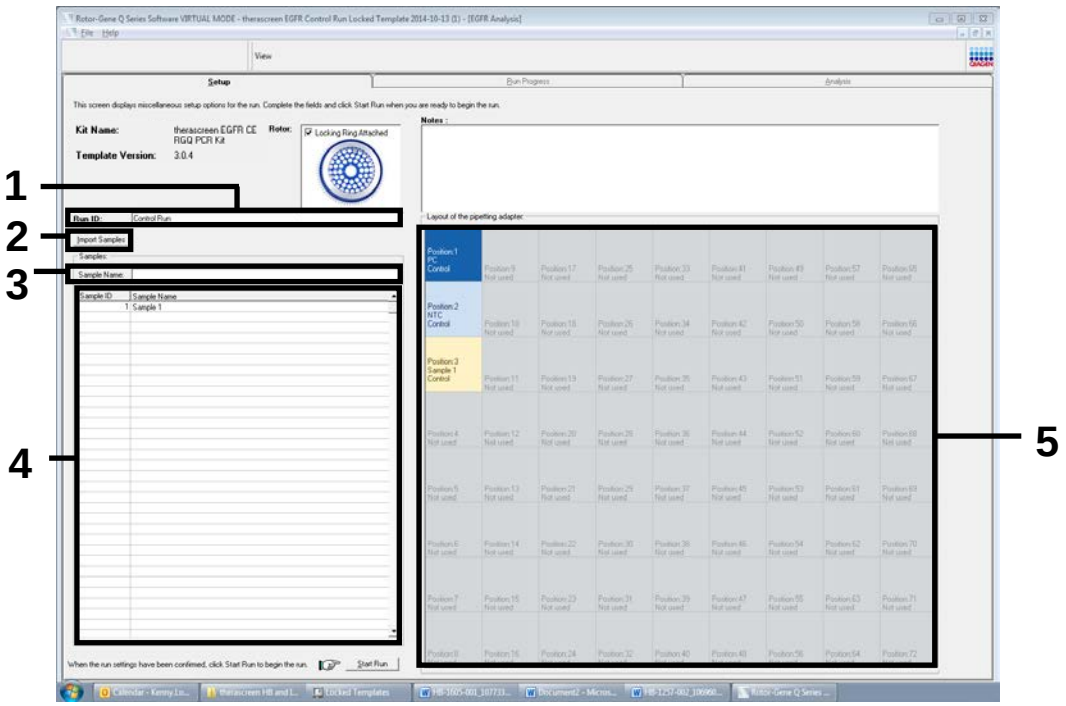

Figure 3. Entering the "Run ID" and "Sample Name" (1 = "Run ID" dialog field; 2 = "Sample Name" dialog field; 3 = "Sample List"; 4 = "Layout of the pipetting adaptor" panel; 5 = "Sample Import" panel).

15. **Repeat step 14 to enter the names of all additional samples (Figure 4).**

    Note: To edit a sample name, click on "Sample Name" in the sample list and the selected sample will appear in the "Sample Name" dialog field above. Edit the sample name according to your local naming convention and press the return key to update the name.

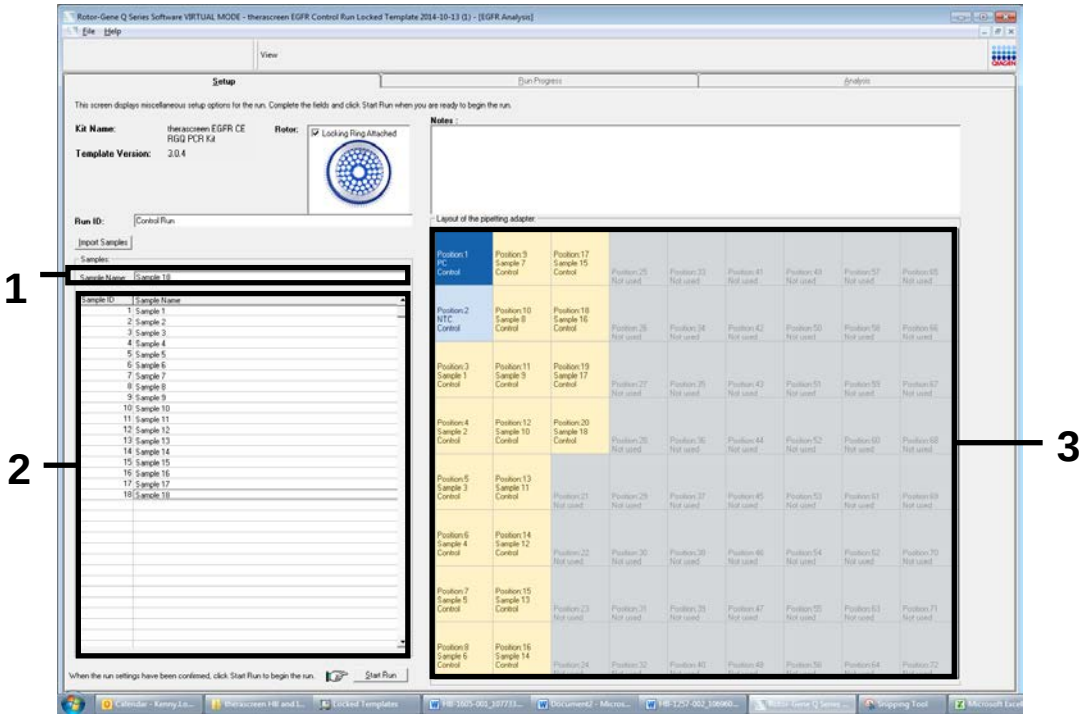

Figure 4. Entering additional sample names in the "Sample Name" dialog field (1 = "Sample Name" dialog field; 2 = "Sample List"; 3 = "Layout of the pipetting adaptor" panel).

16. **When all sample names are entered, check they are correct. Add any additional information in the "Notes" dialog field if necessary and then click "Start Run" (Figure 5).**

    Note: If any rotor position is unused, a "Warning" will appear (Figure 5) to remind the user that all unused positions on the rotor must be filled with capped, empty tubes. Check that all unused rotor positions are filled with capped, empty tubes and click "OK" to proceed.

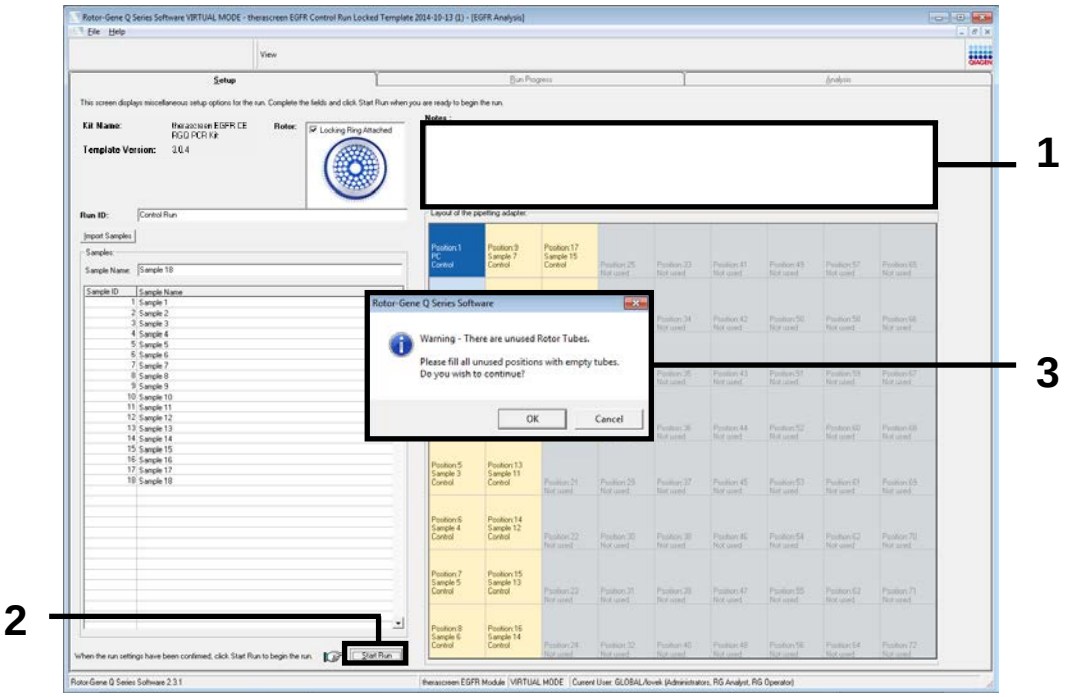

Figure 5. "Notes" dialog field (1), the "Start Run" button (2), and "Warning" of unused rotor positions (3).

17. The "Save As" window opens. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location. Click "Save" (Figure 6).

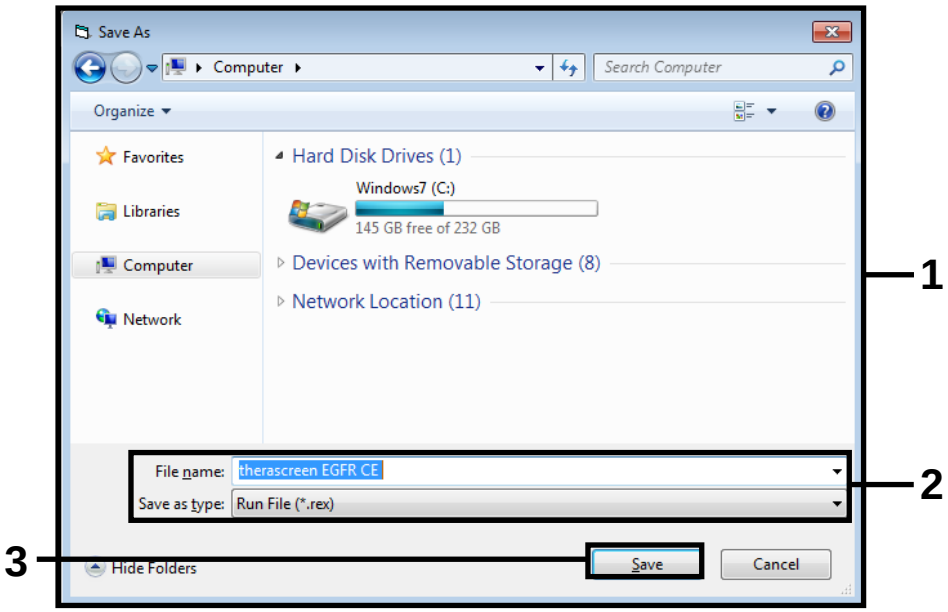

Figure 6. "Save As" window (1) (2 = "File Name" and "Save as type" fields; 3 = "Save" button).

18. The PCR run starts.

Note: When the run starts, the "Run Progress" tab opens to show the temperature trace and remaining run time (Figure 7).

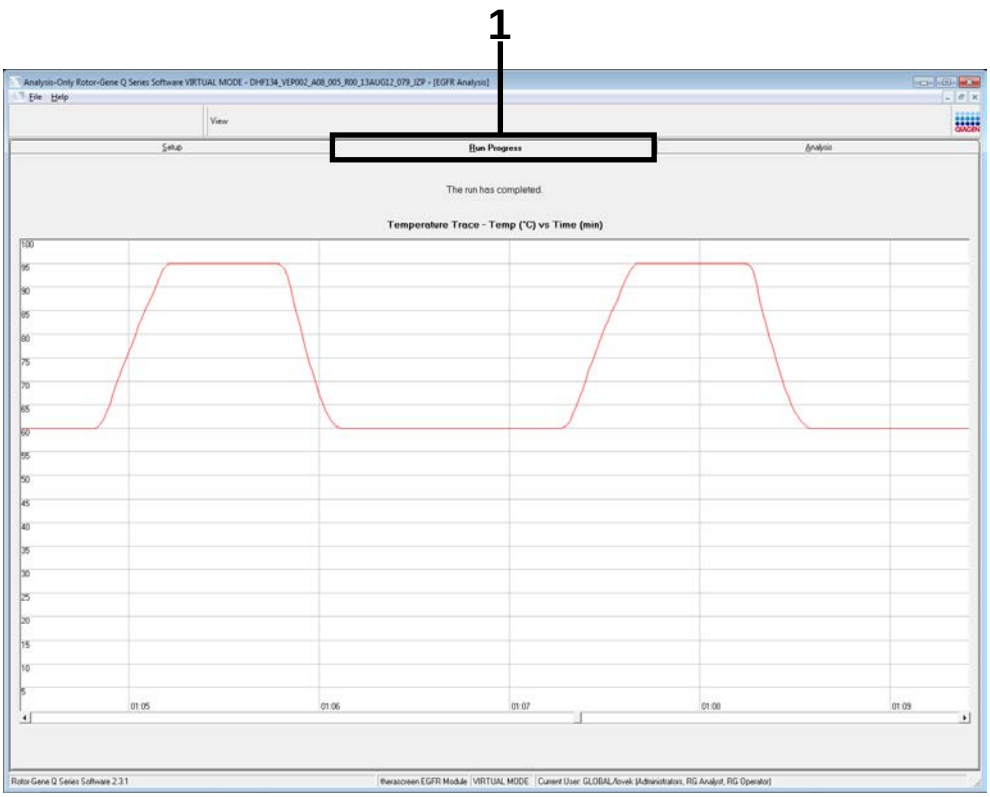

Figure 7. The "Run Progress" tab (1).

19. After the run is finished, the "Analysis" tab opens.

Note: If the "Analysis" tab fails to open, click the "Analysis" tab (Figure 8).

Note: An explanation of the calculation method is presented in the "Interpretation of Results (Automated)" section, page 37.

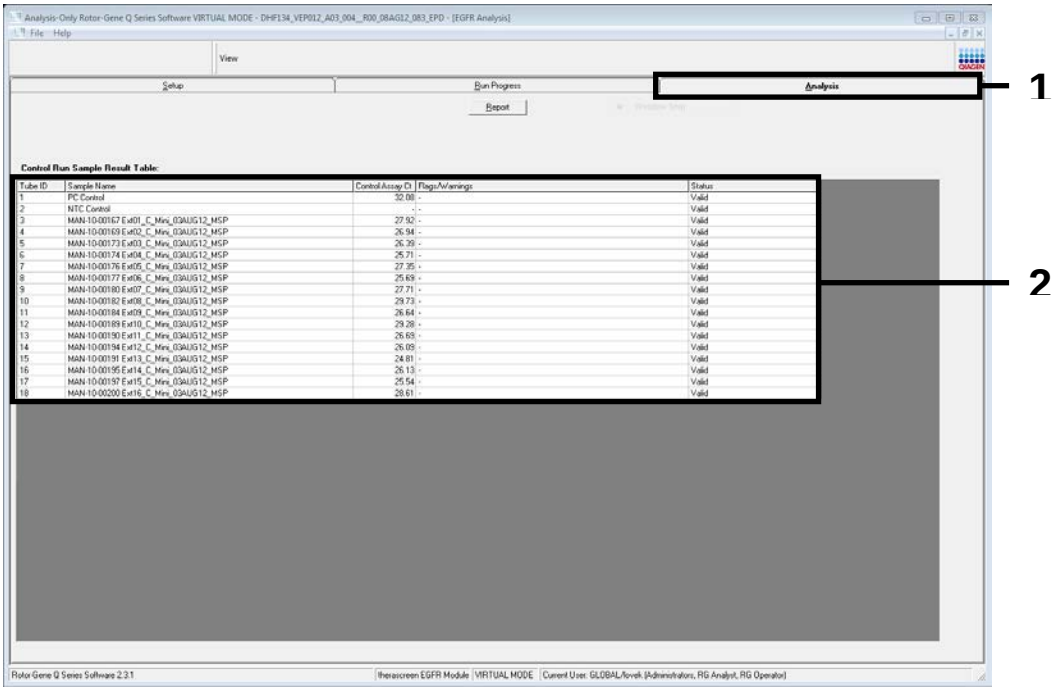

Figure 8. The "Analysis" tab (1) and reporting of results (2 = "Sample QC Result Table").

20. Control results are reported as follows in the "Sample QC Result Table" (Figure 8).

- **Run controls (PC and NTC, tube positions 1 and 2 respectively).**
  If the results are within acceptable ranges, each displays as "Valid". Otherwise, an "Invalid" result appears.
- **Sample control reaction C_T >31.10; displays as "Invalid".**
  Quantity of DNA is not sufficient for mutation analysis. Retest the sample. If the quantity of DNA is still insufficient, extract more tumor tissue if available.
- **Sample control reaction C_T <23.70; displays as "Invalid".**
  DNA concentration is too high for mutation analysis. Dilute with Nuclease- Free Water for Dilution (Dil.) and retest. Dilute to a C_T of 23.70–31.10. Dilution of 1:1 increases the C_T value by approximately 1.0.
- **Sample control reaction C_T of 23.70–31.10 (23.70≤ Control C_T ≥ 31.10); displays as "Valid".**
  DNA concentration is suitable for mutation analysis.

Note: If re-extraction or dilution is required, repeat the control reaction to confirm that the DNA concentration is suitable for use.

21. Click "Report" to produce a report file. The "Report Browser" window opens. Select "EGFR CE Analysis Report" under "Templates" and then click "Show" (Figure 9).

Note: To save reports to an alternative location in Web Archives format, click "Save As" on the top left corner of each report.

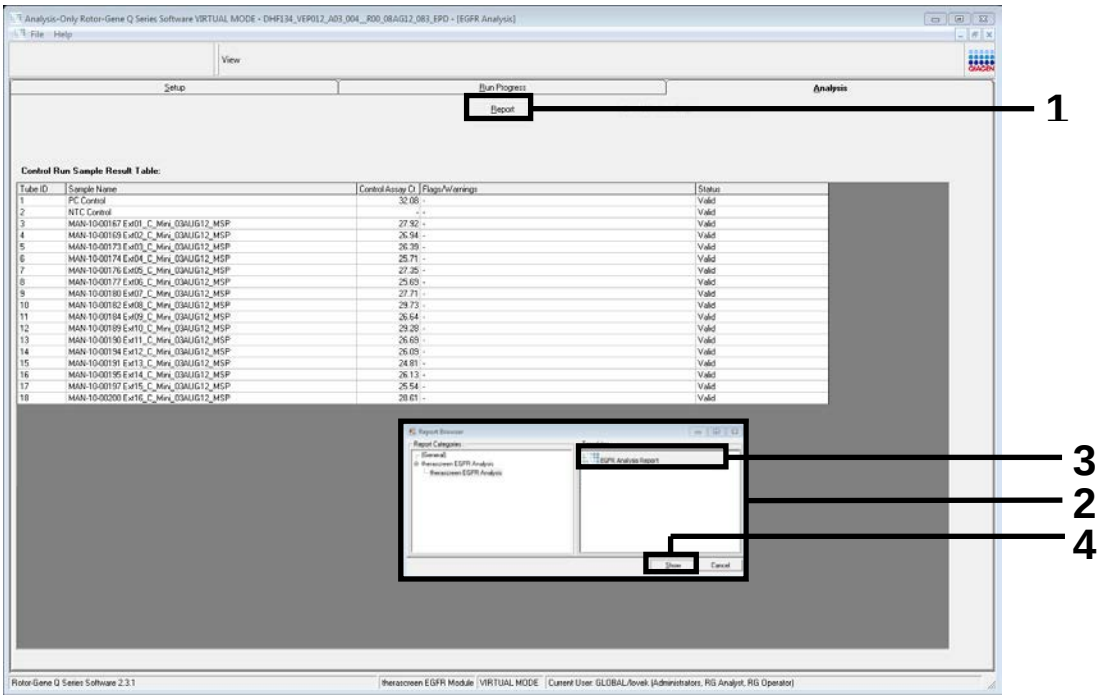

Figure 9. Selecting the "EGFR CE Analysis Report" (1 = "Report" button; 2 = "Report Browser" window; 3 = "EGFR Analysis Report" selection; 4 = "Show" button).

Protocol: EGFR mutation detection

This protocol is for the detection of EGFR mutations. When a sample has passed DNA sample assessment, it can be tested with the EGFR mutation assays using automated software.

Note: For manual mutation detection, refer to "Appendix A: *therascreen* EGFR RGQ PCR Kit Manual Protocol", page 57.

Important points before starting

- Before beginning the procedure, read "General precautions", page 13.
- Take time to familiarize yourself with the Rotor-Gene Q MDx instrument before starting the protocol. See the instrument user manual.
- A sample can be tested using the EGFR mutation assays when it has passed the DNA sample assessment.
- For efficient use of the *therascreen* EGFR RGQ PCR Kit, samples must be grouped into batches of 7. Smaller batch sizes mean that fewer samples can be tested with the *therascreen* EGFR RGQ PCR Kit.
- A sample must be tested using all reaction mixes provided in the *therascreen* 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

- Ensure that the *therascreen* EGFR CE Assay Package software is installed before first use of the Rotor-Gene Q MDx instrument (see "Appendix B: Installation of the *therascreen* EGFR CE Assay Package", page 78).
- 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 5.

   Table 5. 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
1h	4.5 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 MDx 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 14). 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 6. Include reagents for one extra sample to allow sufficient overage for the PCR setup.**

   The master mixes contain all of the components needed for the PCR, except the sample.

Table 6. Preparation of assay master mixes

Assay	Reaction mix tube	Volume of reaction mix	Volume of *Taq* DNA polymerase (tube *Taq*)
Control	CTRL	19.5 µl x (n+1)*	0.5 µl x (n+1)*
T790M	T790M	19.5 µl x (n+1)	0.5 µl x (n+1)
Deletions	Del	19.5 µl x (n+1)	0.5 µl x (n+1)
L858R	L85R	19.5 µl x (n+1)	0.5 µl x (n+1)
L861Q	L861Q	19.5 µl x (n+1)	0.5 µl x (n+1)
G719X	G719X	19.5 µl x (n+1)	0.5 µl x (n+1)
S768I	S768I	19.5 µl x (n+1)	0.5 µl x (n+1)
Insertions	Ins	19.5 µl x (n+1)	0.5 µl x (n+1)

*n = number of reactions (samples plus controls). Prepare enough master mix for one extra sample (n + 1) to allow for sufficient overage 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 7. Immediately add 20 µl of the appropriate assay master mix to each PCR strip tube.**

   Caps remain in the plastic container until required.

Table 7. Layout of control and mutation assays in the loading block.
Numbers denote positions in the loading block and indicate final rotor position.

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

5. **Immediately add 5 µl water for NTC to the tubes in positions 9–16 and cap the tubes.**
6. **Add 5 µl of each sample to the sample tubes (tube positions 17–24, 25–32, 33–40, 41–48, 49–56, 57–64, and 65–72) and cap the tubes.**
7. **Add 5 µl of EGFR PC to the tubes in position 1–8 and cap the tubes.**

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

   Each tube should contain a total reaction volume of 25 µl (20 µl of assay master mix prepared in step 3 (Table 6), plus 5 µl 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 MDx 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 7 samples 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 MDx instrument. Make sure that the locking ring (accessory of the Rotor-Gene Q MDx instrument) is placed on top of the rotor to secure the tubes during the run.**

    Note: If using manual EGFR mutation detection, refer to "Appendix A: *therascreen* EGFR RGQ PCR Kit Manual Protocol", page 57.

12. **Start the Rotor-Gene Q software by double-clicking the "therascreen EGFR CE Locked Template" icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument (Figure 10).**

therascreen EGFR CE
Locked Template

Figure 10. EGFR CE Locked Template icon (EGFR mutation detection).

13. **The "Setup" tab opens as the default (Figure 11). Ensure that the locking ring is properly attached then check the "Locking Ring Attached" box. Close the lid of the Rotor-Gene Q MDx instrument.**

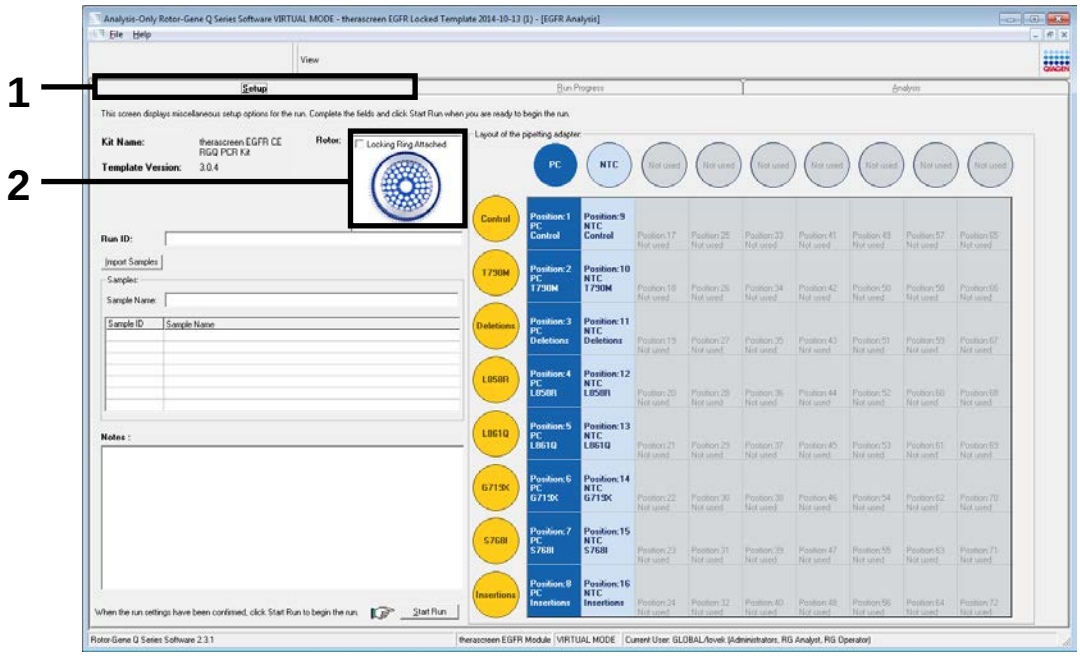

Figure 11. The "Setup" tab (1) and "Locking Ring Attached" box (2).

14. **Enter the run ID in the "Run ID" dialog field according to your local naming convention. Enter the sample name in the "Sample Name" dialog field according to your local naming convention and press the return key.**

    This adds the sample name to the sample list below and assigns the sample a "Sample ID" (1, 2, 3, etc.). In addition, the "Layout of the pipetting

adaptor" panel on the right side updates to include the sample name (Figure 12).

Note: Alternatively, sample names stored in ***.smp** (Rotor-Gene Q sample file) or ***.csv** (comma separated values) formats can be imported using the "Import Samples" button. Sample names are populated automatically using this method.

Note: In the "Layout of the pipetting adaptor" panel, check that addition of the sample name is highlighted by a change in color and the sample name is in the sample position (Figure 12).

Note: A maximum of 7 samples can be added. The sample IDs (in the sample circles) are assigned automatically from 1 to 7.

Note: Sample names with more than 8 characters may not be completely displayed in the "Layout of the pipetting adaptor" panel.

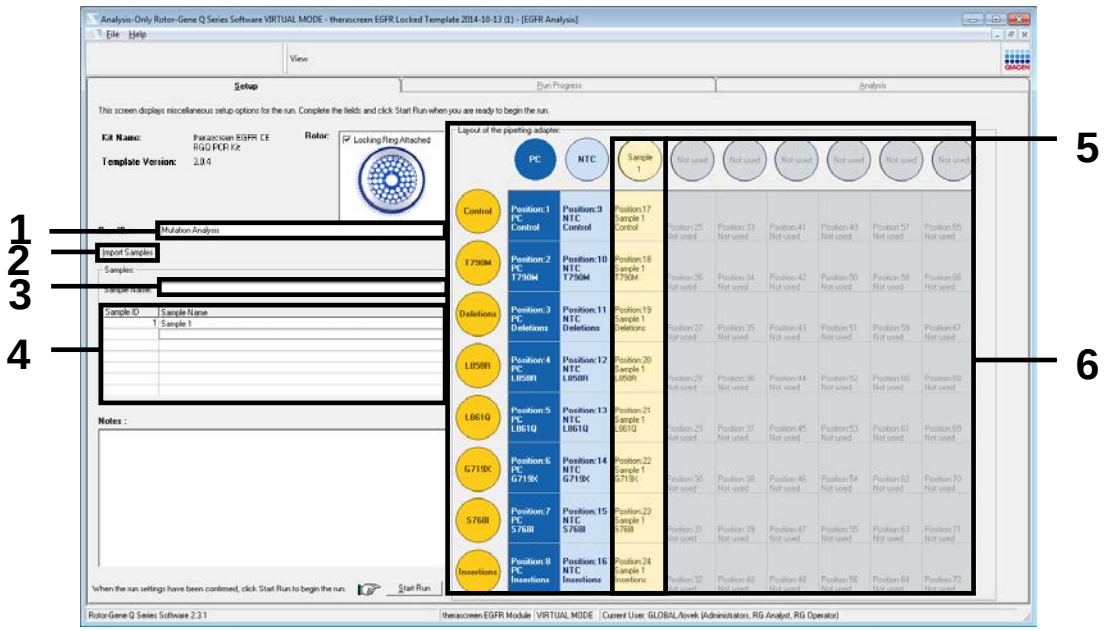

Figure 12. Entering the "Run ID" and "Sample Name" (1 = "Run ID" dialog field; 2 = "Sample Import" button; 3 = "Sample Name" dialog field; 4 = "Sample List"; 5 = "Layout of the pipetting adapter" panel; 6 = Highlighted sample circle and column of 8 assays underneath).

15. **Repeat step 14 to enter the names of all additional samples (Figure 13).**

    Note: To edit a sample name, click on "Sample Name" in the sample list and the selected sample appears in the "Sample Name" dialog field above. Edit the sample name according to your local naming convention and press the return key to update the name.

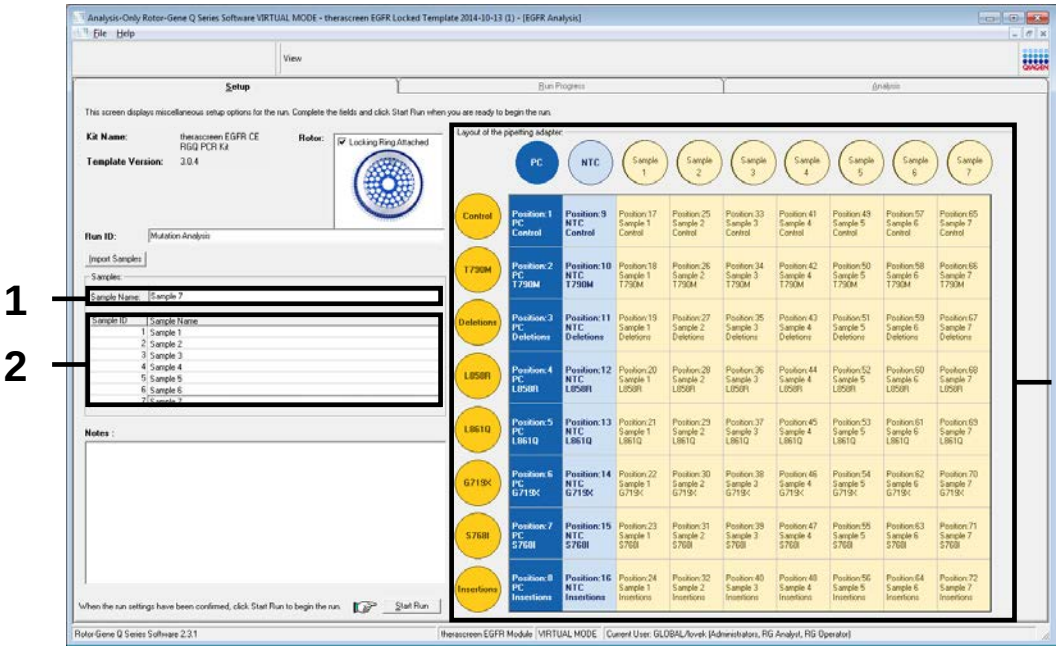

Figure 13. Entering additional sample names in the "Sample Name" dialog field (1 = "Sample Name" dialog field; 2 = "Sample List"; 3 = "Layout of the pipetting adaptor" panel).

16. **When all sample names are entered, verify they are correct. Add any additional information in the "Notes" dialog field if necessary and then click "Start Run" (Figure 14).**

    Note: If any rotor position is unused, a "Warning" will appear (Figure 14) to remind the user that all unused positions on the rotor must be filled with capped, empty tubes. Check that all unused rotor positions are filled with capped, empty tubes and click "OK" to proceed.

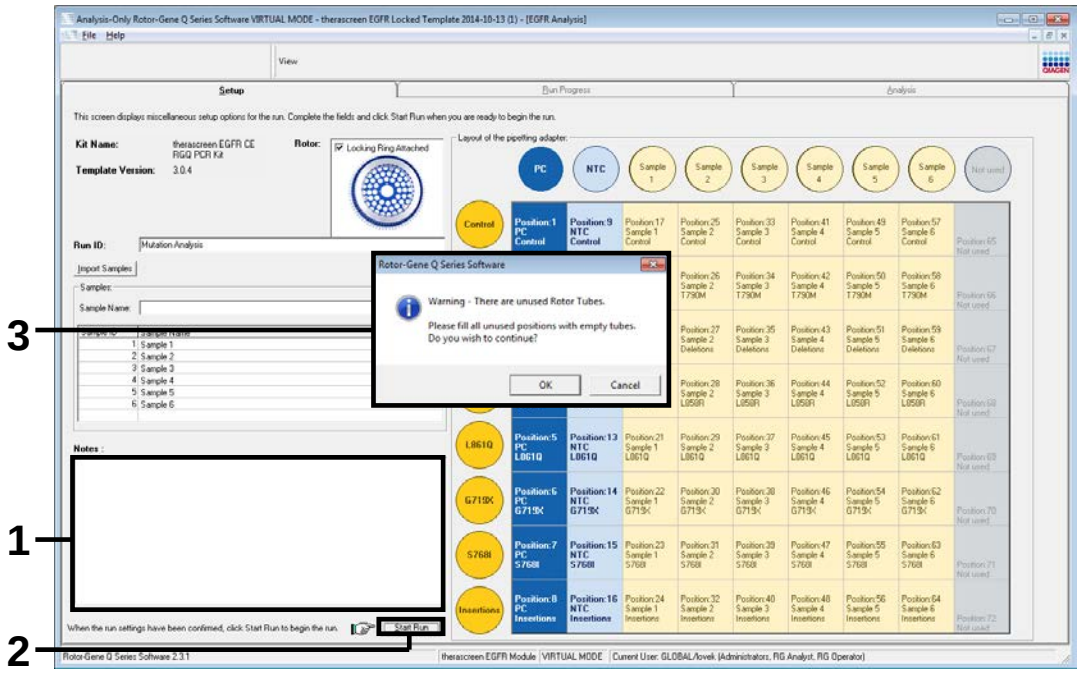

Figure 14. "Notes" dialog field (1), the "Start Run" button (2), and "Warning" of unused rotor positions (3).

17. **The "Save As" window opens. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location. Click "Save" (Figure 15).**

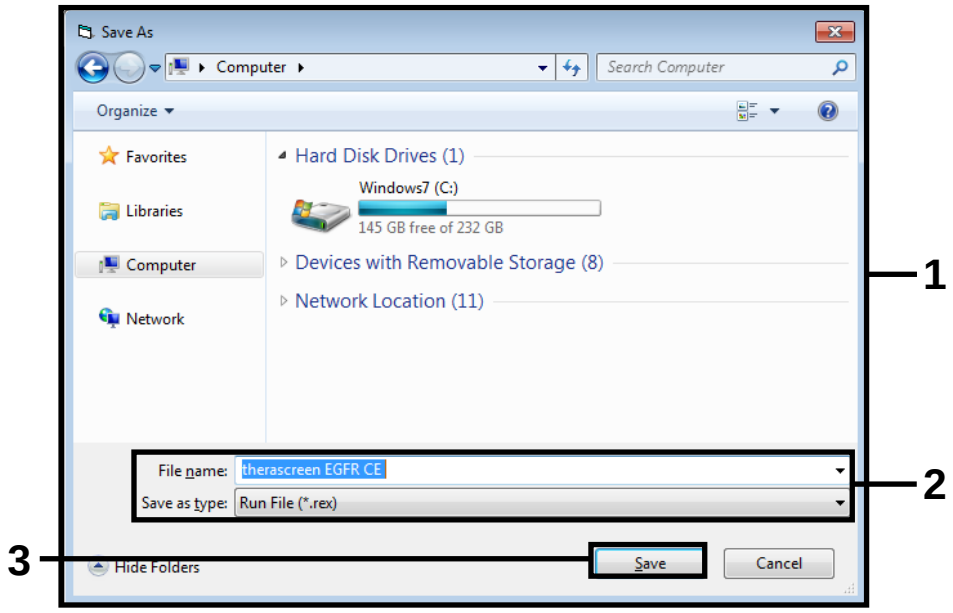

Figure 15. "Save As" window (1) (2 = "File Name" and "Save as type" fields; 3 = "Save" button).

18. **The PCR run starts.**

Note: When the run starts, the "Run Progress" tab opens to show the temperature trace and remaining run time (Figure 16).

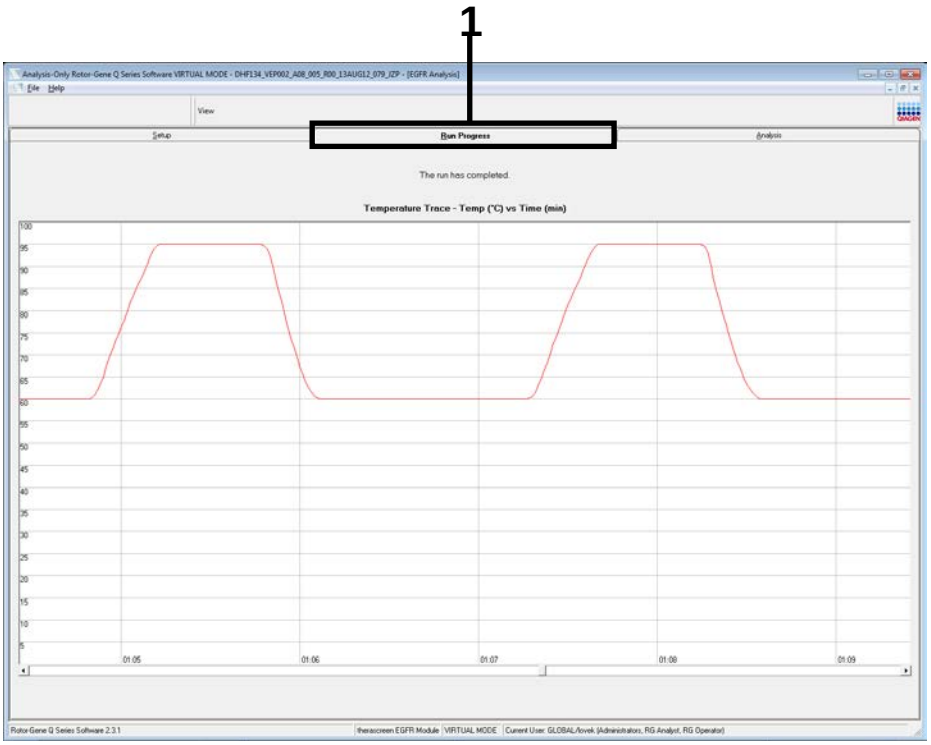

Figure 16. The "Run Progress" tab.

19. After the run is finished, the "Analysis" tab opens.

Note: If the "Analysis" tab fails to open, click the "Analysis" tab (Figure 17).

Note: An explanation of the calculation method is presented in the "Interpretation of Results (Automated)" section, page 37.

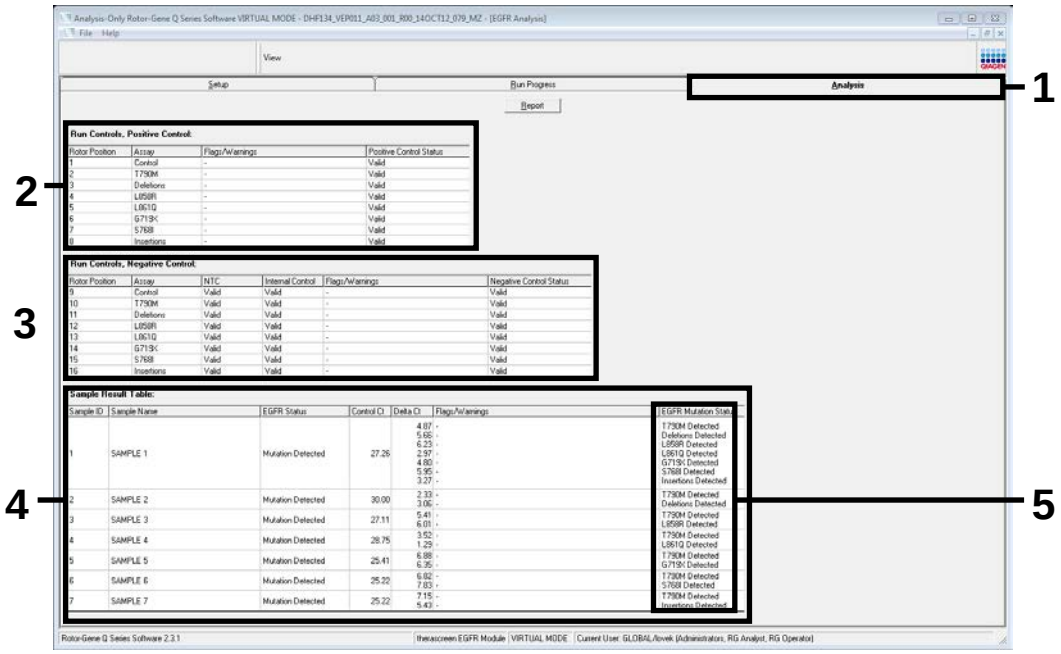

Figure 17. The "Analysis" tab (1) and reporting of results (2 = "Run Controls, Positive Control" panel; 3 = "Run Controls, Negative Control" panel; 4 = "Sample Result Table"; 5 = "Mutation Status" panel).

20. **Assay results are reported as follows (Figure 18).**

- **Run Controls, Positive Control**
  If the results are within the acceptable range, the "Positive Control Status" will display "Valid", otherwise an "Invalid" result will appear.
- **Run Controls, Negative Control**
  If both the "NTC" and "Internal Control" results are within acceptable ranges, the "Negative Control Status" will display "Valid", otherwise an "Invalid" result will appear.
- **Sample Result Table**
  Specific mutations are reported for the Mutation Positive samples under the "EGFR Mutation Status" column.

21. **Click "Report" to produce a report file. The "Report Browser" window opens. Select "EGFR CE Analysis Report" under "Templates", and then click "Show" (Figure 18).**

Note: To save a report to an alternative location in Web Archives format, click "Save As" on the top left corner of each report.

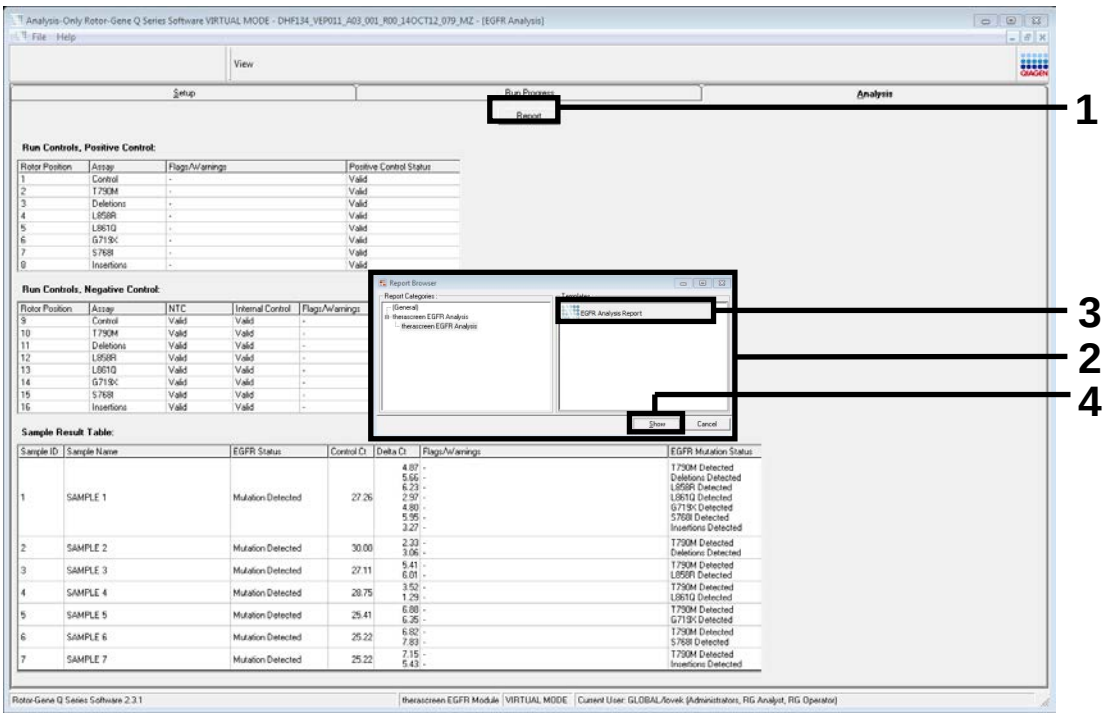

Figure 18. Selecting the "EGFR CE Analysis Report" (1 = "Report" button; 2 = "Report Browser" panel; 3 = "EGFR CE Analysis Report" button; 4 = "Show" button).

Interpretation of Results (Automated)

Analysis and mutation calls are performed automatically by the *therascreen* EGFR Assay Package when a run is completed. The following information explains how the *therascreen* EGFR Assay Package makes the analysis and mutation calls.

Note: For manual analysis of results, refer to "Interpretation of Results (Manual)", page 70.

The PCR cycle at which the fluorescence from a particular reaction crosses a threshold value is defined as the C_T value. C_T values indicate the quantity of specific input DNA. Low C_T values indicate higher input DNA levels and high C_T values indicate lower input DNA levels. Reactions with a C_T value are classed as positive amplifications.

The Rotor-Gene Q software interpolates fluorescence signals between any 2 recorded values. The C_T values can therefore be any real number (not limited to integers) within the range of 0 to 40. For the *therascreen* EGFR RGQ PCR Kit, the threshold value is set at 0.075 relative fluorescence units for the green (FAM) channel and 0.02 for the yellow (HEX) channel. These values are automatically configured in the *therascreen* EGFR Assay Package. The run controls (PC and NTC, and the IC) are assessed to ensure that acceptable C_T values are met and the reactions are performing correctly.

Sample ΔC_T values are calculated, for each mutation assay using the equation:

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

Samples are classed as mutation positive if they give a ΔC_T less than or equal to the cutoff ΔC_T value for that assay. Above this value, the sample may either contain less than the percentage of mutation able to be detected by the *therascreen* EGFR RGQ PCR Kit (beyond the limit of the assays), or the sample is mutation negative and is reported as "No Mutation Detected".

No amplification in mutation reactions is scored as "No Mutation Detected". ΔC_T values calculated from background amplification are expected to be greater than the cutoff ΔC_T values, and the sample is classed as "No Mutation Detected".

The assay results are displayed as "Mutation Detected", "No Mutation Detected", "Invalid", or, if a run control fails, "Run Control Failed". For the mutation positive samples, specific mutations will be reported. A tumor may contain more than one mutation. In such instances, more than one mutation will be reported.

Rotor-Gene Q *therascreen* EFGR Assay Package flags

Possible flags generated by the Rotor-Gene Q *therascreen* EGFR Assay Package, their meaning, and recommended actions are listed in Table 8.

Flag names are constructed to provide information on the affected component of the kit, the sample or control affected, and the failure mode.

For example:

- PC_CTRL_ASSAY_FAIL = The Positive Control (PC), Control Assay (CTRL_ASSAY) has failed (FAIL)
- NTC_INT_CTRL_FAIL = The No Template Control (NTC), Internal Control (INT_CTRL) has failed (FAIL)
- SAMPLE_CTRL_HIGH_CONC = The sample (SAMPLE), Control Assay (CTRL) has a High Concentration (HIGH_CONC)

Table 8. Flags, meaning, and actions to be taken

Flag	Meaning	Action
PC_CTRL_ASSAY_FAIL	PCR run invalid — FAM C_T out of range for positive control in control reaction.	Repeat the entire PCR run.
PC_MUTATION_ ASSAY_FAIL	PCR run invalid — FAM C_T out of range for one or more mutation control reactions.	Repeat the entire PCR run.
PC_CTRL_INVALID_ DATA	PCR run invalid — fluorescence data in positive control (Control Reaction Mix) cannot be interpreted.	Repeat the entire PCR run.
PC_MUTATION_ INVALID_DATA	PCR run invalid — fluorescence data in positive control (mutation reaction mix) cannot be interpreted.	Repeat the entire PCR run.

Flag	Meaning	Action
NTC_INT_CTRL_FAIL	PCR run invalid — internal control above range for negative control.	Repeat the entire PCR run.
NTC_INT_CTRL_ EARLY_CT	PCR run invalid — internal control is below range for negative control.	Repeat the entire PCR run.
NTC_INVALID_CT	PCR run invalid — FAM invalid (smaller than limit) for negative control.	Repeat the entire PCR run.
NTC_INVALID_DATA	PCR run invalid — fluorescence data in negative control cannot be interpreted.	Repeat the entire PCR run.
SAMPLE_CTRL_ INVALID_DATA	Sample invalid — fluorescence data in sample control cannot be interpreted.	Set up new PCR run to repeat the relevant sample(s).
SAMPLE_CTRL_ HIGH_CONC	Sample Invalid — FAM C_T too low in sample control.	Dilute sample to increase control C_T value. This dilution should be calculated on the assumption that diluting 1:1 with the water supplied in the kit will increase the C_T by 1.0; once sample is diluted, set up new PCR run to repeat sample.

Flag	Meaning	Action
SAMPLE_CTRL_FAIL	Sample invalid — FAM C_T too high in sample control reaction.	Set up new PCR run to repeat sample. If invalid on repeat PCR run, extract two further FFPE tissue sections if available. Set up a new PCR run to test this extraction. If invalid, repeat the PCR run on the second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.
SAMPLE_INT_CTRL_ FAIL	C_T too high (or no C_T) for internal control (HEX), FAM channel mutation-negative.	If sample given valid status — no action. If sample is given invalid status: Set up new PCR run to repeat sample. (If the sample control assay C_T allows, dilute sample with the water supplied in the kit using the assumption that diluting 1:1 will increase the C_T of the control reaction by 1.0.) If invalid on repeat PCR run, extract two further FFPE tissue sections if available. Set up a new PCR run to test this extraction. If invalid, repeat the PCR run on the second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

Flag	Meaning	Action
SAMPLE_INT_CTRL_EARLY_CT	Mutation tube invalid — C_T HEX too low for sample (internal control)	If sample is given valid status — no action. If sample is given invalid status:
	Set up new PCR run to repeat sample. If invalid on repeat PCR run, extract two further FFPE tissue sections if available. Set up a new PCR run to test this extraction. If invalid, repeat the PCR run on the second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.	
SAMPLE_INVALID_DATA	Mutation tube invalid — fluorescence data in internal control can't be interpreted.	If sample is given valid status — no action. If sample is given invalid status:
	Set up new PCR run to repeat sample. If invalid on repeat PCR run, extract two further FFPE tissue sections if available. Set up a new PCR run to test this extraction. If invalid, repeat the PCR run on the second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.	

Flag	Meaning	Action
MUTATION_EARLY_CT	Mutation tube invalid — C_T FAM too low for sample.	If sample is given valid status — no action. If sample is given invalid status:
	Set up new PCR run to repeat sample. If invalid on repeat PCR run, extract two further FFPE tissue sections if available. Set up a new PCR run to test this extraction. If invalid, repeat the PCR run on the second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.	
SAMPLE_POSITIVE_AND_INVALID	One or more mutations for a sample are valid and positive; at the same time one or more mutations for the same sample are invalid (warning, not an error).	None. **Note**: This flag does not mean the sample data are invalid but highlights that information is not available for all assays.

Troubleshooting guide

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

	Comments and suggestions
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 MDx 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.

	Comments and suggestions
d) The storage conditions for one or more kit components did not comply with the instructions given in "Reagent Storage and Handling" (page 14)	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
e) The *therascreen* 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.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the *therascreen* EGFR 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 Rotor-Gene Q MDx instruments.

The product is intended for use only on a Rotor-Gene Q MDx real-time PCR cycler.

Strict compliance with the therascreen *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 *therascreen* 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.

Performance Characteristics

Analytical performance

The specific performance characteristics of the *therascreen* EGFR RGQ PCR Kit were determined by studies using FFPE tissue specimens collected from NSCLC patients and FFPE human cell lines (FFPE cell lines). The FFPE cell lines were generated using a lung carcinoma cell line (A549) to produce cell lines harboring the desired specific EGFR mutations. When tissue specimen or cell lines were not available plasmid DNA was used.

Limit of blank (LOB), working range, and cutoff values

A total of 417 FFPE samples were tested in a study following guidance in NCCLS EP17-A (2004) (12) to determine the LOB and cutoff values for each mutation assay. In addition, the working range was determined. The cutoff values were established and are shown in Table 9.

Table 9. Established cutoff values for each mutation assay

Assay	Cutoff (ΔC_T)
T790M	≤7.40
Deletions	≤8.00
L858R	≤8.90
L861Q	≤8.90
G719X	≤8.90
S768I	≤8.90
Insertions	≤8.00

The control reaction C_T range was established as 23.70 to 31.10 C_T.

The assay cutoffs and working ranges were verified using standards and further FFPE samples. During verification, the cutoffs were assessed for ability to distinguish the correct mutation in a background of wild type DNA by assessing each assay with high input genomic DNA and high input mutation DNA (see "Cross-reactivity", page 46). The effect of input DNA on mutation call was also assessed (see "Effect of DNA input on ΔC_T values", page 46).

To assess performance of the *therascreen* EGFR 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 might indicate a low concentration of mutation, samples with no template and NSCLC EGFR wild type DNA were evaluated. The results demonstrated no positive mutation calls for NTC samples and for FFPE wild-type samples.

Effect of DNA input on ΔC_T values

The 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 RGQ PCR Kit is consistent across the control reaction C_T range (23.70–31.10), all 7 EGFR mutation assays were tested against a 6-point, 1-in-3 dilution series (DNA extracted from FFPE cell lines). The target C_T for dilution 1, 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 values measured at different total DNA input levels were consistent across the working range of the *therascreen* EGFR RGQ PCR Kit.

Cross-reactivity

Wild-type EGFR DNA at high DNA input was tested to assess non-specific amplification. The results demonstrated that the lowest ΔC_T values exceeded the established cutoffs, indicating no non-specific amplification.

FFPE cell lines at high DNA input were tested against all reaction mixes to assess potential cross-reactivity. The results demonstrated no impact due to cross-reactivity between mutant reactions. The minimum ΔC_T values were all higher than the respective assay cutoff values for all non-matching reaction mixes and DNA samples.

Accuracy: Comparison to the analytical reference method

A study demonstrated the concordance in mutation detection of the *therascreen* EGFR RGQ PCR Kit relative to bi-directional Sanger sequencing. In this study, 360 FFPE samples were tested.

Samples with both Sanger and *therascreen* EGFR RGQ PCR Kit valid results were analyzed to assess the Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), and Overall Percent Agreement (OPA). These percentages, together with the corresponding two-sided 95% confidence intervals (CI), are summarized in Table 10.

Table 10. Analysis of agreement

Measure	Percent agreement (N)	95% CI
Positive Percent Agreement	99.4% (157/158)	96.5%–100.0%
Negative Percent Agreement	86.6% (175/202)	81.2%–91.0%
Overall Percent Agreement	92.2% (332/360)	89.0%–94.8%

For the 28 discordant Overall Percentage Agreement results:

- 1 (3.6%) sample was wild-type (i.e., no mutation detected) by the *therascreen* EGFR RGQ PCR Kit but mutation detected results by Sanger sequencing.
- 27 (96.4%) samples were mutation detected by the *therascreen* EGFR RGQ PCR Kit but wild-type results by Sanger sequencing.

Limit of detection (LOD) values

A study was conducted to determine the LOD of each of the 29 EGFR mutations. LOD was defined as the lowest amount of mutant DNA in a background of wild-type DNA at which a mutant sample will provide mutation positive results in 95% of the test results (C_{95}).

To determine the LOD for each mutation, samples with different percentages of mutation were prepared at low and high input DNA concentrations and tested with the *therascreen* EGFR RGQ PCR Kit (Table 11). The LOD for each assay was calculated by logistic regression. To verify the LOD, mutation samples at the determined LOD were tested and the positive test rate verified.

Table 11. LOD established using low and high DNA input FFPE clinical specimens, FFPE cell lines, or plasmids

				LOD (% mutant)	
Exon	**Mutation**	**COSMIC* ID**	**Base change**	**Low**	**High**
18	G719A	6239	2156G>C	7.41‡	1.57‡
	G719S	6252	2155G>A	5.08†	7.75~
	G719C	6253	2155G>T	10.3†	-§
19	Deletions	12384	2237_2255>T	1.58~	0.49~
		12387	2239_2258>CA	4.91‡	1.48‡
		12419	2238_2252>GCA	16.87‡	12.47‡
		12422	2238_2248>GC	3.24‡	1.65‡
		13551	2235_2252>AAT	4.24‡	1.41‡
		12678	2237_2251del15	0.55~	0.24~
		6218	2239_2247del9	8.47‡	-§
		12728	2236_2253del18	2.43‡	-§
		12367	2237_2254del18	2.72‡	-§
		6210	2240_2251del12	4.09‡	-§
		6220	2238_2255del18	2.70‡	0.82‡
		6223	2235_2249del15	6.40‡	1.63‡
		6225	2236_2250del15	2.80‡	1.42‡
		6254	2239_2253del15	0.86~	0.47~
		6255	2239_2256del18	0.14~	0.05~
		12369	2240_2254del15	4.94~	1.56~
		12370	2240_2257del18	8.10~	2.08~

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

† LOD values were established using plasmids

‡ LOD values were established using cell lines

~ LOD values were established using clinical samples

§ Not assessed

Table continued on next page

Table 11. Continued

Exon	Mutation	COSMIC* ID	Base change	LOD (% mutant) Low	LOD (% mutant) High
19	Deletions	12382	2239_2248TTAAGAGAAG>C	0.25~	0.10~
		12383	2239_2251>C	4.58~	1.74~
20	S768I	6241	2303G>T	7.66‡	2.18‡
	Insertions	12376	2307_2308ins GCCAGCGTG	11.61‡	-§
		12378	2310_2311ins GGT	4.91‡	1.31‡
		12377	2319_2320ins CAC	2.40‡	0.65‡
	T790M	6240	2369C>T	9.72‡	5.09‡
21	L858R	6224	2573T>G	5.94‡	1.13‡
	L861Q	6213	2582T>A	2.22‡	0.66‡

* COSMIC: Catalogue of somatic mutations in cancer: http://cancer.sanger.ac.uk/
† LOD values were established using plasmids
‡ LOD values were established using cell lines
~ LOD values were established using clinical samples
§ Not assessed

Interference

Effects of necrotic tissue

NSCLC FFPE clinical specimens with necrotic tissue content up to 50% for both EGFR mutant and wild-type specimens did not interfere with call results of the *therascreen* EGFR RGQ PCR Kit.

Exogenous substances

Potential interfering substances present in the DNA extraction process were tested in mutant and wild-type samples at 10x concentration: paraffin wax, xylene, ethanol, and proteinase K. The results demonstrated that these substances did not interfere with the call results of the *therascreen* EGFR RGQ PCR Kit.

Reproducibility

Lot-to-lot reproducibility

The *therascreen* EGFR RGQ PCR Kit test system utilizes 2 separate kits: the QIAamp DNA FFPE Tissue Kit (functionally equivalent to the QIAamp DSP DNA FFPE Tissue Kit) for isolation of DNA, and the *therascreen* EGFR RGQ PCR Kit for the amplification of DNA and detection of EGFR mutation status. Lot-to-lot reproducibility and interchangeability were demonstrated using 3 lots of the QIAamp DSP DNA FFPE Tissue Kit and 3 lots of the *therascreen* EGFR RGQ PCR Kit. The overall percentage of correct calls across lots for EGFR mutation assay was 97.8% (317/324) and that for wild-type samples was 100% (379/379).

Specimen handling

The reproducibility of the QIAamp DSP DNA FFPE Tissue Kit was examined using sections taken from 3 FFPE specimen blocks, specifically the exon 19 deletion mutation (2235-2249 del15), the exon 21 L858R mutation, and one wild type. For each specimen, extractions were carried out in duplicate at 3 sites and tested on 3 nonconsecutive days over a period of 6 days, yielding a total of 18 data points per specimen. At each site, 2 operators conducted the testing using one lot of the QIAamp DSP DNA FFPE Tissue Kit (one lot per site, 3 lots total) in combination with the same lot of the *therascreen* EGFR RGQ PCR Kit reagents across sites. All mutant and wild type specimen results were valid and yielded the expected call result (correct call = 100%, 18/18 for each specimen), supporting the reproducibility and repeatability for the *therascreen* EGFR RGQ PCR Kit at the pre-analytical step of DNA isolation.

Precision and reproducibility

The precision and reproducibility of the *therascreen* EGFR RGQ PCR Kit was investigated by testing DNA extracted from NSCLC FFPE clinical specimens or FFPE cell lines, representing all 7 mutation assays in the *therascreen* EGFR RGQ PCR Kit. NSCLC wild-type FFPE clinical specimens were also included in the study (Table 12).

A matrix study design was implemented to assess assay reproducibility by testing samples at 3 laboratories (sites), with 3 lots of *therascreen* EGFR RGQ PCR Kit (3 lots across 3 sites), using 2 operators per site, on 2 instruments per site, with each sample (prepared at a level close to the LOD) tested in duplicate, over a total of 16 days. Reproducibility for each individual mutation was conducted over nonconsecutive days at each site. The proportion of correct calls is shown in Table 12.

Table 12. Assay reproducibility — Proportion of correct calls for EGFR mutations tested

Exon	Mutation	COSMIC * ID	Calls		% Correct
			Correct/total	% Correct	Lower one sided 95% CI
18	G719A	6239	77/78	98.72	94.06
19	Deletions	12384	93/93	100	96.83
		12387	92/92	100	96.8
		12419	95/95	100	96.9
		12422	83/83	100	96.46
		13551	94/94	100	96.86
		6220	96/96	100	96.93
		6223	96/96	100	96.93
		6225	95/95	100	96.9
		6254	91/95	95.79	90.62
		6255	92/92	100	96.8
		12369	94/96	97.92	93.59
		12370	95/95	100	96.9
		12382	62/63	98.41	92.69
		12383	92/95	96.84	92.04
20	S768I	6241	82/82	100	96.41
	Insertions	12378	93/93	100	96.83
		12377	92/92	100	96.8
	T790M	6240	94/94	100	96.86
21	L858R	6224	92/92	100	96.8
	L861Q	6213	83/84	98.81	94.48
Wild-type			84/84	100	96.5

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

A variance component analysis was used to estimate the standard deviation and 95% confidence intervals for within-run, between-run, between-day, between-lot, and between-site variability. Across all variance components, the total coefficient of variation (CV) was ≤14.11% for all EGFR mutations tested. Across all mutant panel members, the percentage CV was in general <6% for between-lots, between-days, and between-runs. The percentage CV for within-run variability (repeatability/precision) ranged from 5.99% to 13.49%.

Clinical Performance

Clinical outcome data

The LUX-Lung 3 clinical trial was an international, multicenter, open label, randomized Phase 3 trial of afatinib versus chemotherapy as first-line treatment for patients with stage IIIB or IV adenocarcinoma of the lung harboring an EGFR-activating mutation (ClinicalTrials.gov number NCT00949650). The eligibility of a patient for enrollment onto the trial was determined by testing the EGFR mutation status of the patient using the Clinical Trial Assay (CTA). Retrospective testing of tissue specimens was performed using the *therascreen* EGFR RGQ PCR Kit. A bridging study was conducted to assess the concordance between the *therascreen* EGFR RGQ PCR Kit and the CTA.

Based on the CTA test results, 345 patients were in the randomized set (afatinib: 230 patients; chemotherapy: 115 patients). The primary efficacy outcome was progression-free survival (PFS) as assessed by an independent review committee (IRC). Among the 345 randomized patients, tumor samples from 264 patients (afatinib: 178 patients; chemotherapy: 86 patients) were tested retrospectively using the *therascreen* EGFR RGQ PCR Kit. A statistically significant improvement in PFS as determined by the IRC was demonstrated for patients randomized to afatinib compared to those randomized to chemotherapy, in overall CTA+ population and the *therascreen* EGFR RGQ PCR Kit+/CTA+ population. The overall efficacy results are summarized in Table 13 and Figure 19.

Table 13. Clinical benefit of patients tested with the *therascreen* EGFR RGQ PCR Kit in the LUX-Lung 3 clinical trial population

	***therascreen* EGFR RGQ PCR Kit+/CTA+ population n = 264**		**CTA+ population n = 345**	
Parameter	**Chemotherapy n = 86**	**Afatinib n = 178**	**Chemotherapy n = 115**	**Afatinib n = 230**
Progression-free survival PFS				
Number of deaths or progressions, N (%)	53 (61.6%)	120 (67.4%)	69 (60.0%)	152 (66.1%)
Median PFS (months)	6.9	11.2	6.9	11.1
Median PFS 95% CI	[5.3, 8.2]	[9.7, 13.7]	[5.4, 8.2]	[9.6, 13.6]
Hazard ratio	0.49		0.58	
Hazard ratio 95% CI	[0.35, 0.69]		[0.43, 0.78]	
P-value (stratified log-rank test)*	<0.0001		<0.001	

* Stratified by EGFR mutation status and race.

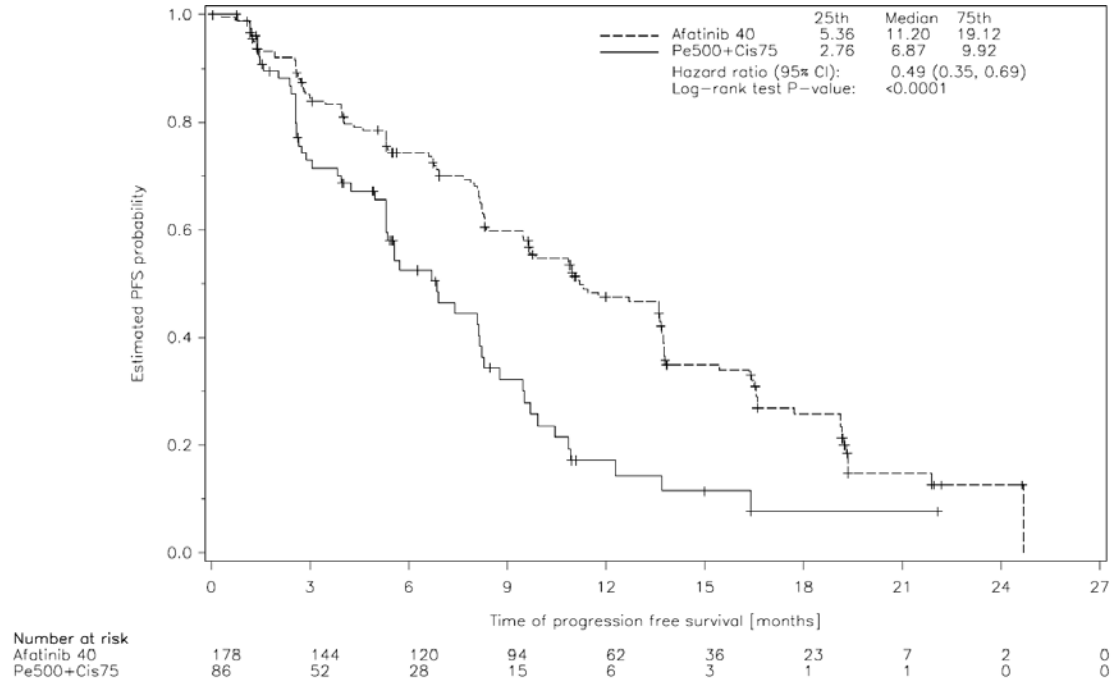

Figure 19. Kaplan-Meier curve of progression-free survival (PFS) by independent review by treatment group (*therascreen* EGFR RGQ PCR Kit+/CTA+ population).

Analysis of the *therascreen* EGFR RGQ PCR Kit+/CTA+ subset (n = 264) revealed that those patients treated with afatinib had a significant increase in PFS time (median PFS 11.2 versus 6.9 months) and are less likely to have an event of progressive disease or death (HR = 0.49, 95 % CI [0.35; 0.69], p<0.0001) than patients treated with chemotherapy. The observed clinical benefit in the subset of patients tested with the *therascreen* EGFR RGQ PCR Kit was comparable to that observed in the full study population (n = 345).

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Symbols

The following symbols may appear on the packaging and labeling:

 <N>	Contains reagents sufficient for <N> reactions
	Use by
IVD	In vitro diagnostic medical device
REF	Catalog number
LOT	Lot number
MAT	Material number
	Keep away from sunlight
GTIN	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

Appendix A: *therascreen* EGFR RGQ PCR Kit Manual Protocol

This section contains instructions for using the *therascreen* EGFR RGQ PCR Kit with Rotor-Gene Q software version 2.3 in the open mode (i.e., without using the Rotor-Gene Q *therascreen* EGFR CE Assay Package).

General information

- For a list of materials required, refer to "Materials Required but Not Provided", page 11.
- For full instructions on sample preparation and sample layout, refer to "Protocol: Sample assessment", page 17 and "Protocol: EGFR mutation detection", page 27.
- Ensure cycling parameters are correct before starting each run.

Protocol: Creating a temperature profile

Before starting, create a temperature profile for the *therascreen* EGFR RGQ PCR Kit analysis. The cycling parameters are the same for DNA sample assessment and EGFR mutation detection.

Procedure

A summary of cycling parameters is shown in Table 14.

Table 14. 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

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

3. Select 72-well rotor as the rotor type. Confirm that the locking ring is attached and check the "Locking Ring Attached" box. Click "Next" (Figure 20).

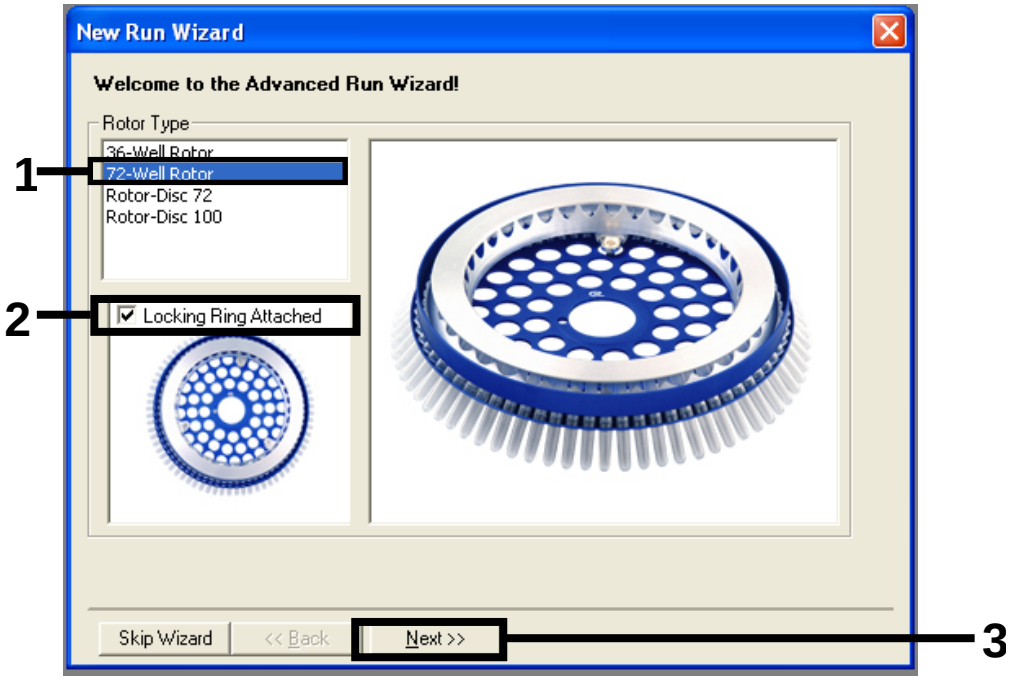


Figure 20. The "New Run Wizard" dialog box (1 = "Rotor type"; 2 = "Locking Ring Attached" box; 3 = "Next" button).

4. Enter the name of the operator. Add any notes and enter the reaction volume as 25. Ensure that "Sample Layout" reads "1, 2, 3…". Click "Next" (Figure 21).

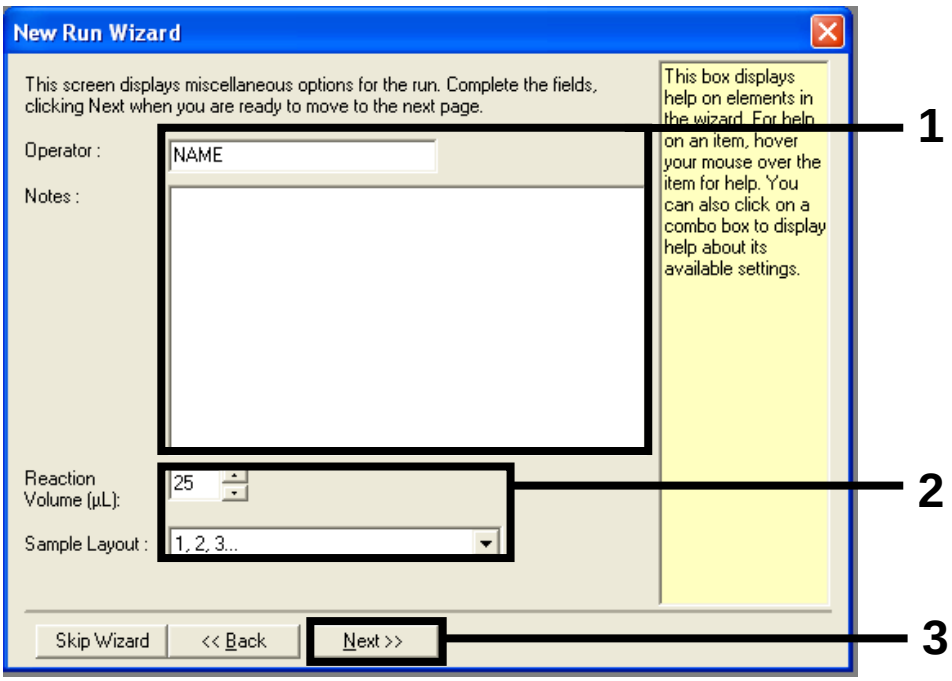


Figure 21. Entering operator name and reaction volumes (1 = "Operator" dialog field and "Notes" dialog field; 2 = "Reaction Volume" field and "Sample Layout" field; 3 = "Next" button).

5. **Click "Edit Profile" in the "New Run Wizard" dialog box (Figure 22) and check the run parameters according to the following steps.**

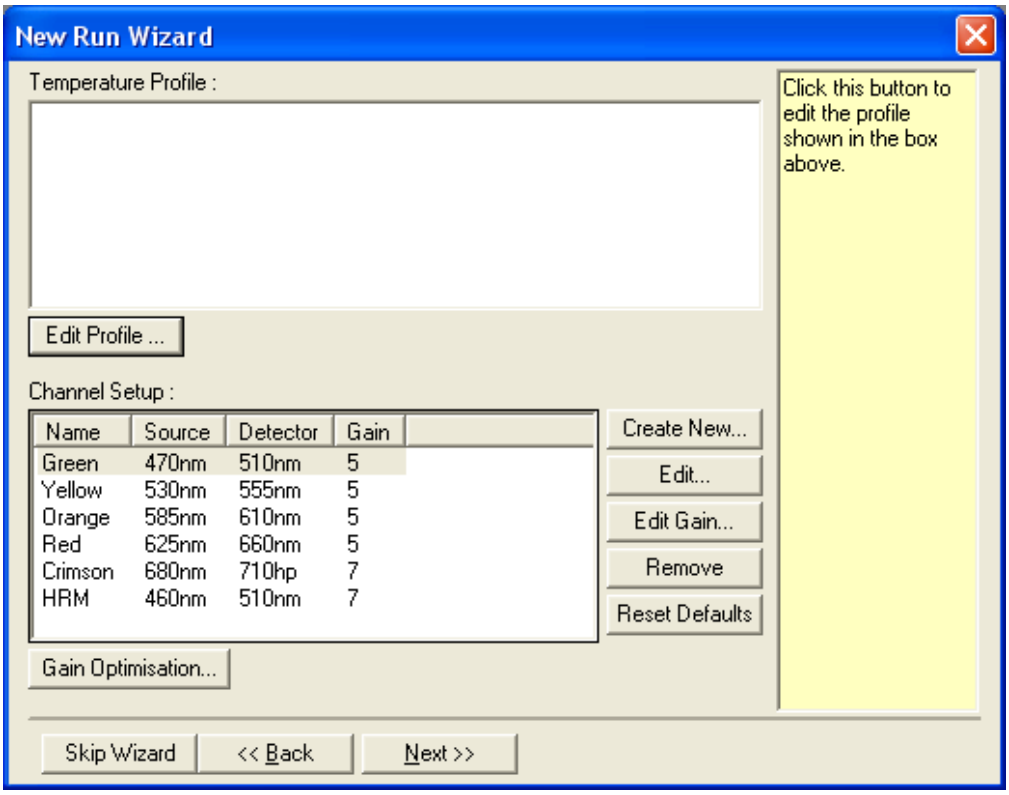


Figure 22. "Edit Profile" in the "New Run Wizard".

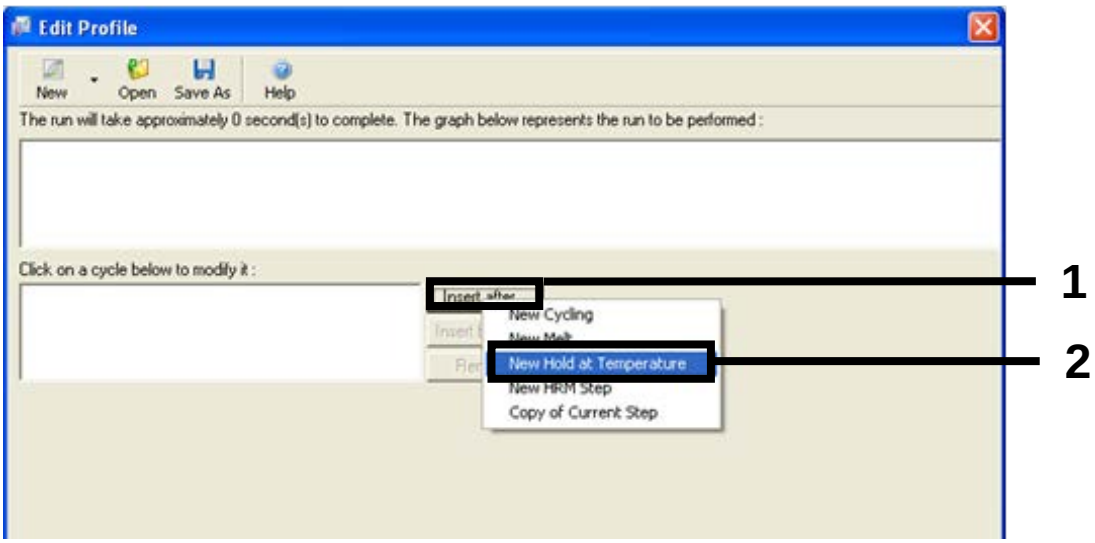


Figure 23. Inserting an initial incubation step (1 = "Insert after" button; 2 = "New Hold at Temperature").

6. **Click the "Insert after" button and select "New Hold at Temperature" (Figure 23).**

7. Change "Hold Temperature" to 95°C and "Hold Time" to 15 mins 0 secs. Click "Insert After" and then select "New Cycling" (Figure 24).

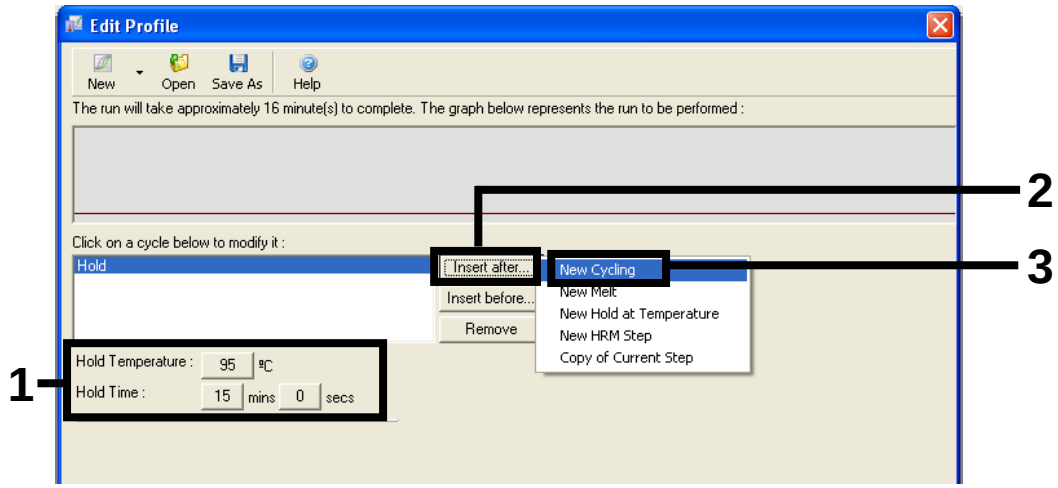


Figure 24. Initial incubation step at 95˚C (1 = "Hold Temperature and Hold Time" buttons; 2 = "Insert after" button; 3 = "New Cycling").

8. Change the number of cycle repeats to 40. Select the first step and set to 95˚C for 30 secs (Figure 25).

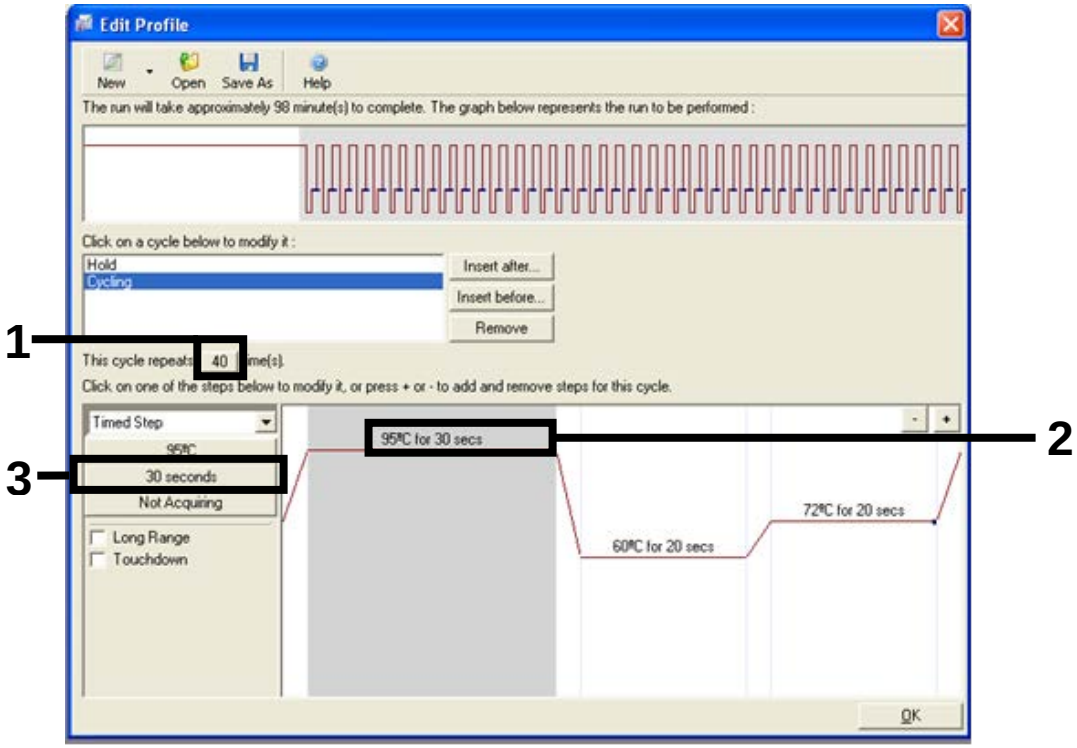


Figure 25. Cycling step at 95˚C (1 = "Cycle repeats" box; 2 = Step one: temperature setting; 3 = Step one: time setting).

9. Highlight the second step and set to 60°C for 60 secs. Enable data acquisition during this step by selecting the "Not Acquiring" button (Figure 26).

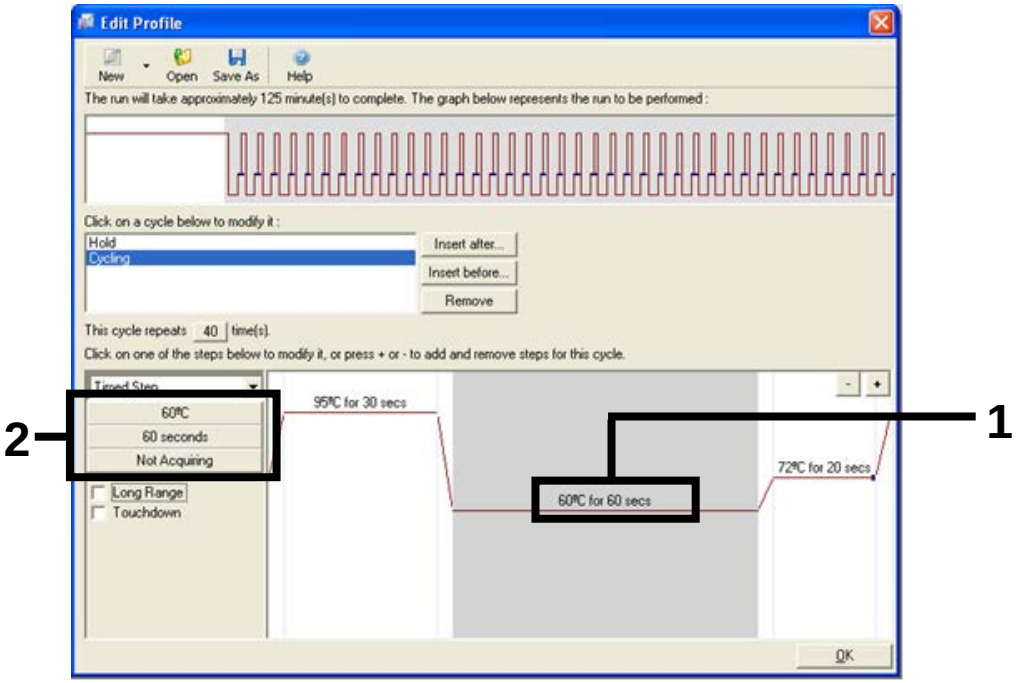


Figure 26. Cycling step at 60°C (1 = Step two: temperature and time setting; 2 = "Not Acquiring" button).

10. Set Green and Yellow as acquiring channels by selecting the ">" button to transfer these channels from the "Available Channels" list. Click "OK" (Figure 27).

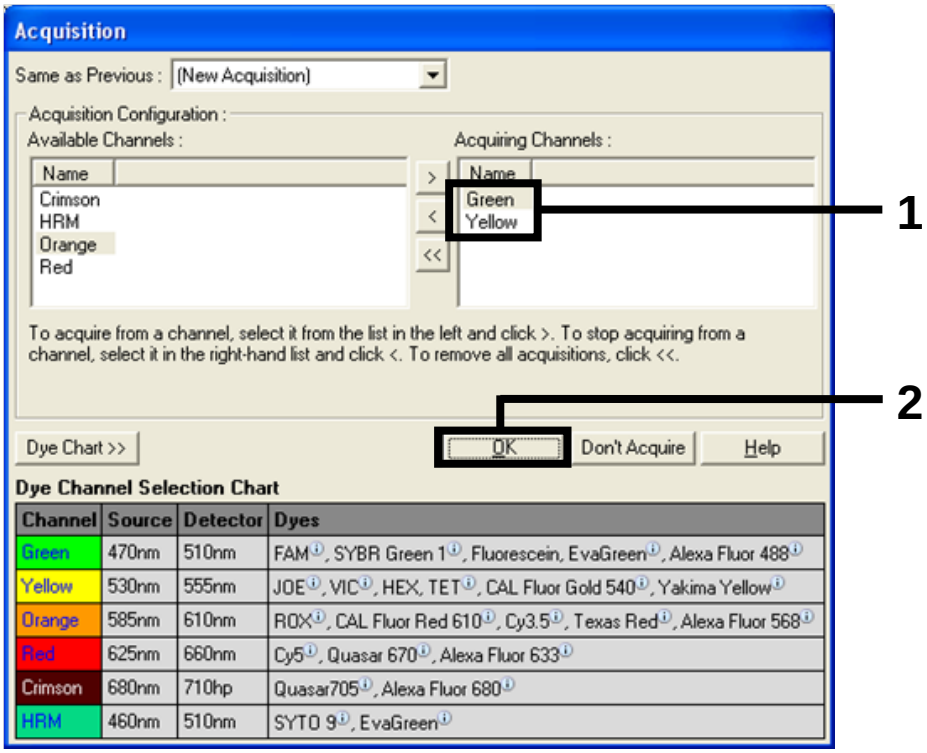


Figure 27. Acquiring at cycling step of 60°C (1 = Selected channels; 2 = "OK" button).

11. Highlight the third step and delete by clicking the "-" button. Click "OK" (Figure 28).

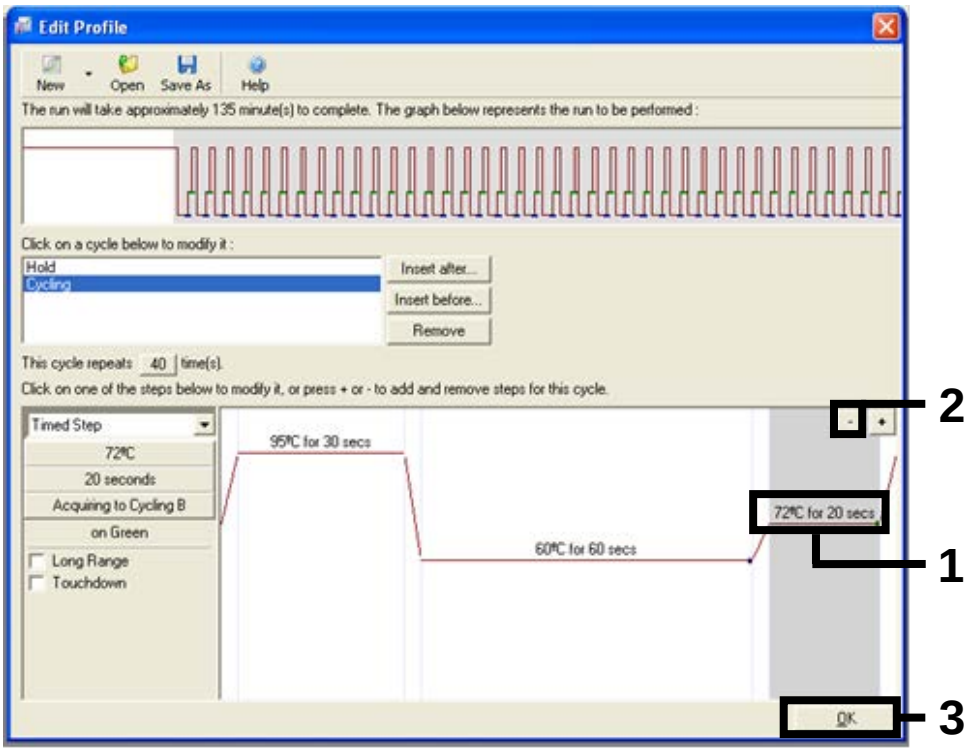


Figure 28. Removal of the extension step (1 = Third step; 2 = Delete button; 3 = "OK" button).

12. In the next dialog box, click "Gain Optimisation" (Figure 29).

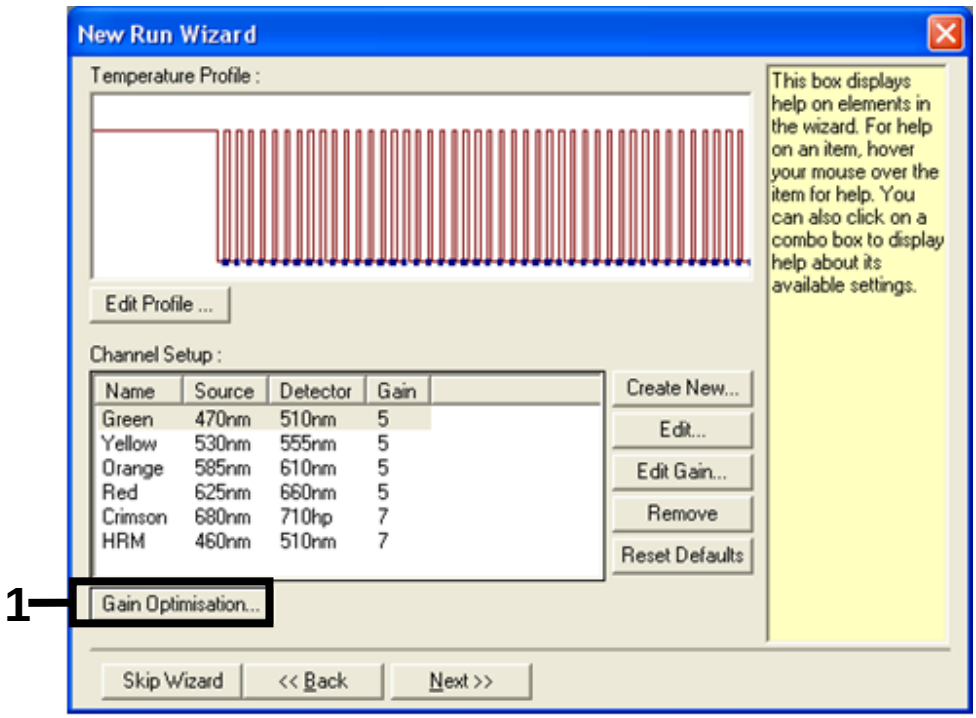


Figure 29. Gain optimization (1).

13. Click the "Optimise Acquiring" button. Channel settings are displayed for each channel. Accept these default values by clicking "OK" for both channels (Figure 30).

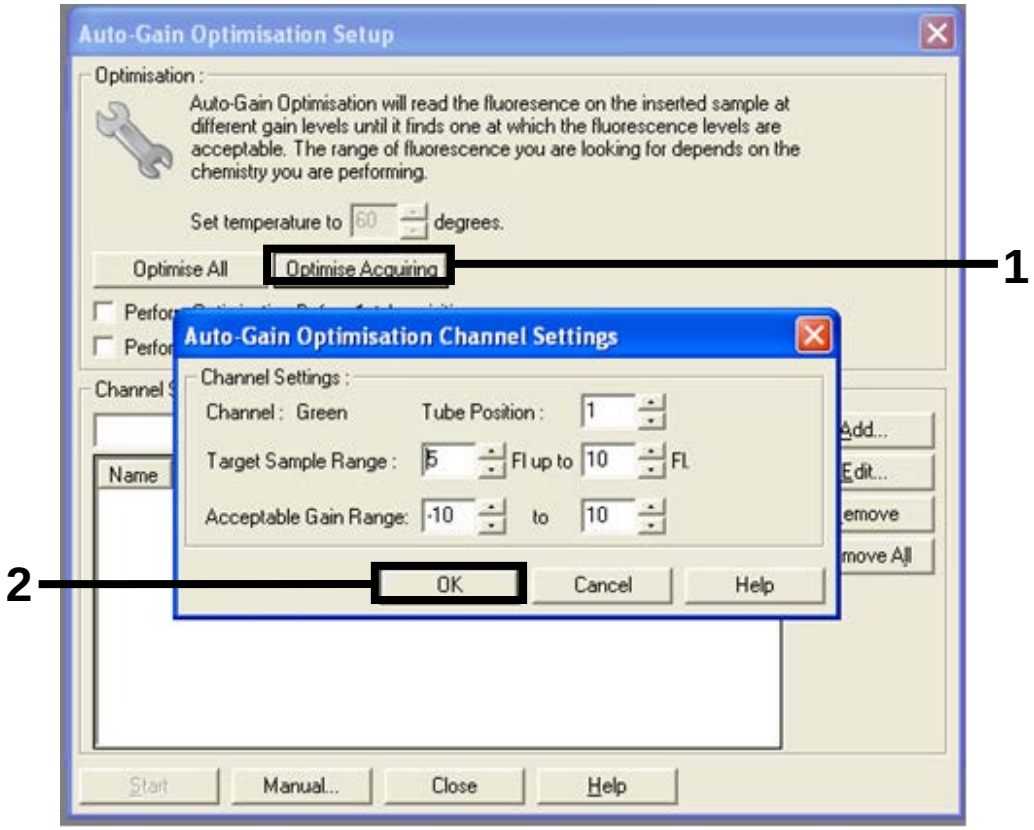


Figure 30. Auto-gain optimization for the Green channel (1 = "Optimise Acquiring" button; 2 = "OK" button).

14. Check the "Perform Optimisation before 1st Acquisition" box, and then click the "Close" button to return to the wizard (Figure 31).

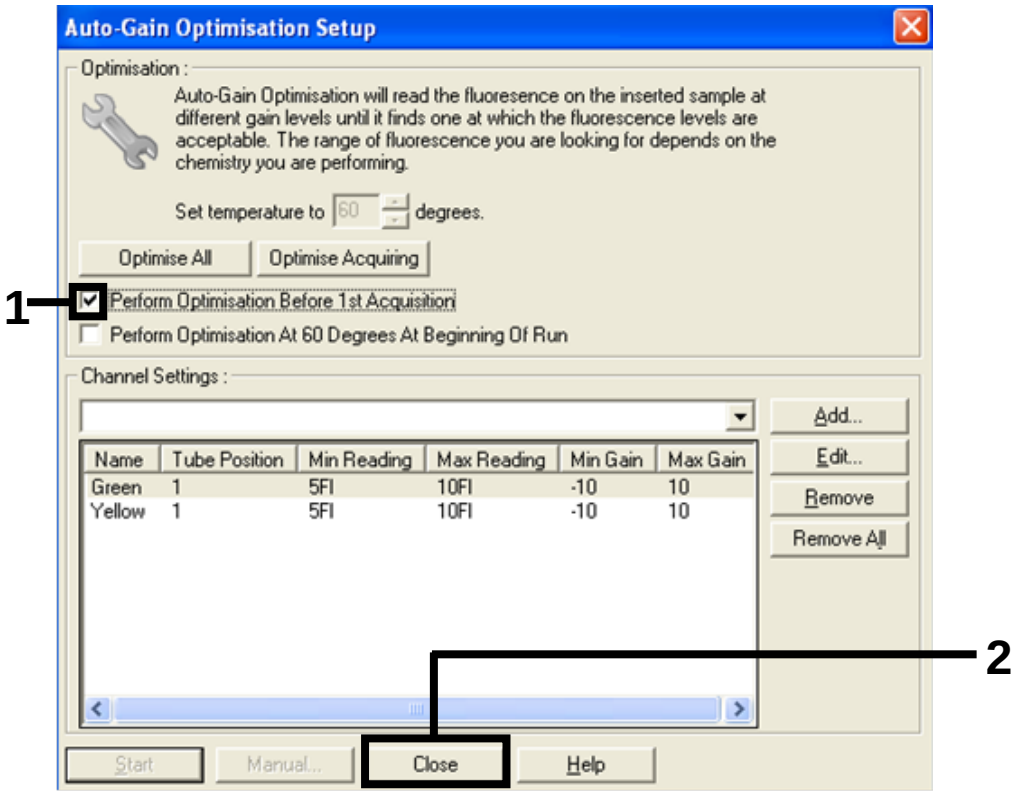


Figure 31. Selection of Green and Yellow channels (1 = "Perform Optimisation Before 1st Acquisition" tick box; 2 = "Close" button).

15. Click "Next" (Figure 32) to save the *therascreen* EGFR RGQ PCR Kit template (*.ret file) in an appropriate location by selecting "Save Template".

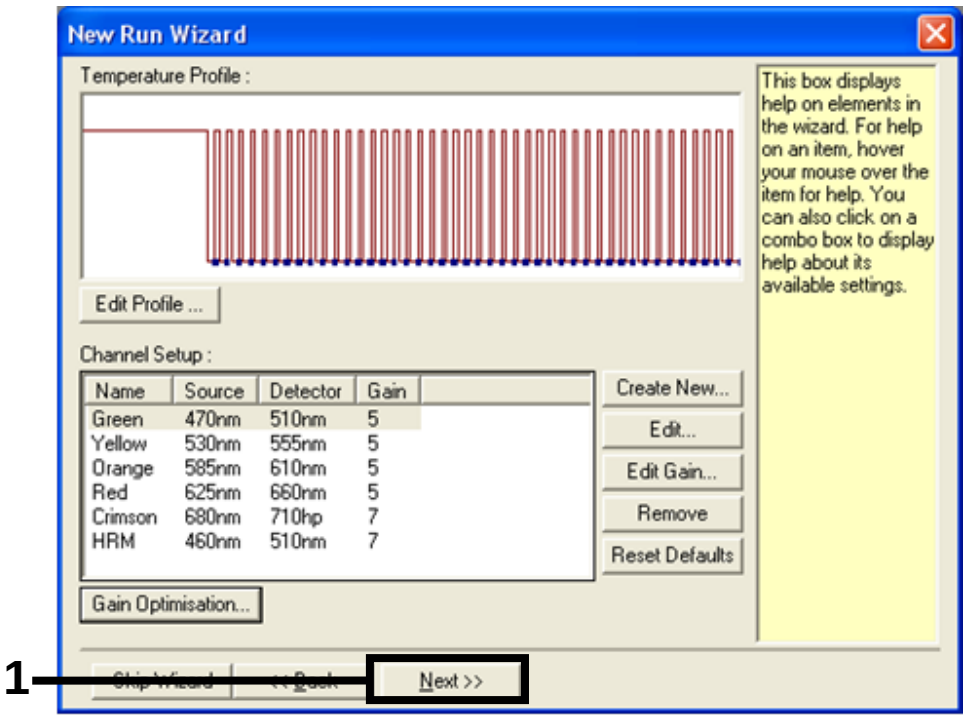


Figure 32. "Next" button (1).

Procedure (Manual)

Protocol: Sample assessment (manual)

This protocol is used to assess the total amplifiable DNA in samples and should be performed prior to EGFR mutation analysis.

- Prepare samples as described in section "Protocol: Sample assessment" on page 17, up to step 11.
- Set up the PCR run on a Rotor-Gene Q MDx instrument as described in section "Protocol: *therascreen* EGFR RGQ PCR Kit Rotor-Gene Q setup" on page 66.
- After the run is complete, analyze the data according to the instructions in section "Sample assessment data analysis" on page 71.

Protocol: EGFR mutation detection (manual)

Once a sample has passed the sample assessment, it can be tested to detect EGFR mutations.

- Prepare samples as described in section "Protocol: EGFR mutation detection" on page 27, up to step 11.
- Set up the PCR run on a Rotor-Gene Q MDx instrument as described in section "Protocol: *therascreen* EGFR RGQ PCR Kit Rotor-Gene Q setup" on page 66.
- After the run is complete, analyze the data according to the instructions in section "EGFR mutation detection data analysis" on page 72.

Protocol: *therascreen* EGFR RGQ PCR Kit Rotor-Gene Q setup

Procedure

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

   For instructions on creating the temperature profile and checking the run parameters, see "Protocol: Creating a temperature profile" on page 57.

2. **Ensure that the correct rotor is selected, and check the box to confirm that the locking ring is attached. Click "Next" (Figure 33).**

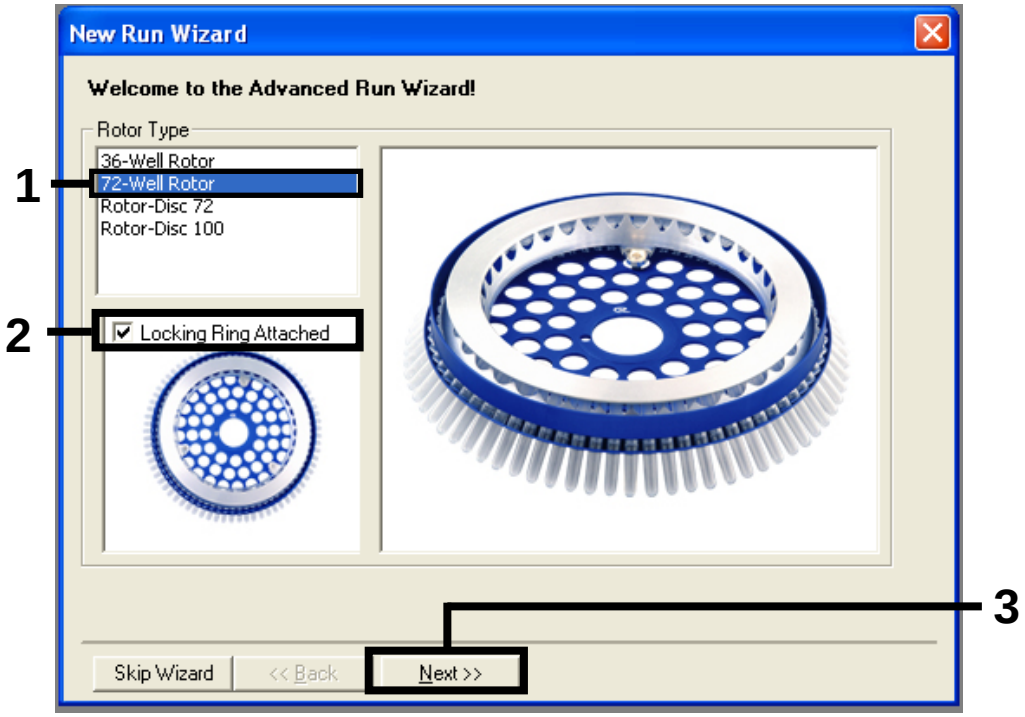


Figure 33. The "New Run Wizard" dialog box and welcome screen (1 = "Rotor type"; 2 = "Locking Ring Attached" box; 3 = "Next" button).

3. **Enter the name of the operator. Add any notes, check that the reaction volume is set to 25 and "Sample Layout" reads "1, 2, 3…". Click "Next" (Figure 34).**

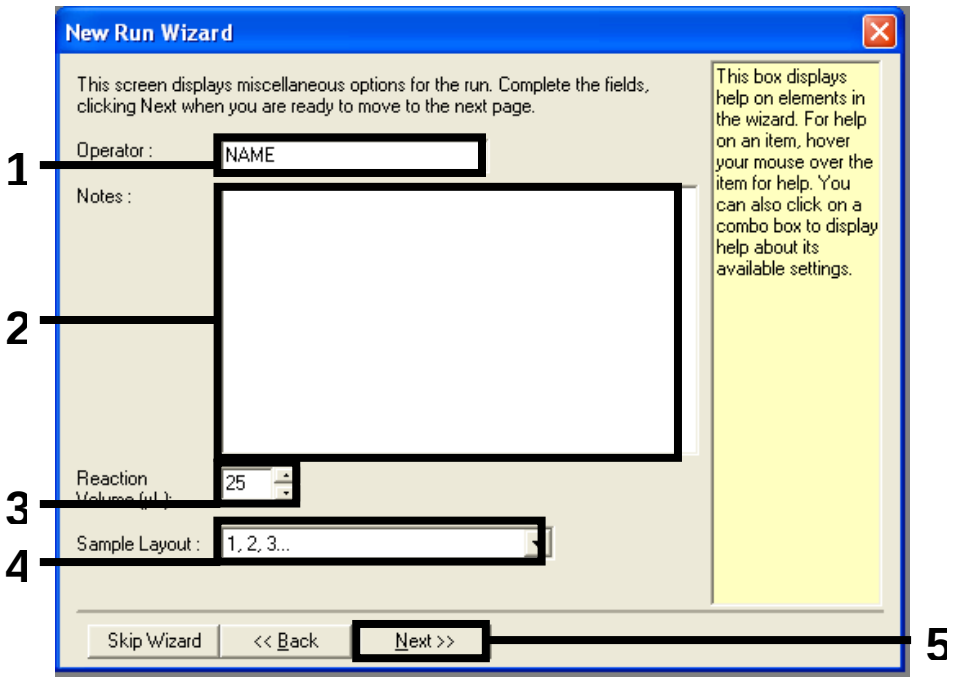


Figure 34. The "New Run Wizard" option screen (1 = "Operator"; 2 = "Notes" field; 3 = "Reaction Volume"; 4 = "Sample Layout" field; 5 = "Next" button).

4. **The next window allows editing of the temperature profile. (No editing is required as the temperature profile was created according to the instructions in "Protocol: Creating a temperature profile", page 57.) Click "Next" (Figure 35).**

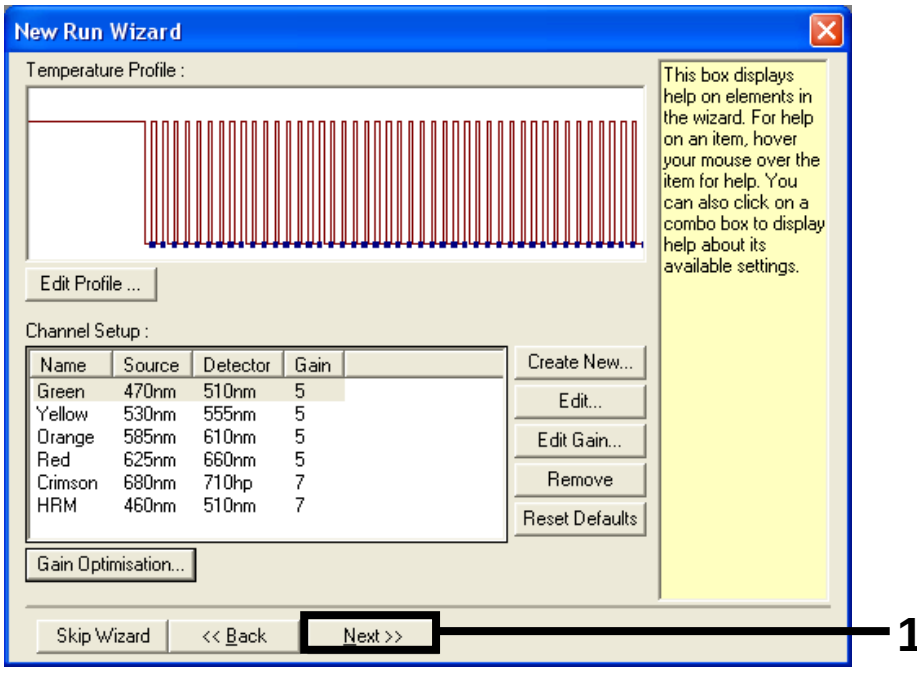


Figure 35. The "New Run Wizard" dialog box and temperature editing screen (1 = "Next" button).

5. **Check the summary and click "Start Run" to save the run file and start the run (Figure 36).**

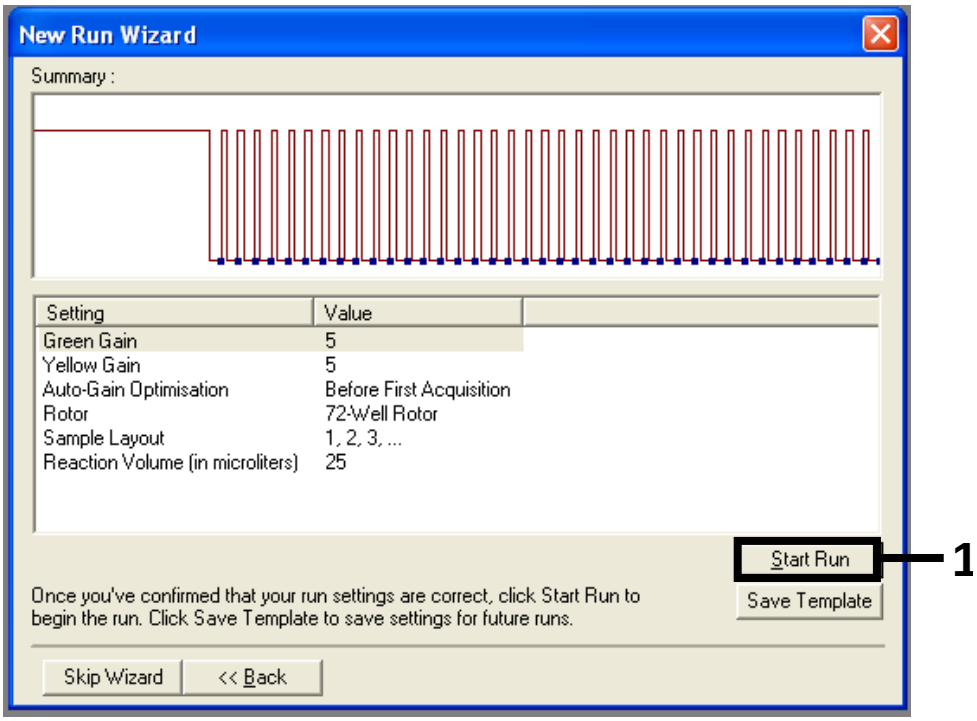


Figure 36. The "New Run Wizard" dialog box and summary screen (1 = "Start Run" button).

6. **After the run starts, a new window opens. You can enter sample names now, or click "Finish" and enter them later by selecting the "Sample" button during the run, or once the run is complete.**

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

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

7. **After the run is complete, analyze the data according to sections "Sample assessment data analysis", page 71, or "EGFR mutation detection data analysis", page 72, as appropriate.**
8. **If quantitation reports are required, click the "Reports" icon on the toolbar in the Rotor-Gene Q run file.**

9. In the report browser, click "Cycling A Green (page 1)" under "Report Categories" (Figure 37).

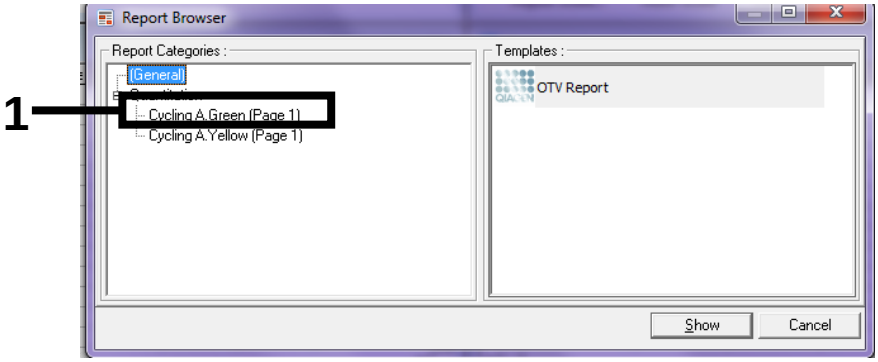


Figure 37. Report browser (1 = "Cycling A. Green (Page 1)" button).

10. Select "Quantitation (Full Report)" under "Templates" (Figure 38).

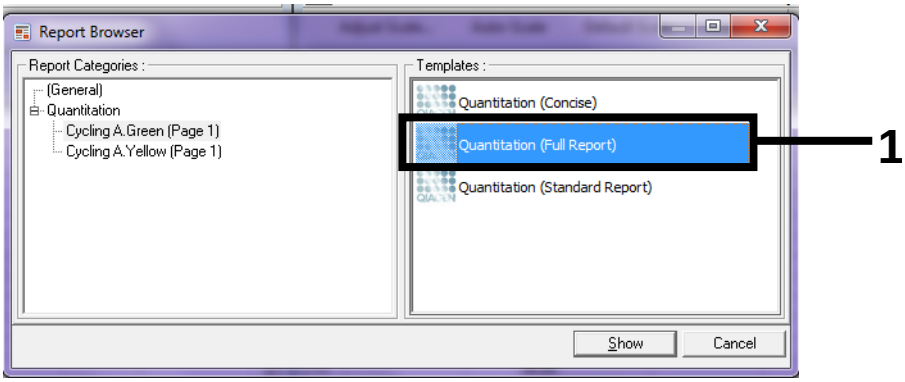


Figure 38. Quantitation report (Full Report) (1).

11. Click "Show" to generate the report.
12. Click "Save As" to save an electronic version.
13. Repeat for "Cycling A Yellow (Page 1)".

Interpretation of Results (Manual)

After the *therascreen* 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 (manual)

  Note: See Table 4, page 19, for tube layout
- EGFR mutation detection analysis (manual)

  Note: See Table 7, page 30, 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 were not already 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 has finished, refer to "Software analysis settings", page 70, and analyze the data as follows. (See Table 4, page 19, 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, then 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

A sample must pass DNA sample assessment before it can be tested to detect EGFR mutations (see "Sample assessment data analysis", page 71).

After the EGFR mutation detection run has finished, refer to "Software analysis settings", page 70, and analyze the data as follows. (See Table 7, page 30, for tube layout.)

Run control analysis

Refer to the run control analysis flowchart in Figure 39.

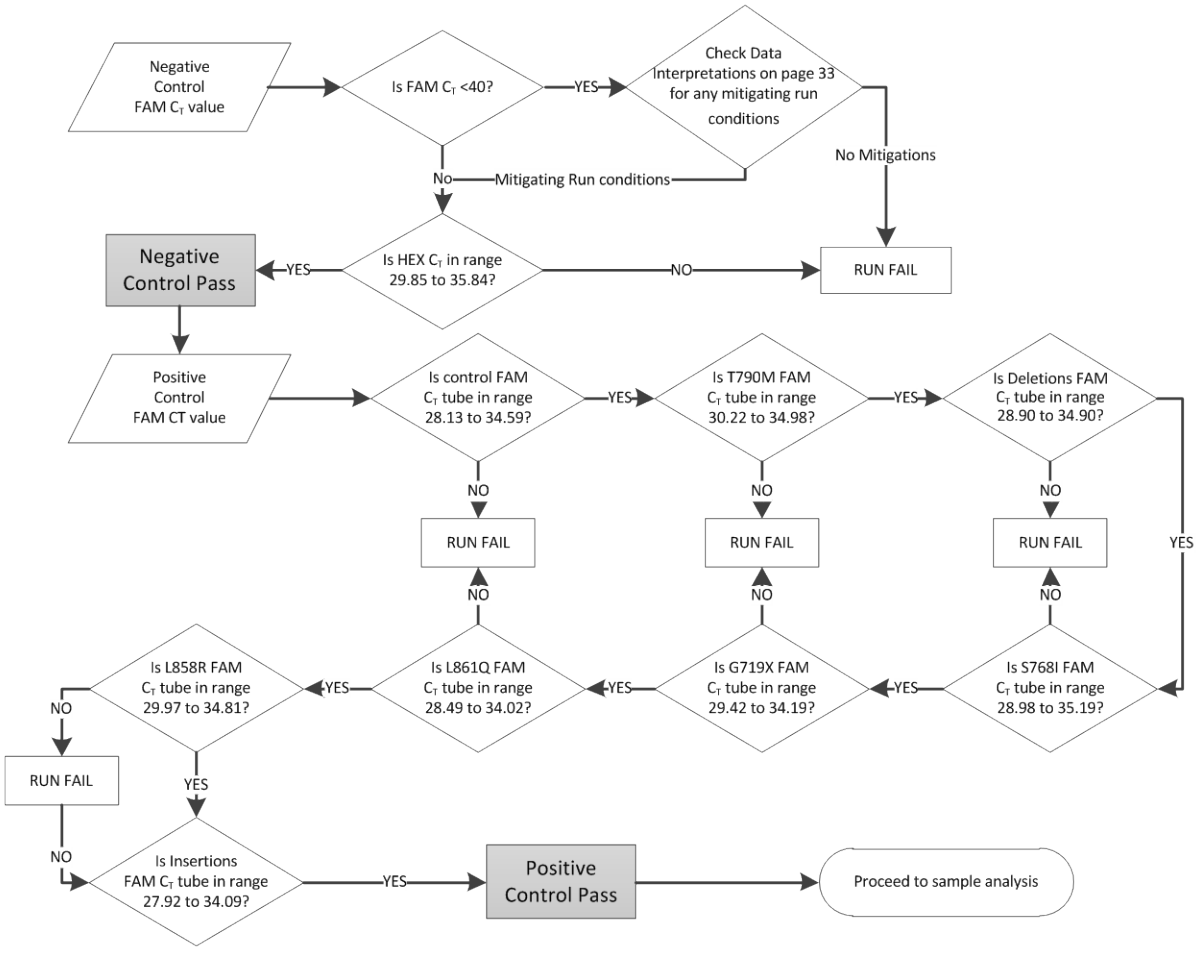


Figure 39. Run control analysis flowchart for EGFR mutation detection.

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 15. 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 15. 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
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 7, page 30, 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 40.

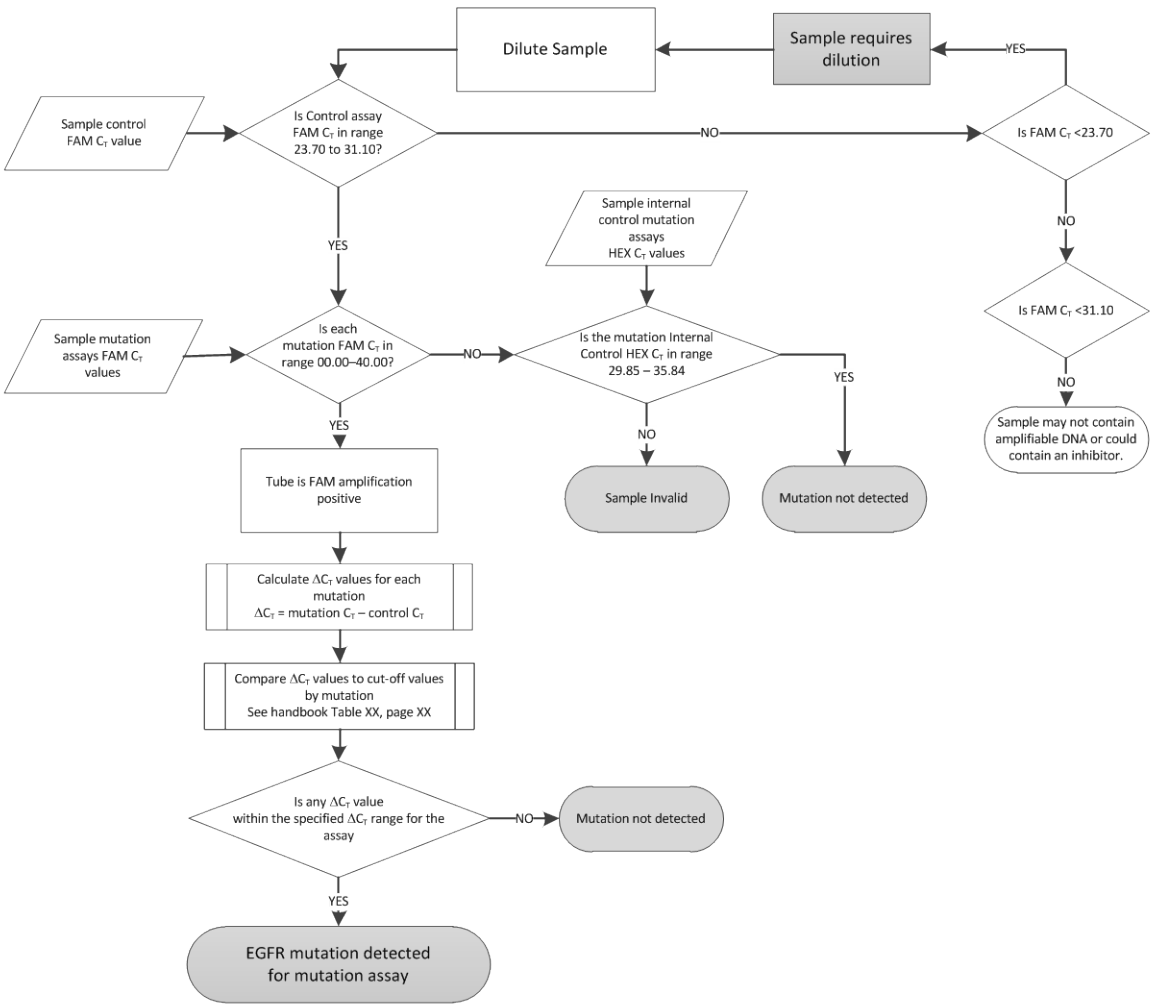


Figure 40. Sample analysis flowchart for EGFR mutation detection.

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

Refer to the sample analysis flowchart for EGFR mutation detection in Figure 40.

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 2 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.

  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 16. The values specified are within and including the values shown. (See Table 7, page 30, for tube layout.)

Table 16. 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	≤7.40
Deletions	0.00 to 40.00	≤8.00
L858R	0.00 to 40.00	≤8.90
L861Q	0.00 to 40.00	≤8.90
G719X	0.00 to 40.00	≤8.90
S768I	0.00 to 40.00	≤8.90
Insertions	0.00 to 40.00	≤8.00

- 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 7, page 30, 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 point for the assay in question (Table 16). Ensure that the correct cutoff point is applied.

The cutoff point 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 cutoff point for an assay, the sample is classed as negative, or beyond the limits of detection of the kit for that assay.

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 at, or below, the cutoff value. 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.

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.

Appendix B: Installation of the *therascreen* EGFR CE Assay Package

The *therascreen* EGFR RGQ PCR Kit is designed for use with the Rotor-Gene Q MDx instrument and a 72-well rotor. The *therascreen* EGFR CE Assay Package is available separately on CD (cat. no. 9023537). The assay package includes the "therascreen EGFR CE Control Run Locked Template" and "therascreen EGFR CE Locked Template".

Note: The *therascreen* EGFR CE Assay Package is compatible only with Rotor-Gene Q software version 2.3. Make sure that the correct version of Rotor-Gene Q software is installed before proceeding with installation of the *therascreen* EGFR CE Assay Package. If your Rotor-Gene Q MDx instrument was delivered with an earlier software version, upgrade by downloading Rotor-Gene Q software version 2.3 from the Rotor-Gene Q MDx product page, in the "Product Resources" section under "Operating Software" on www.qiagen.com/p/rgq-mdx.

Procedure

1. **Order the *therascreen* EGFR CE Assay Package CD (cat. no. 9023537).**
2. **Insert the CD into the CD drive of the computer connected to the Rotor-Gene Q MDx instrument.**
3. **If the CD auto-loads, start the installation by double-clicking the file therascreen_EGFR_CE_Assay_Package_3.0.5.exe.**

   Alternatively, locate and start this executable file using the file browser on the connected computer.

4. The *therascreen* EGFR CE Assay Package setup wizard opens. Click "Next" to continue (Figure 41).

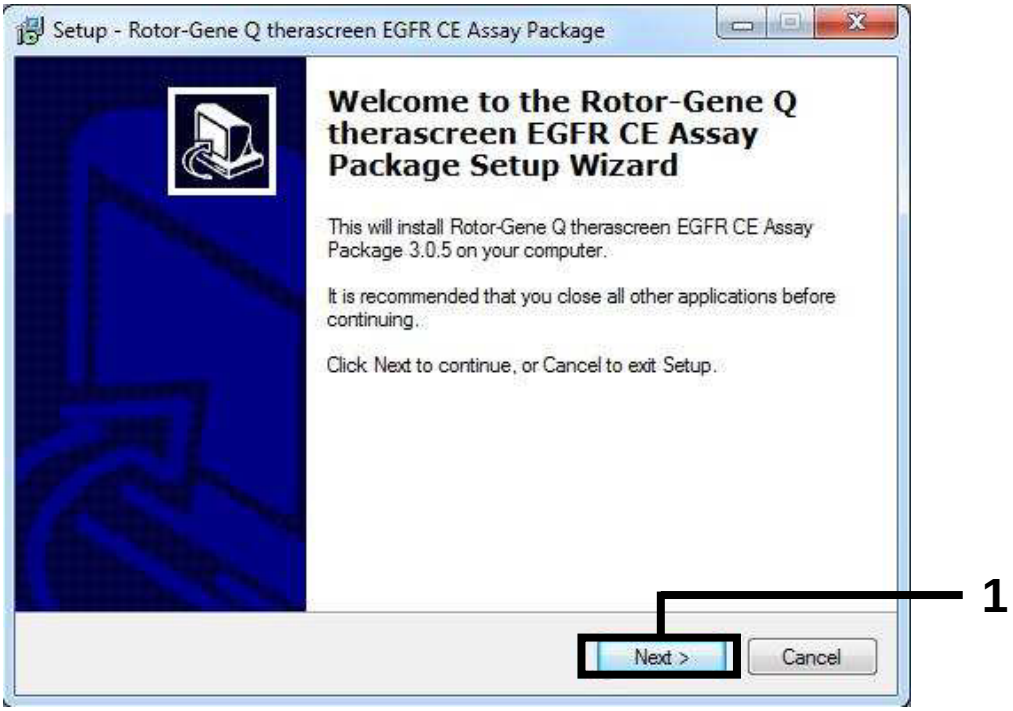


Figure 41. The "Setup Wizard " dialog box (1 = "Next" button).

5. Read the License Agreement in the dialog box and accept the agreement by checking the statement "I accept the agreement". Click "Next" to continue (Figure 42).

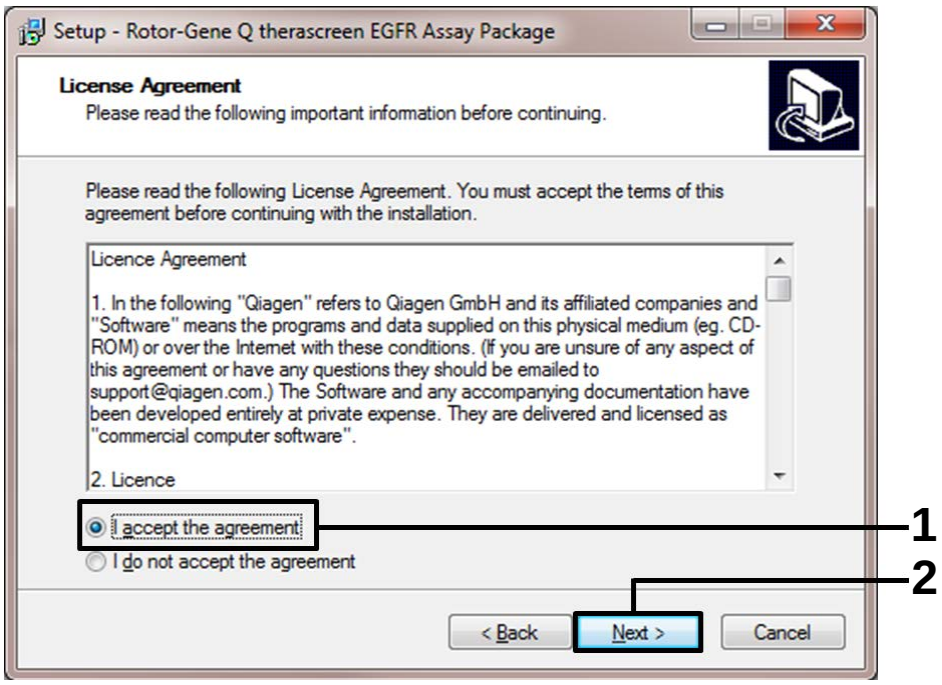


Figure 42. The "License Agreement" dialog box (1 = "I accept the agreement"; 2 = "Next" button).

6. **The setup starts automatically. When installation is complete, a final "Setup Wizard" dialog box opens. Click "Finish" to exit (Figure 43).**

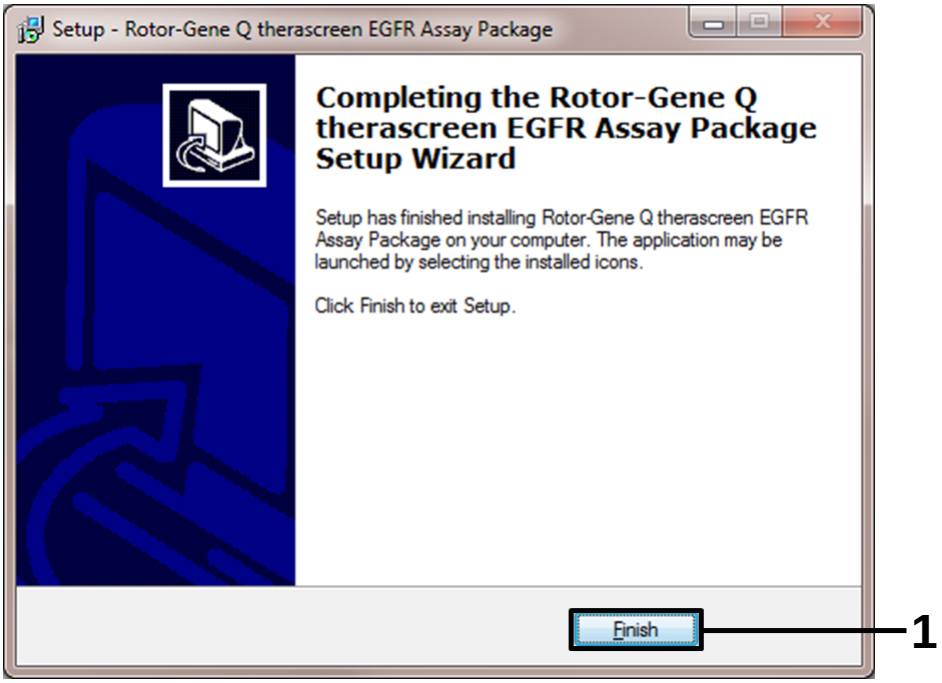
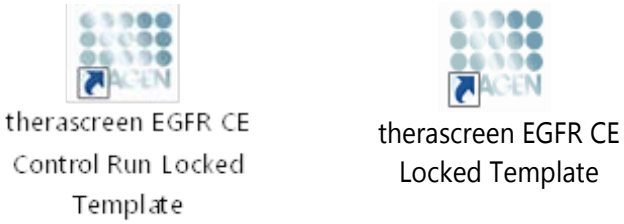


Figure 43. Completing the setup wizard (1 = "Finish" button).

7. **Restart the computer.**

   Shortcuts to both the "therascreen EGFR CE Control Run Locked Template" and "therascreen EGFR CE Locked Template" are generated automatically and appear on the desktop.



Contact Information

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support, call 00800-22-44-6000, or contact QIAGEN Technical Services or local distributors (see back cover or visit www.qiagen.com).

Ordering Information

Product	Contents	Cat. no.
therascreen 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	874111
QIAamp DNA FFPE Tissue Kit		
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 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	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: includes 1-year warranty on parts and labor, installation and training not included	9002032
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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The purchase of this product allows the purchaser to use it for the performance of diagnostic services for human in vitro diagnostics. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby.

The *therascreen* EGFR RGQ PCR Kit is a CE-marked diagnostic kit according to the European In Vitro Diagnostic Directive 98/79/EC. Not available in all countries.

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